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MAPPING THE MICROBIAL CONTAMINATION IN FOOD AND FOOD PROCESSING ENVIRONMENT

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ABSTRACT

The quality and food safety has always been an object of study for microbiology. For this reason, research is focused on both microorganisms of technological interest responsible for metabolic processes, such as fermentation, and important to define the sensory characteristics of the finished product, and food-spoilers microorganisms, that are responsible for the food spoilage. The microorganisms that inhabit the food processing environments play also an important role, since cross-contamination phenomena can occur during the processing phases. Monitoring the presence of spoilage microorganisms in food-processing environment, can be very usefull in order to prevent the spread along the processing chain and consequently the transition to the finished product, preserving the food quality and safety. The methodological approach to study the microbiota has changed and microbial species and strains can be identified and monitored with higher levels of speed, reliability and sensitivity

The aim of the present work was the study of the microbiota of different dairy manufactures, fresh meat and surface samples from the related processing environments by using the new culture-independent method based on high-performance sequencing (high-throughput sequencing, HTS).

Different ecosystems were studied, in order to investigate the microbiota composition and the specific role of the microorganisms in each food matrix and the possible overlap with the microbiota on utilized tools, equipment and processing surfaces. Several kind of dairy manufactures and meat, as well as environmental samples from surfaces and associated processing tools were taken into account. Moreover, a novel approach for of the identification of oligotypes present in foods and environments was used, focusing on the *Pseudomonas* genus. Oligotyping is a computational method that allows to explain the ecologically significant differences between closely related organisms, going over the species identification.

Thanks to the different HTS approaches it was possible to obtain a complete image of the typical microbiome of certain food matrices and their processing environments. In the present study the presence of a selected core microbiota was identified, consisting of a few species well adapted to the considered environment. However a different distribution and varable relative abindance among the samplkes was observed between food and environment, and can be speculated that there is an influence by the environmental microbiota on the food matrices.

The high-throughput sequencing demonstred to be a suitable approach to the study of the microbiota of dairy manifactures and meat, as well as for environmental samples from food processing surfaces, allowing to monitor the microbiota during the various stages of production.

The characterization of the environmental microbiota and the understanding of the correlation between ecological factors and the microbiota of food are of crucial importance for the control of food quality and safety. Getting a description of the surface microbiota in the food processing plant and monitoring changes over time could represent a good start point to map the sources of contamination.

Also understand the composition of the microbiota calling it beyond the species identification is a crucial step to investigate the diversity of microorganisms recognized as spoilers in the food industry. In this context, the dell'Oligotyping use allows in-depth analysis of microbial consortia in food and related environments.

RIASSUNTO

La qualità e sicurezza degli alimenti è da sempre oggetto di studio per la microbiologia. A tal proposito si pone attenzione sia su microrganismi d'interesse tecnologico, responsabili di processi metabolici quali la fermentazione e fondamentali nella definizione delle caratteristiche sensoriali del prodotto finito, sia su microrganismi cosiddeti "spoilers", responsabili del deterioramento degli alimenti. Di fondamentale importanza sono anche i microrganismi che popolano gli ambienti di processamento degli alimenti dal momento che fenomeni di cross-contamination possono verificarsi durante le fasi di lavorazione. Monitorare la presenza di microrganismi alterativi negli ambienti di processamento di alimenti, prevenendone la diffusione lungo la catena di lavorazione e di conseguenza il passaggio al prodotto finito è di fondamentale importanza per preservarne la qualità e la sicurezza. Lo studio del microbiota ambientale presente e la sua influenza sul prodotto finale o sugli intermedi di lavorazione è stato d'interesse per decadi ma recenti studi in ecologia microbica hanno avvalorato l'utilizzo di tecniche per lo studio della contaminazione microbica degli alimenti e degli ambienti di processamento degli stessi.

L'obiettivo del presente lavoro di tesi è stato lo studio del microbiota di diversi prodotti lattiero-caseari, di carne fresca e dei relativi ambienti di lavorazione utilizzando il nuovo metodo coltura-indipendente basato sul sequenziamento ad alto rendimento (high-throughput sequencing, HTS). Pertanto, sono stati studiati diversi ecosistemi per comprenderne la composizione del microbiota e il ruolo specifico dei microrganismi in ogni matrice alimentare e nei relativi ambienti di lavorazione e processamento. Sono state considerate diverse tipologie di prodotti lattieri-caseari e carnei, nonché campioni ambientali relativi a superfici e strumenti di lavorazione associati. Infine, è stato investigato per la prima volta lo studio del microbiota di matrici alimentari utilizzando un nuovo metodo computazionale (Oligotyping) che permette di spiegare le differenze ecologicamente significative tra organismi strettamente correlati arrivando a una identificazione oltre la specie. Tale metodo è stato utilizzato per l'identificazione degli oligotipi presenti negli alimenti e ambienti, descritti precedentemente, appartenenti al genere *Pseudomonas.*

Grazie ai differenti approcci di HTS è stato possibile ricavare un'immagine completa del microbioma tipico di determinate matrici alimentari e dei relativi ambienti di lavorazione. Nei campioni studiati si è potuta osservare la presenza di un microbiota "core" selezionato, costituito da poche specie ben adattate all'ambiente considerato. Tuttavia si è notata una differente distribuzione e abbondanza di tali specie tra alimenti e ambiente lasciando pensare che ci sia un'influenza del microbiota ambientale su quello alimentare.

L'*high-throughput sequencing* si è dimostrato un approccio adatto allo studio del microbiota dei prodotti lattiero-caseari e carnei, nonché dei campioni provenineti dalle superfici di lavorazione degli alimenti, permettendo di monitorare il microbiota nel corso delle varie fasi di produzione.

Dai risultati ottenuti è emerso che lo studio del microbiota ambientale degli impianti di lavorazione degli alimentari può essere molto importante per il mantenimento degli standard di qualità. Quindi, definire una mappatura del microbiota negli ambienti di lavorazione e sorvegliarne i cambiamenti nel tempo può essere un valido approccio per monitorare le contaminazioni ambientali e l'efficacia delle pratiche di pulizia al fine di assicurare i parametri di sicurezza microbiologica negli impianti di lavorazione.

Inoltre comprendere la composizione del microbiota definendolo con un'accuratezza che va oltre l'identificazione della specie è un passo fondamentale per investigare la diversità di *genera* riconosciuti come *spoilers* in ambito alimentare. In questo contesto, l'utilizzo dell'Oligotyping permette un'analisi approfondita dei consorzi microbici nei prodotti alimentari e relativi ambienti.

0 PREFACE

Food safety is a priority for foodservice organizations because inappropriate handling or food spoilage can result in serious problems for both foods and consumers (Cairo et al., 2008; Egan et al., 2007; de Oliveira et al., 2014). Optimal conditions for microbial growth can occur in food processing facilities (Carpentier & Cerf 1993). The growth of microorganisms in a food processing environment and the establishment of certain microbial communities can lead to the development of a well-defined environmental microbiota(Stellato et al., 2015; De Filippis et al., 2013). The study of the microbial ecology of foods has undergone a major revolution, and the advent in microbial ecology of sensitive culture-independent tools allows a rapid and effective evaluation of microbial contamination in many sorts of environments (Ercolini et al., 2012).

In the past, study about microbiota in food has been based on traditional microbiology approaches, such as standard plate counts (Bell 1997). However, standard plate counts might result a lower colony-forming units or numbers compared to actual viable population (Jay et al.,, 2005). This is due to some limitations. First, this technique might not be able to detect novel microorganisms that are not cultivable using known media. Second, it does not capable to recover stressed or viable but non-cultivable (VBNC) microorganisms (Giraffa 2004).

Natural stress, such as starvation, incubation outside the temperature range of growth, elevated osmotic concentrations, oxygen concentration or exposure to white light might lead cells to enter VBNC state (Oliver 2005). One of the meat spoilage bacteria that have been reported demonstrating the VBNC state is *Pseudomonas fluorescens*. A study showed that some non-cultivable *Ps. fluorescens* cells are dividing on the surface that imitate an open surface at meat processing premises although the cell division could not continue to the stage of macro-colony formation on agar (Peneau et al., 2007). Therefore, more elaborated methods based upon molecular biology have been applied to study microbial populations without cultivation (Giraffa 2004; Mayo et al., 2014).

Culture-independent techniques can be used to determine the microbial diversity in natural ecosystems and to observe the evolution of microbial populations over space or time. These methods could overcome problems associated with selective cultivation such as (i) inability to detect some bacteria on the known media, (ii) lack of knowledge of the real conditions under which most of bacteria are growing in their natural habitat and (iii) difficulty to develop media for cultivation accurately resembling these conditions (Doulgeraki et al.,, 2012). These techniques are generally based on the analysis of the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) that is extracted directly from the sample. Subsequently, the nucleic acids are amplified by polymerase chain reaction (PCR) and subjected either to cloning and sequencing or to profiling techniques, such as denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE), or single strand conformation polymorphism (SSCP) (Cocolin et al., 2008). Another alternative method that does not rely on nucleic acids extraction from the sample is called fluorescence in situ hybridization (FISH). Some extensive methods to characterize the strains isolated by culture-dependent methods in molecular level have also been developed, such as randomly amplified polymorphic DNA (RAPD)-PCR, repetitive bacterial DNA elements (Rep)-PCR and enterobacterial repetitive intergenic consensus (ERIC)-PCR (Cocolin et al.,, 2008).

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Next-generation sequencing (NGS) technologies can overcome these limitations (Bokulich & Mills 2012) including high-throughput sequencing approaches. The workflow, limits, and perspectives in applying culture-independent high-throughput sequencing to study food microbiota has been reviewed (De Filippis et al., 2013). A recent study showed that performing pyrosequencing of 16S rRNA gene amplicons extracted directly from meat stored in different packaging conditions led to much higher microbial diversity than PCR DGGE (Ercolini et al., 2011). In another study, high-throughput barcoded parallel 454 pyrosequencing was used to characterize bacterial and fungal changes in vacuum-packed chilled pork (Zhao et al., 2015). The high-throughput sequencing approach can also give a quantitative estimation of the abundance of a single taxon in each sample based on the number of reads of its particular 16S rRNA gene sequence (Doulgeraki et al., 2012).

However, an important microbiological aspect to take in count is that food spoiling is considered a species-specific peculiarity. It was demonstrated that different strains of the same species can behave differently in exactly the same food matrix and storage conditions; accordingly, different biotypes can have distinctive metabolic behaviours that will drive or not to food spoilage. (Casaburi et al., 2014; Casaburi et al., 2011; Ercolini et al., 2011). Such biodiversity of particular genera beyond the species is certainly revealed by colonization capability of the food-processing environment, which is the major source of contamination. Basing on these considerations, the ecological relevance of the biodiversity within defined genera in the food environment has to be consider in defining a microbiota. Unfortunately, HTS does not allow this kind of informations because the analysis of microbial communities via 16S rRNA gene data generally relies upon classification-based approaches that make taxonomic assignments by comparing each DNA sequence to reference data bases (Wang et al., 2007; Huse et al., 2008; Liu 2008), or clustering-based methods that identify taxon-independent operational taxonomic units (OTUs) using a sequence similarity threshold (Schloss et al., 2009; Huse et al., 2008)(Schloss et al., 2009; Huse et al., 2010); so this methods often fail to resolve ecologically meaningful differences between closely related organisms in complex microbial data sets.

This limitation can be overcame by using Oligotyping: a novel supervised computational method that allows investigate the diversity of closely related but distinct bacterial organisms in final operational taxonomic units identified in environmental data sets through 16S ribosomal RNA gene data by the canonical approaches. This computational method can resolve the distribution of closely related organisms across environments and unveil previously overlooked ecological patterns for microbial communities (Eren et al., 2013).

This put the basis to explore in deep the microbial composition of food matrices and related environment in order to investigate the overlap between food and food production environment, the existence of food and environment-specific types that can be possibly linked to resiliency in the food-processing environment and to possible food spoilage occurrence; with the final aim of constantly improving food safety and quality.

1 STATE OF THE ART

1.1 Food spoilage

Spoilage is the process of food deterioration leading to a reduction of its quality, till the point of not being edible for humans. Signs of spoilage may include a different appearance of the food compared to its fresh form and the alteration of the sensorial quality of the product, in particular the aspect (including texture and color) and the presence of off odour (Remenant et al., 2015; Nychas et al., 2008; Gram et al., 2002; Borch et al., 1996).The presence of microorganisms on the surface of the food or of the tools used during food processing will determine food spoilage poilage upon their interaction and growth in optimal conditions (Gram et al., 2002; Doulgeraki et al., 2012).Microbial growth to high numbers is a prerequisite for meat spoilage that can be considered an ecological phenomenon encompassing the changes of the available substrata during the proliferation of bacteria (Olusegun & Iniobong 2011; Nychas et al., 2008). Food spoilage is a complex process in wich a combination of microbial and biochemical events make a food product unacceptable for human consumption: abiotic factors such as temperature, gaseous atmosphere, pH and NaCl will select for certain bacteria, allowing the colonization of the meat surface by different spoilage-related species and strains (Brooks & Flint 2008; Ercolini et al., 2006). Despite the heterogeneity in raw materials and processing conditions, the microflora that develops during storage and in spoiling foods can be predicted based on knowledge of the origin of the food, the substrate base and a few central preservation parameters such as temperature, atmosphere, aw and pH. These methods are still used today, albeit using less and less preservation and combining various lightly preservation procedures to inhibit growth of microorganisms. Signs of microbial spoilage may be characterized by many changes in the food, manifesting in a different appearance of the product compared to its fresh form and the alteration of the sensorial quality of it; preciselly, modification in the aspect (including texture and color), visible growth (slime, colonies), and the presence of off odour (Remenant et al., 2015; Nychas et al., 2008; Gram et al., 2002; Borch et al., 1996) and changes in the structure (degradation of polymers) can occur. The presence of microorganisms on the food surface intermediate or on the final products will determine spoilage upon their interaction and growth when optimal conditions ensue (Gram et al., 2002; Doulgeraki et al., 2012). Investigating the microbial composition of food matrices and microbial consortia, that can populate food-processing environments, is essential if the growth and the activity of spoilage microorganisms are to be reduced. Also, knowledge of the microorganisms involved in spoilage and the metabolites associated with spoilage is needed to develop microbiological and chemical methods for evaluation of quality and shelf life. Generally, the growth and metabolic activity of microbial spoilage is described as strictly dependent on the substrate and modulated by chemical and physical parameters such as temperature, pH, a_w and oxygen availability, and food represents a complex niche with chemical and physical properties that allow the colonization and development of a variety of microorganisms, especially bacteria (De Filippis, La Storia, et al., 2013; Doulgeraki et al., 2012); moreover environmental conditions of food processing plants offer favourable conditions for the growth of a well defined microbial consortia that can led to the establishments of a resident microbial community (Hultman et al., 2015; Stellato et al., 2015; De Filippis et al., 2013). Despite the variability in all of the food matrices and related orocessing environments, the importance of these settings for the selection of a spoilage microbiota cannot be undervalued, in order to understand interactive behaviour between the microorganisms that can determine selection and/or metabolism responses and subsequently spoilage (Gram et al.,, 2002).

1.1.1 The main food-spoiling microorganisms

Most food products are highly perishable since are rich nutrient source for microbial development, with chemical and physical properties which allow the colonization and development of a variety of microorganisms. Considering all the microorganisms able to contaminate food, a part of them present metabolic activities leading to spoilage. Every kind of food product is characterized by a specific microflora that can show a well-defined structure at any point in time during production and storage phases. The microflora's composition is strongly dependent upon raw material microflora, food processing plant microflora, preservation and storage conditions. The potential spoilage ability lof a microorganism is the capability to drive metabolics pathways that lead to the production of typical tmetabolites that are associated with the spoilage of a particular food product.

Basically, all the groups of microorganisms, under some conditions, can contribute to spoilage of foods. Hypothetically, it can be assumed that all microorganisms are initially present on a food product where after a combination of specific conditions, undergo to a selection based first on nutrient assortment and availability and then on the chemical and physical parameters. Although despite the large variety of food matrices, the main bacterial populations involved in spoilage are common. Hence, *Pseudomonas spp*. and some other psychrotrophic organisms usually dominate proteinaceous foods stored aerobically and at cold temperatures and this happens for vegetables, meat, poultry, pork, milk, dairy products and fish (Doulgeraki et al., 2012; Ercolini, Ferrocino, et al., 2010; Martin et al., 2011; Marchand et al., 2012; Franzetti & Scrpellini 2007).

1.1.2 Classification of microbial food spoilers

Spoilage microorganisms can be divided into principals categories: Gram-negative rod shaped bacteria, Gram-positive spore forming bacteria, lactic acid bacteria, other Gram-positive bacteria, yeasts and moulds (Huis In't Veld 1996).

1) Gram-negative rod shaped bacteria

Pseudomonas spp. are the most common spoilage organisms, particularlyin aerobically stored foods with a high water content and sub-alchaline pH, (Doulgeraki et al., 2012; Ercolini, Ferrocino, et al., 2010; Martin et al., 2011; Marchand et al., 2012; Franzetti & Scrpellini 2007) e.g. vegetables, meat, poultry, pork, milk, dairy products and fish (Remenant et al., 2015; Nychas et al., 2008; De Jonghe et al., 2011). *Pseudomonas* is the most heterogeneous and ecologically meaningfull genus of food importance, and includes Gram-negative motile aerobic rods that are widely spreaded in the environment, since shows a relevant metabolic versatilityand remarkable adaptation to different substrates (Franzetti & Scrpellini 2007).

The majority of Pseudomonas group members are psychrotrophic, can be *fluorescent* or *non-fluorescent*, and are largely recognized as being responsible for chilled food spoilage. *Pseudomonas fluorescens, Pseudomonas putida*, and *Pseudomonas fragi* are the most frequently found species, even with a different distribution of the species within the food environment (Arnaut-rollier et al., 1999). *Pseudomonas* spp. plays a significant role in milk spoilage since during the storage of raw milk they are responsible of the production of many lipolytic and proteolytic enzymes reducing both the quality and shelf life of processed milk (Dogan & Boor 2003) or of cheese manufactures (Martin et al., 2011; Andreani et al., 2015). Furthermore, members of *Pseudomonas* spp. have been also isolated from spoiled meat: once they colonize the food matrix, they can be responsible of slime and off odour production that finally compromise food quality and consumer's acceptability of the product (Casaburi et al., 2015; Pothakos, Devlieghere, et al., 2015; Doulgeraki et al., 2012; Nychas et al., 2008).
Other Gram-negative rod shaped bacteria such as *Aeromonas, Photobacterium, Shewanella and Vibrio* may also grow rapidly at low temperatures and, are recognized to contribute to the spoilage of chilled red meat, cured meats, poultry, fish, milk and dairy manufactures. Gram-negative bacteria mentioned above, are also responsible of food spoilage due to protein metabolism (like the pseudomonads) resulting in odour and flavour defects, slime production and the formation of visible often pigmented colonies (Andreani et al., 2015; Martin et al., 2011)

Cold-tolerant *Enterobacteriaceae*, such as *Serratia liquefaciens, Enterobacter agglomerans and Hafnia alvei*, can also grow in aerobically-stored meats but hardly account for more than a small proportion of the total microbiota (Dainty & Mackey 1992). Moreover, they are more prevalent in higher temperature (5-10°C) (Labadie 1999; Huis In't Veld 1996) and on pork and lamb, especially on fat surfaces (Dainty & Mackey 1992). Generally, Enterobacteriaceae take over the spoilage process from Pseudomonas spp. at temperature of 5-10°C. The natures of spoilage by *Enterobacteriaceae* are the production of gas, acid, slime. rope, bitter flavours and off-odors (Huis In't Veld 1996). Due to its presence in spoiled meat and meat products, *Enterobacteriaceae* are often used as food safety indicators (Huis In't Veld 1996; Nychas et al., 2008). Lowering storage temperature can inhibit the growth of *Enterobacteriaceae and Brochothrix thermosphacta* (Borch et al., 1996).

2) Gram-positive spore forming bacteria

A variety of foods undergo pasteurisation process or heating step but there are microorganisms able to survive high temperatures. *Bacillus* spp. are spore forming bacteria that are commonly distributed in

nature, and associated with a range of food products such as milk and dairy manufactures, meat and meat products, pasta and spices. They resistance to extreme conditions especially to high temperature is for their sporing activity. Usually these microorganisms are associated with the spoilage of food products but recently they are considered responsible of food poisoning issues (Fernández-No et al., 2011). *Clostridium* is a rod-shaped cell with a gram-positive membrane and grows at anaerobic conditions. It produces large amounts of gas in packaged meat. It is usually coupled up with foul odors and causes the package to appear in a blown pack (Nölling et al., 2001). Even if it is largely recognized as meat spoiler, it can also be found in soil, sewage and animal intestines.

3) Lactic acid bacteria

Lactic acid bacteria (LAB) are largely associated with fresh meat and cooked meat products. Vacuum-packing atmosphere also cause a microbiota shift in meat to lactic acid bacteria (LAB) due to the inhibition of pseudomonads (Dainty & Mackey 1992). LABs are also recognized as spoilage bacteria in cured meat due to its ability to survive over nitrite compounds(Borch et al., 1996). LAB produce slime and off- flavors due to sugar fermentation. However, they have a tendency to grow slower at refrigeration temperature and be out-competed by pseudomonads under aerobic conditions (Huis In't Veld 1996).

They both contribute to food spoilage because able to product undesidered metabolites and causing the subsequent organoleptic decline of meat (Labadie 1999; Huis In't Veld 1996) or serve as biopreservatives agents with strains of certain species demonstrating reduced spoilage capacities and inhibitory activity against spoiling microbiota (Fall et al., 2012; Vasilopoulos et al., 2010). Hence, a distinction among biotypes is necessary in order to identify spoilage potential strains. Subsequently, this suggests that presence of lactic acid bacteria in food do not necessarily lead to quality defects.

Leuconostoc is one of the lactic acid bacteria, grows optimally at 20-30°C and in modified atmospheres; it is heterofermentative, which means that use a combination of pentose phosphate and phosphoketolase pathways. It produces D-lactate and ethanol and is responsible for the discoloration, gas production, and buttery smell of spoiled meat (Borch et al., 1996). *Leuconostoc* is widely found in fresh meat and meat products, but can also grow in plants, fermenting vegetables, milk, dairy products, wine, and even human blood (Koort et al., 2005).

4) Other Gram-positive bacteria

Brochothrix thermosphacta is a psychotrophic bacteria and can be found dominating in meats in modified atmosphere and vacuum packaging at refrigerated condition (Borch et al., 1996; Huis In't Veld 1996). A study showed that *Brochotrix thermosphacta* increased two log cycles regardless of the initial contamination in meat that spoiled after 7 days of storage at 5°C (Russo et al., 2006). *B. thermosphacta* degrades glucose and glutamate in beef (Gill & Newton 1978). The characteristics of spoilage by *B. thermosphacta* are sour and acid flavors and a slightly sweet, cheesy obnoxious odor. This is particularly found in meat products that have initially been stored anaerobically and subsequent to opening the package in an aerobic atmosphere (Borch et al., 1996).

Carnobacterium is gram-positive genus and its alterative activity consist in defects such as sour off flavours, discolouration, gas or slime production and decrease in pH (Nychas et al., 2008; Doulgeraki et al., 2012; Casaburi et al., 2015; Pothakos, Devlieghere, et al., 2015). *Carnobacterium* genus contains nine species: *C. divergens*, *C. maltaromaticum*, *C altherfunditum*, *C. funditum*, *C. gallinarum*, *C. mobile*, *C. inhibens*, *C. pleistocenium*, and *C. viridans*. However, only *C. divergens* and *C. maltaromaticium* can frequently be isolated in meat products. In food, they are often found responsible for producing antimicrobial peptides and bacteriocin. *C. divergens* and *C. maltaromaticum* grow anaerobically at high CO_2 concentration, low concentration and high pressure. They are able to metabolize arginine and other carbohydrates, which may contribute to their adjustment to the environment. Their catabolic activities were observed to play a role in sensory spoilage of meat products. The other seven species are not commonly encountered. One example of its effects on food is that, *C. divergens* can produce H_2O_2, and when it encounters *C. vriridans*, it would result in a green discoloration of ham. However, this genus has not been fully understood. Its full genome sequence has yet been determined. Thus more studies are needed for a full understanding of *Carnobacterium* effects in food (Leisner et al., 2007).

1.1.3 Food processing enviroment and food-spoilage

Microbial contamination in food processing plant environments can play a primary role in food quality and safety. In industrial environments a specific microbiota can colonize specifically each site of the plant and the variability of the microbiota composition is strictly influenced by the characteristics of the surface. The concept of food safety is of primary importance for industrial organizations because inappropriate handling or food spoilage can result in serious problems for both foods and consumers (Cairo et al., 2008; Egan et al., 2007; de Oliveira et al., 2014). Considering the large variety of food we can find a specific microbiota that is firmly dependent on different variables: the characteristics of the raw materials, the environment where the food is processed and ultimate, the storage and consumption conditions. Foods can be contaminated during all the preparation steps by microorganisms that colonize the processing and storage environments, surfaces, tools, equipment and personnel employed in management and production phases (Worsfold & Griffith 2001; Legnani et al., 2004). When microorganisms colonize foods it can depend not only on the ecological conditions that occur in the food itself, but also from the interactions between food and the environment. For this reason is of fundamental importance for both food quality and microbiological safety the application of the standards of microbial ecology to food systems. Food contact surfaces are good substrates for the proliferation of microorganisms. Observing frequent cleaning and disinfection procedures can generally guarantee acceptable hygienic conditions in the food processing environments, but they may be not totally effective in the elimination of a well-estabilished microbiota. Such microbial populations tend to develop on surfaces that are particularly difficult to clean due to difficult access, surface irregularities or retention of sticky raw materials. The most challenging hazard for food quality and safety remain the transfer of environmental microbiota from surfaces or tools to foods, even after their hygienization (Verran et al., 2008; Shi & Zhu 2009). Environmental conditions play an important role in the selection of microorganisms, and optimal conditions for microbial growth can occur in food processing. In the food processing environments, organic residues from food manipulation can create microenvironments for growth and accumulation of microorganisms and representing a significant source of cross-contamination (Brooks & Flint 2008; McLandsborough et al., 2006; Simoes et al., 2010). Cross- contamination during food preparation can depend on trimming, cutting, washing, rinsing, dewatering and packaging, which are all considered as contributing to microbial food safety hazards (Stellato, et al., 2015)When microbial cells are transferred from the equipment, tools or food contact surfaces to food, other ecological factors such as pH, temperature, osmolarity, O_2 availability, nutrient composition and coexistence of other microorganisms will play a fundamental role in the selection of which microorganisms can develop and eventually cause concerns. The investigation of the environmental microbiota and the understanding of the correlation between ecological factors and the food microflora are of fundamental importance for the control of food quality and safety. Despite the overall high microbial diversity usually found in food processing environments, the composition of the microbiota is related to the specific surfaces (Flores et al., 2013; Scott 2000; Medrano-Felix et al., 2011) and it is also influenced by the architecture of the spatial structure (Kembel et al., 2014). These are all factors affecting the possibility for the bacteria to develop and become resident. In fact, since the characteristics of every type of surfaces, environmental parameters and food processing conditions can influence the structure of the microbiota it is expected that a well-defined resident microbiota can occur in each site of a food-processing plant. Moreover, other environmental source of food contamination can be human or animal skin. Is well known that cross-contaminations can occur directly via the hands of personnel (Konecka-Matyjek et al., 2012). Inappropriate practices of food handling and low microbiological quality standard of the final products can be related to the lack of hygiene of personnel; consequently, food hygiene training for operators (Richardson & Stevens 2003) can be considered as a solution whereby food safety can be increased. The majority of microorganisms belonged to phyla generally identified as dominant in food-processing environments environments are: *Actinobacteria, Firmicutes and Proteobacteria*, that were all found as abundant in several studies (Aydogdu et al., 2010; Flores et al., 2013; Kembel et al., 2014; Stellato, De Filippis, et al., 2015; Stellato, La Storia, et al., 2015).

Facility ecosystem investigation by mapping the microbiota may assure a valuable approach to monitor environmental contamination in order to increase the global quality management in the food-processing plants. Furthermore, understanding the interactions between food products and related environmental microbiota can represent a decisive step to guarantee food manufactures of a high quality level. Sensitive culture-independent tools for rapid and effective evaluation of microbial contamination are actually available thanks to recent advances in microbial ecology appling to many sorts of environments (De Filippis, La Storia, et al., 2013). In the specific case of food-processing plant, a rapid mapping of the

contamination of surfaces, tools and equipment that come in contact with food can be of fundamental interest in order to identify possible sources of food contamination and to evaluate the effectiveness of clean

1.2 Microbial ecology of dairy manufacturing

Approaches for studying microorganisms in food have absolutely changed. Recent advances in molecular biology have provided more information on food-associated bacteria, and have also provided the scientific community with sound, reliable and effective methods for detection, identification and typing of microorganisms from food. The main interest of dairy microbiologists is to study the diversity and dynamics of microorganisms in dairy productions and, possibly, to correlate the occurrence of certain microbial species and strains with desired flavor and sensorial traits of the products. Various molecular methods can be used depending on the level of information required by research. Microbiologists can be interested in identification, detection or typing of bacteria from a certain environment. Identification and detection can benefit from the availability of both culture-dependent and culture-independent techniques, whereas typing is an analysis performed on isolates and is, thus, strictly related to culture-dependent methods (Temmerman et al., 2004).
Moreover, the large variety of dairy manufactures inspire the microbiologist and food technologist to find new solutions ways to prevent the microbial contamination. Spoilage microorganisms include aerobic psychrotrophic Gram-negative bacteria, spore-forming bacteria, heterofermentative lactobacilli, yeasts and molds. Psychrotrophic bacteria are able to produce large amounts of extracellular hydrolytic enzyme, and the risk of recontamination of pasteurized liquid milk products with these bacteria is a major issue for their shelf life. Fungal species may cause spoilage of dairy foods manifesting the presence of a wide variety of metabolic by-products, causing off-odors and flavors, and even producing visible changes in color or texture. Furthermmore, all the microorganisms cited above can all cause gassing defects in cheeses. The are many treatments that can be applicated in order to control the percentage of spoilage of many dairy foods such as reduction the pH by fermenting the lactose to lactic acid; addition of acids or other approved preservatives; introduction of a desirable microflora that restricts the growth of undesirable microorganisms; addition of sugar or salt to reduce the water activity (a_w); removing water; packaging in absence of oxygen and freezing. The variety of spoilage microorganisms change extensively among dairy foods depending on the selective effects of the applied process, composition, packaging, storage, distribution, and handling. The study of microbial ecology associated with dairy fermentation is fundamental to understand the bases of important traits of dairy products.

1.2.1 Dairy manufacturing

The production of all varieties of cheese involves a generally similar protocol (Figure 1); various steps can be modified to give a product with the desired characteristics.

During dairy product production, milk is first pasteurized to kill bacteria that cause unwanted spoilage of the milk and of the downstream milk products. Primary microflora consists of certain kinds of *Lactococcus, Lactobacillus* and *Streptococcus* that are intentionally added to pasteurized milk and grown at 30°C or 37°C (temperature depends on the bacteria added). Secondary microflora include several different types of bacteria (*Leuconstoc, Lactobacillus*, and *Propionibacterium*), yeasts and molds; they are only used for some types of surface ripened and mold ripened cheeses. The various combinations of microflora determine what milk product you will end up with. Although most industrial dairy products are produced from pasteurized milk, a large number of raw milk cheeses are increasingly described as celebrated traditional on-farm-made cheeses and commercially proposed as gastronomic specialities, emphasizing their distinctive flavor and suggesting the best way to consume them.
Different unripened milk products are created by using various starting products and bacteria. For buttermilk production, *Lactobacillus bulgaris* (named for its country of discovery, Bulgaria) is added to skim milk to curdle it. Leuconostoc is then added to thicken it. Sour cream is made the same way except cream is used instead of skim milk. During yogurt production, dry milk protein is added to milk to concentrate the milk before addition of actively growing *Streptococci* and *Lactobacilli*. Butter is produced by curdling and slight souring from *Streptococci* growing in sweet cream. Leuconostoc is then added so it can synthesize diacetyl, a compound that gives butter its characteristic aroma and taste. The milk is then churned to aggregate the fat globules into solid butter. Thus milk type and bacteria will determine the dairy product produced.

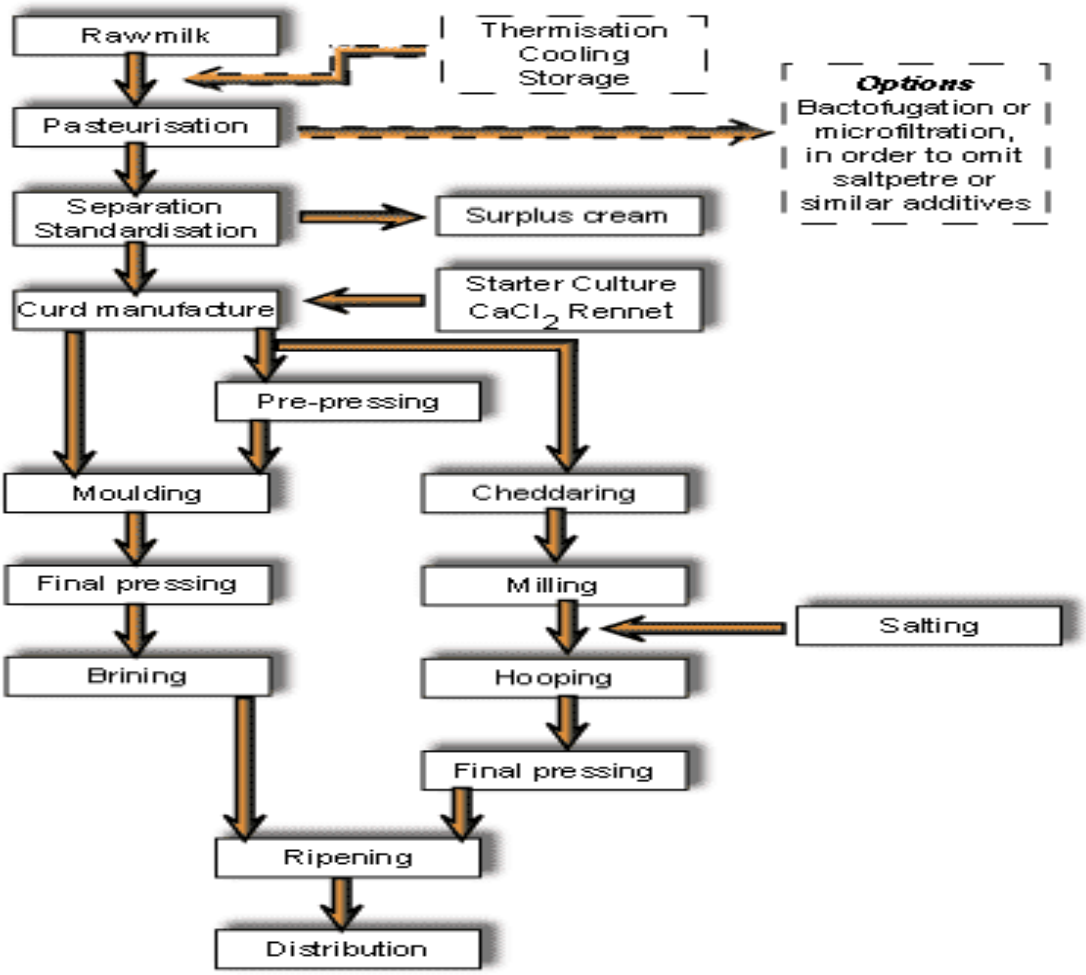

Figure 1. Cheese production process

Cheese is an important product of fermentative lactic acid bacteria. Particularly in the past, cheese was valued for its long shelf life. Due to its reduced water content, and acidic pH, bacterial growth is severely inhibited. This causes cheese to spoil much more slowly than other milk products. Consequently, the art of cheese production has spread throughout Europe, each country manufacturing many different types of cheeses.

Cheese production has three steps: curd formation, curd treatment and curd ripening.

1. A crucial part of the cheesemaking process is the conversion of the milk (liquid) into a solid material (the curd), which contains the casein and fat of the milk, but has expelled the main part of the water and, usually, the whey proteins and part of the lactose. The coagulation of milk may be induced by selective hydrolysis of the k-casein at the phenyalanine$_{105}$–methionine$_{106}$ peptide bond by the addition of acid proteinases, referred to generically as rennets (chymosin, pepsin); or by acidification (using food-grade acids), at a temperature of 20–40°C, to a pH value close to the isoelectric pH of casein, i.e. ~4.6. There is a close relationship between the amount of fat and the amount of protein in milk and the higher the fat, the higher the protein. The protein falls into two major groups: caseins (80%) and whey proteins (20%). Casein is always present in the form of calcium caseinate. It is present in the suspicion/colloidal form in the milk.
   Curd formation can use mare, ewe, cow or goat milk to produce “sour” or “sweet” curd. Sour curd is produced by fermentative lactic acid bacteria as mentioned above. Sweet curd is produced by adding an enzyme called renin instead of bacteria to curdle the milk. The curd is separated

from the whey by draining. The curd can be used directly to make unripened cheeses such as ricotta or cottage cheese or can undergo further processing to make a ripened cheese.

2. Curd treatment consists of condensing and squeezing to form dense, hard curd. Following curd coagulation, the resultant milk gel is subjected to a number of operations that promote the release of whey, an approximate tenfold concentration of the casein, fat and micellar calcium phosphate components, and a transformation to a curd with much higher dry matter content than the original milk gel. These operations include cutting the gel into pieces (referred to as curd particles, ~0.5–1.5 cm cubes), heating the particles in whey, reducing the pH by fermentation of lactose to lactic acid by the lactic acid bacteria (LAB) in the starter culture added to the milk prior to rennet addition, and physical draining of the whey by pressing the curd particle–whey mixture. It is then molded into the desired shape, salted and mixed with different types of secondary microflora.

3. Secondary microflora ripen the cheese and will determine the final texture and aroma of each type of cheese. For hard ripened cheeses such as Cheddar, curds are further compressed and the bacteria particular for the cheese is added. The Cheddar is wrapped in wax or plastic to prevent contamination and then incubated to allow the bacteria to do its work. For soft ripened cheeses such as Camembert and Limburger, a microbe, usually mold, is added to the surface of the cheese that produces a protein-digesting enzyme. This enzyme breaks apart the curds and causes the cheese to become creamy and spreadable. Many cities have long held traditions and nuances for producing a particular cheese i.e. the limestone caves in Roquefort, France which have constant heat and humidity that create unique and delightful cheeses.

A short summary of the types of dairy products and typical spoilage microorganisms associated with them is shown in Table 1 (from Sperber, 2009)

Table 1. Dairy products and typical types of spoilage microorganisms or microbial activity

Food	Spoilage microorganism or microbial activity
Raw milk	A wide variety of different microbes
Pasteurized milk	Psychrotrophs, sporeformers, microbial enzymatic degradation
Concentrated milk	Spore-forming bacteria, osmophilic fungi
Dried milk	Microbial enzymatic degradation
Butter	Psychrotrophs, enzymatic degradation
Cultured buttermilk, sour cream	Psychrotrophs, coliforms, yeasts, lactic acid bacteria
Cottage cheese	Psychrotrophs, coliforms, yeasts, molds, microbial enzymatic degradation
Yogurt, yogurt-based drinks	Yeasts
Other fermented dairy foods	Fungi, coliforms
Cream cheese, processed cheese	Fungi, spore-forming bacteria
Soft, fresh cheeses	Psychrotrophs, coliforms, fungi, lactic acid bacteria, microbial enzymatic degradation
Ripened cheeses	Fungi, lactic acid bacteria, spore-forming bacteria, microbial enzymatic degradation

1.2.2 Microbiological spoilage of dairy products

The bacterial load and composition of the bacterial flora in raw milk is directly influenced by the handling and sanitary condition of the equipment with which the milk comes in contact. The bacterial flora are composed of the natural microflora of raw milk which are not significant, but other undesirable types are likely to be present, represented by Pseudomonas and other psychrotrophic organisms that are able to multiply at refrigeration temperatures. Many psychrotrophs produce heat-resistant lipases and proteinases. In efficiently refrigerated pasteurised milk, the shelf life is largely determined by the growth of psychrotrophic bacteria. In milk that is virtually free from post-pasteurisation

contamination, subsequent spoilage is regarded as being due to psychrotrophic thermodurics deriving from the raw milk. Any increase in the psychrotrophic count by recontamination due to unsanitary equipment and practices between the exit of the pasteurizer and the sealing of the package, will substantially reduce keeping quality. Consequently, recontamination of pasteurised milk is the predominant cause of early spoilage. As temperature increases during distribution, mesophilic bacteria will play a more significant part in the spoilage process. This applies to any delivery system of limited cooling capacity. Shelf life times will not exceed a few days and raw milk bacteria surviving pasteurisation will be as important as recontamination. Thus, depending on the average storage temperatures, the responsible organisms for pasteurised milk spoilage may vary.

Psychotrophic bacteria

In raw milk, psychrotrophic microorganisms are a substantial portion of the bacteria including pseudomonads and related aerobic, Gram-negative, rod-shaped bacteria being the predominant groups. Psychrotrophic refers to microorganisms which are able to grow at temperatures less than 7°C. Cold milk storage and transport selects for psychrotrophic bacteria which are often proteolytic and lipolytic. Common psychrotrophic bacteria in milk are species of *Micrococci, Bacilli, Staphyloccoci, Lactobacilli, Pseudomonas,* and *coliforms*. *Pseudomonas* species are the most common and typically have the most impact on quality. At temperatures of 2 – 4°C, bacterial growth in milk is mainly due to strains of *Pseudomonas flourescens.* Little growth occurs at temperature less than 2°C. Most of the psychrotrophs isolated from raw milk are *Pseudomonas* species (De Jonghe et al., 2011) Pseudomonads are able to grow at low temperatures (3–10°C) and to hydrolyze and metabolize large molecules of proteins and lipids for growth. Also members of the genera *Bacillus, Micrococcus*, *Aerococcus, and Lactococcus* and of the family Enterobacteriaceae are other important psychrotrophs associated with raw milk include. Pseudomonads can decrease the diacetyl rate of buttermilk and sour cream (De Jonghe et al., 2011), and leading to a "green" or acid flavor from an imbalance of the diacetyl to acetaldehyde amount. Considering cottage cheese, the typical pH is marginally favorable for the growth of Gram-negative psychrotrophic bacteria, with the pH of cottage cheese curd ranging from 4.5 to 4.7 and the pH of creamed curd being within the more favorable pH range of 5.0–5.3. In the cottage is generally added salt content insufficient to limit the growth of spoilage bacteria; consequently, the shelf life of cottage cheese is principally limited by the presence of psychrotrophs bacteria; particularly, when their in cell numbers is greater than 10^6 CFU/ml in rraw milk, they affect the quality of cheese curd (De Jonghe et al., 2011)

Coliforms

Coliforms can also reduce the diacetyl content of buttermilk and sour cream (De Jonghe et al., 2011),producing an acid flavor. During cheese production, lactic acid production by starter cultures promote the growth and production of gas by coliform bacteria in a slow process, since coliforms have short development under such conditions. In such types of cheese as soft and mold-ripened cheeses, the pH rises during ripening ncreasing the growth of potential of coliform bacteria (Hayaloglu & Kirbag 2007).

Lactic Acid Bacteria

When the growth of encapsulated, slime-producing lactococci occur, the increment of viscosity can appear in buttermilk and sour cream. As for the coliforms, also LAB can reduce the diacetyl content of buttermilk and sour cream at 7◦C (De Jonghe et al., 2011), resulting in a acid flavor. LAB with heterofermentative metabolism such as lactobacilli and *Leuconostoc* can cause off-flavors and gas in ripened cheeses. These microorganisms use lactose as substrate to produce lactate, acetate, ethanol, and CO2 (Chaves-Lopez et al., 2006) and their optimal temperature of growth is at 15◦C during ripening. The role of heterofermentative bacteria that is completing the fermentation, producing gas and off-flavors, when the homofermentative LAB not metabolize all of the fermentable sugar in a cheese. Amino acids metabolism in cheese, is operated by lactobacilli, propionibacteria, and *Lactococcus lactis* subsp. *lactis* that can also produce a certain amount of gas in cheeses (Beuvier et al., 1997) . When extra quantity of gas is produced by certain strains of *Streptococcus thermophilus* cracks in cheeses can happen by forming CO2 and 4-aminobutyric acid by decarboxylation of glutamic acid (Ahmed et al., 2005) .

Fungi

Yeasts can grow in buttermilk and sour cream at the low pH and can produce off-flavors. Moreover, yeasts can metabolize diacetyl in these products (Prillinger et al., 1999), thereby leading to a yogurt-like flavor. *Geotrichum candidum* is a common yeast contaminant of cottage cheese often results in a decrease of diacetyl content; it can halves the diacetyl concentrations in lowfat cottage cheese after about 15 days of storage at 4–7◦C. In certain milk based products such as yogurt and fermented milks Yeasts are a major cause of spoilage thanks to the low pH consitions.

Yogurts produced under conditions of good manufacturing practices should contain no more than 10 yeast cells and should have a shelf life of 3–4 weeks at 5◦C. However, yogurts having initial counts of >100 CFU/g tend to spoil quickly. Yeasty and fermented off-flavors and gassy appearance are often detected when yeasts grow to 105–106 CFU/g. Previous studies described the role of galactose in the spoilage of yogurt by yeasts and stated that galactose, was fermented by galactose-positive strains of yeasts such as *Saccharomyces cerevisiae* (Prillinger et al., 1999) . Most of cheeses are advantageous environment for the growth of spoilage yeasts for the low pH and the nutritional composition; moreover surface moisture, also containing lactic acid, peptides, and amino acids, supports their rapid growth. Numerous yeasts are able to produce alcohol and CO_2, giving to cheese the typical "yeasty" taste. Vacuum packaging conditions or modified atmospheres can report sometimes bubble as a result of the increment of CO2 production operated by yeast. Many proteolytic yeast strains produce sulfides, while from lipolitic catabolism are obtained short-chain fatty acids that combine with ethanol forming fruity esters. The most common cheese contaminating yeasts are *Candida spp., Kluyveromyces marxianus, Geotrichum candidum, Debaryomyces hansenii and Pichia spp*. (Lopandic et al., 2006). With aerobic condition and low pH offered by the cheese surfaces, molds can grow, while in packaged cheeses, mold growth is limited by anaerobic conditions, even some molds can grow under low oxygen availability. *Penicillium spp. and Cladosporium spp.* commonly growth in vacuum-packaged cheeses (Hayaloglu & Kirbag 2007).

Spore-Forming Bacteria

Spore forming bacteria are able to exist in a highly stable form called "spores". In the spore state, these bacteria are able to withstand greater extremes of acidity, temperature and dessication. Raw milk is commonly source of spore-forming bacteria in dairy manufactures. Even their load is low before pasteurization, they can also contaminate milk after processing (Coorevits et al., 2008). Example of spore-forming bacteria largely found in dairy manufactures are *Bacillus licheniformis, B. cereus, B. subtilis, B. mycoides, and B. megaterium*. In previous study, *B. cereus* was isolated in more than 80% of raw milks sampled (Coorevits et al., 2008) and showed that temperature of pasteurization was optimal for the surviving spores so they were prepared to germinate at that favorable growth temperature. During aging of ripened cheeses, lsignificative proteolysis activity can occur, determining the release of amino acids and concomitant increase in pH that can support the growth of clostridia, especially *Clostridium*, and also the increment of gas production (Nölling et al., 2001). Usually in cheese curd we can find spores causing gassiness in some cheeses. In many case cheeses, such as in the case of Swiss, Emmental, Gouda, and Edam, have a high pH, low salt content and a certain moisture. Sometimes, also *C. butyricum or C. sporogenes* can cause gassy defects in cheeses.

Other Microorganisms

Clostridium tyrobutyricum is a thermoduric (survives pasteurization) spore-forming organism of legendary fame among cheese makers. *C. tyrobutyricum* causes gas formation (carbon dioxide) during the later stages of ripening of Swiss and Dutch type cheeses. The resulting craters and cracks in the cheese are called "late gas defect". European cheese makers frequently check raw milk for thermoduric and/or spore forming bacteria to estimate potential for late gas defects. Five hundred spores per litre of milk are sufficient to cause late gas defect.

Propioni bacterium produces the desirable gas formation in Swiss type cheese. Some lactic cultures, called heterofermentative, also produce carbon dioxide.
Ropy bacteria cause stringy milk due to excretion of gummy polysaccharides. Usually ropy bacteria such as *Alcaligenes viscolactis* are undesirable. However, in some fermented dairy products, ropy lactic acid bacteria such as certain subspecies of *Lactococcus lactis* are used to develop texture. Sweet curdling bacteria produce rennet-like enzymes which may coagulate milk. Common examples are the psychrotrophic spore formers *Bacillus subtilis* and *Bacillus cereus.*
Numerous off flavours have been associated with specific milk contaminates. Some examples are: Malty (*S. lactis var maltigenes)*; Bitter (Proteolytic bacteria); Rancid (Lipolytic bacteria); Unclean (coliform bacteria); Fishy (*Pseudomonas);* Fruity *(Pseudomonas).*
Sweet curdling bacteria produce rennet-like enzymes which may coagulate milk. Common examples are the psychrotrophic spore formers *Bacillus subtilis* and *Bacillus cereus.*

1.2.3 Factors Affecting Keeping the Quality of Heat-Treated Milk

Milk is an excellent substrate for the growth of many microorganisms, including lactic acid bacteria, pathogens and spoilage organisms, because of its complex biochemical composition, near-neutral pH and high water content (Gori et

al., 2013; Myszka & Czaczyk 2011). On average, cow milk is composed of approximately 87.4% water, 3.7% fat, 4.8% lactose, 3.4% protein and 0.7% mineral substances (Hayaloglu & Kirbag 2007). Differences in the principal constituents are found among milk from different animals (sheep, goat, etc.). In healthy animals the secretory tissue of the udder is free of microorganisms. However, the mucosal membrane of the streak canal has a microflora that includes streptococci, staphylococci, micrococci (normally >50%), *Corynebacterium* spp., coliforms, lactic acid bacteria, and other bacteria. Moreover, milk is further contaminated by microorganisms from the farm or milking barn environment and from people and equipment (Ahmed et al., 2005). In industrialized countries since the 1980s, practices such as cold storage of milk and udder-cleaning and teat-disinfecting procedures have improved the hygienic quality of raw milk and concomitantly decreased its microbial load (Beuvier E, Buchin S, Fox P, McSweeney P, Cogan T 2004). The loads of most microbial groups have remained stable in raw cow's milk since the mid 1990s (Quigley et al., 2013)and standard plate counts currently range from 10^3 to 10^4 colony forming units per mL (CFU/mL). Usually, bacterial counts are far higher than fungal counts. For all microbial groups, inter-farm variability is wide while intra-farm variability is generally much lower except from season to season (Lafarge et al., 2004). Despite this low count, raw milk still exhibits substantial microbial diversity. They are mainly Gram-negative bacteria, Gram positive and catalase positive bacteria, lactic acid bacteria (LAB), yeasts and moulds. For historical and technological reasons, most studies focused on LAB often regarded as the main bacteria in raw milk. Recent advances in analysis techniques have made it possible to detect many more species besides LAB. Raw milk microbiota proves to be very rich. Strain diversity in raw milk is also substantial but varies between species and between farms. Raw milk is often conserved at refrigeration temperature before cheesemaking, especially when it is not processed directly at the farm. Psychrotrophic bacteria are naturally present in milk, where they can reach counts up to 10^5 CFU/mL (Pennacchia et al., 2009). Most of these are Gram-negative bacteria. *Pseudomonas* spp. are the most commonly occurring psychrotrophs in raw milk, along with *Acinetobacter* spp. and *Enterobacteriaceae* (Pennacchia et al., 2009; Hantsis-Zacharov & Halpern 2007; Martins et al., 2006). They are recognized as a cause of milk spoilage, which may be due to their proteolytic and lipolytic activities (Hantsis-Zacharov & Halpern, 2007). Storage of milk at refrigeration temperature alters milk microbial balance, as shown by changes in the DGGE and TGGE banding patterns of bacterial communities after milk incubation at 4°C for 24h (Lafarge et al., 2004). Counts of culturable psychrotrophic bacteria in milk increased of more than 3 logCFU/mL within 3 days of storage at 8°C and after 7 days at 4°C. Different storage temperatures and durations led to different species balances in farm and dairy tanks. Upon refrigeration at 4 °C for at least 70 h, dominance in dairy tank milk populations shifted from Gram positive (*Macrococcus*) to Gram negative bacteria (*Pseudomonas*, *Acinetobacter*, *Chryseobacterium*) (Fricker et al., 2011; Raats et al., 2011; Rasolofo et al., 2010). To a processor the desirable shelf life is often very much a function of the length of the distribution and marketing networks and frequency of deliveries to shops and consumers. For the consumer, the shelf life of pasteurised milk is determined by its organoleptic qualities, which are visual appearance, smell and flavour. A number of factors influence the organoleptic quality.The character of the bacterial flora in the raw milk, at the time of processing, has a significant influence on organoleptic quality. The composition of the bacterial flora is dependent on the hygienic quality of the milk at the point of production and the care and attention it has received during handling until processing. The bacterial load present in the raw milk is a function of the hygienic condition of the personnel and equipment used for handling the milk, and the elapsed time between milking and cooling the milk to below 5ºC. The elapsed time between milking and pasteurization profoundly influences the bacterial load in the raw milk. Consequently, the hygienic quality will be influenced by the bacteriological condition of the surfaces of plant and equipment with which the milk comes in contact. The sanitary condition of items of equipment, including milking clusters, teats cups, pipes and tubing, milk lines, farm storage tanks, pumps, transport tankers, milk churns together with the time/temperature duration of the contact with these items of equipment are the determining factors in determining the bacterial load in the raw milk. To this must be added the state of health of the udder, where excessive numbers of microorganisms in the milk cistern, duct and teat canal udder will lead to the presence of high numbers of microorganisms in the milk as it is milked. Milk when produced in the secretor cells within the alveoli of the udder tissue, is virtually free of microorganisms. Microorganisms accumulate in the milk as it passes through the milk ducts within the udder. Further influences upon the quality of the milk are due to the type, nature and concentration of the naturally occurring and/or bacteriological developed lipases and proteases that are present in the raw milk, and the effects of the heat-stable enzymes that survive the heat treatment and continue their activity in the post pasteurised milk. The rate and intensity of the enzymatic activity is related to the composition of the bacterial flora and the resulting enzymatic content in the milk. These are influenced by the time and temperature conditions that were imposed upon the milk before and after pasteurisation.

The efficiency of handling and treatment of the milk, which includes the preparation of the plant for use and the suitability of the process plant equipment together with the overall level of technological skill of the operators, are additional factors that influence the product quality.

The essential aim of the processor must be to reduce the level of bacteriological contamination in the post-pasteurised milk to the minimum possible. The final packaged product must be handled with care to reduce multiplication of the bacterial flora to the minimum possible. Milk is a biological medium. It is not stable but is constantly undergoing change due to the accumulation of metabolic products resulting from microbial growth and enzymatic activity deriving from bacterial sources. This activity causes changes in the physical-chemical character of the milk.

Effects of pretreatment handling and storage

Milk on arrival at the dairy plant is either processed immediately or passed through a plate cooler and stored at low temperature until processed. The milk is generally clarified before pasteurisation. The keeping quality of the finished pasteurised milk will be influenced by its bacteriological load and chemical composition at the time of milking, the mechanical and thermal treatment it subsequently receives and the microbiological condition of the equipment. Severe mechanical agitation, as produced by pumping and turbulent flow conditions, especially when combined with fluctuating thermal conditions, can have a pronounced detrimental effect on certain milk components. Such treatment can cause churning and damage to the milk fat globule membrane and so encourage lipolytic activity by the naturally occurring lipase present in the milk. The resulting hydrolysis of some of the fat molecules will lead to a flavour defect, detected as a bitter taste that subsequently may become more pronounced due to further activity by bacterial lipases. In practice, activation of the enzyme usually arises through excessive turbulence caused by incorrectly designed and poorly installed pipelines systems at the both the farm and the processing dairy levels. It can also take place when the milk is allowed to splash in the farm tank, due to insufficient depth of the milk above the agitator, and when a second milking is dumped onto the surface of a previous milking at about 10-12°C. Freezing and thawing, together with excessive and prolonged agitation in storage tanks are conducive to lipolysis.

Flavor and odor defects in milk and dairy products

Good quality milk should have a pleasantly sweet and clean flavor with no distinct aftertaste. Because of the perishability of milk and the nature of milk production and handling procedures, the development of off-flavors/odors is not uncommon. To prevent flavor/odor defects in milk, proper milk handling procedures from the farm to the consumer are essential. Is possible to describe the common flavor and odor defects found in milk and their potential causes.. These defects may be classified in three groups of off-flavors:

-Absorbed/Transmitted

-Bacterial/Microbial

-Chemical/Enzymatic/Processing

Absorbed. Feedy, barny, cowy, weedy, unclean, lacks freshness, stale, refrigerator/cooler odors. Raw or pasteurized milk products can absorb flavors during production, storage and distribution. On the farm, off-flavors can be absorbed, or more correctly transmitted, through the bloodstream of the cow from the lungs and/or rumen into the milk in the udder (e.g., onion/garlic, feedy, barny, and cowy). Similar off-flavors may be absorbed into the milk during farm storage if ventilation is poor and the milk is not protected. Pasteurized milk can absorb flavors during refrigeration storage, especially in paperboard or low barrier cartons. Examples of off-flavors that might be absorbed include volatile compounds of fruits or vegetables or unclean odors associated with poorly cleaned milk coolers. Absorption of flavors by packaged milk can occur at the plant, in the supermarket or in the consumers' home refrigerators.

Bacterial. Acid, bitter, malty, lacks freshness, unclean, fruity/fermented, putrid and rancid. Bacterial and other microbial (i.e., yeast or molds) off-flavors result from the growth of microorganisms that are present in milk due to poor sanitation and/or milk handling practices. Bacteria that are able to grow at refrigeration temperatures (≤45°F/7.2°C), or psychrotrophic bacteria, are most often responsible for spoiling refrigerated milks. The type of spoilage (e.g., fruity, rancid, acid) depends on the predominant type(s) of bacteria present and generally occurs when bacterial numbers (i.e., Standard Plate Count) exceed one to ten million per milliliter. The time it takes for bacteria counts to reach spoilage levels depends on the initial numbers of bacteria and the temperature of storage; the warmer the storage temperature, the quicker bacteria grow and produce off-flavors and the shorter the shelf-life. If the raw milk quality is good and post-pasteurization contamination is prevented during processing, the numbers of microorganisms should not reach spoilage levels before 14-21 days when milk is held under proper refrigeration. Bacterial and other microbial defects can occur in raw or pasteurized milk and in other dairy products.

Chemical. Cowy (ketosis), salty, rancid, bitter, oxidized, sunlight, foreign, astringent, medicinal, flat, cooked. Chemical and enzymatic defects can occur in both raw and pasteurized milk. The cows may be suffering from ketosis (rare) or mastitis, which can affect milk flavor. Abusive handling of raw milk may result in a rancid flavor from the action of the naturally occurring lipase enzyme, which breaks down butterfat to free fatty acids (i.e., butyric acid is perceived as "rancid"). Oxidized flavors can be induced by heavy metals, particularly copper, or by exposure to sunlight and fluorescent lights. Chemical or foreign off-flavors can also occur due to contamination with cleaning chemicals, sanitizers, medicines, or other substances during production or processing. Processing parameters, if not managed properly, can result in off-flavors including cooked (from high heat) or flat (from added water).

1.2.4 Sources of Spoilage Microorganisms

Contamination of Raw Milk

Dairy products are good media for the growth of microorganisms for their highly nutritious composition. Milk contains abundant water and nutrients and has a nearly neutral pH.
Water is major constituent of milk as it constitute more than 80% of whole milk. The amount of water in milk is regulated by the amount of lactose synthesized by the secretary cells of the mammary gland. The water that goes into the milk is delivered to the mammary gland by the blood. Milk production is very rapidly affected by a shortage of water and drops the same day drinking water is limited or unavailable. This is one reason why the cow should have free access to a plentiful supply of drinking water at all times
The principal carbohydrate in milk is lactose. The concentration of lactose in the milk is relatively constant and averages about 5% (4.8-5.2%). As opposed to the concentration of fat in milk, lactose concentration is similar in all dairy breeds and cannot be altered easily by feeding practices. Milk protein is very exclusive in nature and it is the natural source of amino acids. More than 95 amino acids of total declared amino acids are available in the milk protein. Milk protein is regarded as high resource and matchless resource of amino acids. Particularly it contains many essentials amino acids that are not synthesized in our body and required for normal metabolism. The concentration of protein in milk varies from 3.0 to 4.0%. The percentage varies with the breed of the cow and in proportion to the amount of fat in the milk. There is a close relationship between the amount of fat and the amount of protein in milk the higher the fat, the higher the protein. The protein falls into two major groups: caseins (80%) and whey proteins (20%). Casein is always present in the form of calcium caseinate. It is present in the suspicion / colloidal form in the milk. Normally, fat (or lipid) makes up from 3.5 to 6.0% of milk, varying between breeds of cattle and with feeding practices. A ration too rich in concentrates that do not elicit rumination in the cow may result in milk with a depressed percentage of fat (2.0 to 2.5%). Fat is present in milk in small globules suspended in water. Each globule is surrounded by a layer of phospholipids, which prevents the globules from clumping together by repelling other fat globules and attracting water. As long as this structure is intact, the milk fat remains as an emulsion. The majority of milk fat is in the form of triglycerides formed by the linking of glycerol and fatty acids. The proportions of fatty acids of different lengths determine the melting point of fat and thus the consistency of the butter derived from it. Milk fat contains predominantly short-chain fatty acids (chains of less than eight carbon atoms) built from acetic acid units derived from fermentation in the rumen. This is a unique feature of milk fat compared with other kinds of animal and plant fats. The long chain fatty acids in milk are primarily the unsaturated acids, with the predominant one being oleic (18-carbon chain), and polyunsaturated linoleic and linolenic acids. Milk is an excellent source of most minerals and vitamins required for the growth of the young. Differnet minerals found in milk are potassium, calcium, chloride, phosphorus, sodium, sulfur, magensium and small amounts of trace minerals. The digestibility of calcium and phosphorus are unusually high, in part because they are found in association with the casein of the milk. Vitamin A, D, E, K, C and B complex are also found in milk.
In addition to being a nutritious food for humans, milk provides a favourable environment for the growth of microorganisms. Yeasts, moulds and a broad spectrum of bacteria can grow in milk, particularly at temperatures above 16°C. Microbes can contaminate milk via the cow, air, feedstuffs, milk handling equipment and the milker. Once microorganisms get into the milk their numbers increase rapidly. It is more effective to exclude microorganisms than to try to control microbial growth once they have entered the milk. Milking equipment should be washed thoroughly before and after use, rinsing is not enough.

Pasteurized milk can be contaminated by microorganisms originated primarily from water, air, tools, equipment or inhabit the surfaces and can be well established in the environment for long time (Bokulich et al., 2013; De Filippis, La Storia, et al., 2013; Stellato, De Filippis, et al., 2015). The refrigerated storage of raw milk throughout the dairy chain prior to heat treatment creates selective conditions for growth of psychrotolerant bacteria. These bacteria, mainly belonging to the genus *Pseudomonas*, can cause spoilage and texture failings in pasteurized and ultra-high-temperature-treated milk, by production of extracellular proteases and lipases. Previous study tried to map the effect of refrigerated

storage conditions on the growth of these microorganisms, by analyzing different milk samples at simulated storage conditions. (De Jonghe et al., 2011). The result was observed by cultivation-independent denaturing gradient gel electrophoresis (DGGE). Isolates were identified by a polyphasic approach revealing that development of Pseudomonas species occurred from the beginning of the dairy chain (farm tank) in storage conditions. Moreover, the most abundant species identified were *Pseudomonas gessardii, Pseudomonas gessardii-like, Pseudomonas fluorescens-like, Pseudomonas lundensis, Pseudomonas fragi, and Pseudomonas fragi-like.* Those taxa show an important spoilage potential as determined on elective media for proteolysis and lipolysis, in fact the psychrotrophic nature of *Pseudomonas* can help to withstand the competing microbial populations in milk and in fresh cheeses (De Jonghe et al., 2011; Franciosi et al., 2011; Martin et al., 2011; Morales et al., 2005) possibly determining changes in food structure or discoloration such as the case of "blue Mozzarella cheese" (Stellato, De Filippis, et al., 2015; Martin et al., 2011) . *Acinetobacter, Pseudomonas* and *Psychrobacter* can be involved in food spoilage, and they are recognized as undesired bacteria in food processing environments (Doulgeraki et al., 2012).

Contamination of Dairy Products

Washed curd types of cheeses are especially susceptible to growth of coliforms (De Jonghe et al., 2011), so great care must be taken to monitor the quality of water used in these processes. A high incidence of contamination of brine-salted cheeses by yeasts results from their presence in the brines (Prillinger et al., 1999) . Many mold species are particularly well adapted to the cheese-making environment and can be difficult to eradicate from a production facility. Fungi causing a "thread mold" defect in Cheddar cheeses (Hocking & Faedo, 1992) were found in the cheese factory environment, on cheese-making equipment, in air, and in curd and whey. In a study of cheese-making facilities in Denmark, *Penicillium commune* persisted in the cheese coating and unpacking areas over a 7-year period (Lund et al., 2003). A major cause of failure of processing and packaging systems is the development of biofilms on equipment surfaces. These communities of microorganisms develop when nutrients and water remain on surfaces between times of cleaning and reuse. Bacteria in biofilms (sessile form) are more resistant to chemical sanitizers than are the same bacteria in suspension (planktonic form); consequently chemical sanitizers may be rendered ineffective by biofilms leaving viable bacteria to be dislodged into milk product.
(Carpentier & Cerf 1993

Spoilage of liquid milk products

The shelf life of pasteurized milk can be compromised by the presence of a large numbers of somatic cells in raw milk that are correlated with the amount of a heat-stable protease (plasmin) and of lipoprotein lipase in fresh milk (Samet-Bali et al., 2013). Consequently, it's intuitive that the activities of these enzymes can increase the enzymatic bacterial activity and hence accelerate the time to spoilage. The initial cell numbers of psychrotrophic bacteria, in raw milk is a good indicator for the presence of these enzymes. The presence of a large numbers of lipase producers, (Samaržija et al., 2012) the stability of the enzyme to the thermal process, the long-term storage and the favorable conditions of temperature, pH, and water activity are all favourable conditions for lipolyzed flavor to develop from residual lipases in processed dairy foods.

Spoilage of Cheese products

Spoilage of cheeses is depending on several factors such as water activity, pH, salt, temperature, amount of the lactic starter culture, types and viability of contaminating microorganisms, presence and abundance of residual enzymes. The combination of these variables will start deteriorative reactions, that will affect the cheeses through vary widely spoilage characteristics.
The types of cheese that generally have the highest pH values, and with the lowest salt amount, spoil most quickly and is this the case of soft or unripened cheeses. On the contrary, aged, ripened cheeses mantein their sensorial qualities for long periods thanks to their comparatively low pH, low water activity, and low redox potential. Moreover, in pasta filata cheeses, the low salt amount and high brining temperature (18°C) permitted the growth of coliforms, which caused gas formation in the cheese (Manolopoulou et al., 2003).

Spoilage from dairy environment and equipment

During cheese production, the product encounters many equipment surfaces on its journey from milk to cheese, all acting as potential vectors for microbes (Bokulich & Mills 2013a). Hence, the processing environment may serve as an important reservoir for bidirectional microbial transfer between fermentations, and microbial surveillance of this environment is critical for understanding the complete microbial ecosystem of cheese production. In modern cheese production facilities, biofilms of psychrotrophic bacteria and non-starter lactic acid bacteria can form on equipment surfaces, acting as a source of contamination in successive batches of cheese (Brooks & Flint 2008; Kumar & Anand 1998; Myszka & Czaczyk 2011). Wooden processing surfaces, including aging boards and milk vats (Licitra et al.,

2007; Lortal et al., 2009; Didienne et al., 2012) are also rich sources of microbes that are important for cheese acidification and ripening. In traditional cheesemaking facilities, adventitious microbes inhabiting such equipment surfaces can represent a "house" microbiota important for the development of specific cheese characteristics (Mounier et al., 2005) . The wooden surfaces of the vats used to produce PDO Salers and PDO Ragusano cheeses are a reservoir of microorganisms, active acidifying LAB in particular. The group/species composition of a biofilm was found to be stable over several seasons once it had become established on a vat surface, but varied widely between vats (Didienne et al., 2012; Lortal et al., 2009). Wooden vats can increase microbial loads in the milk compared to those in milk before pouring into the vat. Both strain and species richness of the LAB dominating a raw milk increased of 50% after a few minutes in the wooden vat (Settanni et al., 2012).Wooden ripening shelves are a reservoir of surface microbiota that can be transferred directly to cheese surface. Yeasts, moulds and coryneform bacteria can colonize the surface of cheese, and also dominate the biofilms of shelves used for ripening (Valdés-Stauber et al., 1997). These biofilms, which do not change with season or shelf age, are a possible source of surface microflora for smear cheeses, often not deliberately inoculated with surface microorganisms. Therefore, even in facilities incorporating defined, commercial inocula, the production environment remains a pertinent source of microbes throughout the course of the manufacture, likely subtly shaping product quality (Bokulich & Mills 2013a).

1.3 Microbial ecology of meat processing

1.3.1 Spoilage of raw meat

Meat spoilage bacteria contaminations can occur in the processing environment. Food spoilage generally indicate the deterioration of food products due to the growth of microbial contaminants. Microbial spoilage manifest principally through sensory changes, such as offodours and off-flavours, slime production, texture change, discoloration and gas production. Food spoilage events influence the shelf life of food products, since the food products will be stored until the acceptable level of off-odour/off-flavours not decrease (Borch et al., 1996). Shelf life is defined as the period of time during the product properties persist acceptable to the product user (Lambert et al., 1991). Meat is susceptible to microbial spoilage because of its high concentration of nutrients and high water activity (Stoops et al., 2015; Ercolini et al., 2011). The deteriorative effects caused by bacterial growth are discolouration, off-odours, and slime production (Iulietto et al., 2015; Doulgeraki et al., 2012; Casaburi et al., 2015). The degree of deteriorative changes depends primarily on the meat composition, the hygienic practices during the processing and the storage conditions (Olusegun & Iniobong 2011; Andritsos et al., 2012). The development of microbial flora in food systems during storage is affected by many intrinsic and extrinsic factors. The intrinsic factors depend by the characteristics of the food itself (nutrient composition, water activity, pH, natural antimicrobial substances, etc.), mode of processing, initial load of the contaminants, storage temperature and packaging methods. Packaging methodologies play a fundamental role in the raw chilled meat shelf life (Labadie 1999; Ammor et al., 2009; Ercolini et al., 2011). Water content, color, microbial quality, lipid stability, and sensorial accettability are the main intrinsic properties of meat that will determine the shelf life. During meat storage, deteriorative events can be determined by metabolic reactions in the biological membrane and biochemical oxidative processes (Addis 2015; Dave & Ghaly 2011; Huis In't Veld 1996). Meat is a good substrate for bacterial growth especially for its composition: 75% water and large variety of metabolites such as amino acids, peptides, nucleotides, and charbohydrates. Slaughtering operations influence the microbial contamination of carcasses (Warriner et al., 2002; Gustavsson & Borch 1993). Spoilage is characterised by any change in a food product that renders it unacceptable to the consumer in terms of sensorial characteristics. Microbial numbers are not always related to degree of spoilage, but microbial activity is considered to be of great importance for the manifestation of spoilage. Discoloration, offodors, and slime production are among the deteriorative factors caused by bacterial growth (Casaburi et al., 2015). The most abundant microorganisms on the raw meat surface are *Brochothrix thermosphacta*, *Lactobacillus spp., Leuconostoc spp., Carnobacterium spp., Pseudomonas spp.* and *Enterobacteriaceae* (Casaburi et al., 2014; Casaburi et al., 2011; Pothakos, Snauwaert, et al., 2014; De Jonghe et al., 2011; Gustavsson & Borch 1993). The heterogeneous initial colonizating microflora can show a certain variety that will be selected by refrigerated meat storage conditions (Chaillou et al., 2015) and in the most of cases the dominant spoilage flora in proteinaceous raw foods are *Pseudomonas spp.,* particularly *P. fluorescens, P. putida and P. fragi* (De Jonghe et al., 2011). Pseudomonas fragi is well-known as the most causative agent of meat spoilage (Lebert et al., 1998) and very frequently isolated from fresh and spoiled meat products (Ercolini et al., 2009; Ercolini et al., 2006).

1.3.2 Composition of meat: water, carbohydrates, minerals and vitamins

The most abundant chemical in meat is water followed by protein and fats. Carbohydrates, minerals and vitamins occur on much smaller amounts but nevertheless are very important metabolically and nutritionally. Adipose tissue contains little moisture; therefore, the fatter the animal, the lower the total water content of its carcass or cuts. Beef muscle from mature and relatively fat animals may contain as little as 45% moisture, while veal muscle from very youthful and relatively lean animals may contain as much as 72% moisture. Texture, color and flavor of muscle are affected by the water amount in muscle tissue. A large percentage of the water in muscle tissue exists as free molecules within the muscle fibers; a smaller percentage is located in the connective tissue. it is possible for some of the water to remain (during storage, curing and heat treatment) within muscle fibers because of the three-dimensional structure of the fibers; water retained under forces of pressure and temperature increase is termed “bound water;" that which is lost is called “free water’ The water-holding capacity of the muscle can be decreased by disruptions of muscle structure. Grinding, chopping, freezing, thawing, salting, degradation of connective tissue by enzymatic or chemical means, the application of other chemicals or organic additives that change acidity (pH), and heating are treatments that can affect the final water content of meat products.

The primary carbohydrate reservoir for the animal body is the liver. That organ contains about one-half of the carbohydrates found in the body. Carbohydrates are stored as glycogen in the liver and in muscles. The remaining SO percent of carbohydrates are distributed throughout the body, largely in the muscles, but with substantial quantities in the blood (usually as glucose) and in other tissues, organs and glands. The changes that occur in energy metabolism, e.g., the conversions of glycogen to glucose and glucose to lactic acid, are complex; all such changes are controlled and mediated by enzymes and hormones. The lactic acid content of the muscles in a carcass increases during initial stages of aging or ripening, lowering the pH (muscle acidity). The pH of muscle considered “normal” is 5.6 (pH is the negative log of the hydrogen ion concentration; the higher the pH, the less acidic is the muscle). Muscle color, texture, water-holding capacity and tenderness are influenced by pH. If an animal experiences vigorous stress or exercise of the muscles immediately prior to slaughter and has no opportunity to restore its normal glycogen levels, the glycogen content within the muscles at slaughter will be reduced substantially. Because so little glycogen is available to be converted (after death) to lactic acid, a higher ultimate pH (e.g., a final pH of 6.2) will occur in this animal's muscles after slaughter, and the muscles will be dark, firm and dry (DFD). This is a reasonably rare occurrence in beef (perhaps 2 percent of carcasses are affected) and those carcasses are termed “dark cutters’ The DFD condition also occurs in pork and lamb carcasses. It is thought that the dark color of muscles with a high pH is due to its higher water-holding capacity, which causes muscle fibers to be swollen. The swollen state of the fibers causes more incidents light to be absorbed, rather than reflected, by the meat surface, and thus the color appears to be darker. “Dark cutters" are severely discounted in price by packers and retailers, due to poor consumer appeal of this meat; therefore, stress and rough handling of animals is minimized prior to slaughter. A too rapid postmortem (after death) drop in muscle pH (to a final pH of 5.1, for example) is associated with the pale, soft, and exudative (PSE) conditions that are somewhat common in pork muscle. PSE muscle is characterized by soft and mushy texture, low water-holding capacity and pale muscle color. The looser muscle structure of PSE muscle associated with its lower water-holding capacity is responsible for a greater reflectance of incident light and hence it has a pale color.

In addition to protein and fat, meat (beef, veal, pork and lamb) is a significant source of several other nutrients in the U.S. diet. These include the minerals iron and zinc, and most of the B-vitamin complex (B1, B2, niacin, B6 and B12).

1.3.3 Microbial spoilage of meat

Meat is derived from animal tissues. The internal tissues of healthy, not exhaustive slaughter animals can be considered free of bacteria (Jay et al., 2005). However, a study regarding microbiological status of fresh beef cuts showed that the cuts yielded levels of 4.0 to 6.2, 1.1 to 1.8, and 0.8 to 1.0 log CFU/g for total aerobic plate count, total coliform count and E. coli count, respectively (Stopforth et al., 2006). The primary sources of microorganisms in fresh meats are as follow: knife that is used to slit the jugular vein, animal hide, gastrointestinal tract, hands of handlers, containers, handling and storage environment and lymph nodes (Bell 1997; Jay et al., 2005). Therefore, the biota of meats often reflects the slaughtering and processing environments. Gram-negative bacteria are predominantly found in meat and, among Gram-positives, enterococci and lactobacilli are most often found. Some mold genera, such as Penicilium, Mucor and Cladosporium, could also be found due to its abundant presence in meat-processing environment, while the mainly found yeast are members of the genera Candida and Rhodotorula (Jay et al., 2005). Foods, both plant and animal origin, have several intrinsic parameters that can affect microbial growth. These intrinsic parameters include pH, moisture content, oxidation-reduction potential, nutrient content, antimicrobial constituents, and biological structures. Meats have rich nutrient content, which supports microbial growth, thus increase the probability of undergoing microbial spoilage (Jay et al., 2005). Meat spoilage is an ecological phenomenon that includes the changes of the

available substrates during bacteria proliferation. Generally, determining SSO requires combination of microbiology, sensory analyses and chemistry (Gram et al., 2002).

Bacterial groups on fresh meat

The most frequent bacteria to occur on fresh meat are bacteria of the genera *Acinetobacter, Pseudomonas, Brochothrix, Flavobacterium, Psychrobacter, Moraxella, Staphylococcus and Micrococcus,* lactic acid bacteria and various genera of the *Enterobacteriaceae* family. The survival and growth of these microbes is influenced, to a great extent, by the composition of the atmosphere surrounding the meat. According to

Pennacchia et al., (2011), bacteria that are present on aerobically spoiled chilled meat most frequently are:

1) *Pseudomonas spp.*

2) *Enterobacteriaceae*

3) *Brochothrix thermosphacta*

4) *Lactic acid bacteria*

Pseudomonas spp. are known to be responsible for the spoilage of meat stored aerobically at low temperatures (Arnaut-rollier et al., 1999).Though *Pseudomonas spp.* are usually found in a small proportion of the initial microbiota of fresh foods, they are extensively found in the environment. They can utilize a broad range of materials as substrates to grow (Huis In't Veld 1996). Three main species of *Pseudomonas*, which are *Ps. fragi*, *Ps. fluorescens* and *Ps. lundensis*, are important during meat spoilage. Among those three species, *Ps. fragi* grows faster at low temperature than the others (Labadie 1999). Off odors become evident when *Pseudomonas* population has reached the level of 107-8 CFU/g. This is due to *Pseudomonas* ability to metabolize nitrogenous compounds into offensive sulfurous volatiles when glucose and lactate have been exhausted in meat (Ellis & Goodacre 2001; Nychas et al., 2008). At later stage, extracellular slime becomes visible (Huis In't Veld 1996). Cold-tolerant *Enterobacteriaceae*, such as *Serratia liquefaciens*, *Enterobacter agglomerans* and *Hafnia alvei*, can also grow in aerobically-stored meats but hardly account for more than a small proportion of the total microbiota (Dainty & Mackey 1992). Moreover, they are more prevalent in higher temperature (5-10oC) (Huis In't Veld 1996; Labadie 1999) and on pork and lamb, especially on fat surfaces (Dainty & Mackey 1992). Generally, *Enterobacteriaceae* take over the spoilage process from *Pseudomonas spp.* at temperature of 5-10^{o}C.

The natures of spoilage by *Enterobacteriaceae* are the production of gas, acid, slime, rope, bitter flavours and off-odors (Huis In't Veld 1996). Due to its presence in spoiled meat and meat products, *Enterobacteriaceae* are often used as food safety indicators (Huis In't Veld 1996; Nychas et al., 2008). Lowering storage temperature can inhibit the growth of *Enterobacteriaceae* and *Brochothrix thermosphacta.*

Many species of *Enterobacteriaceae* are found naturally in the environment of meat processing plant and their presence in fresh meats is well known (Jay et al., 2002).

However, EC has a direct association with Salmonella contamination in pork at retail level (Prendergast et al., 2009). Salmonella is a common pathogen found in food that can be hazardous if the food is not cooked thoroughly. Salmonella testing is advised when EC is greater than 3.0 log10 CFU/g (Prendergast et al., 2009). In the present study, although the mean EC values of meats were lower than previous studies (Kilonzo-Nthenge et al., 2013; Prendergast et al., 2009), there was a butchery that had EC value of pork greater than 3.0 log10 CFU/g. Hence, the pork samples in that butchery should be tested for Salmonella until the EC are reduced to acceptable level.

Brochothrix thermosphacta is a psychotrophic bacteria and can be found dominating in meats in modified atmosphere and vacuum packaging at refrigerated condition (Borch et al.,, 1996; in't Veld, 1996). A study showed that *Br. thermosphacta* increased two log cycles regardless of the initial contamination in meat that spoiled after 7 days of storage at 5°C (Russo et al., 2006). *Br. thermosphacta* degrades glucose and glutamate in beef (Gill & Newton, 1977). The characteristics of spoilage by *Br. thermosphacta* are sour and acid flavors and a slightly sweet, cheesy obnoxious odor. This is particularly found in meat products that have initially been stored anaerobically and subsequent to opening the package in an aerobic atmosphere (Borch et al., 1996). LAB are also recognized as spoilage bacteria in cured meat due to its ability to survive over nitrite compounds (Borch et al., 1996). Vacuum-packing atmosphere also cause a microbiota shift in meat to lactic acid bacteria (LAB) due to the inhibition of pseudomonads (Dainty & Mackey 1992). LABs produce slime and off-flavors due to sugar fermentation. However, they have a tendency to grow slower at refrigeration temperature and be out-competed by pseudomonads under aerobic conditions (Huis In't Veld 1996).

1.3.4 Mechanisms of spoilage for meat and meat products

There are three main mechanisms for meat and meat products spoilage after slaughtering and during processing and storage:

(a) microbial spoilage
(b) lipid oxidation
(c) autolytic enzymatic spoilage

Microbial spoilage

Meat, with chemical and physical properties, is a preferential substrate for the colonization and development of a variety of microorganisms, especially bacteria (De Filippis, La Storia, et al., 2013; Doulgeraki et al., 2012). Several factors can influence the occurrence of microbes in meat. After slaughtering, meat can be contaminated by microorganisms from the water, air and soil as well as from the workers and equipment involved in the manufacturing. In the further actions of the fresh meat chain (handling, cutting, storage), abiotic factors such as temperature, gaseous atmosphere, pH and NaCl will select for certain bacteria, allowing the colonization of the meat surface by different spoilage-related species and strains (Brooks & Flint 2008; Ercolini et al., 2006). Microbial growth to high numbers is a prerequisite for meat spoilage that can be considered an ecological phenomenon encompassing the changes of the available substrata during the proliferation of bacteria (Olusegun & Iniobong 2011; Nychas et al., 2008). Spoilage is the process of food deterioration leading to a reduction of its quality, till the point of not being edible for humans. Signs of spoilage may include a different appearance of the food compared to its fresh form and the alteration of the sensorial quality of the product, in particular the aspect (including texture and color) and the presence of off odour (Remenant et al., 2015; Nychas et al., 2008; Gram et al., 2002; Borch et al., 1996). The presence of microrganisms on the surface of the cut meat and meat products will determine meat spoilage upon their interaction and growth in optimal conditions (Gram et al.,, 2002; Doulgeraki et al.,, 2012). Although there are many different types of meat, the main bacterial populations involved in spoilage are common. The most abundant bacteria causing spoilage of refrigerated beef and pork are *Brochothrix thermosphacta*, *Carnobacterium* spp., clostridia, *Enterobacteriacea*e, *Lactobacillu*s spp., *Leuconostoc* spp., *Pseudomonas* spp., and *Weissella* spp, and their alterative activity consist in defects such as sour off flavours, discolouration, gas or slime production and decrease in pH (Nychas et al., 2008; Doulgeraki et al., 2012; Casaburi et al., 2015; Pothakos, Taminiau, et al., 2014). Moreover, the environmental microbiota from the processing plant has been often addressed as source of microbes potentially affecting the quality attributes of meat (Hultman et al., 2015; De Filippis, La Storia, et al., 2013; Lambert et al., 1991).

Lipid Oxidation

Lipid oxidation reduces meat quality by a number of ways, including off-flavour formation, drip loss, colour changes etc. During lipid oxidation poly-unsaturated fatty acids are degraded to volatile short-chain oxidation products, which lead to off-odour and off-flavour formation (Addis 2015). The oxidation process is strongly enhanced during cooking and storage of the meat. The formation of volatile lipid oxidation products strongly reduces the consumer's acceptability of the product. Oxidative processes can also affect the ability of the membranes to hold water and may contribute to drip loss (Addis 2015).

After slaughtering, the fatty acids in the animal tissues undergo oxidation (Pennisi Forell et al., 2010). Lipid oxidation in meat is promoted by several factors including: fatty acid composition, the level of the antioxidant vitamin E and prooxidants such as the free iron presence in muscles. Poly saturated fatty acids are more inclined to lipid oxidation (Tang et al., 2001). Their breakage produce secondary oxygenated compounds products of lipid oxidation such aldehydes and ketones can cause loss of color and nutritive value due to sever effects on lipids, pigments, proteins, carbohydrates and vitamins. The lipid oxidation in meat is showed in the Figure 2

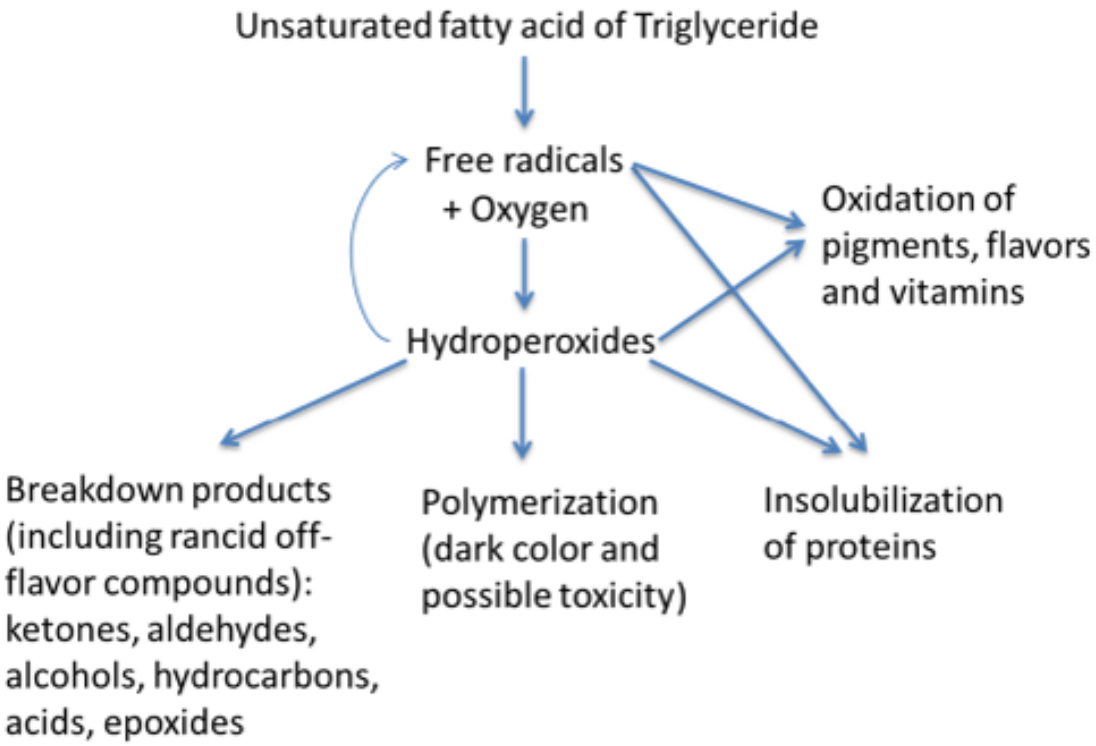

Figure 2. Lipid oxidation mechanism (Santos-Fandila A et al.,, 2014)

Autolytic Enzimatic Spoilage

Enzymatic reactions naturally occurr in the animal muscle after slaughtering and are they can lead to meat deterioration. The enzymes are able to use the organic compounds of meat and work as catalysts for chemical reactions that are responsible of meat deterioration. During the enximatic autolysis process, the organic compounds composing the meat tissues, such as carbohydrates, fats and protein, are fragmented into simpler ones resulting in visual phenomens of discoloration and texture loss of the meat. These autolysis reactions proceed through proteolysis and fat hydrolysis mechanisms that are responsible of microbial decomposition. These complex or reactions start post mortem (Toldrá & Flores 2000) . The mechanism of proteolysis post mortem is showe in the Figure. 3 (Dave & Ghaly 2011). Proteolytic enzymes are active at low temperatures and lead to decrease of meat quality due to growth of microorganisms responsible for biogenic amines production.

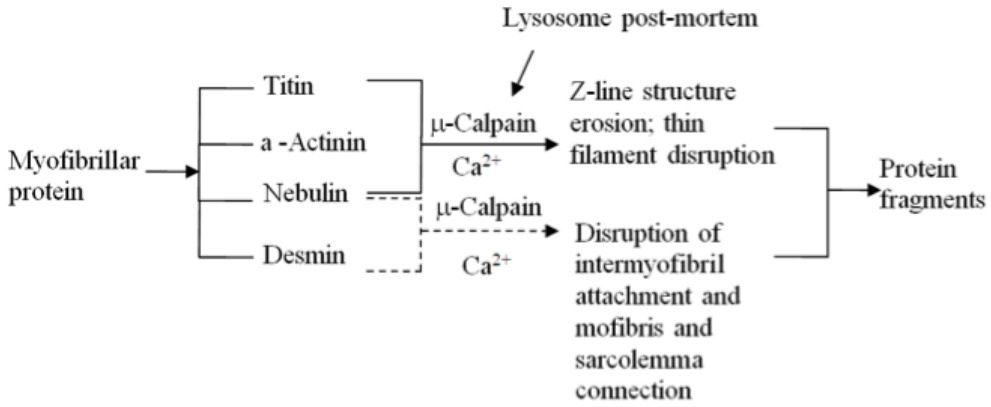

Figure 3. Mechanism of proteolysis post mortem.

1.3.5 Causes of decrease of meat quality

Pre-Slaughter Handling Effects on Meat Quality

The energy required for muscle activity in the live animal is obtained from sugars (glycogen) in the muscle. In the healthy and well-rested animal, the glycogen content of the muscle is high. After the animal has been slaughtered, the glycogen in the muscle is converted into lactic acid, and the muscle and carcass becomes firm (rigor mortis). Lactic acid is produced due to the breakdown of glycogen content of animal muscles via an anaerobic glycolytic pathway as shown in the Figure 4.

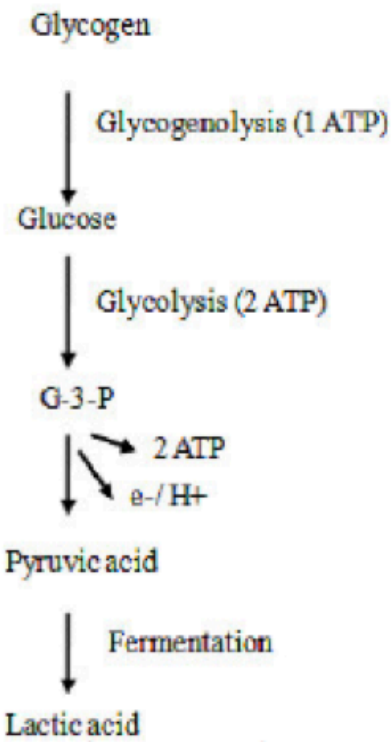

Figure 4. Anaerobic glycolytic pathway

The lactic acid is necessary to produce meat, which is tasteful and tender, of good keeping quality and good colour. If the animal is stressed before and during slaughter, the glycogen is used up, and the lactic acid level that develops in the meat after slaughter is reduced. This will have serious adverse effects on meat quality (Ferguson & Warner 2008). The acid lactic level will influence the Ph value and consequently the meat quality; for this reason an elevated ultimate PH results in beef of lower overall quality although some of the individual quality characteristics may be improved for certain purposes. Pale soft Exudative meat (PSE) in the animal is caused by severe, short-term stress just prior to slaughter, for example during off-loading, handling, holding in pens and stunning. Here the animal is subjected to severe anxiety and fright caused by manhandling, fighting in the pens and bad stunning techniques. All this may result in biochemical processes in the muscle in particular in rapid breakdown of muscle glycogen and the meat becoming very pale with pronounced acidity (pH values of 5.4-5.6 immediately after slaughter) and poor flavour. In this case meat is difficult to use or cannot be used at all by butchers or meat processors and is wasted in extreme cases. Allowing the animal to rest for one hour prior to slaughter and quiet handling will considerably reduce the risk of PSE.

Relative to beef with a normal rested ultimate pH of about 5.5, beef with an elevated ultimate PH will be characterized by:

- darker color

- blander and usually less acceptable flavor

- higher water-holding capacity

High levels of pH (6.4-6.8) result in Dark, Firm and Dry (DFD) meat (Figure 5). This condition can be found in carcasses of cattle or sheep and sometimes pigs and turkeys soon after slaughter. The carcass meat is darker and drier than normal and has a much firmer texture. The muscle glycogen has been used up during the period of handling, transport and pre-slaughter and as a result, after slaughter, there is little lactic acid production, which results in DFD meat. This meat is of inferior quality as the less pronounced taste and the dark colour is less acceptable to the consumer and has a shorter shelf life due to the abnormally high pH-value of the meat (6.4-6.8). DFD meat means that the carcass was from an animal that was stressed, injured or diseased before being slaughtered

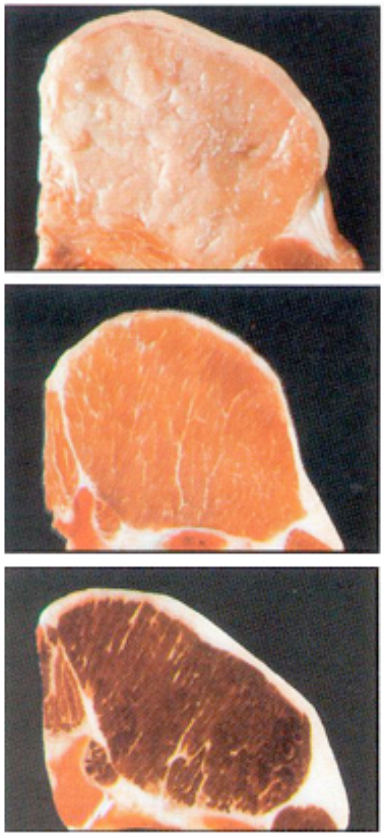

Fig.1: A. Pale Soft and Exudative (PSE) meat
B. Normal meat
C. Dark Firm and Dry (DFD) meat

Figure 5: Meat texture and color (Chambers and Grandin 2001)

Bruising and injury

Bruising is the escape of blood from damaged blood vessels into the surrounding muscle tissue. This is caused by a physical blow by a stick or stone, animal horn, metal projection or animal fall and can happen anytime during handling, transport, penning or stunning. Bruises can vary in size from mild (approx. 10-cm diameter) and superficial, to large and severe involving whole limbs, carcass portions or even whole carcasses. Meat that is bruised is wasted as it is not suitable for use as food because:

- is not acceptable to the consumer;

- It cannot be used for processing or manufacture;

- It decomposes and spoils rapidly, as the bloody meat is an ideal medium for growth of contaminating bacteria;

- It must be, for the above reasons, condemned at meat inspection.

Bruising is a common cause of meat wastage and can be significantly reduced by following the recommended correct techniques of handling, transport and slaughter (Mpakama et al., 2014).
Injuries such as torn and haemorrhagic muscles and broken bones, caused during handling, transport and penning, considerably reduce the carcass value because the injured parts or in extreme cases the whole carcass cannot be used for food and are condemned. If secondary bacterial infection occurs in those wounds, this causes abscess formation and septicaemia and the entire carcass may be condemned.

Transportation

Animals in the most of cases have to be transported over some distances to these locations for sale and slaughter, since grow on farms that can be be situated far away from slaughter plants and market. Transportation has to be done in the most comfortable conditions for the animal (Gade & Christensen 1998). During transportation, animals suffer many environmental stresses such as heat, cold, humidity, noise and overcrowding that may increase the damage of carcass and meat quality defects. Through the transportation phase the animal welfare is compromised. Moreover panic for the handling procedures and for an unknown place makes the animal feel pain (Ohl & van der Staay 2012). For the reasons mentioned above, transportation phase is a critical point in the life of an animal prior to slaughter. Furthermore, many other conditions such as starvation, dehydration, injury and temperature stress occur during transportation and they can lead to a loss of meat quality (Warriss et al., 2010).

1.4 Molecular approaches to food microbial diversity

Knowledge of microorganisms in the environment has depended in the past mainly on studies of pure cultures in the laboratory. Rarely are microbes so captured, however. Studies of several types of environments estimate that more than ninety-nine percent of organisms observed by microscopy are not cultivated on classical laboratory medium (Alain & Querellou 2009). Microbial organisms occupy a peculiar place in the human life as they take part to biogeochemical cycles, and are involved in numerous symbiotic association with plants and animals. Moreover, they play a fundamental role in all the biochemical cycles of conversion and production of inorganic elements as nitrogen, carbon and oxygen into biological compounds accessible by other form of life. They are also involved in desired food process such as fermentation or in undesired food deterioration processes.

In the past, study about microbiota in meat has been based on traditional microbiology approaches, such as standard plate counts (Bell 1997; Sumner et al., 2003) (Figure 6). However, standard plate counts might result a lower colony-forming units or numbers compared to actual viable population (Jay et al., 2005). This is due to some limitations. First, this technique might not be able to detect novel microorganisms that are not cultivable using known media. Second, it does not capable to recover stressed or viable but non-cultivable (VBNC) (Giraffa & Neviani 2001)microorganisms .

Natural stress, such as starvation, incubation outside the temperature range of growth, elevated osmotic concentrations, oxygen concentration or exposure to white light might lead cells to enter VBNC state (Oliver 2005). One of the meat spoilage bacteria that have been reported demonstrating the VBNC state is *Pseudomonas fluorescens*. A study showed that some non-cultivable *Pseudomonas fluorescens* cells are dividing on the surface that imitate an open surface at meat processing premises although the cell division could not continue to the stage of macro-colony formation on agar (Peneau et al., 2007). Therefore, more elaborated methods based upon molecular biology have been applied to study microbial populations without cultivation (Giraffa & Neviani 2001).

Culture-independent techniques can be used to determine the microbial diversity in natural ecosystems and to observe the evolution of microbial populations over space or time (Giraffa & Neviani 2001). These methods could overcome problems associated with selective cultivation such as the inability to detect some bacteria on the known media, the lack of knowledge of the real conditions under which most of bacteria are growing in their natural habitat and the difficulty to develop media for cultivation accurately resembling these conditions (Doulgeraki et al., 2012). These techniques are generally based on the analysis of the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) that is extracted directly from the sample. Subsequently, the nucleic acids are amplified by polymerase chain reaction (PCR) and

subjected either to cloning and sequencing or to profiling techniques, such as denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE), or single strand conformation polymorphism (SSCP) (Cocolin et al., 2008). Another alternative method that does not rely on nucleic acids extraction from the sample is called fluorescence in situ hybridization (FISH).

Some extensive methods to characterize the strains isolated by culture-dependent methods in molecular level have also been developed, such as randomly amplified polymorphic DNA (RAPD)-PCR, repetitive bacterial DNA elements (Rep)-PCR and enterobacterial repetitive intergenic consensus (ERIC)-PCR (Cocolin & Ercolini 2008). Figure 1 shows a polyphasic approach which defined as a combination of many different methodologies that has been directed toward analyzing phenotypic, genomic, and phylogenetic characteristics for taxonomic purposes (Pontes et al., 2007).

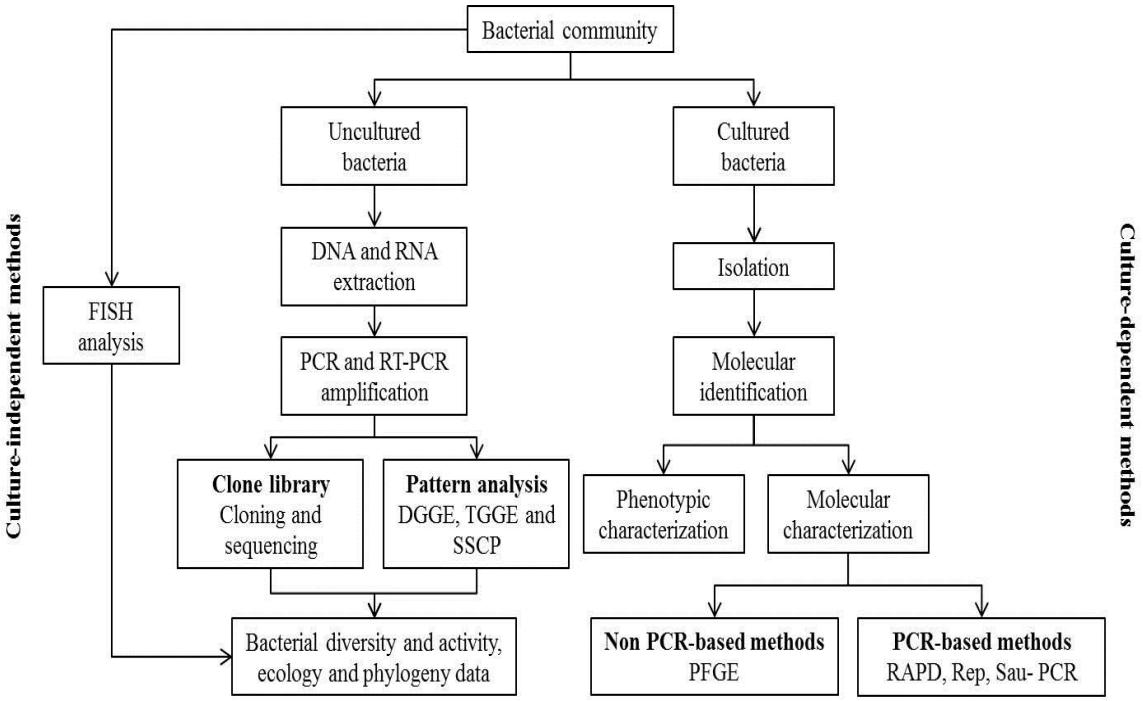

Figure 6. Culture-independent and dependent methods (Cocolin et al.,, 2008; Pontes et al.,, 2007).

1.4.1 Culture-independent methods to detect and identify food spoilage bacteria

PCR-denaturing gradient gel electrophoresis (DGGE) of ribosomal RNA (rRNA) is possibly the most commonly used among the culture-independent fingerprinting techniques. DGGE can detect differences between DNA fragments of the same size but with different sequences. These fragments are separated in a denaturing gradient gel based on their denaturation (melting) profile. DGGE result provides a fingerprint of the bacterial community in an environmental sample (Ercolini 2004).

PCR-DGGE is a beneficial method to monitor changes in microbial composition in meat during storage. Each meat sample in a particular storage condition can be associated to a specific microbial profile where the identification of the bands will provide the exact species composition of that particular sample without the need for cultivation. Therefore, any changes can be easily detected (Doulgeraki et al., 2012). PCR-DGGE has been widely used in spoilage-related microbiota in meat; monitoring microbial spoilage of beef during 14 d storage at 5°C under modified-atmosphere packaging conditions (Ercolini et al., 2006); assessing the microbial populations causing chilled beef spoilage during storage(Ercolini, Casaburi, et al., 2010). In another study, PCR-DGGE was used to analyze bulk cells from selective media and detected some bacterial species that have important role in meat spoilage though they were not found in DNA extracted directly from meat (Pennacchia et al., 2011).

However, DGGE has some limitations that make it not suitable for large-scale studies of microbial ecology. First, DGGE is time consuming and technically challenging method, as the denaturing gradient gels are difficult to set and run properly. Second, it is a non-quantitative method. It can detect the presence of the dominant species present but

cannot reliably determine relative abundance (Bokulich & Mills 2012).

Next-generation sequencing (NGS) technologies can overcome these limitations (Bokulich & Mills 2012), including high-throughput sequencing approaches. The workflow, limits, and perspectives in applying culture-independent high-throughput sequencing to study food microbiota has been reviewed (Ercolini 2013). A recent study showed that performing pyrosequencing of 16S rRNA gene amplicons extracted directly from meat stored in different packaging conditions led to much higher microbial diversity than PCR DGGE (Ercolini et al., 2011). In another study, high-throughput barcoded parallel 454 pyrosequencing was used to characterize bacterial and fungal changes in vacuum-packed chilled pork (Zhao et al., 2015). This study found hundreds of different microbes on the surface of vacuum-packed chilled pork and the changed microbial components through the 21 days of storage. The high- throughput sequencing (HTS) approach can also give a quantitative estimation of the abundance of a single taxon in each sample based on the number of reads of its particular 16S rRNA gene sequence (Doulgeraki et al., 2012).

1.4.2 High-throughput sequencing approach in food microbial ecology

The most important application of HTS in food microbial ecology is study of the microbiota based on the single-gene amplicon sequencing (van Hijum et al., 2013). Since the application of HTS was incremented, the need of new bio-informatics tools for data analysis arise, leading to collect a variety of bioinformatical packages appositely ideated for each step in standardized pipelines, such as QIIME (Caporaso et al., 2010), Phyloseq (McMurdie & Holmes 2013), Mothur or Oligotyping (Eren et al., 2013), with the aim to satisfy the microbial ecologists' requests approaching to this kind of investigations. Starting from a 16S rRNA approach, the bioinformatic analysis is focused on the individuation of genes of taxonomic interest, also thanks to the accessibility of furnished databases (Caporaso et al., 2010; Pruesse et al., 2007; Cole et al., 2003). The internal transcribed spacer is the most utilized target for the eucariota too (O'Sullivan et al., 2015; Bokulich & Mills 2013b; Bokulich et al., 2014). Anyway, it should be considered that, the irregular ITS length among species can generate preferential amplification (Bokulich & Mills 2013b) and consequently, incorrect estimation of OTUs abundances. Thus, a good solution for thi issue is proceding using different targets, as demonstred by (Garofalo et al., 2015)in the study of the fungal population of milk kefir grains.

HTS of 16S rRNA gene found its application in different fields of food microbiology such as the study of the microbiota in different dairy ecosystems (Ercolini et al., 2012; De Filippis et al., 2014; O'Sullivan et al., 2015; Calasso et al., 2016; Stellato, De Filippis, et al., 2015; Dolci et al., 2014) allowing the identification of new microbial consortia, not previously associated with cheese manufacturing (Lisa Quigley et al., 2012) and describing the complex microbiota of raw milk (Dolci et al., 2014; Quigley et al., 2013; McInnis et al., 2015), exploiting th high sensitivity of this thechnology. The microbiota involved in cheese production was also investigated. Previous study showed that thermophilic lactic acid bacteria (LAB), added through the natural whey culture (NWC), dominated Water buffalo mozzarella cheese and intermediates of production (Ercolini et al., 2012). On the other hand, the ripenind phases, was dominated by the development of mesophilic lactobacilli, not added by starter, but finally present in the core of a medium-ripened pasta filata cheese and, moreover, they were found responsible for the production of amino acid and many volatile compounds (DePasquale et al.,, 2014). Furthermore, in an other type of ripened cheese the resident microbiota showed different level of complexity depending on the time of cheese manufacturing: when the cheese production started in the evening, the ripening microbiota showed a higher complexity compared to compared to the early day production ones (O'Sullivan et al., 2015), underling how even the hour can influence the microbiota composition. HTS was also used to study fermentation processes of meat (Polka et al., 2014), fresh meat (Hultman et al., 2015); olives (Cocolin et al., 2013), dairy manufactures (De Filippis et al., 2014), and possible overlap between food matrices and food processing environment (Calasso et al., 2016; Stellato, La Storia, et al., 2015; Stellato, De Filippis, et al., 2015). Recently, HTS found a large application to food spoilage microbiota and many studies were also carried out. Psychrotrophic LAB were found as main spoilers of packaged and chilled-stored food products in Northern Europe (Pothakos, Taminiau, et al., 2014). Besides, the microbiota involved in meat and seafood products spoilage was also explored (Remenant et al., 2015; Doulgeraki et al., 2012; Coorevits et al., 2008), and specific OTUwere found strictly associated to different investigated samples. Nevertheless, a common core microbiota was was shared by the spoiled samples, and pshychrotrophic microorganisms prevailed. Actually, several studies demonstred that also also different packaging conditions can select a specific spoilage microbiota in beef (Pennacchia et al., 2011). HTS was also considered a suitable method for tracking back the sources of food contamination, since food processing plants and equipment are a significant reservoir of microorganisms, that can undergo to cross-contamination events during manufacturing and processing and could perform as spoilers or be positively involved in process steps. Several studies demonstrated that in many cases the environmemntal microbiota can influence positively the final product; many examples are cited such as in the case of cheese manufactures, beer and sake (Bokulich & Mills 2012; Bokulich et al., 2012; Bokulich & Mills 2013a) where a resident plant microbiota was found to drive the fermentations process.

Furthemore, the microbial consortia composition involved in the wine production were found to be influenced, both by the seasonal environmental conditions and also by the winery localization (Bokulich et al., 2013), emphasizing the presence of a al specific territorial microbiota and giving interesting evidences on the possible presence of a well defined microbiota derived by grapes' origin. As previously stated, food processing plant can also be a source of spoilage microorganismss as showed for cheese (Stellato, De Filippis, et al., 2015; Calasso et al., 2016), beef (Hultman et al., 2015; De Filippis, Pennacchia, et al., 2013), beer (Bokulich et al., 2012), and ready-to-eat meals (Pothakos, Snauwaert, et al., 2014). Furthermore, other studies investigated also residential kitchen and foodservice plant surfaces and they were found to be contaminated by bacteria associated with the skin, animals, and foods (Flores et al., 2013; Stellato, La Storia, et al., 2015). Nowdays, the most challenging aim for the HTS application is the investigation of microorganisms beyond the species level (De Filippis et al., 2014), since is well knowed the strict association of potential spoilage to many phenotypic (Ercolini, Casaburi, et al., 2010; Casaburi et al., 2014; Casaburi et al., 2011) or the potential good contribuition to food processing (Gori et al., 2012; Zago et al., 2011) can be also strain-specific. Amplicon sequencing of target genes showing high sequence heterogeneity within a species may allow a quantitative moni- toring of biotypes during fermentation or spoilage processes. In the only report about this application, lacS gene amplicon sequencing was used to monitor Streptococcus thermophilus beyond the species during curd fermentation of different cheeses(De Filippis et al., 2014). Microbial ecologists are approaching to a new idea of organizing, merging and elaborate the already available informations about food and food processing plant microbiota a, in order to enrich the related knowledgment. Actually the availability of more data-collecting platform, allows to investigate the food microbiota variability merging and comparing information from a variety of previous studies, obtaining a rapid and easy visual comparison of different food and food environment samples (Huse et al., 2014; Parente et al., 2016)

1.4.3 The study of food microbial ecology through different HTS approaches

In the field of the microbial ecology the HTS approach can be grouped into two branches: amplicon metagenomics (sequencing of libraries of a PCR-amplified gene of interest), and shotgun metagenomics (sequencing of libraries of randomly isolated DNA fragments) (Figure 7). In th e amplicon metagenomics case, a PCR step is performed after total DNA extraction (RNA has to be retrotrascribed to complementary DNA), in order to select the gene to be sequenced, usually a gene of taxonomic interest. The use of ribosomal RNA (rRNA) amplicon sequencing is the most exploited HTS application in microbial ecology. This led to the identification of the "microbiota", that is the taxonomic composition of the microbial community that inhabit the sample and the relative abundance of each OTU (Operational Taxonomic Unit). Moreover, HTS of specific target genes can provide the identification beyond the genus andconsequently a strain monitoring in food samples.

In the shotgun metagenomics method, no PCR selection is requested and total DNA is fragmented and directly sequenced. Consequently, after a computational assembly and a comparison with databases, you can have the abundance of all the genes present in the environment, so the potential activities that the microbiota could find out. With this approach, we will identify the "microbiome", the complex of the microorganisms and their genomes in the investigated environmen, with subsequent identification of the microbial genes occurring in that specific environment and their relative abundances (Figure 1). If we want to investigate which genes are really expressed, we will need to proceed through the RNA-seq (Mutz et al., 2013). For the RNA-seq method, first a total RNA extraction has to be performed, after that, the ribosomal RNA is essential (considering that the rRNA represents more than 80% of total RNA). Next, the RNA enriched of messenger RNA (mRNA) is retrotrascribed and it is sequenced with the shotgun approach as described above.

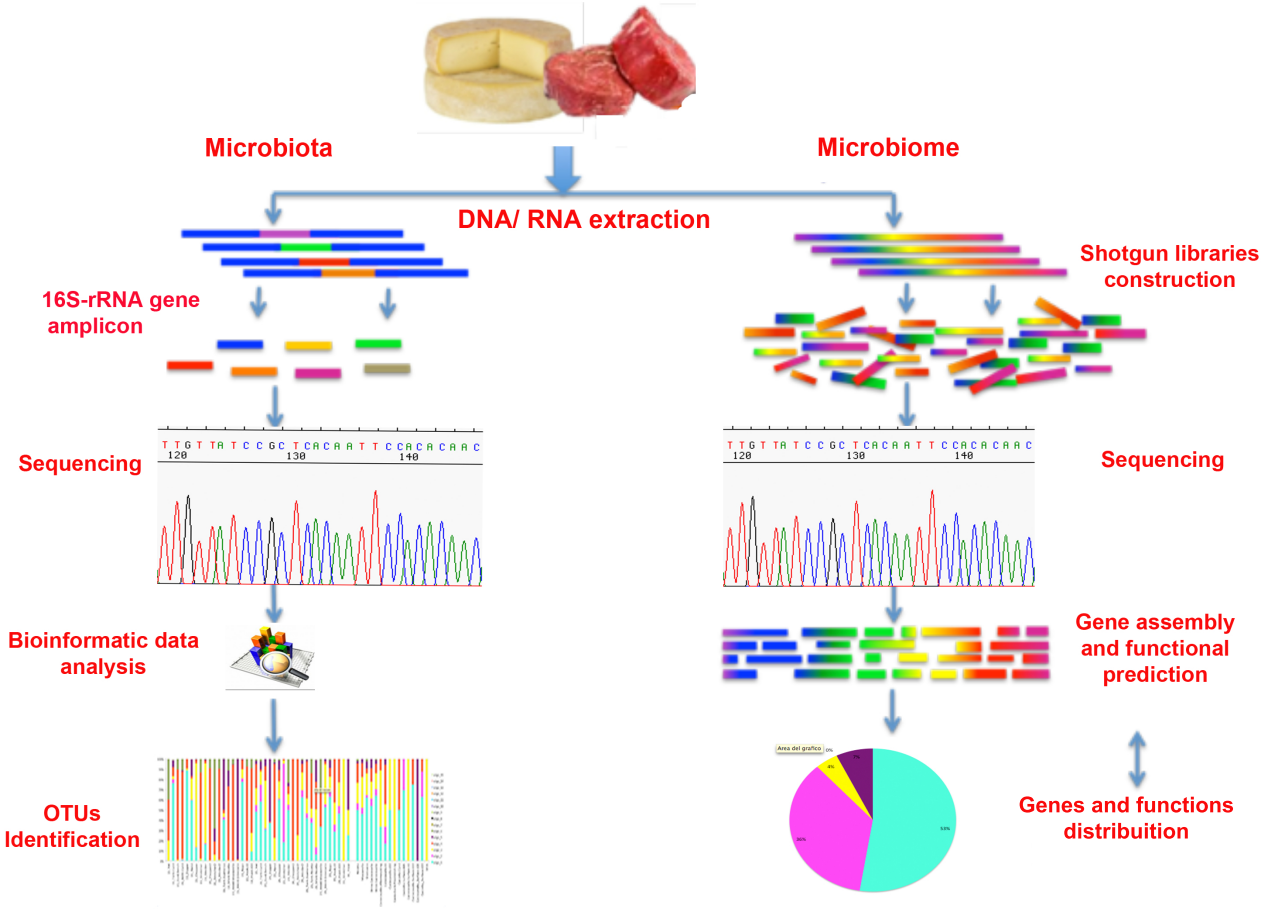

Figure 7. HTS workflow to study food microbiota and microbiome.

1.4.4 Next generation sequencing technology

Nowdays, the massively parallel DNA sequencing read production is performed principally by using two platform: the Roche/454 (http://www.454.com/enablingtechnology/the-system.asp) and the Illumina/Solexa (http://www.illumina.com/pages.ilmn?ID=203) (Mardis, 2008). Both platforms represent the results of the principles of enzymology, chemistry, high-resolution optics, hardware and software engineering. The genomic libraries are obtained by using platform-specific linkers. Since adapter sequences are used, the molecules then can be selectively amplified by PCR, and moreover no bacterial cloning step is necessary to amplify the genomic fragment in a bacterial intermediate as is made in traditional sequencing approaches. Furthermore, considering the difference between these implements and capillary platforms, has to be underlined the run time required to generate data. Next-generation sequencers require longer run times, between 8 h and 10 days, depending upon the platform and read type (single end or paired ends). The sequence length can reach more than 1000 bp and the yield in number of reads can be highly variable from several hundred thousand reads (Roche/454) to tens of millions of reads (Illumina and Applied Biosystems SOLiD) (Mardis, 2008).

Roche/454 FLX Pyrosequencer

The Roche/454 FLX Pyrosequencer was the first sequencer commercialized in 2004 and utilizes an unusual sequencing technology known as pyrosequencing. In pyrosequencing, a DNA polymerase promote the incorporation of each nucleotide resulting in the release of pyrophosphate, which initiates a series of downstream reactions that finally produces light by the firefly enzyme luciferase. The amount of light produced is proportional to the number of nucleotides incorporated (up to the point of detector saturation). In the Roche/454 approach Figure 8 library fragments are mixed with a population of agarose beads whose surfaces carry oligonucleotides complementary to the 454-specific adapter sequences on the fragment library, so each bead is associated with a single fragment. Each of these fragment-bead complexes is isolated into individual oil: water micelles that also contain PCR reactants, and thermal cycling (emulsion PCR) of the micelles produces approximately one million copies of each DNA fragment on the surface of each bead. These amplified single molecules are then sequenced en masse. First the beads are arrayed into a picotiter

plate (PTP; a fused silica capillary structure) that holds a single bead in each of several hundred thousand single wells, which provides a fixed location at each sequencing reaction that can be monitored. Enzyme-containing beads that catalyze the downstream pyrosequencing reaction steps are then added to the PTP and the mixture is centrifuged to surround the agarose beads. The PTP acts as a flow cell into which each pure nucleotide solution is introduced in a stepwise fashion, with an imaging step after each nucleotide incorporation step. The PTP is seated opposite a CCD camera that records the light emitted at each bead. The first four nucleotides (TCGA) on the adapter fragment adjacent to the sequencing primer added in library construction correspond to the sequential flow of nucleotides into the flow cell. This strategy allows the 454 base-calling software to calibrate the light emitted by a single nucleotide incorporation. However, the calibrated base calling cannot properly interpret long stretches (>6) of the same nucleotide (homopolymer run), so these areas are prone to base insertion and deletion errors during base calling. By contrast, because each incorporation step is nucleotide specific, substitution errors are rarely encountered in Roche/454 sequence reads. The FLX instrument currently provides 100 flows of each nucleotide during an 8 h run, which produces an average read length of 700 nucleotides. These raw reads are processed by the 454 analysis software and then screened by various quality filters to remove poor-quality sequences, mixed sequences (more than one initial DNA fragment per bead), and sequences without the initiating TCGA sequence.

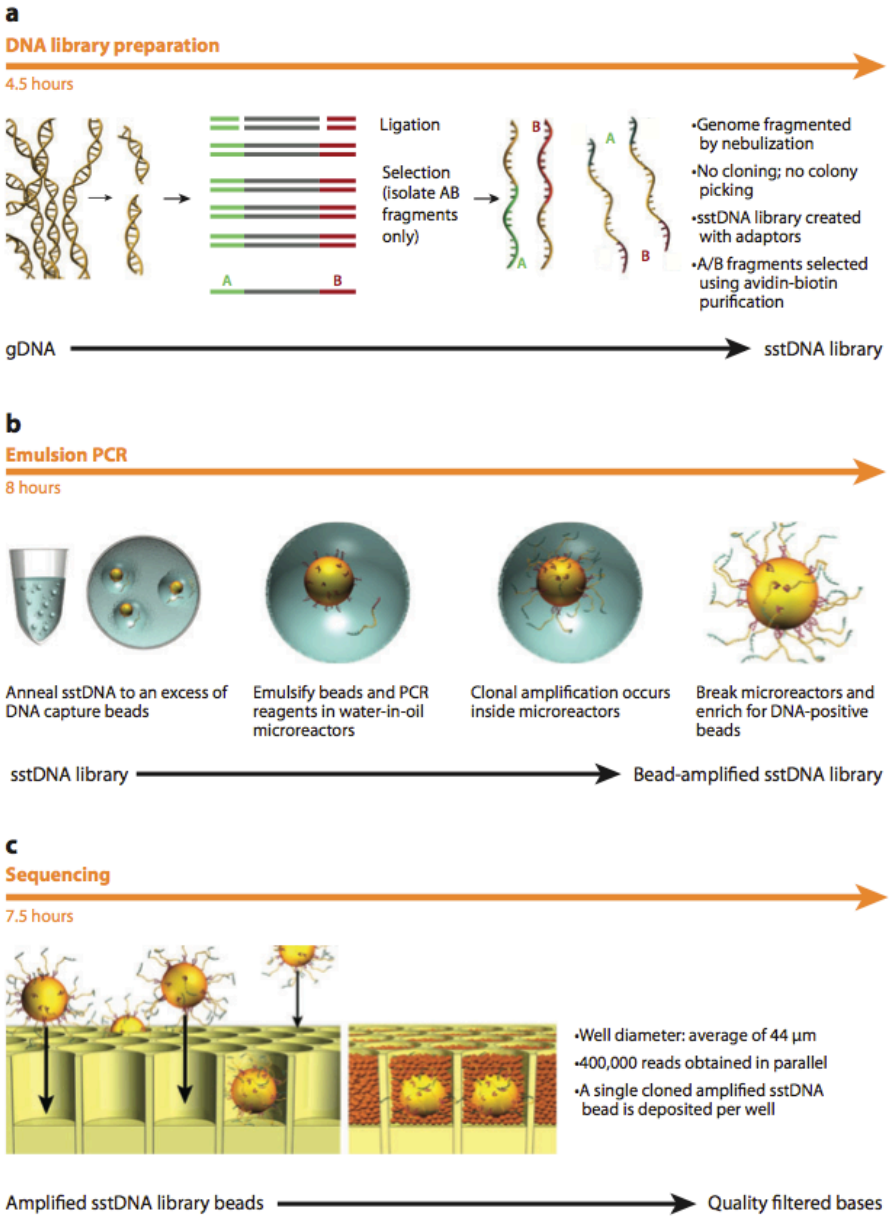

Figure 8. The method used by the Roche/454 sequencer to amplify single-stranded DNA copies from a fragment library on agarose beads (from Mardis 2008).

Illumina Genome Analyzer

The Illumina Genome Analyzer starts the amplification step with an Illumina-specific adapter library that stand on the oligo-derivatized surface of a flow cell, and proceed by using an automated device called a Cluster Station. The flow cell is an 8-channel sealed glass microfabricated device that allows bridge amplification of fragments on its surface, and uses DNA polymerase to produce multiple DNA copies, or clusters, that each represent the single molecule that initiated the cluster amplification. Each cluster contains approximately one million copies of the original fragment, which is sufficient for reporting incorporated bases at the required signal intensity for detection during sequencing. The Illumina system works through a sequencing-by-synthesis approach in which all four nucleotides are added at the same time to the flow cell channels, along with DNA polymerase, for incorporation into the oligo-primed cluster fragments (Figure 9). Particularly, the nucleotides carry a base-unique fluorescent label and the 3'-OH group is chemically blocked such that each incorporation is a unique event. An imaging step follows each base incorporation step, during which each flow cell lane is imaged in three 100-tile segments by the instrument optics at a cluster density per tile of 30,000. After each imaging step, the 3'- blocking group is chemically removed to prepare each strand for the next incorporation by DNA polymerase. This series of steps continues for a certain number of cycles, as determined by user-defined instrument settings, which permits discrete read lengths of 50–250 bp. A base-calling algorithm assigns sequences and associated quality values to each read and a quality checking pipeline evaluates the Illumina data from each run, removing poor-quality sequences.

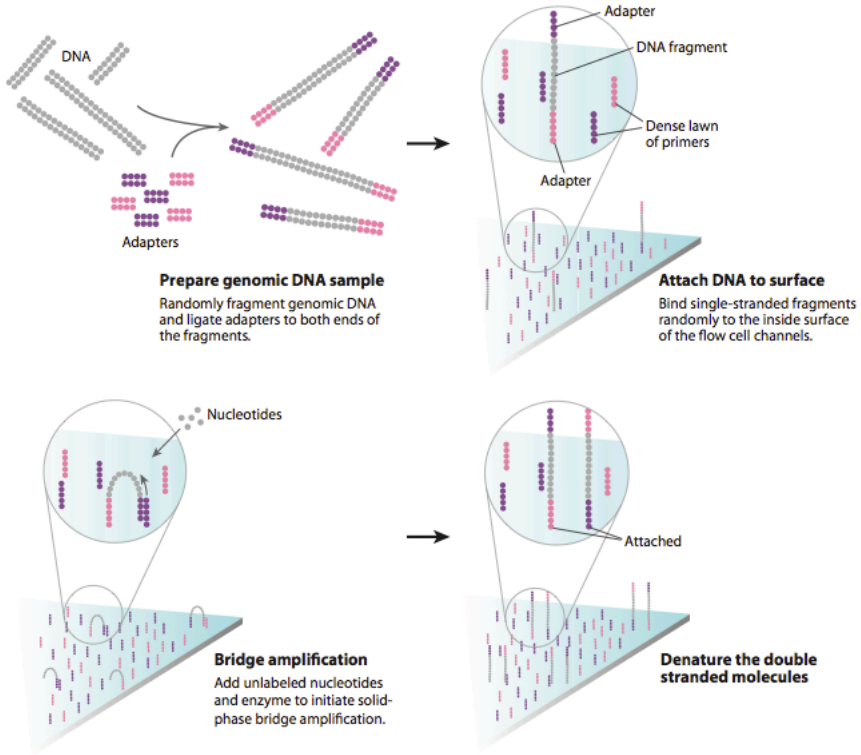

Figure 9. The Illumina sequencing-by-synthesis approach. Cluster strands created by bridge amplification are primed and all four fluorescently labeled, 3′-OH blocked nucleotides are added to the flow cell with DNA polymerase. The cluster strands are extended by one nucleotide. Following the incorporation step, the unused nucleotides and DNA polymerase molecules are washed away, a scan buffer is added to the flow cell, and the optics system scans each lane of the flow cell by imaging units called tiles. Once imaging is completed, chemicals that effect cleavage of the fluorescent labels and the 3′-OH blocking groups are added to the flow cell, which prepares the cluster strands for another round of fluorescent nucleotide incorporation (from Mardis, 2008).

1.5 High-throughput sequencing

1.5.1 Current and prospective applications of HTS

The choice of environment is unlimited but a well-studied environment will enable to make better sense of the metagenomic data. Unravelling the genetic and molecular mechanisms at work within and across microbes of whole ecosystem communities will provide new insights for fundamental research, and new tools for applications in environment, agriculture, human health and nutrition and bioindustry. In the table XXX are listed the current and propositive application of the HTS method.

Fundamental research: microbe diversity, ecology and biology

- Discovery of new bacteria and archae divisions, discovery of new genes and new functions (e.g., discovery of a new phototrophic bacterium: Candidatus Chloracidobacterium thermophilum, (Bryant et al., 2007)). (It is worth noting that, in terms of evolution and biodiversity analysis, the study of nucleotide polymorphism in the gene coding for the 16S subunit of ribosomal RNA preceded metagenomic analysis and provided sufficient information back in 1977 to re-draw the basic phylogenetic tree of life into three domains: bacteria, archae and eukarya. This technology remains instrumental as a preliminary step to evaluate the diversity of samples prior to metagenomic analysis.)
- Insight into microbial community balance, resilience and adaptation to environmental changes
- Meta-pathways or microbial cooperation resulting in collective metabolism, communication between specialised bacteria, co-evolution / specialisations / multi-cellular super-organism or community of individuals?
- Analysis of the role of microbes in biogeochemical cycles (eg C and N cycles)
- Host/microbe symbiosis and evolution, evaluate the interactions between animal genome, gut metagenome (the microbiome), environment, nutrition and health.

Environment

- Evaluation and monitoring of microbe biodiversity
- Identification of markers or biosensors of environmental change by monitoring microbe diversity (specific individuals, specific genes, or specific functions in microbe communities, potentially using array-based analyses)
- Identification and exploitation of specialised microbe metabolisms for:
- Bioremediation of soils contaminated with toxic substances (e.g., heavy metals)
- Industrial treatment of waste (e.g., exploitation of anammox bacteria to remove nitrogen in wastewater treatment plants (Strous et al., 2006))

Agriculture

- Optimisation of plant interaction with rhizobia, mycorrhizae and other soil microbes for better nutrition and reduced artificial fertilisation
- Improvement of crop health from a better understanding, control and exploitation of soil microbial communities that have a protective effect on plants
- Rapid identification of pathogens potentially responsible for emerging diseases (e.g., honey bee colony collapse disorder (Cox-Foster et al., 2007))
- Identification and exploitation of microbial markers for early disease detection in plants or animals
- Monitoring and control of food safety

Human nutrition and health

- Search for new drugs, new antibiotics, new sources of beneficial nutrients (e.g. vitamins)
- Extension of nutrigenomics to nutri-metagenomics taking into account the role of intestinal flora in human nutrition, towards better determination of the benefits of probiotics, functional foods, nutraceuticals and the metabolism and safety assessments of food additives
- The same concept is applicable to personalised medicine, with better assessment of drug metabolism and bioavailability
- Validation and exploitation of relationships between obesity and gut microbiome to fight obesity (concept applicable to other pathologies)
- Identification and use of markers of disease or disease susceptibility for earlier diagnostics, potential treatment or prevention of disease.

Bioindustry: discovery of novel active compounds or enzymatic activities by functional metagenomic approaches

- Targeted approach based on identification of previously know enzymeencoding sequences (specific PCR screen) and subsequent functional test
- Unbiased approach by systematic functional characterisation of whole metagenome libraries for new therapeutic activities (antibacterial, antifungal, antiviral) or new chemical activities
- Bioenergy production: alternative energies based on the conversion of biomass to biofuel require more cost-efficient technologies to be economically viable.

Microbes can be a source of efficient enzymes to degrade cellulose and hemicellulose into sugars, and to ferment those sugars into alcohol (ethanol, preferably butanol) usable as biofuel. Potentially interesting enzymatic system was found in spirochetes (helical bacteria) from tropical termite guts (Warnecke et al., 2007)

- Enzymes of anaerobic metabolism and their exploitation to produce methane and hydrogen

-Biocatalysts for green chemistry (e.g., hydrolases)

1.5.2 HTS approach for the study of the microbiota associated to food microbial ecology

The high-throughput sequencing of 16S rRNA gene-based is largely employed for the investigation of the microbiota in a specific fermented food, considering both the final microbial consortia populating the final product at the end of production, or for supervising the microbial populations that alternating occurr during the fermentation process from raw materials to the final step.

Dairy microbiology is the branch of food microbiology that most swiftly takes up the novel approaches successfully employed in other fields of microbial ecology. The HTS approach was used to establish the relationships between house, rind and core microbiotas of cheese varieties manufactured at the same industrial dairy plant (Calasso et al., 2016). Caciotta and Caciocavallo Pugliese cheeses were chosen as model systems. Mesophilic lactobacilli, cocci and, especially, thermophilic cocci were the most abundant cultivable bacteria found on equipment, which were located in the production area. According to cell counts, catabolic profiles of microbial communities deriving from equipment, and cheese core and rind differed. As shown by 16S rRNA targeted metagenomics, *Streptococcus thermophilus* dominated the communities from knife surface, brine tank, curds and core cheeses as well as it was the main colonizing bacterium from drain table, rinds and ripening room of Caciocavallo Pugliese cheeses. Compared to S. thermophilus, the other starters used (*Lactococcus lactis, Lactobacillus delbrueckii subsp. lactis and Lactobacillus helveticus*) showed low relative abundance in cheeses and/or colonization capability. A set of other genera/species, which varied depending on the equipment surfaces and cheese making, contributed to the formation of a rather heterogeneous house microbiota. Representatives from such communities had (e.g., *Lactobacillus casei, Lactobacillus plantarum* group) or not (e.g., *Actinobacteria*) the capacity to colonize cheeses, which depended on the variety (Caciocavallo Pugliese or Caciotta cheese) and layer (rind or core). Other genera/species were mainly associated to the rind and ripening room of Caciotta (*Staphylococcus* species and *Brochothrix* spp.) or Caciocavallo Pugliese (*Chromohalobacter and Sphingomonas*) cheeses.

Determining the composition of the microbiota in the final products by HTS is commonly used. A set of samples including 60 Irish soft, semi-hard or hard cheeses was used to performed a smetagenomic study by HTS to describe differences in bacterial diversity according to type of cheese, milk and production technology (Quigley et al., 2013). The dominant OTU in all the cheeses *was Lactococcus*, although in hard cheeses type the abundance decreased with increasing *Lactobacillus* ones. Moreover, a low abundance of contaminants was found in cheese rinds including genera such as *Faecalibacterium*, *Prevotella* and *Helcococcus* (L. Quigley et al., 2012).

As reported in a previous study conducted by HTS on kefir grains, more than 90% of the OTUs were associated to the genus *Lactobacillus* and remaining LAB only represented a minor portion of the populations (Leite et al., 2012). Moreover, the microbial population of an Irish kefir grain was dominated by *Lactobacillaceae*, while *Streptococcaceae* were basically found in the kefir-fermented milk (Dobson et al., 2011).

1.5.3 Microbiota associated to food spoilage

The 16S rRNA gene analysis by culture-independent HTS can be easily applied to look at the structure of a microbial population developing in fresh food during storage and to identify the microbiota responsible for the spoilage of certain foods.

HTS approach was used to evaluate the microbial diversity in beefsteaks before and after aerobic storage at 4°C and to investigate the sources of microbial contamination by examining the microbiota of carcasses wherefrom the steaks originated and of the processing environment where the beef was handled (De Filippis, La Storia, et al., 2013)Carcass, environmental (processing plant) and meat samples were analyzed by culture-independent high-throughput sequencing

of 16S rRNA gene amplicons. The microbiota of carcass swabs was very complex, including more than 600 operational taxonomic units (OTUs) belonging to 15 different phyla. A significant association was found between beef microbiota and specific beef cuts ($P<0.01$) indicating that different cuts of the same carcass can influence the microbial contamination of beef. Despite the initially high complexity of the carcass microbiota, the steaks after aerobic storage at 4°C showed a dramatic decrease in microbial complexity. *Pseudomonas sp. and Brochothrix thermosphacta* were the main contaminants, and *Acinetobacter, Psychrobacter and Enterobacteriaceae* were also found. Comparing the relative abundance of OTUs in the different samples it was shown that abundant OTUs in beefsteaks after storage occurred in the corresponding carcass. However, the abundance of these same OTUs clearly increased in environmental samples taken in the processing plant suggesting that spoilage-associated microbial species originate from carcasses, they are carried to the processing environment where the meat is handled and there they become a resident microbiota.

The microbiota of ready-to-eat food was also investigated by HTS approach to study the contamination levels of psychrotrophic lactic acid bacteria (LAB) (Pothakos, Taminiau, et al., 2014). The study was conducted on 33 retail, packaged food products stored at chilling temperature when the mesophilic enumeration technique was implemented as reference shelflife parameter. In the present study, the microbial diversity of the dominant psychrotrophic LAB recovered after incubation of plates at 22 °C for 5 days was determined using a polyphasic taxonomic approach. A total of 212 LAB isolates were identified using a combination of rep-PCR fingerprinting, amplified fragment length polymorphism (AFLP) analysis and pheS gene sequencing. *Leuconostoc gasicomitatum, Leuconostoc gelidum, Leuconostoc spp., Lactococcus piscium and Lactobacillus algidus* proved to be the most competent and predominant species that may go undetected by the widely applied mesophilic enumeration protocols (ISO 4833:2003 and ISO15214:1998).

In a further application to meat storage, the relative abundance of different OTUs was determined during chill storage of beef in air; modified atmosphere packaging (MAP); vacuum packaging; bacteriocin-activated antimicrobial packaging (Ercolini, Ferrocino, et al., 2010). The initial meat prior to packaging was found contaminated by at least 21 different taxonomic units. Using the sequencing approach, it was found that this diversity changed dramatically depending on the storage conditions. Microbial taxa never associated with meat, such as *Ralstonia* sp. and *Limnobacter* sp., were the most abundant in the beef at time zero. However, in each type of packaging the microbiota evolved differently. *B. thermosphacta* and *Pseudomonas* sp. dominated in the first and second part of air storage, respectively, while *B. thermosphacta* and *C. divergens* developed in the first and second period of MAP storage, respectively. More bacteria were observed during vacuum pack storage, such as *Streptococcus* sp., *Lactobacillus* sp., *Lactococcus* sp. *C. divergens* and *Carnobacterium* sp. The highest variety of species was observed in meat stored in antimicrobial packaging. However, while at the early stages microorganisms such as *Ralstonia* sp., *Limnobacter* sp. *Limnobacter thiooxidans, Bradyrhizobium* sp., *Rudaea cellulosilytica* and *Rhodococcus* sp. were found, after three weeks of storage in active packaging these bacteria dramatically decreased and a high incidence of *C. divergens* up to 95% characterized the beef stored in antimicrobial packaging at the final stages of storage (Ercolini et al., 2011). Surprisingly, from the same initial meat microbiota, very different OTUs can develop depending on the specific storage conditions, and such studies can have a strong impact in evaluating different storage systems for the specific inhibition of certain spoilage-associated microbes.

Finally, the bacterial biogeographical patterns in a hospital cooking center was studied by 16S rRNA-based culture-independent highthroughput amplicon sequencing in order to provide a comprehensive mapping of the surfaces and tools that come in contact with foods during preparation (Stellato, La Storia, et al., 2015). Across all area, surface swab-samples from work surfaces of different zones were taken: food pre-processing rooms (dedicated to fish, vegetables, and red and white meat), storage room and kitchen. The microbiota of environmental swabs was very complex, including a large variety of gerea with extremely variable relative abundances (0.02–99%) depending on the species. A corevmicrobiota was found that was common to more than 70% of the samples analyzed and that included microbial species that were common across all areas such as *Acinetobacter, Chryseobacterium, Moraxellaceae, and Alicyclobacillus*, although their abundances were below 10% of the microbiota. Some surfaces were contaminated by high levels of *Pseudomonas, Psychrobacter, Paracoccus* or *Kocuria.* However, basing on the composition of the microbiota, the environmental samples grouped according to the sampling time but not according to the specific area of sampling except for the case of samples from the vegetable pre-processing room that showed a higher level of similarity. Most of the microbial taxa found are not those commonly found in food as spoilers or hazardous bacteria, which indicates that food and storage conditions can be very selective in the growth of possible contaminants.

1.5.4 HTS limitations and critical issue

Since the culture-independent HTS analysis of microbiota is a quantitative method, all the possible issues that can determine an alteration of the original amount of microbial cells (or DNA extracted therefrom) in a specific food sample

must be avoided because it may lead to defective description of the microbiota. The HTS method can be considered a quantitative method because it is based on the concept that there is a proportion between abundance of a specific microorganism in the food, amount of nucleic acid extracted, amount of amplicons gained and the final number of sequences obtained belonging to the specific microorganism. Anyway, the number of sequences found is proportional to the abundance of the microorganism in question. All possible efforts are necessary to avoid the alteration of the mentioned proportion.

Sampling issues linked to unknowledge or instability of environment can occur. These kind of issues can led to the impossibility to have a sampling representativity and reproducibility, compromising the interpretation of experiments or comparative studies. Moreover, sample handling is also a frequent problem linked to the analytical approach used. After sample collection, high attention is needed during the nucleic acid extraction in order to avoide the proportion mentioned above. Every kind of alterations will result in notable changes in the proportion between sequence numbers and OTU abundance, with the alteration of the estimation of the proportions of microbial populations in the original food sample. For this reason, each step included in the sampling phase, such as aerobic or anaerobic storage, transport, freezing or chilling can influence the development of the microorganisms in the food by altering the number and species that we are going to detect.

The variability setermined by the nucleic acid extraction steps can depend by different factors. Microbial species have different sensitivity to lytic agents, the differences are mainly due to the cell wall structure. This influence the analyses based on *in situ* nucleic acid extraction because a high yield in pure DNA/RNA is required as well as the detection of all the species occurring in that sample. For this reason, depending on the complexity of the matrix, it can be more or less difficult to obtain good extraction and to discard all the contaminations that can negatively affect the PCR amplification step. In the case of food matrices this issue is really frequent; the presence of natural components such as lipids, proteins, carbohydrates and salts may convert the extraction very hard and some of the impurity can persist until the end of the extraction and be found in the extract where they might act as PCR inhibitors (Wilson & Wilson 1997). Choosing an adequate extraction procedure is consequently very important in order to havea good extraction product and provide templates from all the microbial entities occurring in the sample. Previous studies showed procedures of optimization of DNA extraction from food matrices (Pirondini et al., 2010). Even the PCR procedure can compromise the culture-independent food samples analysis.

Last, the HTS approach is deeply influenced, by the bioinformatics analysis of sequences (Scholz et al., 2012). For processing 16S amplicon HTS data, numerous open-source programs are available (Caporaso et al., 2010; Schloss et al., 2009; Meyer et al., 2008). The choice of certain bioinformatics tools in HTS-based microbial ecology has been reviewed (Zaneveld et al., 2011; Kuczynski et al., 2011). A prerogative of the data analysis is the accuracy and reliability of the final investigated composition of the food microbiota, and it is directly dependendt on the quality of the reference database used to assign the taxonomy (Tedersoo et al., 2011; Caporaso et al., 2010). There is a variety of databases for prokaryotes assign taxonomy (Pruesse et al., 2007; Cole et al., 2003) and all containing high-quality 16S rRNA gene sequences.

1.5.5 HTS approach: disantvantages and advantages

Nowdays the high-throughput sequencing method is largely employed for research laboratories in many fields. Besides, advantages and disantvantages need to be analysed in order to better evaluate the possible use of this method on large-scasle research. None of the culture-independent methods currently employed to study food products has a throughput comparable to HTS. Thousands of sequences are available from HTS analysis that can be readily analyzed to ensure swift, reliable identification of the majority of microorganisms occurring in food samples. Depending on the desired level of sample coverage, many food samples can be sequenced at the same time, saving much time compared to the approaches currently used. In addition, when microbiomes are studied by shotgun library sequencing, insights into microbial activities can be obtained from the sequences of microbial genes present in the original food sample, which offers important advances in studying microbial ecology of foods. The HTS approach entails a safer bench-activity with reduced exposure to unsafe reagents used, for example, for electrophoresis. Moreover, with some sequencing technologies or by using automated liquid handlers there is an almost negligible contribution of the operators and much bench-time is saved in the laboratories. However, the drawbacks of HTS include the need for bioinformatics analysis of data and, depending on the choice of the specific working conditions, the cost of analysis per sample. The final output of HTS is thousands of sequences that need to be studied in order to translate them into useful information for food-associated microbial ecology. The bioinformatics part of the study cannot be performed by any laboratory worker: managing large numbers of sequences does not just require simple “blast” procedures that many students have learned in molecular biology laboratories. Skilled bioinformaticians must be specially trained for this activity and therefore HTS technologies cannot just be acquired in a laboratory and used immediately. Costs of analysis are decreasing

significantly as a result of new lower-cost technologies becoming operative and competition between the different HTS platform suppliers. However, the initial cost of the equipment is rather high especially compared with the cost of electrophoretic equipment used for traditional culture-independent approaches. In light of the above considerations, it is unlikely that the food industry will readily acquire equipment and know-how to use HTS analyses of foods. The food industry will probably not need routine use of the technology and will therefore call on external services to process their own food samples under specific requirements and for specific project needs.

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2 RESULTS AND DISCUSSION

2.1 Mapping the bacterial distribution in a cooking centre for hospital foodservice

2.1.1 Introduction

Food safety is a priority for food service organizations because inappropriate handling or food spoilage can result in serious problems for both foods and consumers (Cairo et al., 2008; Egan et al., 2007; de Oliveira et al., 2014).

Every food has a specific microbiota that is strictly dependent on the nature of the raw materials, the environment in which the food is produced, and on the processing, storage and consumption conditions. During their preparation, foods can be contaminated by microorganisms from the processing and storage environments, surfaces, tools, equipment and personnel engaged in handling and production activities (Legnani et al., 2004; Worsfold & Griffith 2001).

Hospital meals have a remarkable associated safety risk because they are prepared for vulnerable people (children, elderly, pregnant women and immune-compromised people) who are more susceptible to food-borne illness than the rest of the population. Microbiological hazard is one of the most significant food safety hazards because microorganisms cannot be easily detected, they are widespread in the environment and can easily come in contact with humans through foods.

Taking into account the peculiarity of hospital catering systems, which is intended to susceptible consumers, a scrupulous compliance with European food safety regulations is essential, (EU Regulation 178/2002, 852/2004, 853/2004). In particular, the regulation (EC) 852/2004 about the hygiene of foodstuffs, lays down the requirements of the premises intended for the production of meals and equipment that come into contact with food and indicates the preventive measures and procedures for sanitizing of equipment and kitchen tools.

Colonization of foods by microorganisms depends not only on the ecological conditions that occur in the food itself, but also from the interactions between food and the environment. Mishandling of food in establishments where food is served, such as restaurants, hotels, schools and hospitals can be a remarkable issue for consumers. In the case of hospital cooking services, safety is a priority at every stage of the production from the arrival of the raw materials to the final delivery of meals to the patient (Cairo et al., 2008). Application of the principles of microbial ecology to food systems is of fundamental importance for both food quality and microbiological safety.

Food contact surfaces are good substrates for the proliferation of microorganisms. Although frequent cleaning and disinfection procedures can generally assure acceptable hygienic conditions in the food processing environments, they may fall short in the elimination of a well-developed microbiota. Such microbial populations tend to settle on sites that are especially difficult to clean due to difficult access, surface irregularities or retention of sticky raw materials. The transfer of environmental microbiota from surfaces or tools to foods, even after their hygienization, is a hazard for food quality and safety (Verran et al., 2008; Shi & Zhu 2009).

Environment plays an important role in the selection of microorganisms, and optimal conditions for microbial growth can occur in food processing. In such food handling environments, organic residues from food processing can create microenvironments for growth and accumulation of microorganisms and can represent a relevant source of cross-contamination (Brooks & Flint 2008; McLandsborough et al., 2006; Simoes et al., 2010). Operations that can determine cross-contamination during food preparation are trimming, cutting, washing, rinsing, dewatering and packaging, which are all considered as contributing to microbial food safety hazards (Gil et al., 2013). Once microbial cells are transferred from the environment or food contact surfaces to food, other ecological factors such as pH, temperature, osmolarity, O_2 availability, nutrient composition and presence of other bacteria will determine which microorganisms can develop and eventually cause concerns. Food quality and safety are fundamental in collective foodservice systems. Various bacterial species can reside in the kitchen, preparation rooms and storage facilities and can be a direct source of food contamination. This is a threatening issue especially in food services that prepare many meals per day and when the end users are categories at risk such as hospital patients. In fact, resident microbiota is not always cause of disease in healthy individuals, but it may represent a remarkable issue in immunocompromised patients and newborns (Cairo et al., 2008). The characterization of the environmental microbiota and the understanding of the correlation between ecological factors and the microbiota of food are of crucial importance for the control of food quality and safety.

Since characteristics of specific surfaces, environmental conditions and food processing can affect the structure of the microbiota it is expected that a specific resident microbiota can occur in each site of a food service facility. Although several studies have reported on the microbial contamination in mass catering establishments, and have indicated environment, equipment and personnel as key sources of meals contamination (Cairo et al., 2008; Egan et al., 2007;

Legnani et al., 2004; de Oliveira et al., 2014; Konecka-Matyjek et al., 2012), no studies have explored in depth the structure of the microbiota in foodservice environments to highlight the possible sources of contamination of the prepared meals.

Recent advances in microbial ecology have provided sensitive culture-independent tools for rapid and effective evaluation of microbial contamination in many sorts of environments (Ercolini 2013). In the specific case of foodservice kitchens, a rapid mapping of the contamination of surfaces and equipment that come in contact with food can be of great interest in order to identify possible sources of food contamination and to evaluate the effectiveness of cleaning procedures.

In this study, the environmental microbiota in a hospital foodservice was investigated over two separate days of production by using a culture-independent amplicon sequencing approach in order to describe the potential sources of contamination during food handling and preparation.

2.1.2 Materials and methods

Sampling

Two separate environmental sampling (December 2013 and two months after) in the same hospital cooking centre were performed. Across all area, surface swab-samples from work surfaces of different zones were taken: food pre-processing rooms (dedicated to fish, vegetables, red and white meat), storage room and kitchen (Figure 1). The sampling took place on cleaned surfaces, 2 h after the routine cleaning and more precisely at the time when they were ready to come in contact with food for the processing. The sampling was performed after the cleaning in order to avoid an overestimation of the microbial diversity that could come in contact with food during preparation. In addition, the samplings were performed in days when all the pre-processing rooms had been previously used for food preparation. Surfaces were sampled with sterile cotton-tipped swab that was moistened with sterile PBS and rubbed vertically, horizontally and diagonally across the sampling site (100 cm^2) delineated by a template, rotating the swab to ensure full contact of all parts of the swab tip and surface (Bokulich & Mills 2013a)

DNA extraction

After collection, swabs were placed into sterile 10 ml polyethylene tube containing 1 ml of sterile PBS, cooled at 4°C for the necessary time of transport to laboratory and analyzed within 3 hours. Prior to DNA extraction, tubes were vigorously stirred in a vortex to transfer the cells from the swab to solution. Total DNA extraction from the swab samples was carried out by using a Biostic Bacteremia DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). The extraction protocol was applied to the pellet (12,000 x g) obtained from 2 ml of suspension.

16S rRNA gene amplicon library preparation and sequencing

The microbial diversity was studied by pyrosequencing of the amplified V1–V3 region of the 16S rRNA gene amplifying a fragment of 520 bp by using primers Gray28F 59-TTTGATCNTGGCTCAG and Gray519r 59-GTNTTACNGCGGCKGCTG (Ercolini et al.,, 2012). 454-adaptors were included in the forward primer followed by a 10 bp sample-specific Multiplex Identifier (MID). Each PCR mixture (final volume, 50 ml) contained 60 ng of template DNA, 0.4 mM of each primer, 0.50 mmol l21 of each deoxynucleoside triphosphate, 2.5 mmol l21 MgCl2, 5 ml of 10 PCR buffer and 2.5 U of native Taq polymerase (Invitrogen, Milano, Italy). The following PCR conditions were used: 94°C for 2 min, 35 cycles of 95°C for 20 s, 56°C for 45 s and 72°C for 5 min, and a final extension at 72°C for 7 min. After agarose gel electrophoresis, PCR products were purified twice by Agencourt AMPure kit (Beckman Coulter, Milano, Italy), quantified using the QuantiFluorTM (Promega, Milano, Italy) and an equimolar pool was obtained prior to further processing. The amplicon pool was used for pyrosequencing on a GS Junior platform (454 Life Sciences, Roche, Italy) according to the manufacturer's instructions by using a Titanium chemistry (Ercolini et al., 2012).

Bioinformatics and data analysis

In order to remove sequences of poor quality, 16S rRNA raw reads were first filtered according to the 454 processing pipeline. Sequences were then analyzed and further filtered by using QIIME 1.8.0 software (Caporaso et al., 2010)and a pipeline previously described (De Filippis et al., 2014). Briefly, raw reads were demultiplexed and further filtered through the splitlibrary.py script of QIIME. To guarantee a higher level of accuracy in terms of Operational Taxonomic Units (OTUs) detection, the reads were excluded from the analysis if they had an average quality score lower than 25, if there were ambiguous base calls, if there were primer mismatches and if they were shorter than 300 bp. Sequences that passed the quality filter were chosen and singletons were excluded. OTUs defined by a 97% of similarity were picked using the uclust method (Di Bella et al., 2013) and the representative sequences were submitted to the RDPII classifier (Wang et al., 2007)to obtain the taxonomy assignment and the relative abundance of each OTU using the Greengenes

16S rRNA gene database (McDonald et al., 2012). Depending on sequence length and matches with the database, species-level identifications were attempted when possible. Alpha diversity analysis was performed through QIIME to investigate the diversity within the samples, generating Good's coverage, Chao1 richness (Chao and Bunge, 2002) and Shannon diversity indices (Shannon and Weaver, 1949). Beta diversity was evaluated by weighted and unweighted UniFrac analysis (Lozupone and Knight, 2005) and Principal Coordinates Analysis (PCoA) was performed to show the diversity between the samples according to their specific microbiota. The core microbiota occuring in the environments analyzed was defined at 70%. Venn diagrams were obtained by using the Bioinformatics & Evolutionary Genomics software (Shade & Handselman 2012).

2.1.3 Results

A total of 278,347 reads passed the filters applied through the QIIME splitlibrary.py script, with an average value of 3,303 reads/sample and an average length of 442 bp. The number of OTUs, the Good's estimated sample coverage (ESC), the Chao1 and Shannon indices were obtained for all the samples (Table 1). Overall, the ESC indicated that a satisfactory coverage was reached for most of the samples in the two experiments. The microbial diversity in the different samples was quite heterogeneous. Some were satisfactory covered with about 2000 reads, such as PPRCutter and KBalance while others required more than 3000 sequences to reach an ESC > 95%, such as the sink in the PPW and sink samples. The diversity indices varied much depending on the samples and there was no significant association between areas and alpha diversity parameters ($P>0.05$).

In order to assess the differences between samples according to their microbiota, Principal Coordinate Analysis (PCoA) was implemented according to the calculated UniFrac distance matrices. At first, UniFrac analysis was performed separately for each sampling and showed that in both experiments the samples did not cluster according to the zones except for the PPV samples, which in both cases showed a trend to group more closely compared to other zones (Figure 2). Moreover, the weighted and unweighted UniFrac analyses were also performed including all the samples and it was shown that samples from the two sampling experiments showed a certain degree of separation on the basis of their microbiota (Figure 3). Overall, a core microbiota, including OTUs occurring in 70% of the samples, was shared among the samples (Table 2). The number of OTUs shared between the different zones is represented in Figure 4. As shown in the Venn Diagram, 19 OTUs (Figure 4A) were shared between all the zones of the pre-processing area but only *Acinetobacter, Acinetobacter johnsonii* and *Alyciclobacillus* showed an average relative abundance higher than 4% (Table 2). Thirteen OTUs were common to fish and white meat pre-processing areas, 11 of which were also common to the red meat zone (Figure 4A). Similarly, 32 OTUs were shared by the kitchen, storage area and tools (Figure 4B), with *Acinetobacter johnsonii, Alyciclobacillus* and *Chryseobacterium,* being the most abundant (Table 2). Finally, a total of 14 species occurred in all the samples (Figure 4C) although the average relative abundance was never higher than 10% except in the case of *Acinetobacter johnsonii* (Table 3).

The abundance of microbial genera and species can be observed in the Tables 4 and 5. Only some members of the core microbiota occurred with a relative abundance >0.01%. *Paracoccus* occurred in both sampling experiments but with a different distribution across the zones: a predominance was found in the PPV (70 and 80%, in the first and second sampling respectively), followed by a remarkable presence in the PPF zone (40 and 60% in the first and second sampling respectively). *Psychrobacter* was found in both experiments with a variable abundance ranging from 0.2 to 34% in the zones S, K, T, PPR, and PPV with the highest relative abundance in the KBalance and in the TSteel Tray in the first and second experiment, respectively. Although it was more frequently found in the second sampling, *Acinetobacter johnsonii* was also among the most abundant OTUs shared in both experiments for the zones CS, PPV, K and T. It was more abundant in the TColander in both samplings and dominated in several samples in the second experiment such as PPVCuttingBoard and TSteelShovel (Table 4 and 5). *Kocuria* was found in all the Sink samples belonging of the Pre-Processing zones, although with a variable relative abundance ranging from 2 to 49%. Moreover, *Acinetobacter,* occurred in the Sink and Workbench samples belonging to PPV and PPW with an abundance above 40% regardless of the sampling. Similarly, *Pseudomonas* was found in the Sink and Workbench samples belonging to K, PPR and PPV zones (first experiment) and PPV zone (second experiment). A higher presence of *Pseudomonas* was found on the PPRCutter in the first experiment as well as the BCWall and KBalance in the second experiment. Many samples contained a 30% average of *Massilia timonae* such as the Sink and Workbench samples of PPF and PPW zones, PPVCuttingBoard and TKnife. Finally, some OTUs were characteristic of specific samplings. For example, *Brochothrix* occurred only in the second sampling and it was particularly abundant in CSShelf sample. Similarly, *Serratia* was found only in the first experiment in more than 50% of the samples. A relevant abundance of *Alyciclobacillus* occurred mostly in the first sampling for all the zones especially in the T, BC and CS samples, while *Streptococcus* prevailed in T samples.

2.1.4 Discussion

In this study, the biogeographical distribution of the microbiota in a hospital cooking centre was studied by 16S-based culture-independent high-throughput amplicon sequencing. In order to obtain a mapping of the potential sources of contamination, food contact surfaces were examined. In accordance with European Regulation (EC) No.2073/2005, food-contact surfaces are a major concern for foodservice facilities. Food service areas are considered critical to health, and the evaluation of their microbiological quality is recognized as fundamental (Konecka-Matyjek et al., 2012). A detailed analysis of all the spaces used for the food processing is necessary to focus on that factors that may lead to bacterial contamination of the surfaces, and subsequently of the food, finally affecting the hygienic safety of the meals prepared at mass catering establishments (Legnani et al., 2004). In previous studies, the microbiological quality of surfaces was recognized as a useful indicator for the control of the procedures of cleaning and disinfection (Legnani et al., 2004; de Oliveira et al., 2014). Environmental swabs from different zones including food pre-processing rooms, storage room, kitchen, cold storage and blast chiller were studied. The microbiota of the environmental samples was very complex, including more than 500 OTUs in total. Accordingly, other studies describing the microbial community across home's surfaces reported that the microbiota from the kitchen environments had the highest variability in composition (Dunn et al., 2013).

Despite the overall high diversity found, the composition of the microbiota was related to the specific surfaces sampled. This is basically due to characteristics of the surfaces, ecological factors, nutrient composition and cleaning procedures (Flores et al., 2013; Scott 2000; Medrano-Felix et al., 2011), and it is also influenced by the architecture of the spatial structure (Kembel et al.,, 2012). These are all factors affecting the possibility for the bacteria to develop and become resident. In this study, the majority of microorganisms belonged to phyla generally identified as dominant in indoor environments: *Actinobacteria, Firmicutes* and *Proteobacteria* were all found as abundant in other studies that sampled indoor surfaces (Aydogdu et al., 2010; Flores et al., 2011; Kembel et al., 2014). Microbiological contamination of food production utensils and other equipment may be caused by ineffective methods of cleaning (Moore & Griffith 2002). Thus food production utensils, other equipment, and all the hard-to-reach places can be a potential risk of food contamination, directly through their contact with food or indirectly via the hands of personnel or other potentially clean surfaces (Konecka-Matyjek et al., 2012). Interestingly, the storage zones showed a high microbial diversity, which can be associated to a less frequent cleaning compared to other zones (Dunn et al., 2013) and to the material of the surfaces composing the storage cells as previously reported (Flores et al., 2013). According to the literature, these differences may result from the different ways to implement good hygienic practices (GHP) and Hazard Analysis and Critical Control Points (HACCP) across the different zones during the different cleaning routines (Legnani et al., 2004). Furthermore, a low microbial diversity (especially in terms of number of OTUs) was associated to the steel surfaces such as KBalance, PPRCutter, TSteel Shovel, TSteel Tray, suggesting that those surfaces can be more efficiently cleaned. The results indicate that the level of contamination and the alpha diversity are extremely variable and could be indeed influenced by the surface material and cleaning frequency and procedures, which is a fundamental issue for the maintenance of standard hygiene in the food services.

In agreement with previous reports (Grice et al.,, 2011), members of the core microbiota such as *Probionbacterium, Corynebacterium, Staphylococcus* and *Streptococcus* can be all associated to human skin. The presence of skin-associated bacteria confirms that human or animal skin can be an important source of food contamination from the environment. Improper practices of food manipulation, and poor microbiological quality of the final products can be related to hygiene of personnel; therefore, food hygiene training for operators (Richardson & Stevens 2003)can be considered as one strategy whereby food safety can be increased (Doyle & Erickson 2006). *Acinetobacter, Pseudomonas* and *Psychrobacter* can be involved in food spoilage, and they are recognized as undesired bacteria especially in fresh meat (Doulgeraki et al., 2012). In this study, these bacteria were found to be abundant in several samples confirming some of our previous evidence that suggested they could be members of the resident microbiota of processing plants (De Filippis, La Storia, et al., 2013). Other studies also demonstrate that different resident microbial populations contaminate environments in the same food facilities (Flores et al., 2013). However, the relative abundance of these bacteria was not constant in the two samplings and was not always found in the same category of surfaces.

The beta diversity analysis highlighted that there was no clustering of the samples according to the specific zone of origin except for the PPV zone whose samples tended to group together on the basis of their microbiota. In the case of the PPV zone, the similarity of the microbiota could derive from the specific raw materials or be due to an establishment of a resident microbiota in that specific environment. The fact that a zone-specific microbiota could not be observed suggests that the microbial biogeography in such environments is determined not only by the specific food matrix that is manipulated in a specific area but again to other factors (surface composition, ecological factors, nutrient availability, cleaning frequency) that play a fundamental role in defining the microbiota. Therefore, it can be speculated that the microbial composition of each single microenvironment is only partially affected by the food handled.

The environmental swabs analyzed in this study were taken after the routine cleaning. We found some microorganisms (e.g*., Kocuria palustris, Pseudomonas, Paracoccus, Psychrobacter*) in areas that were geographically distant. The dissemination across different areas can be promoted by the aerosolized bacteria (Dunn et al., 2013; Burroes et al.,, 2009). In addition, cleaning procedures can have a very strong impact on the spatial distribuition of the microbial communities as the use of the same cleaning tools can be even a possible vector of bacterial diffusion. This will depend on the composition of the cleaning tools, level of humidity, detergents used, availability of nutrients and frequency of their washing that could all affect the microbial proliferation and dissemination (Medrano-Felix et al., 2011; Mattick et al.,, 2003). Therefore, frequent washings or use of disposable items would be highly recommended for the cleaning practices in food service environments. Accordingly, the Unifrac analysis also showed the dissimilarity between the two different samplings. In fact, the samples from the two samplings tended to be distinguished and this result further supports the role of cleaning procedures on the environmental microbiota. The dissimilarity between samples is even reflected in occurrence of some specific OTUs such as *Serratia* that was only found during the first and *Brochothrix* during the second sampling. Variation in occurrence of such spoilage-related species can be an important factor in determining the potential of food contamination and possible spoilage. In our specific case, the preparation tools were used in more areas of the plant. Therefore, if not properly sanatized, they could also be a source of bacterial transfer between different zones. Regardless of the sources, most of the bacterial species found in this study as part of the core microbiota or that were remarkably abundant in some of the surface samples are not all considered as food spoilers and none of them is a foodborne pathogen. The sensitivity of the deep sequencing would have allowed the detection of even sub-dominant populations including pathogens (Ercolini 2013). Although the technique as used in this research is DNA-based and does not allow speculating on the viability of the microbial populations detected, we decided to avoid any selective culturing of pathogens as they were not part of the contaminant microbiota shown by the sequencing results. In future screenings of food preparation rooms or food related environments, such culture-independent approach could be very useful to get a rapid map of the surface contamination in the plant and the analysis could represent a good start point to map the sources of contamination to be necessarily followed by culture-dependent selective identification of pathogens in case they are detected in the environmental samples.

2.1.5 Conclusion

In conclusion, provided that adequate cleaning procedures are applied, environmental contamination in foodservice might not necessarily have a dramatic impact on food quality. The food matrix, cooking procedures and storage conditions can be extremely powerful in selecting the microbiota that can finally affect food quality and safety.

2.1.6 References

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Figures and Tables

Figure 1 – Map of the hospital cooking centre. S, storage area; K, kitchen area; PPF, Fish pre-processing area, PPR, red meat pre-processing area; PPW, white meat pre-processing area; PPV, vegetables pre-processing area; T, tools.

Figure 2

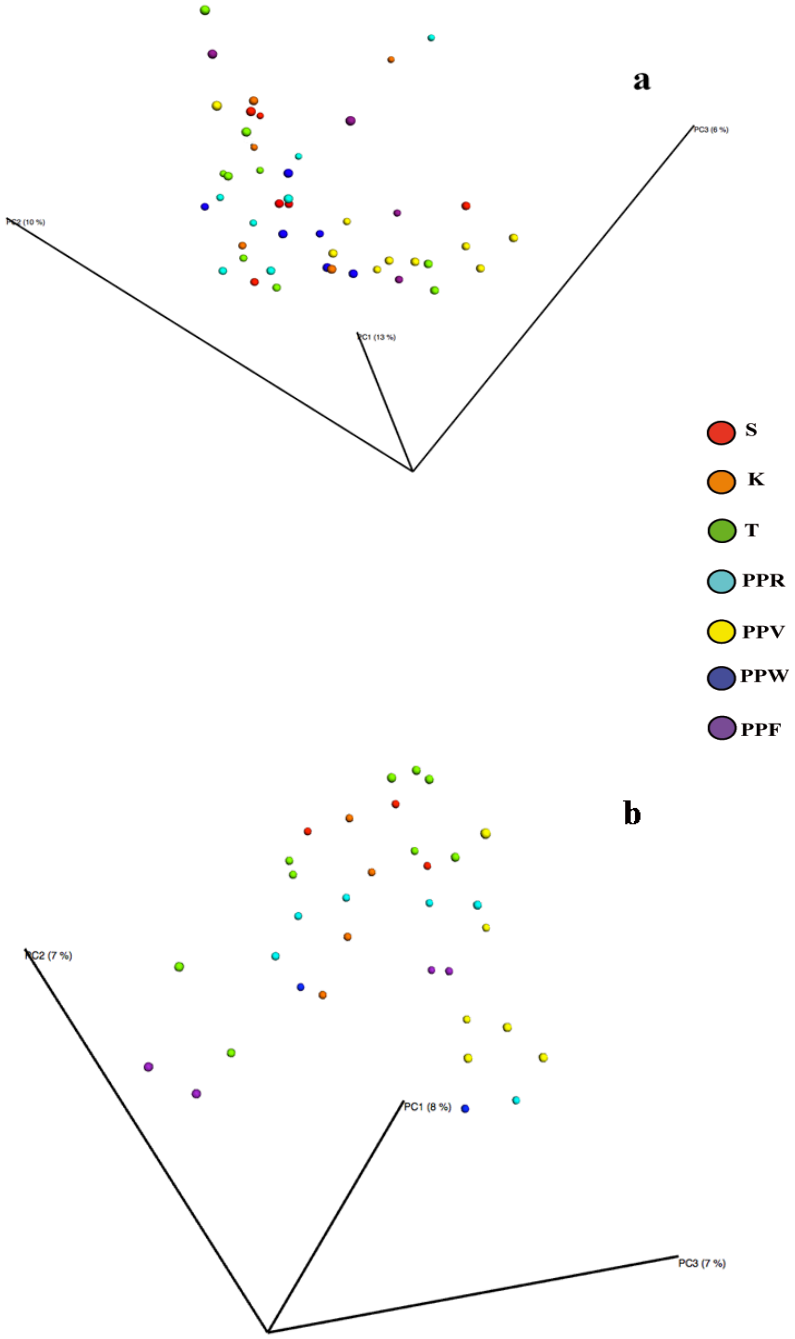

Figure 2 - Principal Coordinates Analysis of unweighted UniFrac distances for 16S rRNA gene sequence data. Samples are labeled with different colors according to the zone. Panel a, samples from experiment 1; Panel b, samples from experiment 2. PC1, PC2 and PC3 along the three axes display the amount of variance in the samples explained by these coordinates.

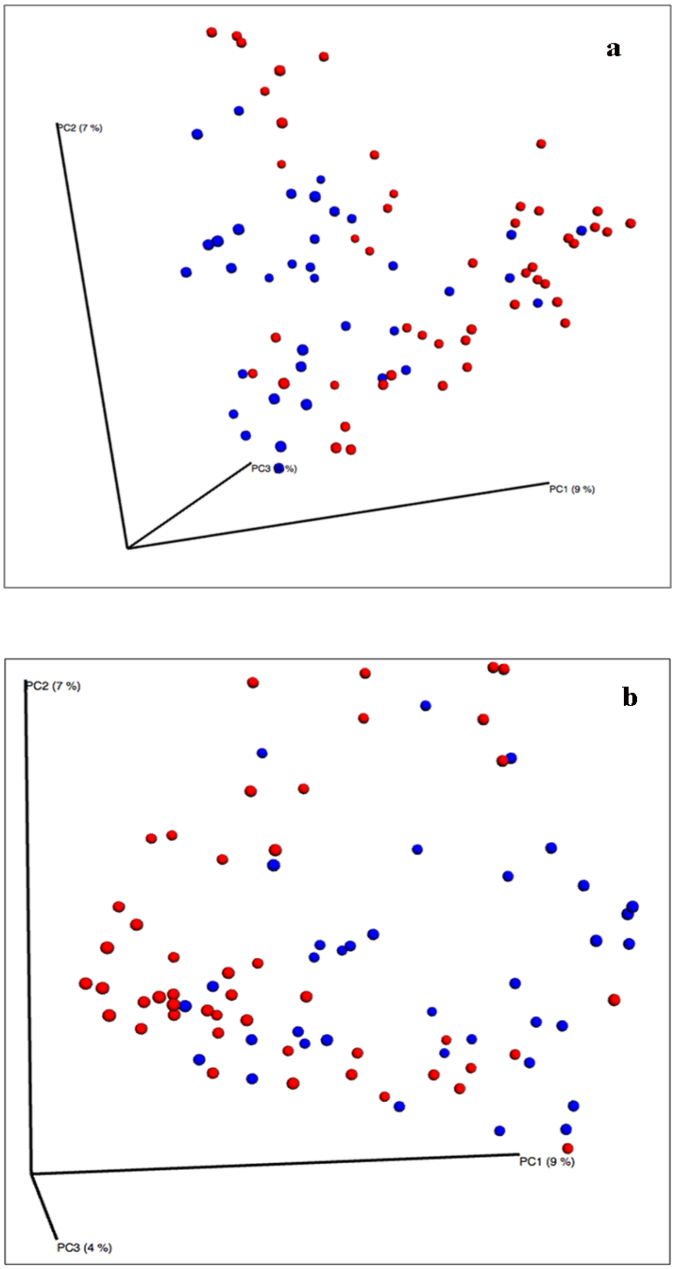

Figure 3

Figure 3 - Principal Coordinates Analysis of weighted (panel a) and unweighted (panel b) Unifrac analysis performed including all the samples from first (red dots) and second (blue dots) sampling. PC1, PC2 and PC3 along the three axes display the amount of variance in the samples explained by these coordinates.

Figure 4

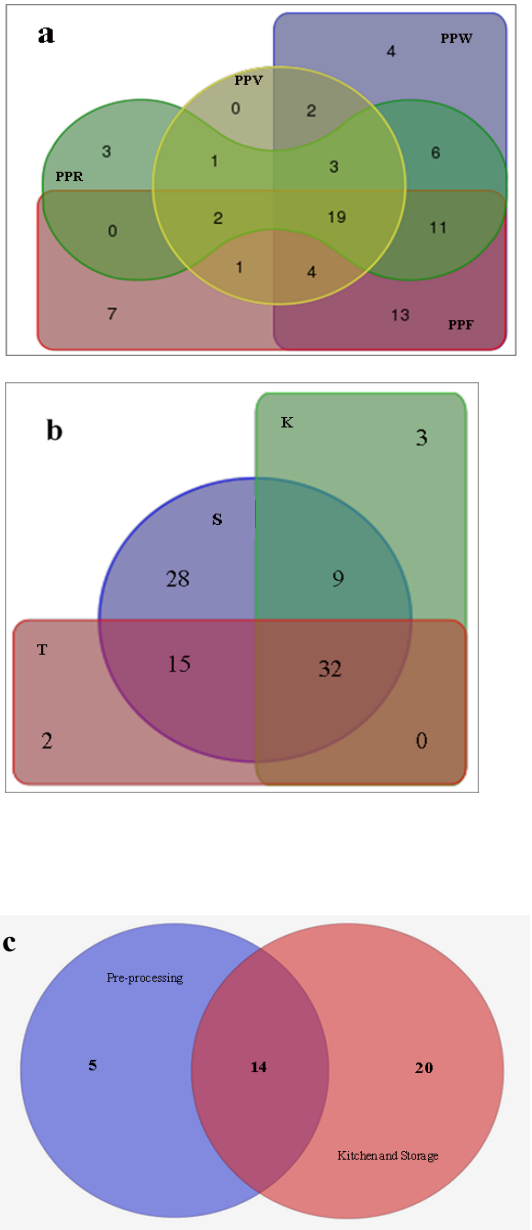

Figure 4 – Venn Diagramm showing the number of shared OTUs between zones belonging to the pre-processing area (panel a), kitchen and storage areas (panel b) and their combination (panel c). S, storage area; K, kitchen area; PPF, Fish pre-processing area, PPR, red meat pre-processing area; PPW, white meat pre-processing area; PPV, vegetables pre-processing area; T, tools.

		Sampling 1					Sampling 2				
Environment	**Sample**	**Reads**	**OTUs**	**Chao1**	**Shannon**	**ESC**	**Reads**	**OTUs**	**Chao1**	**Shannon**	**ESC**
Storage (S)	Blast chiller Wall	2503	179	221,63	4,50	98%	3992	121	176,04	1,78	99%
S	Cold storage Wall	2110	170	200,59	4,80	97%	6067	644	864,07	6,40	96%
S	Cold storage Shelf	3210	136	160,29	3,88	99%	7819	217	308,74	3,10	99%
Kitchen (K)	Balance	4397	70	91,00	2,81	99%	6955	91	106,12	2,08	100%
K	Sink	2505	168	210,10	4,39	98%	3730	427	653,71	5,97	95%
K	Workbench	2275	85	115,27	2,98	98%	2542	324	577,90	5,50	94%
Pre-Processing Fish (PPF)	Sink	2358	101	148,04	3,03	98%	2534	338	492	5,96	92%
PPF	Workbench	2092	178	312,17	5,59	97%	3012	252	379,23	5,38	93%
Pre-Processing Red Meat (PPR)	Cutter	3177	40	53,46	2,24	100%	5945	474	656,84	5,78	97%
PPR	Sink	2834	110	165,31	3,04	99%	6142	393	561,36	4,68	97%
PPR	Workbench	4948	97	133,63	2,59	98%	4201	390	616,25	5,46	96%
Pre-Processing Vegetables (PPV)	Cutting Board	2605	92	114,24	4,53	98%	1471	202	296,89	5,97	94%
PPV	Grinder	2688	106	132,70	3,70	99%	1780	140	248,04	2,75	96%
PPV	Sink	2998	100	125,00	3,75	99%	2163	284	413,72	5,72	94%
PPV	Workbench	967	150	186,49	5,16	95%	1732	385	626,93	7,03	89%
Pre-Processing White Meat (PPW)	Sink	2568	153	193,04	4,79	97%	4776	392	591,08	5,63	95%
PPW	Workbench	2391	213	254,28	4,98	98%	1732	228	354,98	5,55	94%
Tools (T)	Colander	2480	78	141,91	1,76	98%	1516	252	428,70	6,23	92%
T	Cutting Board	1659	69	94,30	3,48	99%	1402	265	440,00	6,09	91%
T	Knife	3376	44	64,00	2,21	99%	978	247	511,00	6,12	85%
T	Pan	2347	150	172,44	3,74	99%	1857	227	367,93	5,16	94%
T	Steel Dish	1934	145	172,98	4,82	99%	19689	435	592,14	4,23	99%
T	Steel Pincers	1759	155	197,09	5,28	97%	2250	358	594,00	6,34	92%
T	Steel Shovel	4943	99	164,33	2,74	97%	1930	207	406,24	5,64	94%

Table 2 – Mean relative abundances of the core OTUs identified from the pre-processing areas and kitchen and storage areas[1].

	Core microbiota of Pre-Processing Zones			Core microbiota of Storage area, Kitchen and tools		
OTU	**Min (%)**	**Max (%)**	**Average**	**Min (%)**	**Max (%)**	**Average**
Acidovorax	-	-	-	0,024	0,23	0,127
Acinetobacter	2,8	7,86	5,33	0,26	8,87	4,565
Acinetobacter Johnsonii	0,63	7,37	4	0,29	26,93	13,61
Actinobacteria	0,05	0,61	0,33	0,007	0,45	0,2285
Alphaproteobacteria	0,06	0,27	0,165	-	-	-
Alyciclobacillus	1,34	7,36	4,35	1,43	15,8	8,615
Bacillus	-	-	-	0,02	0,12	0,07
Brevundimonas	0,13	0,6	0,365	-	-	-
Caulobacteraceae	0,25	1,91	1,08	-	-	-
Chryseobacterium	1,64	5,87	3,755	0,15	6,78	3,465
Corynebacterium	-	-	-	0,13	0,57	0,35
Enterococcus lactis	-	-	-	0,08	0,56	0,32
Flavobacteriaceae	0,06	0,46	0,26	0,01	1,04	0,525
Jonesiaceae	-	-	-	0,31	2,02	1,165
Kocuria	-	-	-	1,08	0,59	0,835
Kocuria palustris	0,23	4,49	2,36	0,08	1,87	0,975
Lactococcus	-	-	-	0,003	0,01	0,0065
Microbacteriaceae	-	-	-	1,07	9,59	5,33
Microbacterium	-	-	-	0,18	0,01	0,095
Micrococcaceae	0,024	0,45	0,237	-	-	-
Micrococcus	-	-	-	2,61	0,003	1,3065
Moraxellaceae	0,01	7,37	3,69	0,02	1,89	0,955
Neisseria	0,001	0,59	0,2955	0,06	0,72	0,39
Pedobacter cryoconitis	0,001	1,87	0,9355	0,004	0,005	0,0045
Porphyromonas	-	-	-	0,02	0,05	0,035
Propionibacterium	-	-	-	0,57	3,38	1,975
Pseudoxanthomonas	0,009	0,06	0,0345	0,002	0,003	0,0025
Ralstonia	0,005	0,23	0,1175	0,007	0,31	0,1585
Rhodococcus	0,009	0,02	0,0145	0,003	0,41	0,2065
Sphingomonadaceae	-	-	-	0,049	0,14	0,0945
Stenotrophomonas	0,15	0,64	0,395	0,024	0,21	0,117
Streptococcaceae	-	-	-	0,01	0,22	0,115
Streptococcus	0,06	0,41	0,235	0,02	0,84	0,43
Streptococcus sanguinis	0,12	2,25	1,185	1,65	4,45	3,05
Streptococcus termophilus	-	-	-	0,09	0,36	0,225

[1]The microbial species in this table are those identified as members of the core microbiota as reported in Figure 4A and 4B.

Table 3 - Mean relative abundances of the core OTUs **that were shared by all the areas.**

OTU	Min (%)	Max (%)	Average
Acinetobacter	0,26	8,87	4,565
Acinetobacter johnsonii	0,29	26,93	13,61
Actinobacteria	0,007	0,61	0,3085
Alicyclobacillus	1,34	15,8	8,57
Chryseobacterium	0,15	6,78	3,465
Kocuria palustris	0,08	4,49	2,285
Moraxellaceae	0,01	7,37	3,69
Neisseria	0,001	0,72	0,3605
Pedobacter cryoconitis	0,001	1,87	0,9355
Pseudoxanthomonas	0,002	0,06	0,031
Ralstonia	0,005	0,31	0,1575
Rhodococcus	0,003	0,41	0,2065
Stenotrophomonas	0,02	0,64	0,33
Streptococcus sanguinis	0,12	4,45	2,285

[1]The microbial species in this table are those identified as members of the core microbiota as reported in Figure 4C.

Table 4
Abundance of bacterial taxa in environmental swabs from the hospital cooking center in sampling 1. Only OTUs with >0.1% abundance in more than 20% of the samples are represented.

Taxon	S_BlastChiller_Wall	S_ColdStorage_Shelf	S_ColdStorage_Wall	K_Balance	K_Sink	K_Workbench	PPF_Sink	PPF_Workbench	PPR_Cutter	PPR_Sink	PPR_Workbench	PPV_CuttingBoard
Acinetobacter	0.003	0.007	0.001	0.004	0.009	0.001	0.000	0.012	0.001	0.017	0.001	0.000
Acinetobacter johnsonii	0.004	0.071	0.007	0.005	0.096	0.001	0.001	0.009	0.001	0.079	0.001	0.000
Alicyclobacillus	0.266	0.029	0.127	0.000	0.020	0.041	0.030	0.107	0.000	0.021	0.049	0.001
Caulobacter	0.001	0.002	0.001	0.000	0.001	0.000	0.002	0.008	0.000	0.001	0.000	0.046
Chryseobacterium	0.000	0.002	0.000	0.001	0.030	0.000	0.000	0.010	0.000	0.014	0.000	0.003
Corynebacterium	0.003	0.001	0.007	0.000	0.009	0.000	0.002	0.000	0.000	0.002	0.000	0.001
Enterococcus lactis	0.000	0.006	0.002	0.001	0.017	0.000	0.000	0.000	0.022	0.040	0.000	0.000
Kocuria	0.011	0.058	0.042	0.000	0.024	0.001	0.116	0.040	0.000	0.070	0.031	0.006
Kocuria marina	0.000	0.002	0.000	0.000	0.000	0.000	0.002	0.002	0.000	0.001	0.002	0.000
Kocuria palustris	0.006	0.010	0.006	0.000	0.012	0.000	0.006	0.000	0.000	0.028	0.002	0.000
Lactobacillus	0.004	0.000	0.007	0.000	0.002	0.001	0.015	0.004	0.000	0.001	0.000	0.000
Massilia timonae	0.004	0.003	0.001	0.000	0.007	0.000	0.100	0.004	0.000	0.000	0.000	0.091
Moraxellaceae	0.000	0.002	0.000	0.000	0.005	0.000	0.006	0.003	0.000	0.002	0.000	0.000
Paracoccus	0.017	0.007	0.009	0.000	0.018	0.000	0.084	0.004	0.003	0.003	0.001	0.104
Propionibacterium	0.025	0.004	0.009	0.000	0.001	0.006	0.004	0.015	0.000	0.001	0.002	0.000
Pseudomonas	0.028	0.024	0.011	0.241	0.258	0.273	0.001	0.002	0.426	0.010	0.603	0.001
Psychrobacter	0.110	0.190	0.005	0.316	0.023	0.236	0.093	0.001	0.110	0.192	0.000	0.000
Ralstonia	0.005	0.000	0.006	0.000	0.001	0.004	0.003	0.007	0.001	0.001	0.001	0.000
Serratia	0.007	0.000	0.019	0.000	0.001	0.002	0.002	0.004	0.000	0.003	0.001	0.000
Sphingomonas	0.006	0.001	0.006	0.000	0.002	0.000	0.015	0.000	0.000	0.001	0.001	0.057
Staphylococcus	0.005	0.008	0.003	0.000	0.007	0.002	0.002	0.000	0.000	0.004	0.000	0.000
Streptococcus	0.002	0.003	0.012	0.000	0.002	0.001	0.004	0.013	0.000	0.002	0.000	0.000
Streptococcus sanguinis	0.051	0.018	0.140	0.000	0.030	0.011	0.018	0.035	0.006	0.070	0.007	0.001
Streptococcus thermophilus	0.005	0.002	0.005	0.000	0.003	0.002	0.002	0.004	0.001	0.004	0.000	0.000
Other	0.018	0.047	0.022	0.011	0.023	0.016	0.023	0.046	0.010	0.014	0.009	0.016

PPV_Grinder	PPV_Sink	PPV_Workbench	PPW_Sink	PPW_Workbench	T_Colander	T_CuttingBoard	T_Knife	T_Pan	T_SteelDish	T_SteelPincers	T_SteelShovel	T_SteelTray
0.015	0.082	0.010	0.018	0.026	0.007	0.001	0.000	0.001	0.000	0.003	0.392	0.001
0.001	0.031	0.033	0.033	0.100	0.793	0.000	0.001	0.012	0.001	0.004	0.004	0.003
0.001	0.000	0.166	0.092	0.210	0.036	0.000	0.005	0.313	0.002	0.121	0.020	0.058
0.001	0.022	0.002	0.005	0.006	0.000	0.000	0.000	0.004	0.000	0.001	0.001	0.003
0.017	0.029	0.005	0.006	0.027	0.005	0.007	0.000	0.000	0.002	0.001	0.006	0.000
0.009	0.000	0.002	0.010	0.002	0.001	0.003	0.005	0.002	0.000	0.001	0.001	0.003
0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.002	0.001	0.000	0.018	0.007	0.000
0.076	0.000	0.008	0.086	0.049	0.000	0.019	0.322	0.002	0.014	0.010	0.001	0.002
0.002	0.000	0.000	0.001	0.002	0.001	0.001	0.005	0.000	0.000	0.001	0.000	0.000
0.198	0.000	0.001	0.022	0.005	0.000	0.000	0.005	0.000	0.002	0.001	0.000	0.001
0.000	0.000	0.011	0.033	0.004	0.001	0.002	0.001	0.023	0.000	0.003	0.001	0.007
0.049	0.137	0.002	0.061	0.006	0.001	0.000	0.000	0.000	0.000	0.001	0.001	0.000
0.001	0.002	0.002	0.043	0.017	0.001	0.101	0.024	0.000	0.000	0.004	0.001	0.001
0.023	0.088	0.009	0.004	0.013	0.000	0.000	0.000	0.002	0.079	0.001	0.012	0.002
0.010	0.000	0.034	0.004	0.008	0.003	0.000	0.000	0.016	0.000	0.010	0.003	0.034
0.001	0.040	0.038	0.007	0.007	0.019	0.092	0.029	0.006	0.000	0.003	0.004	0.002
0.094	0.000	0.008	0.005	0.001	0.004	0.276	0.035	0.000	0.001	0.011	0.001	0.002
0.000	0.000	0.008	0.001	0.009	0.000	0.000	0.000	0.011	0.000	0.007	0.001	0.032
0.000	0.000	0.016	0.001	0.007	0.001	0.000	0.000	0.043	0.000	0.006	0.000	0.003
0.000	0.026	0.003	0.007	0.005	0.001	0.000	0.000	0.004	0.033	0.000	0.001	0.001
0.000	0.000	0.010	0.014	0.004	0.001	0.000	0.000	0.004	0.000	0.001	0.000	0.004
0.000	0.000	0.015	0.005	0.003	0.002	0.000	0.000	0.011	0.000	0.028	0.002	0.032
0.001	0.000	0.088	0.026	0.058	0.032	0.100	0.017	0.267	0.001	0.052	0.032	0.076
0.000	0.000	0.008	0.003	0.005	0.001	0.003	0.000	0.013	0.000	0.007	0.005	0.006
0.031	0.019	0.028	0.025	0.020	0.011	0.014	0.014	0.006	0.049	0.030	0.012	0.017

Table 5
Abundance of bacterial taxa in environmental swabs from the hospital cooking center in sampling 2. Only OTUs with >0.1% abundance in more than 20% of the samples are represented.

Taxon	S_BlastChiller_Wall	S_ColdStorage_Shelf	S_ColdStorage_Wall	K_Balance	K_Sink	K_Workbench	PPF_Sink	PPF_Workbench	PPR_Cutter	PPR_Sink	PPR_Workbench	PPV_CuttingBoard
Acinetobacter	0.000	0.000	0.007	0.000	0.056	0.005	0.017	0.032	0.031	0.006	0.159	0.133
Acinetobacter johnsonii	0.001	0.001	0.024	0.000	0.032	0.002	0.005	0.014	0.020	0.023	0.319	0.656
Alicyclobacillus	0.001	0.004	0.067	0.000	0.002	0.076	0.000	0.015	0.000	0.005	0.001	0.000
Brochothrix	0.002	0.285	0.013	0.024	0.000	0.005	0.000	0.003	0.004	0.030	0.000	0.000
Caulobacteraceae	0.000	0.000	0.001	0.000	0.007	0.004	0.000	0.002	0.000	0.039	0.001	0.000
Chryseobacterium	0.000	0.000	0.004	0.000	0.053	0.000	0.036	0.034	0.002	0.039	0.020	0.000
Corynebacterium	0.000	0.001	0.012	0.000	0.000	0.016	0.000	0.001	0.023	0.000	0.007	0.000
Kocuria	0.009	0.002	0.158	0.000	0.004	0.015	0.488	0.006	0.068	0.230	0.003	0.000
Kocuria palustris	0.001	0.001	0.051	0.000	0.023	0.004	0.012	0.000	0.007	0.056	0.000	0.000
Massilia timonae	0.001	0.000	0.002	0.000	0.008	0.033	0.061	0.032	0.001	0.097	0.008	0.009
Microbacterium	0.000	0.000	0.006	0.000	0.005	0.001	0.000	0.029	0.000	0.022	0.001	0.000
Moraxellaceae	0.000	0.000	0.003	0.000	0.036	0.007	0.001	0.000	0.035	0.039	0.081	0.009
Paracoccus	0.000	0.000	0.057	0.000	0.007	0.042	0.042	0.209	0.018	0.054	0.023	0.000
Propionibacterium	0.003	0.003	0.046	0.000	0.001	0.033	0.000	0.008	0.000	0.001	0.000	0.000
Pseudomonas	0.794	0.020	0.020	0.951	0.005	0.005	0.038	0.006	0.012	0.016	0.002	0.022
Psychrobacter	0.000	0.069	0.004	0.000	0.161	0.145	0.041	0.026	0.224	0.028	0.003	0.002
Rhodobacteraceae	0.000	0.000	0.006	0.000	0.002	0.000	0.000	0.015	0.000	0.016	0.023	0.001
Sphingobacterium faecium	0.000	0.000	0.000	0.000	0.008	0.000	0.058	0.037	0.002	0.000	0.000	0.000
Sphingomonas	0.003	0.000	0.002	0.000	0.001	0.000	0.001	0.056	0.000	0.004	0.001	0.000
Stenotrophomonas	0.000	0.000	0.000	0.000	0.002	0.000	0.020	0.009	0.006	0.004	0.003	0.000
Other	0.035	0.007	0.048	0.005	0.044	0.028	0.026	0.023	0.040	0.034	0.035	0.015

PPV_Grinder	PPV_Sink	PPV_Workbench	PPW_Sink	PPW_Workbench	T_Colander	T_CuttingBoard	T_Knife	T_Pan	T_SteelDish	T_SteelPincers	T_SteelShovel	T_SteelTray
0.000	0.011	0.031	0.045	0.031	0.051	0.003	0.017	0.001	0.087	0.003	0.044	0.004
0.000	0.074	0.100	0.011	0.407	0.516	0.001	0.001	0.007	0.535	0.009	0.545	0.058
0.008	0.000	0.000	0.000	0.001	0.072	0.000	0.000	0.015	0.004	0.091	0.008	0.006
0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.004
0.000	0.016	0.021	0.026	0.000	0.003	0.010	0.000	0.001	0.000	0.000	0.000	0.000
0.002	0.007	0.094	0.083	0.002	0.003	0.303	0.081	0.000	0.163	0.001	0.002	0.003
0.001	0.000	0.001	0.002	0.010	0.000	0.008	0.001	0.001	0.000	0.028	0.002	0.001
0.042	0.018	0.031	0.032	0.013	0.001	0.048	0.008	0.010	0.002	0.070	0.001	0.012
0.007	0.002	0.042	0.009	0.000	0.000	0.001	0.001	0.002	0.000	0.006	0.000	0.000
0.000	0.003	0.018	0.025	0.022	0.003	0.025	0.299	0.000	0.000	0.003	0.001	0.010
0.001	0.042	0.001	0.020	0.001	0.001	0.003	0.000	0.000	0.000	0.002	0.000	0.000
0.000	0.017	0.001	0.045	0.015	0.011	0.003	0.001	0.005	0.002	0.011	0.007	0.003
0.008	0.086	0.098	0.048	0.000	0.003	0.001	0.050	0.008	0.000	0.007	0.001	0.000
0.001	0.000	0.000	0.000	0.001	0.004	0.000	0.000	0.203	0.001	0.010	0.002	0.001
0.001	0.221	0.104	0.036	0.387	0.007	0.117	0.024	0.004	0.072	0.010	0.002	0.001
0.000	0.002	0.001	0.150	0.002	0.009	0.073	0.190	0.295	0.000	0.004	0.006	0.391
0.013	0.039	0.003	0.002	0.000	0.001	0.000	0.001	0.001	0.000	0.002	0.001	0.000
0.000	0.000	0.000	0.019	0.000	0.001	0.080	0.000	0.000	0.000	0.000	0.001	0.000
0.003	0.020	0.012	0.012	0.000	0.000	0.001	0.000	0.000	0.000	0.006	0.000	0.000
0.001	0.010	0.050	0.010	0.001	0.001	0.045	0.004	0.000	0.000	0.000	0.001	0.000
0.187	0.047	0.027	0.037	0.013	0.037	0.037	0.034	0.044	0.011	0.133	0.047	0.025

2.2 Relationships among lactic acid bacteria and potential spoilage microbiota in a dairy-processing environment

2.2.1 Introduction

Cheese manufacture and ripening are affected by the metabolic activity of different types of microorganisms. When milk of optimal hygienic quality is used, the dairy microbial consortia can be simple when starter cultures are employed, or a higher degree of complexity can occur in the case of natural fermentations. The environmental microbiota from the processing plant has been often addressed as a source of microbes that may play a role in the cheese making (Bokulich & Mills 2013a; Ksontini et al., 2013; Irlinger & Mounier 2009; Didienne et al., 2012). When lactic acid bacteria (LAB) are included in the environmental microbiota, they may actively con- tribute to fermentation and ripening of cheese. Conversely, when potential spoilage organisms contaminate the environment, these organisms can play a crucial role because they can be transferred from the environment to intermediates of production and may negatively affect the cheese production process and the quality of the final products. It has been demonstrated that the microbial populations involved in fermentation and ripening are often found on the processing surfaces(Bokulich & Mills 2013a; Ksontini et al., 2013; Irlinger & Mounier 2009) , highlighting the im- portance of the plant environment in potentially contributing to the microbiota of cheese. Depending on the nature of the micro- organisms, the environmental microbiota can exert functional activities important for the fermentative and/or the ripening process but sometimes may also be a hazard for cheese quality and safety (Mounier et al., 2005).

The study of the microbial ecology of foods has undergone a major revolution, and the advent in microbial ecology of sensitive culture-independent tools allows a rapid and effective evaluation of microbial contamination in many sorts of environments (Ercolini 2013). Optimal conditions for microbial growth can occur in food processing facilities. The growth of microorganisms in a food processing environment and the establishment of certain microbial communities can lead to the development of a well-defined environmental microbiota. Various microbial contamination sources can be identified in a dairy processing plant, including the tank, cheese vat, bench, cloths, knives, and other tools (Kousta et al., 2010). In the food industry, the resident microbial communities may create a persis- tent source of product contamination (Lindsay & Holy 2006), causing food spoilage (Brooks & Flint 2008)) and leading to serious hygienic problems (Kumar & Anand 1998; Carpentier & Cerf 1993) and also economic losses (Brooks & Flint 2008; Hood & Zottola 1997). Organic residues from food processing can create microenvironments for growth and accumulation of microorganisms and can represent a relevant source of cross-contamination (Brooks & Flint 2008; Hood & Zottola 1997; McLandsborough et al., 2006) Improperly cleaned or sanitized equip-ment is usually considered the major source of milk and dairy product contamination (Srey and Jahid, 2012). Exploring the relationships between fermentation and facility environment can be very useful to clarify whether the processing environment can actually influence the standard development of the cheese production. Microbial loads in the milk, as well as strain and species richness (Settanni et al., 2012), increase after the milk is poured into a vat (Didienne et al., 2012; Lortal et al., 2009; Settanni et al., 2012). Bokulich and Mills (2013) demonstrated that fermentation-associated microbes dominate most surfaces in dairy environments and can be transferred to the product, influencing the course of the fermentation. The species composition of vat surface biofilms was found to be stable over several seasons but varied widely between vats (Didienne et al., 2012; Licitra et al., 2007). Sometimes, facility-resident biotypes can outcompete the commercial strains. In fact, the bacteria found in ripened washed-rind cheeses were different from those inoculated (Mounier et al., 2005). It can be supposed that an equilibrium exists between dairy products and plant environment where microbial transfer occurs from both parts. Consequently, the dairy-environment relation-ship has the potential to affect the dairy process dynamics and the quality of the final products.

In this study, environmental swabs were collected in a dairy plant, and they were analyzed by using a culture-independent amplicon sequencing approach in order to describe the microbiota populating the dairy environment. Moreover, the microbiota of the cheeses produced was also assessed with the additional aim of investigating the existing overlap between environmental and cheese microbiota and how such a relationship may influence the quality of the manufactures.

2.2.2 Materials and methods

Sampling

The dairy plant considered in the present study is located in the Campania region (Southern Italy) in the province of Salerno, and it is involved in the production of different dairy products: ricotta (R), mozzarella (M), Caciocavallo (C), Grancacio (G), and Scamorza (S) cheeses. All of these cheeses except R are produced by using natural whey cultures (NWCs) as a natural starter for the fermentation according to a back-slopping procedure (Parente and Cogan, 2004). Sample collection was replicated twice at 3-week intervals. More details regarding the samples and sampling

procedures are provided in Table 1. Across all areas, surface swab samples from work surfaces and from all tools usually used during the production process were taken. In addition, samples from the R, M, C, G, and S cheese manufactures were collected. The sampling took place on the surfaces after routine cleaning (Table 1) and before the start of pro- duction. Surfaces were sampled with sterile cotton-tipped swabs that were moistened with sterile phosphate-buffered saline (PBS) and rubbed vertically, horizontally, and diagonally across the sampling site (100 cm^2) delineated by a template, rotating the swab to ensure full contact of all parts of the swab tip and surface (Bokulich & Mills 2013a).

DNA extraction

After sample collection, the swabs were placed into sterile, 10-ml polyethylene tubes containing 1 ml of sterile PBS, cooled at 4°C for the necessary time of transport to the laboratory. The cheeses were sampled after production, transferred into sterile plastic bags, and transported to the laboratory under refrigeration. Environmental swabs and cheese samples were analyzed within 6 h. Prior to DNA extraction, the tubes were vigorously stirred in a vortex to transfer the cells from the swab to solution. Total DNA extraction from the swab and cheese samples was carried out using a Biostic Bacteremia DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA). The extraction protocol was applied to the pellet (12,000 x *g*) obtained from 2 ml of suspension for the swab samples or from 2 ml of a homogenized 2-fold dilution of the cheese samples in one-quarter-strength Ringer's solution (Oxoid, Milan, Italy). All of the samples were collected and used under the surveillance of the dairy manager. No animals were involved in the present study; only animal products were employed.

16S and 26S rRNA gene amplicon library preparation and sequencing.

The bacterial diversity was studied by pyrosequencing of the ampli- fied V1-to-V3 region of the 16S rRNA gene amplifying a fragment of 520 bp (Ercolini et al., 2012). 454 adapters (454 Life Sciences, Roche, Italy) were included in the forward primer, followed by a 10-bp sample-specific multiplex identifier (MID). Each PCR mixture (final volume, 50 ¯l) contained 60 ng of tem- plate DNA, 0.1 □M concentrations of each primer, 0.50 mmol of each deoxynucleoside triphosphate $liter^{-1}$, 2.5 mmol of $MgCl_2$ $liter^{-1}$, 5 microl of 10 microl PCR buffer, and 2.5 U of native *Taq* polymerase (Invitrogen, Milan, Italy). The following PCR conditions were used: 94°C for 2 min, 35 cycles of 95°C for 20 s, 56°C for 45 s, and 72°C for 5 min, with a final extension at 72°C for 7 min. The fungal community was studied by sequencing of the D1-D2 domain of the 26S rRNA gene amplifying a fragment of 540 bp using the primers NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') (Kurtzmann and Robnett, 1998) as recently re-ported (Garofalo et al., 2015). The 454 adapters were included in the forward primer, followed by a 10-bp sample-specific MID. PCR mixtures were prepared as described above. The following PCR conditions were used: 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 52°C for 45 s, and 72°C for 1 min, and then a final extension at 72°C for 7 min and holding at 4°C (Garofalo et al., 2015). After agarose gel electrophoresis, PCR products were purified twice using an Agencourt AMPure kit (Beckman Coulter, Milan, Italy) and quantified using the QuantiFluor system (Promega, Milan, Italy), and then an equimolar pool was obtained prior to further processing. The amplicon pool was used for pyrosequencing on a GS Junior platform (454 Life Sciences, Roche, Italy) according to the manufacturer's instructions, using Titanium chemistry (Ercolini et al., 2012).

Bioinformatics and data analysis

In order to remove sequences of poor quality, raw reads were first filtered according to the 454-processing pipeline. Sequences were then analyzed and further filtered using QIIME 1.8.0 software (Caporaso et al., 2010) and a previously described pipeline (Kurtzman and Robnett, 1998). The reads were excluded from the analysis if they were shorter than 300 or 450 bp for the 16S or 26S rRNA gene, respectively. Sequences that passed the quality filter were chosen and singletons were excluded. Operational taxonomic units (OTUs) defined by 99% similarity were picked using the UCLUST method (Edgar and Baterman, 2010), and the representative sequences were submitted to the RDPII classifier (Wang et al., 2007) to obtain the taxonomy assignment and the relative abundance of each OTU. The Greengenes 16S rRNA gene database (McDonald et al., 2012) was used for the taxonomic assignment of bacteria, and the deepest level of assignment was interpreted as a putative species identification. For the 26S rRNA gene, the centroids of each sequence cluster (i.e., the longest sequence) were compared to the sequences reported in GenBank by using the BLAST (Basic Local Alignment Search Tool) algorithm, in order to obtain the taxonomic assignment. Alpha and beta diversities were studied through QIIME as previously described (De Filippis et al., 2014). Statistical analysis and plot-ting was carried out in the R environment (http://www.r-project.org). Venn diagrams were obtained by using Bioinformatics and Evolutionary Genomics software (Shade & Handselman 2012) in order to describe the microbial community shared by different sets of samples.

Metagenome predictions

PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States [http://picrust.github.io/picrust/]) (Langille et al., 2013) was used to predict the functional profiles in the microbial communities of environmental swabs and cheese samples. For the analysis with PICRUSt, the OTU levels at 97% identity were picked by the closed reference method against the Greengenes database (version 05/2013) using QIIME 1.8. The data were normalized for 16S rRNA gene copy numbers, and the metagenomes were predicted. From the inferred metagenomes, KEGG orthologs were identified, and the obtained was rarefied at the lowest number of sequences per sample. KEGG or- thologs were then collapsed at level 3 of hierarchy, and the resulting table was imported in R (www.r-project.org). Nearest sequenced taxon index (NSTI) values were calculated in order to evaluate the accuracy of meta- genome predictions, which depends on how closely related the microbes in a given sample are to microbes with sequenced genome representatives; NSTIs with lower values indicate a closer mean relationship (Langille et al., 2013). The 16S and 26S rRNA gene sequences are available at the Sequence Read Archive of the NCBI (accession number SRP058584).

2.2.3 Results

A total of 117,490 reads passed the filters applied through the QIIME splitlibrary.py script, with an average length of 454 bp. The re-sults of the alpha diversity analysis are reported in Table 2. For both 16S and 26S rRNA genes, Good's estimated sample coverage (ESC) indicated that a satisfactory coverage was reached for all of the samples (an ESC of 99% in most cases). The diversity indices varied greatly depending on the samples, and there was a signifi cant association between the sample type (cheese or environmen- tal samples) and diversity indices, as confirmed by the Adonis and Anosim statistical tests run by QIIME ($P > 0.001$). The microbial diversity in the different samples was quite het- erogeneous for the 16S rRNA, but overall a higher level of diversity was shown in the swabs than in the cheese samples, with average values of 90 and 70 OTUs, respectively. The principal coordinate analysis (PCoA) based on weighted Unifrac distance showed a clear separation between cheese and environmental microbiota, except in the case of the ricotta cheese, which clustered with the swab samples (Fig. S1a). A lower level of microbial diversity was found in 26S data, as shown by the lower number of fungal OTUs detected (Table 2); however, an analysis of the fungal community confirmed the microbiota driven differences between cheeses and environmental swabs (see Fig. S1b).

Bacterial diversity in cheeses and dairy plant.

Two different samplings in the same dairy were carried out. Since the Kendall's correlation between the matrices obtained in the two samplings was high (Kendall's tau = 0.71; $P = 2 \times 10^{-16}$), the average OTUabundance was used for the subsequent analyses. A common mi- crobiota, including OTUs occurring in 70% of the samples, was shared among the samples, though taxa with variable relative abundances were included (Fig. 1). The most abundant members of the core microbiota were *Streptococcus thermophilus*, *Pseu- domonas*, *Acinetobacter* spp., *Acinetobacter johnsonii*, and *Psychro- bacter* spp., which occurred in 99% of the samples. A predomi- nance of *Streptococcus thermophilus* was found in the cheese samples (average, 70%), except for the ricotta, where the abun- dance was 24%. In the environmental samples, the abundance of *S. thermophilus* was extremely variable, ranging from 3% (tank curd) to 43% (stretcher). *Pseudomonas* was found in all of the samples; however, in the cheese samples, it was a minor contaminant (never above 5%), except for the ricotta, where its abun- dance reached 30%. Moreover, *Pseudomonas* showed a remark- able presence in the environmental samples, achieving the highest levels in the molder and chopper (50%), followed by curd bench, chopper 2, tank, and mold dedicated to ricotta cheese making (average, 30%). *Acinetobacter* occurred in all of the samples, displaying higher levels in vat specimens (33%) and a lower average abundance of 20% in other environmental samples (Fig. 1). Moreover, some OTUs prevailed only in specific samples. For example, *Lactobacil- lus delbrueckii* occurred in 70% of the samples, but it was particu- larly abundant in Caciocavallo samples at the beginning of the manufacture (15%), as well as *Lactococcus lactis*, which had a re- markable abundance in mold Grancacio (20%) but in the rest of the samples was never above 1%. A principal component analysis of the species-level microbiota clearly grouped cheeses and envi- ronmental swabs separately (Fig. 2). Many OTUs of the *Firmicutes* phylum, such as *Lactobacillus helveticus*, *Lactobacillus delbrueckii*, *Lactobacillus casei* group, *Pediococcus* sp., *Leuconostoc* sp., *Weissella* sp., *Lactococcus garviae*, and *S. thermophilus*, had significantly higher abundances in cheeses ($P < 0.01$), whereas *Corynebacte- rium variabile*, *Kocuria* sp., *Microbacterium* sp., *Pseudoclavibacter helvolus* (*Actinobacteria*) and *Pseudomonas* spp., *Psychrobacter* sp., *Acinetobacter* sp., and *Paracoccus marcusii* (*Proteobacteria*) were more abundant in environmental swabs ($P < 0.01$).

With regard to genus-level OTUs found in at least 30% of the samples, the numbers of genera shared between the different groups of samples are represented in Fig. 3. The samples were grouped by cheese type, and for each cheese

type the environmental samples from the specific equipment were included. Thirty- two OTUs were common to all of the groups, and, interestingly, no microbial genus was specifically associated with a single-cheese group of samples (Fig. 3a). Remarkably, seven genera were specific for the environmental samples, while none was exclusively associated with cheeses (Fig. 3b).

Fungal diversity in cheese and dairy plant.

Thirty-six fungal OTUs were identified, and they were distributed among the samples with different relative abundances (Fig. 4). Overall, the most abundant species in environmental swabs were present in the cor- responding cheese samples, although they were never predomi- nant. The most abundant were *Kluyveromyces marxianus*, *Yamadazyma triangularis*, *Trichosporon faecale*, and *Debaryomyces hansenii*, occurring in 90% of the samples. A predominance of *Y. triangularis* was found in the cheese samples (average, 56%), except for the Caciocavallo cheese, where *K. marxianus* was the most abundant; this difference probably caused the differentiation in the PCoA plot showing the separation of samples based on 26S rRNA data (see Fig. S1b in the supplemental material). On the other hand, in the swab samples, the abundance of *Y. triangularis* was extremely variable, ranging from 0.7% (knife curd) to >90% (brine and hook t_0). *T. faecale* was found in 95% of the samples, although it was never >2% in cheese samples and it was much more abundant in the curd-related environmental swabs (Fig. 4). *D. hansenii* had a 90% occurrence rate, with higher levels of relative abundance in R, M, and related environmental samples. Also, in the case of fungal communities, most of the OTUs were shared by the different cheese groups. Twelve genera were common to all cheese groups and related equipment. In this case, some group-specific genera could be identified (Fig. 5a), although they never displayed >1% relative abundance values. Eighteen genera were shared by the environmental swabs and cheese samples (Fig. 5b). Only *Candida sake* was found exclusively in cheese samples, although its average abundance was low (0.01%).

OTU cooccurrence and/or coexclusion.

With regard to the 16S rRNA gene data, OTU cooccurrence was investigated by con- sidering the genus-level taxonomic assignment and including OTUs with at least 0.1% relative abundance in at least five samples and significant correlations with a false discovery rate (FDR) of <0.05 (Fig. 6a). *Lactobacillus* showed the highest number of negative correlations, including the core OTUs of *Pseudomonas*, *Acinetobacter*, and *Agrobacterium*, whereas it cooccurred with *Streptococcus*. The analysis of the relationships within the fungal microbiota showed a coexclusion between *Saccharomyces* and *Debaryomyces* and between *Yamadazyma* and *Trichosporon* (Fig. 6b).

Diversity of predicted metagenomes.

The PICRUSt tool was used to predict the metagenomes from the 16S rRNA gene se- quence database (28). The weighted NSTI for the samples of the present study was 0.015 ± 0.013. More precisely, the cheese sam- ples had the lowest NSTI values (0.003 + 0.07), whereas environ- mental swabs had an average NSTI of 0.018 + 0.013. A clear separation between cheese and environmental samples was achieved also by considering the predicted metagenomes (Fig. 7). In particular, cheeses were characterized by a lower abundance of KEGG pathways belonging to xenobiotic biodegradation and metabolism and biosynthesis of other secondary metabolites. In contrast, galactose metabolism, glycolysis, pentose phosphate pathways, and activities related to several amino acid metabolisms were more abundant in cheeses than in environmental samples.

2.2.4 Discussion

In this study, the microbiota in a dairy processing plant was studied by rRNA gene-based culture-independent high-throughput sequencing to describe the bacterial patterns characterizing the environment and the different cheese manufactures. The micro- biota of the environment was very complex, including more than 500 taxa at the genus/species level. Other studies describing the microbial community across surfaces in cheese-making plants re ported that the microbiota from surfaces had a high variability in composition, and most of the OTUs identified in the cheese manufacture originated from the processing environment (Bokulich & Mills 2013a; Gori et al., 2013; Andreani et al., 2015). Our microbial community structure had several microbial species in common with the previous studies, although the relative abundance of the species can depend remarkably on the specific manufacture studied. The settlement of the resident microbiota can depend on the characteristics of the surfaces, ecological factors, nutrient availability and composition, and the ability of microbes to develop biofilms, as well as on operators and cleaning procedures (Irlinger & Mounier 2009; Andreani et al., 2015; Marchand et al., 2012). The cleaning procedures used in the dairy plant constantly ensure, according to the producers, a standard quality of the cheeses; no spoilage case has

been reported in the last 5 years. The most abundant bacteria in the dairy environment were *Streptococcus thermophilus*, *Pseudomonas* spp., and *Psychrobacter* spp., while *Debaryomyces*, *Yamadazyma*, and *Galactomyces* spp. prevailed among the fungi. These bacterial and fungal genera (excluding *Yamadazyma*) were already reported in other studies describing the microbiota of cheese-making plant surfaces (Bokulich and Mills, 2013; (Irlinger & Mounier 2009; Marchand et al., 2012; L. Quigley et al., 2012). They coexisted in the same environment and are spread both across surfaces and in the dairy intermediates and products. Differences in relative abundance were mainly due to fermentation and pro- cessing, with *S. thermophilus* more abundant in the cheeses and the potential spoilers *Pseudomonas* and *Psychrobacter* more abun-dant on the surfaces. Similarly, *Y. triangularis* and *D. hansenii* were more abundant in cheese, whereas *T. faecale* was more abundant on surfaces. This is likely due to selective conditions imposed by curd fermentation and heating steps (such as curd stretching for the pastafilata cheese) that are unfavorable to the persistence of the spoilers. Such distribution suggests that specific care should be taken in the ordinary cleaning of tools and surfaces that are involved in the postfermentation steps of production. Interestingly, some of the above-mentioned species coexcluded in the dairy environment. *Streptococcus* and *Lactobacillus* indeed had the highest numbers of coexclusions with contaminating and potential spoil- age bacteria, and the same behavior was noticed for *Yamadazyma*. All of the cheeses produced in the two visits to the dairy had standard quality and did not present any defects. It is interesting to speculate that the establishment of LAB in the environment could have a valuable effect toward protection against spoilage bacteria, as supported by our coexclusion patterns, and protection against spoilage could be obtained when LAB colonize the environment. Such coexclusion may be due to differential colonization capability, which depends on several factors .(Ksontini et al., 2013; Andreani et al., 2015; Marchand et al., 2012) Our evidence is based on only one dairy, although it comes from repeated samplings; therefore, other surveys involving other dairies using natural starters will be important to further support our theory. Co-cultivation assays involving LAB and cheese spoilage bacteria would be valuable for studying competition mechanisms in depth, although these trials are not always fully descriptive of the actual relationships that are established in real cheeses (Wolfe & Dutton 2015). The LAB species identified here come from the NWCs used for the fermentation, although strain-level identification was not performed in the present study. Indeed, *Streptococcus thermophilus* and species of *Lactobacillus* are frequently found in the NWCs for cheese production (Ercolini et al., 2012; De Filippis et al., 2014; Cocolin & Ercolini 2008). However, these species are persistently found in the environment even after cleaning, as shown by our results, and this is consistent with the current literature reporting *S. thermophilus* (Settanni et al., 2012) and other LAB to be persistent in the environment (Lortal et al., 2009; Licitra et al., 2007; Beresford et al., 2001). LAB use in manufacture and their occurrence in the environment can be regarded as beneficial for both cheese quality and environmental protection. This is further confirmed by the fact that ricotta, the only cheese analyzed that is not derived by a fermentation procedure, although it comes from the heat treatment of the whey resulting from other manufactures, showed a high microbial diversity, which made it indistinguishable from the environmental samples. In addition, the lower incidence of LAB in ricotta was associated with a higher relative abundance of contaminating bacteria from the surfaces and the related tools of production. The gaskets used for molding and as containers for ricotta are made of plastic material, which is more porous, less adequate for a thorough cleaning, and sensitive to hot water, which can affect the mechanical properties of the plastic, in turn permitting corrosion, leading to an increased possibility of adherence by bacteria (Cocolin & Ercolini 2008; Myszka & Czaczyk 2011). Consistently, all of the cheese molders made of stainless steel showed a lower microbial diversity (in terms of number of OTUs) than the plastic ones, suggesting that these should replace the plastic gaskets for improved quality and safety of the unfermented dairy productions. The variability of the alpha diversity parameters indicated that surface contamination is strictly dependent on the surface material, which represents a fundamental issue for the maintenance of standard hygiene in food processing plants (Marchand et al., 2012). *Pseudomonas* in the environmental samples likely originates from raw milk, which can be contaminated by water and soil or by inappropriate sanitizing of milking surfaces, or contamination can occur during storage and while transporting equipment (Andreani et al., 2015). In addition, persistence on surfaces can be facilitated by the well- known ability of *Pseudomonas* to adhere to food processing surfaces (Andreani et al., 2015; Van Houdt & Michiels 2010; Myszka & Czaczyk 2011; Scott 2000), with the organism growing very rapidly on dairy plant equipment (PJ et al., 2009; Scott 2000). The psychrotrophic nature of *Pseudomonas* can help it to withstand the competing microbial populations in milk and in fresh cheeses (De Jonghe et al., 2011; Franciosi et al., 2011; Martin et al., 2011; Morales et al., 2005), possibly determining changes in food structure or discoloration, such as the case of "blue mozzarella cheese" (Andreani et al., 2015; Nogarol et al., 2013). *Acinetobacter*, *Pseudomonas*, and *Psychrobacter* spp. can be involved in food spoilage, and they are recognized as undesirable bacteria in food processing environments (Doulgeraki et al., 2012). In the present study, these bacteria were found to be abundant in several samples, confirming some of our previous evidence suggesting that they could be members of the resident microbiota of food processing plants (De Filippis, La Storia, et al., 2013; Stellato, La Storia, et al., 2015). The predominance of some specific yeasts can reduce the risk of spoilage by other microorganisms. This was demonstrated in the case of *D. hansenii* (a member of our core microbiota), which was able to control contamination by clostridial species through the production of antibacterial metabolites (Prillinger et al., 1999). Previous studies indicated that *D. hansenii* originates from the dairy house microbiota (Bokulich & Mills 2013a;

Gori et al., 2013; Gori et al., 2012), and this further highlights the possible impact of the environmental microbiota on food processing. Cleaning procedures can have an impact on the spatial distribution of the microbial communities in food processing plants (Prillinger et al., 1999; Simoes et al., 2010; Parkar et al., 2004). Our samplings were performed after the cleaning routine. The results of replicate samplings showed consistent microbial profiles and suggest that these communities are established on the surfaces and on the equipment in spite of frequent cleaning and sanitation. Indeed, our study focused on one dairy plant only, and different results may be obtained in other processing environments where different starter cultures, raw materials, and cheese- making protocols are used. However, the persistence of dairy bacteria in the environment and the occurrence of a cheese environment core microbiota are consistent with previous reports (Bokulich & Mills 2013a), suggesting that this may be the general case.

2.2.5 Conclusion

Environmental microbiota in food processing plants can be very important for the achievement and maintenance of food quality. The persistence of LAB in the environment can be helpful to contrast the development of potential spoilers, and the use of natural starters may represent a valuable source of robust LAB that can spread in the environment. Facility ecosystem surveillance by mapping the microbiota may become a valuable approach to monitor environmental contamination in order to support the overall quality management in the dairy plants. Moreover, understanding the interactions between cheese and specific environmental microbiota can represent a crucial step to ensure cheese manufacture of a standard quality level.

2.2.6 References

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Figures and Tables

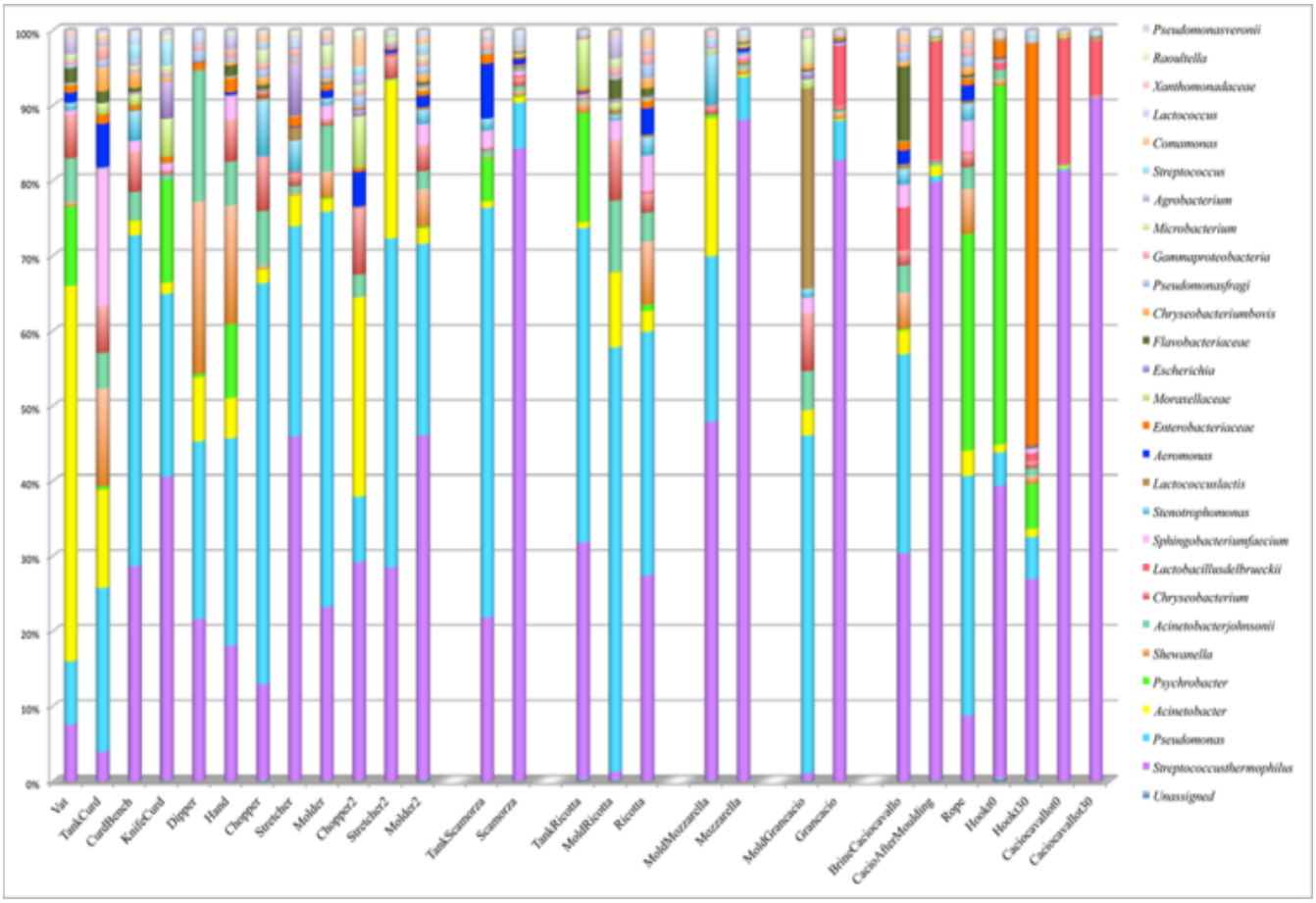


Figure 1 - Abundance of bacterial genera and species in environmental swabs from the dairy plant and from cheese samples obtained by 16S rRNA gene pyrosequencing analysis. Only OTUs occurring in 70% of the samples are reported.

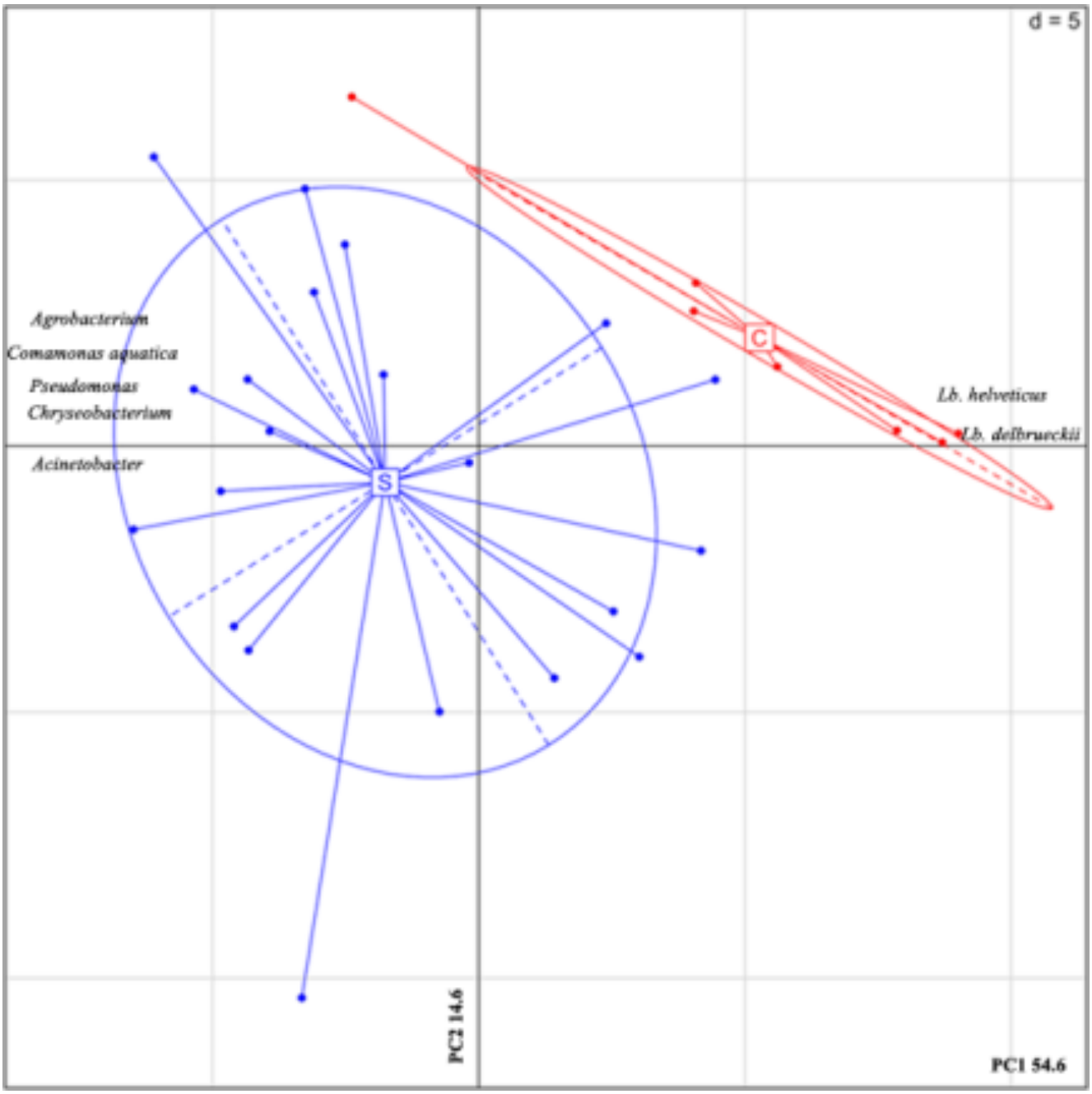


Figure 2 - Principal Component Analysis based on the species-level microbiota. The two principal components were plotted using the made4 package in R. The center of gravity for each cluster is marked by a rectangle indicating the sample type (S, swabs; C, cheeses). Only those OTUs which showed a loading score >=0.7 are shown in the figure.

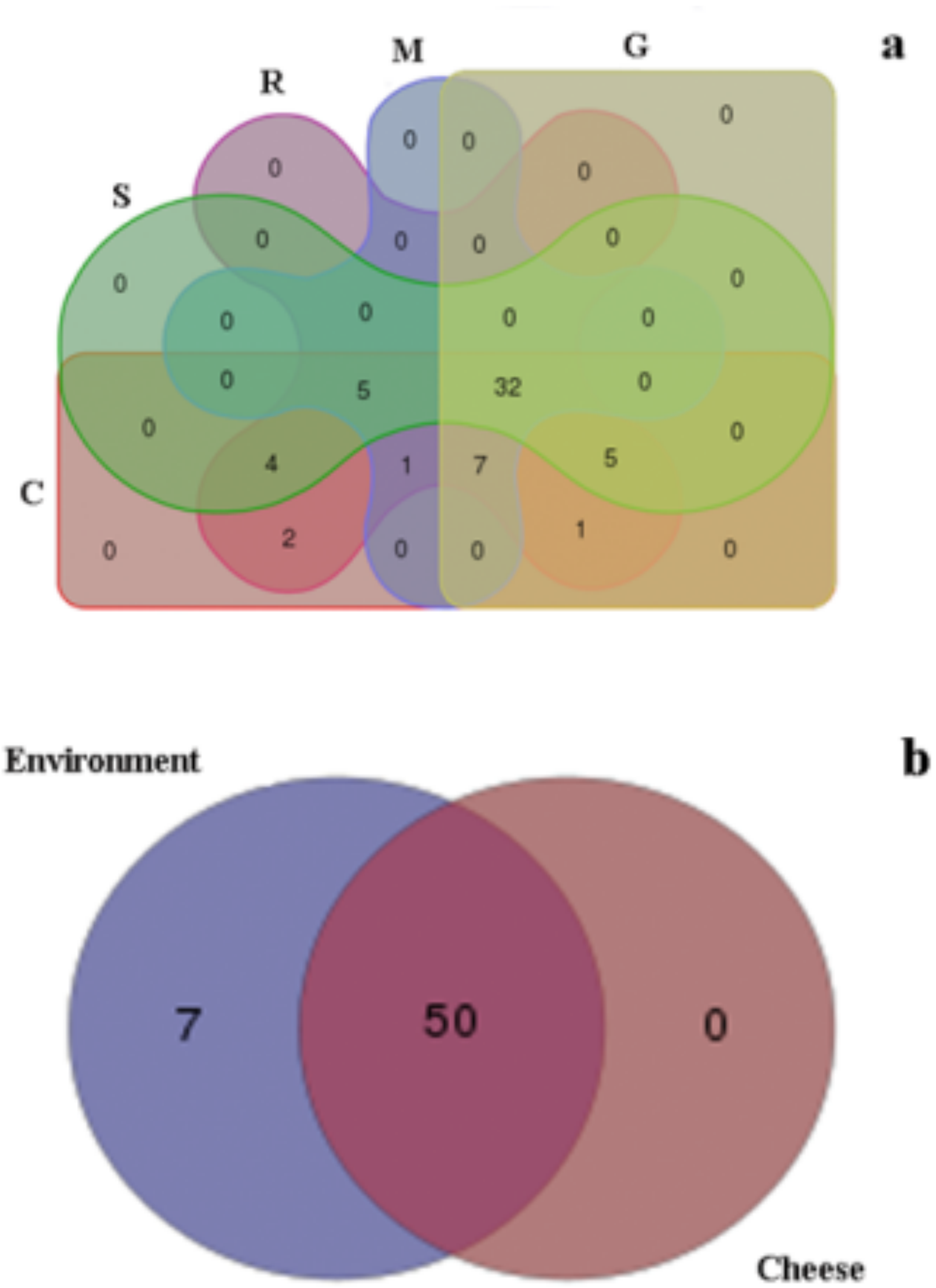


Figure 3 - Venn Diagramm showing the number of shared OTUs between samples obtained by 16S rRNA gene pyrosequencing analysis. Samples were grouped by cheeses type including relative environmental samples from the dedicated equipment (panel a); and their combination separating environmental swab from cheese samples (panel b). C, Caciocavallo; S, Scamorza; R, Ricotta; M, Mozzarella; G, Grancacio.

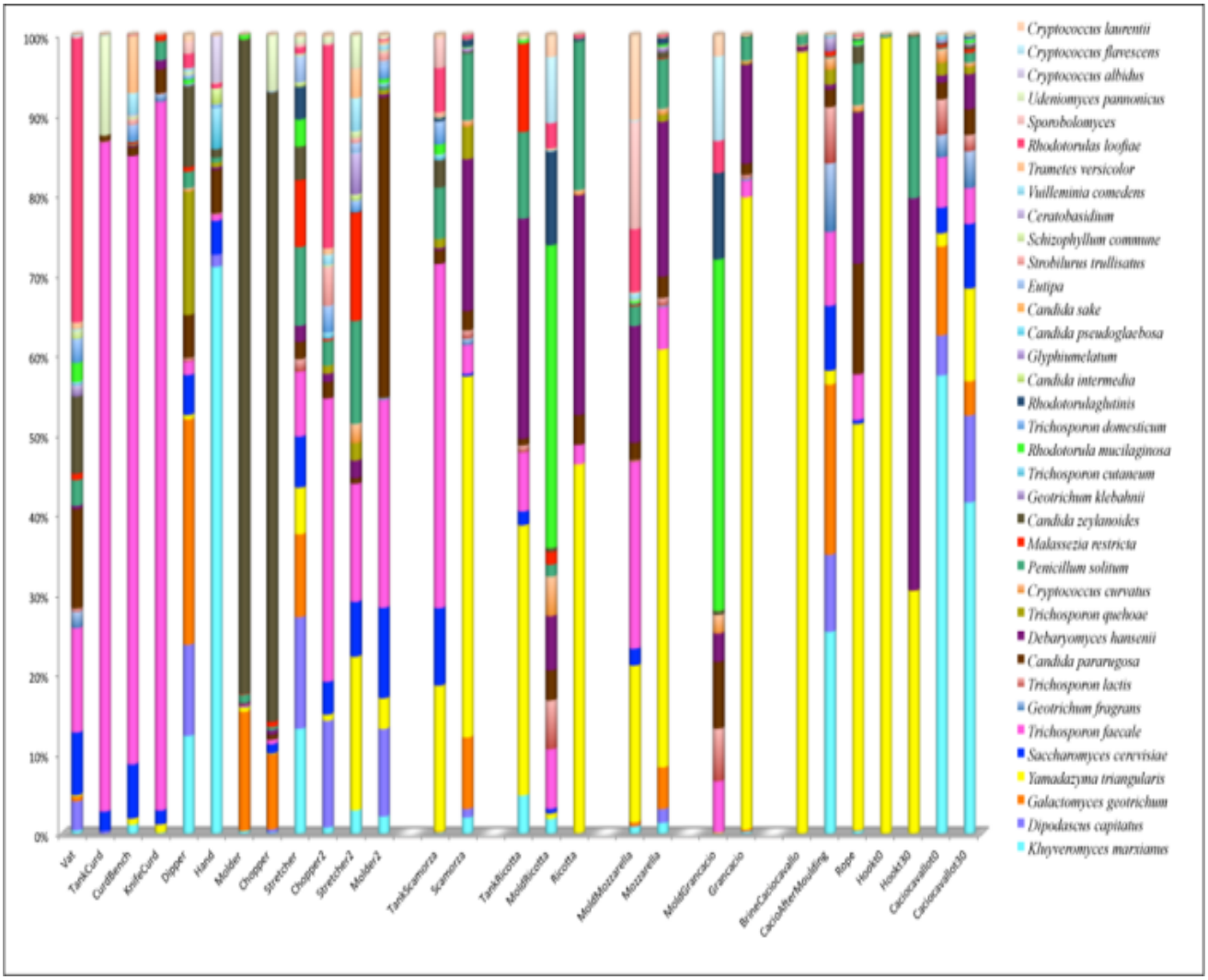


Figure 4 - Abundance of fungal genera and species in environmental swabs from the dairy plant and from cheese samples obtained by 26S rRNA gene pyrosequencing analysis. All the identified OTUs are reported with their different relative abundances.

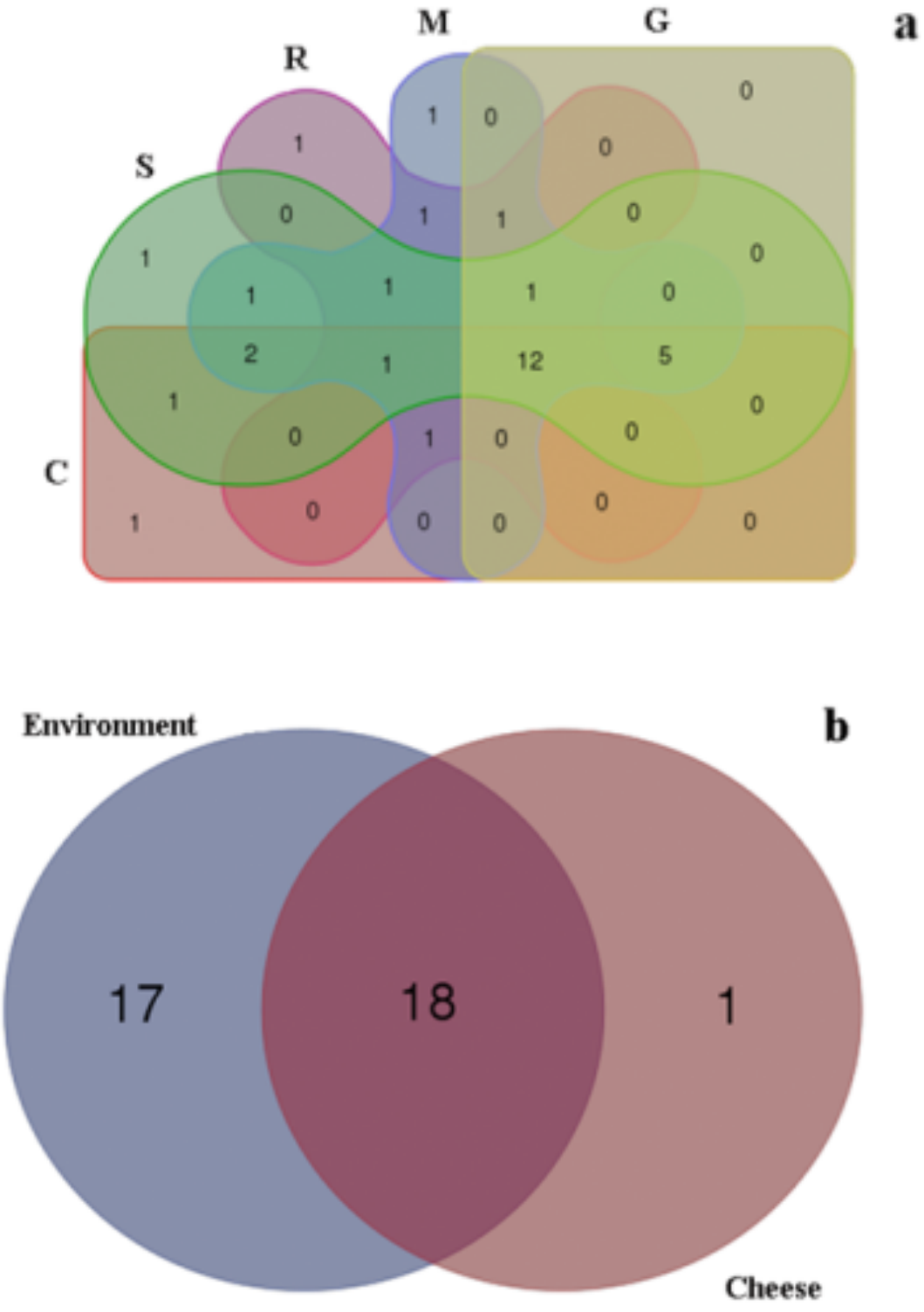
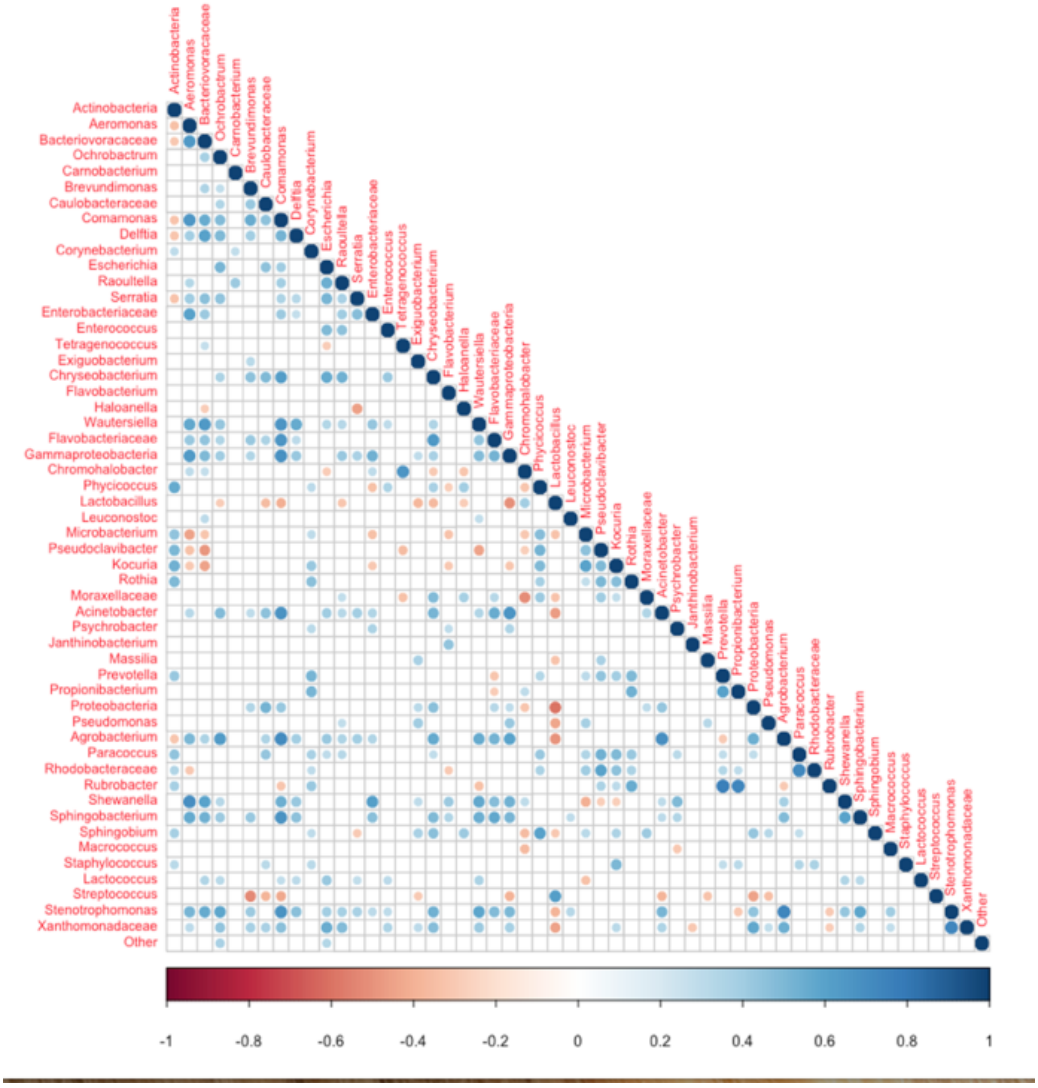
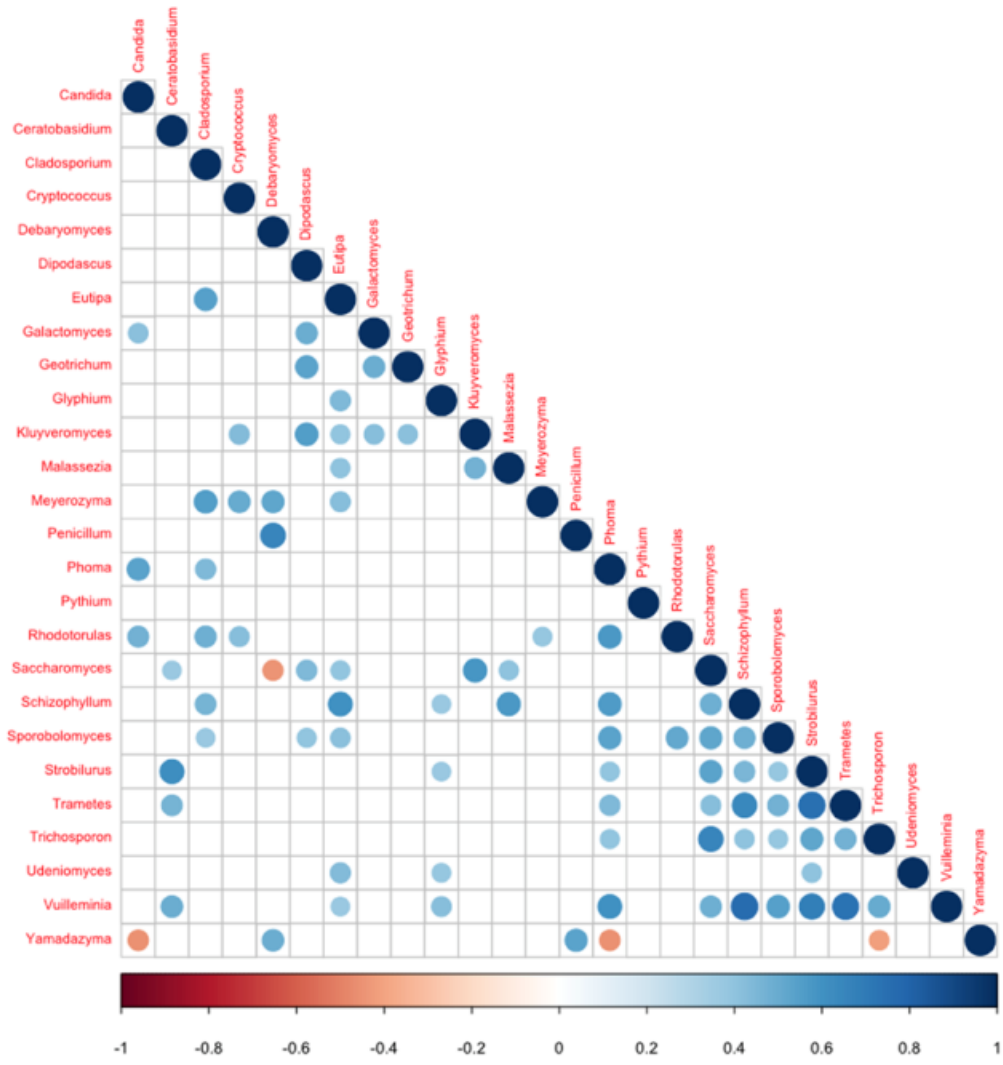


Figure 5 - Venn Diagramm showing the number of shared OTUs between samples obtained by 26S rRNA gene pyrosequencing analysis. Samples were grouped by cheeses type including relative environmental samples from the dedicated equipment (panel a); and their combination separating environmental swab from cheese samples (panel b). C, Caciocavallo; S, Scamorza; R, Ricotta; M, Mozzarella; G, Grancacio.



a

Figure 6a - Spearman's rank correlation matrix of bacterial (panel a) and fungal OTUs with > = 0.1% abundance in at least 5 samples. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (dark blue) and −1 indicating a perfectly negative correlation (dark red) between two microbial genera. Only significant correlations (FDR<0.05) are shown.



b

Figure 6b - Spearman's rank correlation matrix of bacterial and fungal (panel b) OTUs with > = 0.1% abundance in at least 5 samples. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (dark blue) and −1 indicating a perfectly negative correlation (dark red) between two microbial genera. Only significant correlations (FDR<0.05) are shown.

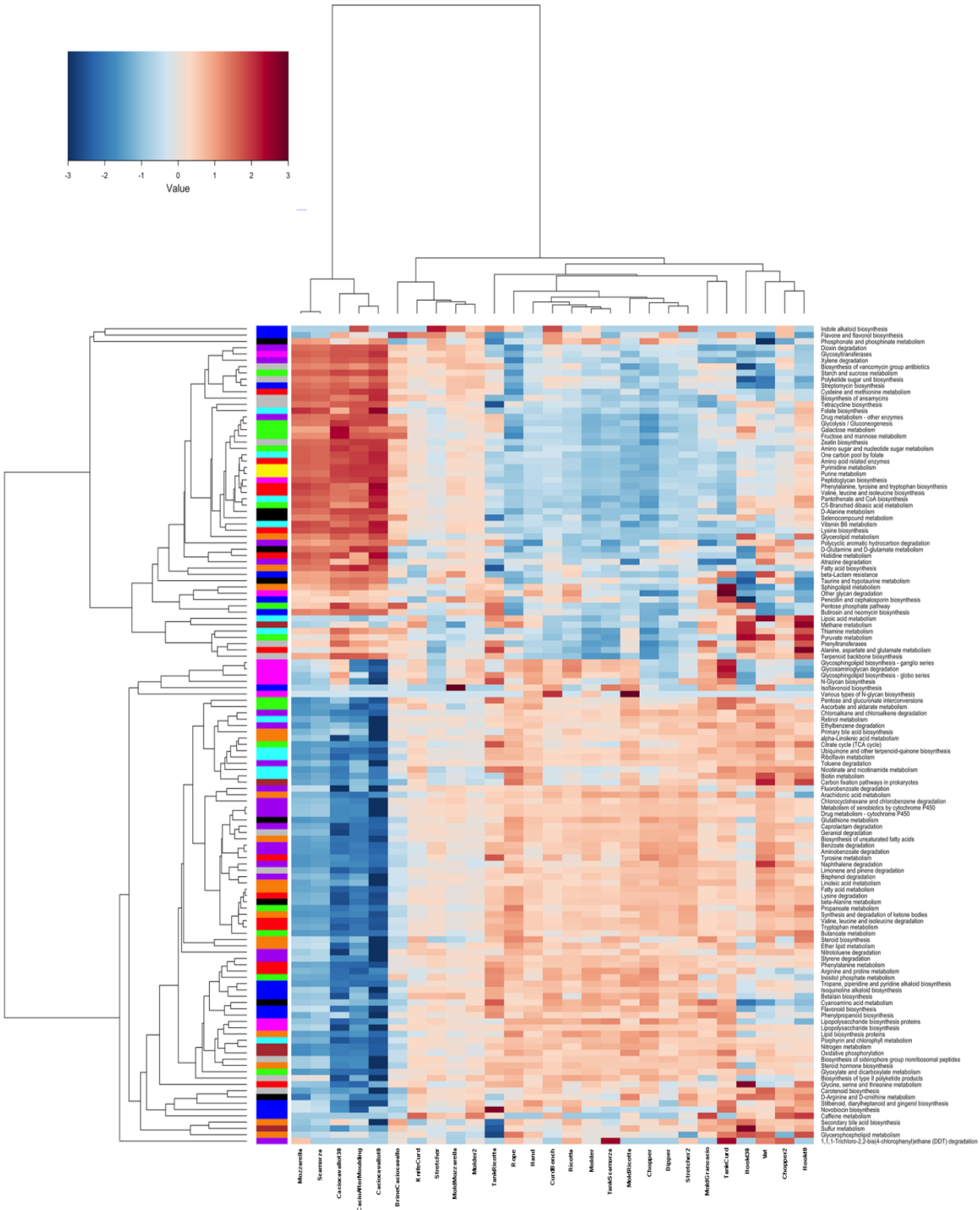


Figure 7 - Average linkage clustering based on the Spearman correlation coefficients of the proportion of KEGG Orthologs collapsed at level 3 of hierarchy, filtered for subject prevalence of at least 10%. Row bar colors denote the higher level of hierarchy (L2): carbohydrate (green), amino acid (red), energy (brown), cofactors and vitamins (cyan), terpenoids and polyketides (grey), lipid (orange), xenobiotics (purple), nucleotide (violet), other amino acids (black) metabolisms, biosynthesis of other secondary metabolites (blue), glycan biosynthesis and metabolism (magenta). Only KEGG Orthologs belonging to metabolism category were considered. The color scale represents the scaled abundance of each gene, denoted as Z-score, with red indicating high abundance and blue indicating low abundance.

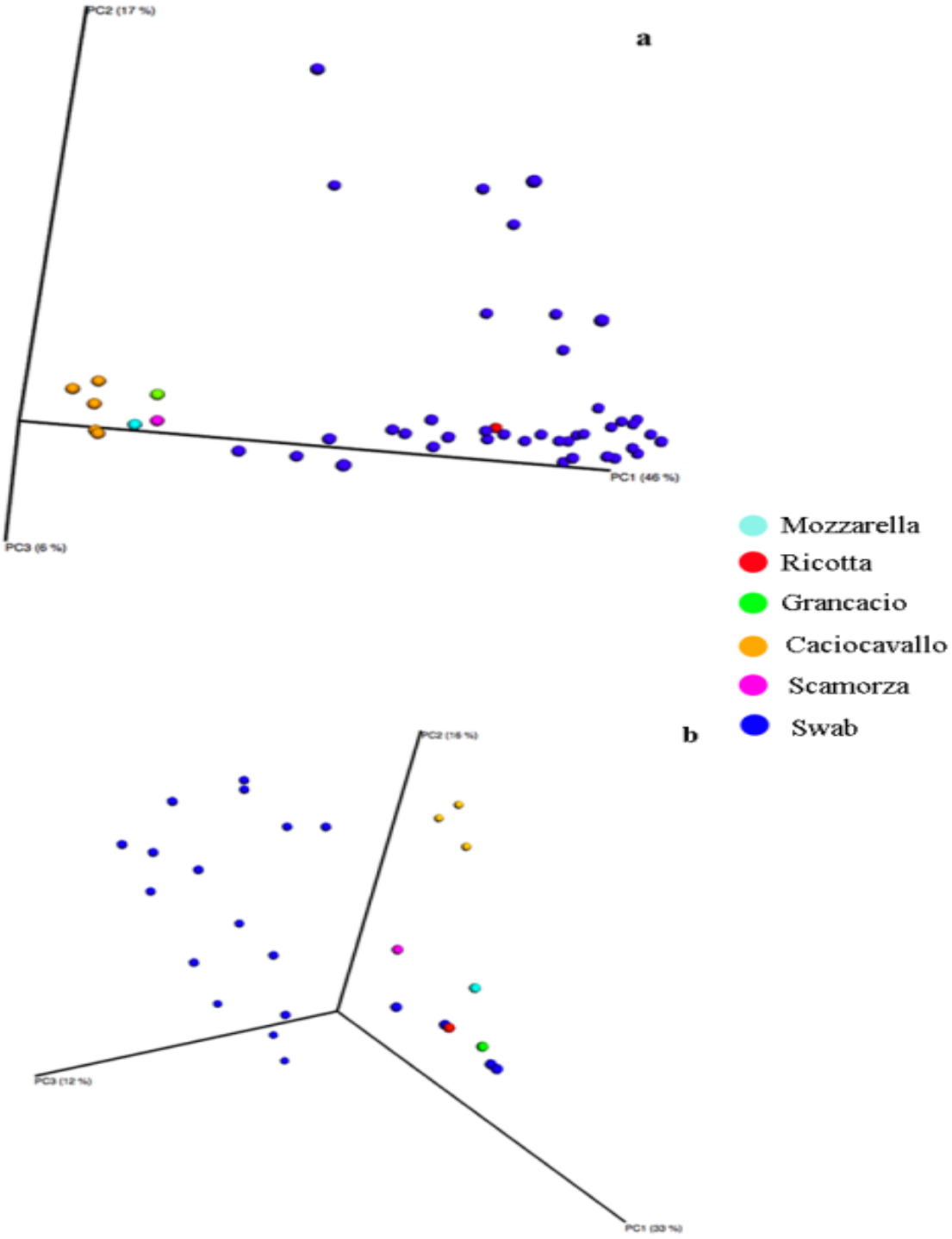


Figure S1 - Principal Coordinates Analysis (PCoA) based on weighted UniFrac distances for 16S rRNA gene sequence data (panel a) and PCoA based on Bray Curtis distances for 26S rRNA gene sequences (panel b). Samples are labeled with different colors according to their origin.

Table 1 - Description of the samples analyzed in this study.

Sample	Description
[a]Brine Caciocavallo	Liquid brine used for salting of Caciocavallo cheese.
[b]Caciocavallo After moulding	Caciocavallo cheese obtained after stretching and molding steps.
[b]Caciocavallo t_0	Caciocavallo cheese obtained after stretching, molding and brining, ready to start the maturation process.
[b]Caciocavallo t_{30}	Caciocavallo cheese after 30 days of ripening.
[c]Chopper	Steel machine used for curd shredding.
[c]Chopper 2	See above.
[c]Curd Bench	Steel table were the curd is left to drain.
[b]Grancacio	Pasta-filata cheese with production technology similar to Caciocavallo but characterized by larger size.
[e]Hand	Hand of the stretching operator.
[f]Hook t_0	Tool used to hang the Caciocavallo by the rope during ripening. It was sampled at the start of the maturation step.
[f]Hook t_{30}	Hook sampled after 30 days of cheese ripening.
[g]Knife Curd	Knife used to cut the curd for the draining.
[d]Mold Grancacio	Mold used for molding of Grancacio.
[d]Mold Mozzarella	Mold used for molding of Mozzarella.
[d]Mold Ricotta	Mold used for molding of Ricotta.
[c]Molder	Steel machine employed to mold the stretched curd for the production of Mozzarella and Caciocavallo
[c]Molder 2	See above.
[b]Mozzarella	Mozzarella cheese.
[b]Ricotta	Fresh, soft cheese that does not undergo fermentation or ripening.
[h]Rope	Used to tie Caciocavallo cheese molds at the neck in order to hang it for the ripening.
[b]Scamorza	Pasta-filata cheese with a semi-soft texture and a typical pear shape.
[c]Stretcher	Steel machine used to stretch the shredded curd.
[c]Stretcher 2	See above.
[c]Tank Curd	Vat where milk is heated and the rennet is added to obtain the curd.
[c]Tank Ricotta	Vat where whey is heated to be curdled to obtain the Ricotta cheese.
[c]Tank Scamorza	Tank containing the brine for Scamorza cheese salting.
[c]Vat	Vat containing the milk to be curdled.

Table 2 - Observed diversity and estimated sample Coverage (ESC, Good's coverage) for 16S and 26S rRNA amplicons analysed in this study.

	OTU		Chao1		Shannon		ESC	
Sample	**16S**	**26S**	**16S**	**26S**	**16S**	**26S**	**16S**	**26S**
Brine Caciocavallo	93	8	155.50	8.50	3.51	0.19	98%	99%
Caciocavallo After moulding	67	18	86.25	18.00	1.94	3.18	99%	99%
Caciocavallo t_0	42	20	50.63	21.00	3.03	2.43	99%	99%
Caciocavallo t_{30}	58	22	71.34	22.00	3.60	2.98	99%	99%
Chopper	79	13	119.89	16.00	3.18	1.21	99%	99%
Chopper 2	81	20	110.86	26.00	3.44	2.83	99%	98%
Curd Bench	98	18	124.69	20.50	3.75	1.47	98%	99%
Grancacio	65	16	79.62	19.00	2.13	1.18	99%	99%
Hand	120	18	142.47	19.50	4.73	1.83	99%	99%
Hook t_0	83	3	115.21	3.00	2.85	0.03	99%	99%
Hook t_{30}	71	4	101.86	4.00	2.99	1.51	98%	100%
Knife Curd	127	10	154.77	11.00	4.22	0.82	98%	99%
Mold Grancacio	85	11	108.54	12.00	3.42	0.88	99%	99%
Mold Mozzarella	60	27	96.50	34.50	2.56	2.65	98%	99%
Mold Ricotta	98	12	130.75	12.00	4.15	2.70	99%	99%
Molder	92	22	115.79	22.60	3.22	3.04	99%	99%
Molder 2	128	18	171.02	18.00	3.62	3.13	99%	99%
Mozzarella	62	20	77.55	20.50	1.70	2.39	99%	99%
Ricotta	114	11	160.43	14.00	4.50	1.91	99%	99%
Rope	102	17	139.53	17.50	3.93	2.19	99%	99%
Scamorza	79	21	100.08	22.50	2.22	2.65	99%	99%
Stretch Dipper	61	23	81.60	26.00	2.93	3.24	99%	99%
Stretcher	68	22	112.52	22.00	2.86	3.82	99%	99%
Stretcher 2	82	23	102.56	29.00	2.96	3.61	99%	99%
Tank Curd	111	7	131.03	8.00	4.60	0.82	99%	99%
Tank Ricotta	79	12	115.73	12.00	2.48	2.53	99%	99%
Tank Scamorza	90	21	139.83	22.50	3.44	2.77	99%	99%
Vat	96	24	122.09	25.00	3.86	3.15	99%	99%

2.3 Comparing the spoilage microbiota between meat and meat processing environment in traditional vs large-scale retail distribution

2.3.1 Introduction

Meat is a complex niche with chemical and physical properties that allow the colonization and development of a variety of microorganisms, especially bacteria (De Filippis, La Storia, et al., 2013; Doulgeraki et al., 2012). Several factors can influence the occurrence of microbes in meat. After slaughtering, meat can be contaminated by microorganisms from the water, air and soil as well as from the workers and equipment involved in the manufacturing. In the further actions of the fresh meat chain (handling, cutting, storage), abiotic factors such as temperature, gaseous atmosphere, pH and NaCl will select for certain bacteria, allowing the colonization of the meat surface by different spoilage-related species and strains (Brooks & Flint 2008; Ercolini et al., 2006).

Microbial growth to high numbers is a prerequisite for meat spoilage that can be considered an ecological phenomenon encompassing the changes of the available substrata during the proliferation of bacteria (Olusegun & Iniobong 2011; Nychas et al., 2008). Spoilage is the process of food deterioration leading to a reduction of its quality, till the point of not being edible for humans. Signs of spoilage may include a different appearance of the food compared to its fresh form and the alteration of the sensorial quality of the product, in particular the aspect (including texture and color) and the presence of off odour (Remenant et al., 2015; Nychas et al., 2008; Gram et al., 2002; Borch et al., 1996). The presence of microorganisms on the surface of the cut meat and meat products will determine meat spoilage upon their interaction and growth in optimal conditions (Gram et al., 2002; Doulgeraki et al., 2012).

Although there are many different types of meat, the main bacterial populations involved in spoilage are common. The most abundant bacteria causing spoilage of refrigerated beef and pork are *Brochothrix thermosphacta*, *Carnobacterium* spp., clostridia, *Enterobacteriacea*e, *Lactobacillu*s spp., *Leuconostoc* spp., *Pseudomonas* spp., and *Weissella* spp, and their alterative activity consist in defects such as sour off flavours, discolouration, gas or slime production and decrease in pH (Nychas et al., 2008; Doulgeraki et al., 2012; Casaburi et al., 2015; Pothakos, Devlieghere, et al., 2015).

The environmental microbiota from the processing plant has been often addressed as source of microbes potentially affecting the quality attributes of meat (Hultman et al., 2015; De Filippis, La Storia, et al., 2013; Lambert et al., 1991). Indeed, several studies demonstrate that the microbiota involved in the food processing steps are often found on the processing surfaces or tools (Bokulich et al., 2013; Pothakos, Devlieghere, et al., 2015; Hultman et al., 2015; Calasso et al., 2016; Stellato, La Storia, et al., 2015; Stellato, De Filippis, et al., 2015) underling the importance of the hygienic practices in influencing the food microbiota. However, no studies have investigated the differences in the contamination types and levels between small versus large-scale retail distributions. Food handling and cleaning practices can be completely different according to size, level of automation and organization of the specific retail.

In the food processing facilities, optimal conditions for the growth of specific microbial populations may occur and this often leads to the establishment of a resident microbial community (Hultman et al., 2015; Stellato, La Storia, et al., 2015; De Filippis, La Storia, et al., 2013). In the meat handling environment, the presence of a resident microbiota represents a constant source of meat contamination, possibly contributing to the occurrence of spoilage (Brooks & Flint 2008) and thus leading to economic losses (Gram et al., 2002; Remenant et al., 2015) and/or safety issues (Hultman et al., 2015; Kumar & Anand 1998; Carpentier & Cerf 1993). Various microbial contamination sources can be identified in a butchery including chopping boards, refrigerators, operators' hands, cloths, knives and other tools (De Filippis, La Storia, et al., 2013; Hultman et al., 2015). The availability of organic residues on the surfaces can lead to growth and aggregation of microorganisms and represents a significant source of cross-contamination (Stellato, La Storia, et al., 2015; Giaouris et al., 2014; Verran et al., 2008; Montville et al., 2001). Good cleaning and sanitization practices of surfaces and equipment are supposed to solve the problem of food contamination since low hygiene standards in the food processing plant are the major cause of contamination of raw meat and meat products (Hultman et al., 2015). The most abundant species present on the processing tools were also found at high levels on meat, suggesting an establishment of a mutual equilibrium between food and environment that affects the quality of the final product (Hultman et al., 2015; De Filippis, La Storia, et al., 2013; Vihavainen et al., 2007). However, the effect of retail size and organization has never been investigated as possible variable affecting the microbiological quality of meat.

In this study, we describe the microbiota in environmental swabs and meat samples collected in small-scale as well as large-scale retail distribution in order to explore the influence of the microbiota in meat handling environment on the initial microbiological quality of meat and to assess the effect of the type of retail on the extent of microbial contamination.

2.3.2 Materials and methods

Sampling

Samples were collected from 20 butcheries (indicated with alphabetic letters) including 10 small-scale retail distribution (SD) and 10 butcher counters in large-scale retail distribution (LD) (indicated with "*" symbol), located in the Campania region (Southern Italy). Sample collection was replicated twice at 3 weeks interval. The sampling took place on the surfaces at least 1 h after the routine cleaning and before the start of the sale. Meat samples collected included fresh beef and pork cuts (indicated with number 4 and 5, respectively) while surface samples were taken from knife, chopping board and operator's hand (indicated with numbers 1, 2 and 3 respectively). The surface sampling was carried out using sterile sponge (Whirl-Pak® Speci-Sponge®, Nasco, Fort Ankinson, WI, USA) pre-moistened with 25ml sterile peptone buffer solution. Sponge was rubbed vertically, horizontally and diagonally across meat chopping board surface (100cm^2), both sides of the knife and palm of the butcher hand. After collection, samples were cooled at 4^{o}C and analyzed within 3h. All the samples were collected with the permission of the butchers. No animals were involved in the present study, but only animal products.

Microbiological analysis

Prior to analysis, 25 g of each meat sample was homogenized in 225 ml sterile quarter-strength Ringer's solution (Oxoid, UK) in a stomacher (Stomacher®400 Circulator, Seward Medical, London, UK) for 1min at 230 rpm at room temperature. The homogenate meat and surface samples were used to perform a 10-fold serial dilution by using sterile Ringer's solution as diluent. Pour plating was used to determine total psychrotrophic counts, lactic acid bacteria and *Enterobacteriaceae* by using, plate count agar (PCA), de Man, Rogosa and Sharpe agar (MRS) and violet red bile glucose agar (VRBGA), respectively (all from Oxoid, UK). Spread plating was used to determine *Pseudomonas* spp. and *Brochothrix thermosphacta* by using, *Pseudomonas* agar base added with cetrimide-fucidin-cephalosporin (CFC) supplement and streptomycin sulphate-thallium acetate-actidione agar (STAA) added with STAA Selective Supplement SR0151E (all from Oxoid, UK). Plates were were incubated at 20^{o}C for 48h (PCA), 20^{o}C for for 48h (MRS agar, *Pseudomonas* CFC agar and STAA agar) and 20^{o}C for 48h (VRBGA). Plate counts were performed in triplicate. Data were analyzed using ANOVA and Tukey post-hoc test at a significance level of 0.05 for sample comparison. The statistical analysis was performed using IBM SPSS Statistics TM software version 16.0.

Dna extraction

Total DNA extraction from sponges and meat samples was carried out by using a Biostic Bacteremia DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). The extraction protocol was applied to the pellet (12,000 x g) obtained from 10-fold dilution in sterile Ringer's solution for meat samples and from 20 ml of sponge buffer for swabs.

PCR amplifications, 16S gene amplicon library preparation and sequencing.

The bacterial diversity was studied by pyrosequencing of the amplified V1–V3 region of the 16S rRNA gene, amplifying a fragment of 520 bp (Ercolini et al., 2012). 454-adaptors were included in the forward primer followed by a 10 bp sample-specific Multiplex Identifier (MID). PCR conditions were previously described (De Filippis, La Storia, et al., 2013). After agarose gel electrophoresis, PCR products were purified twice by Agencourt AMPure kit (Beckman Coulter, Milano, Italy), quantified using the PlateReader AF2200 (Eppendorf, Milano, Italy) and equimolar pools were obtained prior to further processing. The amplicon pools were used for pyrosequencing on a GS Junior platform (454 Life Sciences, Roche, Italy) according to the manufacturer's instructions by using a Titanium chemistry. The same DNA templates were also PCR-screened for the presence of *Toxoplasma gondii* by using the 18S rRNA gene as target (Auriemma et al., 2014)(A; the test was negative for all the samples.

Bioinformatics and data analysis

Raw reads were first filtered according to the 454-processing pipeline. Sequences were then analyzed and further filtered by using QIIME 1.8.0 software(Caporaso et al., 2010) and a pipeline previously described (De Filippis et al., 2014). Alpha and beta diversity were studied through QIIME as previously described (De Filippis et al., 2014). Core microbiota was defined as the microbial genera/species present in at least 80% of the samples. Statistical analysis and plotting were carried out in the R environment (http://www.r-project.org), by using the packages *vegan*, *stats*, *psych*, *corrplot* and *made4*. Permutational Multivariate Analysis of Variance (non parametric MANOVA) based on Jaccard and Bray Curtis distance matrices was carried out by using 999 permutations to detect significant differences in the overall microbial community composition as affected by the type of sample or the type of retail. Pairwise Wilcox tests were used in order to determine significant differences in alpha diversity parameters, in OTU or in predicted pathway abundance between environmental and meat samples. Correction of p values for multiple testing was performed when

necessary(Benjamini & Hochberg 1995). Principal Components Analysis (PCA) was carried out on log transformed abundance tables by using *dudi.pca* function in *vegan* package. Venn diagrams were obtained by using the Bioinformatics & Evolutionary Genomics software(Shade & Handselman 2012) in order to describe the microbial community shared by different sets of samples.

PICRUSt was used (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, http://picrust.github.io/picrust/) to predict the potential functional profiles of the microbial communities in environmental swabs and meat samples. For this analysis, OTUs were picked against the Greengenes database (version 05/2013) using QIIME 1.8. The abundance of the predicted metagenomes was normalized for 16S rRNA gene copy numbers. KEGG orthologs were identified from the inferred metagenomes and collapsed at level 3 of hierarchy. Subsequent analyses were carried out in R as described above.

2.3.3 Results

Enumeration of bacterial populations

The viable counts on appropriate media of the target meat spoilage groups in meat and environmental samples are reported in Tables 1-5. Mean log counts were not significantly different ($P > 0.05$) between beef and pork samples. The mean log counts of Hand and Knife samples were significantly lower than the chopping board ($P<0.05$), while Knife and Hand were not significantly different ($P > 0.05$). Grouping the samples into traditional small-scale retail distribution (SD) and large-scale retail distribution (LD), the effect of butchery type was also not significant (Table 6).

Sequencing data analysis, alpha- and beta-diversity. A total of 658,572 reads passed the filters applied through the QIIME splitlibrary.py script, with an average length of 454 bp. The diversity indices varied much among the samples and there was a significant association between the sample type and the microbial diversity (Figure 1). Interestingly, the Chopping Board showed a significantly higher diversity compared to the other surface swabs (FDR < 0.05), with an average number of 581 OTUs (±303) and an average Chao1 index of 1371 (±700). No difference was found between traditional butcheries and butcher counters in large-scale retail distribution (FDR > 0.05).

The PCoA based on weighted Unifrac distance matrix showed that samples from both samplings did not cluster separately (Figure S1) and the microbial composition was not significantly different between the two samplings ($P<0.001$).

Bacterial diversity in meat and processing environment

The microbial diversity at species level in traditional butcheries and large-sized establishments are shown in Figure 2, where an average value for the two samplings is reported. *Streptococcus* sp., *Pseudomonas* sp., *Brochothrix* sp., *Psychrobacter* sp. and *Acinetobacter* were part of a core microbiota as they were abundant in both types of butcheries and occurring in the 99% of the samples although with different distribution. The highest levels of *Pseudomonas* were observed in the SD environment (avg 84% in "S") (Fig. 2a) and in meat samples from LD (avg. 60% in all meat samples) (Fig. 2c). *Brochothrix* occurred in all the samples (avg. 20%) but showed a remarkable occurrence in "Hand" samples. *Psychrobacter* showed a homogeneous distribution among all the samples having a remarkable relative abundance in the environmental samples of both SDs and LDs (avg. 35% and 40% respectively), and the highest abundance in the Pork meat from LD. Finally, some OTUs were characteristic of specific SD samples although with low relative abundance such as the case of *Acinetobacter* in the beef samples from E, F, S and T retails (avg. 8%) and *Leuconostoc* in samples O3 and N3 avg 4%).

In the Figure 3 the genera shared between the samples are represented. Grouping meat and environmental samples separately for LD and SD, 31 genera, were common to all the samples (Figure 3a). These genera included *Streptococcus*, *Brochothrix*, *Pseudomonas*, *Acinetobacter* and *Psychrobacter* sp. that were also the most abundant in the core microbiota (avg. relative abundances higher than 10%, Table 7). Fifty genera were shared by meat and environmental samples belonging to both type of retails (Figure 3b).

Permutational Multivariate Analysis of Variance based on both Bray Curtis and Jaccard distance matrices showed significant difference in the overall microbiota between swabs and meat samples ($P < 0.001$). On the contrary, no effect of the type of retail was observed ($P > 0.05$). The hierarchical clustering in Figure 4 shows a certain degree of separation between meat and environmental samples, mostly driven by the abundance of OTUs within the Proteobacteria phylum, significantly higher in meat compared to swab samples (FDR < 0.05). *Pseudomonas* and several *Enterobacteriaceae* were significantly more abundant in meat samples, while *Staphylococcus*, *Streptococcus*, *Lactococcus lactis*, *Leuconostoc*, *Brochothrix* and *Psychrobacter,* showed higher levels in environmental samples (FDR < 0.05). Accordingly, a Principal Component Analysis (PCA) based on microbiota composition showed no clustering of the samples according to the retail type (Figure S2), and even when SD or LD samples were analyzed separately, the clustering was consistently driven by the sample type (Figure S2 B and C).

The OTU co-occurrence was investigated by considering the genus-level taxonomic assignment and including OTUs with at least 0.1% of relative abundance in at least 50% of samples. Significant correlations at FDR<0.05 are plotted in Figure S3. *Basfia* showed strong positive correlations with *Bordetella* and *Streptococcus*. *Gammaproteobacteria* co-occurred with OTU-core members such as *Acinetobacter* and *Moraxellaceae,* while *Lactococcus* showed a weak co-occurrence with *Lactobacillus.*

Predicted metabolic activities

Potential metabolic activities of the samples were predicted by using the software PICRUSt. A consistent grouping of the samples on the basis of the sample type (meat vs environment) was achieved also when considering the predicted microbial pathways (Figure 5). Pathways related to carbohydrates metabolism increased in environmental swabs, while aminoacid and lipid metabolisms were more abundant in meat (FDR < 0.05). In particular, arginine, proline and aromatic aminoacid metabolisms, as well as fatty acid metabolism, had higher levels in meat (FDR < 0.05). Spearman's correlations between predicted pathways and OTUs are reported in Figure 6, where only Proteobacteria and Firmicutes phyla are shown. Proteobacteria OTUs, in particular Pseudomonas, several Enterobactericeae and Psychrobacter, were positively correlated to lipid and aminoacid metabolisms, while Firmicutes, such as Brochothrix and lactic acid bacteria co-occurred with carbohydrates-related pathways (FDR <0.05).

2.3.4 Discussion

In this study, the microbiota in twenty butcheries was studied by rRNA gene-based culture-independent high-throughput sequencing in order to identify the relationships between the microbial diversity of processing environment and meat and to compare the microbiota occurring in small-scale retail distribution (SD) and butcher counters in large-scale retail distribution (LD). Results showed no significant effect of butchery types on bacterial counts in agreement with previous reports(Andritsos et al., 2012; Pèrez-Rodrìguez et al., 2010). The microbiota of the environment was extremely complex, including more than 500 taxa at the genus/species level, as well as that of fresh meat cuts and environmental samples (Stoops et al., 2015; Zhao et al., 2015; De Filippis, La Storia, et al., 2013; Ercolini et al., 2011). Meat microbial complexity usually decreases strongly after storage as a consequence of the effect of abiotic factors, such as storage temperature and type of packaging used, which select few species to become dominant and spoil the meat (Stoops et al., 2015; Zhao et al., 2015; De Filippis, La Storia, et al., 2013; Ercolini et al., 2011). A significantly higher microbial diversity and viable counts were observed in the Chopping Board compared to the Knife. This difference suggests that surface contamination is strongly affected by surface material, which represents an important aspect to take into account in order to maintain acceptable levels of hygiene in the food processing plants(Hultman et al., 2015; Stellato, De Filippis, et al., 2015; De Filippis, La Storia, et al., 2013; Faille & Carpentier 2009). Porous material tools such as wooden chopping boards, are less adequate for a thorough cleaning, and increase possibility of adherence by bacteria and establishments of resident microbiota (Stellato, La Storia, et al., 2015; Faille & Carpentier 2009; PJ et al., 2009). Moreover, ecological factors, nutrient availability and composition, capability of microbes to develop biofilms and adhere to the surfaces, cleaning procedures and staff hygiene training all play an important role on the microbiological fresh meat quality (Doulgeraki et al., 2012; Ksontini et al., 2013; Marchand et al., 2012). The microbial community composition across surfaces in meat processing plants is reported as highly variable and most of the OTUs identified in the meat samples (raw, spoiled and processed) originate from the processing environment (Hultman et al., 2015; Giaouris et al., 2014; De Filippis, La Storia, et al., 2013). In the present study, *Pseudomonas* sp*., Brochothrix* sp*., Psychrobacter* sp., *Streptococcus* sp. and *Acinetobacter* sp. were identified as the core microbiota occurring in all the samples analyzed. They were previously reported as contaminants in food processing environments (Møretrø et al., 2013; Brightwell et al., 2006; Bagge-Ravn et al., 2003). Our results indicate that they are part of a resident microbiome, but we showed that their prevalence is not influenced by the type of retail considered. The microorganisms found in the processing facilities sampled here frequently occur on freshly cut and aerobically stored meat (Hultman et al., 2015; Doulgeraki et al., 2012), they are recognized as undesired bacteria in food processing environments (Doulgeraki et al., 2012) and as main contributors to meat spoilage (Hultman et al., 2015; Remenant et al., 2015; Olusegun & Iniobong 2011; Borch et al., 1996). In particular, *Pseudomonas* spp. are recognized as able to form biofilm (Liu et al., 2013; Myszka & Czaczyk 2011; PJ et al., 2009; Van Houdt & Michiels 2010), adhering to surfaces and improving their resistance to sanitation and cleaning procedures (Giaouris et al., 2014; Grobe et al., 2001; Wirtanen et al., 2001).

Predicted metagenomes highlighted the remarkable abundance of aminoacid and lipid metabolisms in meat samples and their strong correlation to Proteobacteria, such as *Pseudomonas* and *Enterobacteriaceae* OTUs. *Pseudomonas fragi* were previously reported as lipolytic and proteolytic, as well as species belonging to *Enterobacteriaceae* family, and they can contribute to spoilage through the production of volatile organic compounds and other undesired metabolites, such as biogenic amines (Ercolini, Ferrocino, et al., 2010; De Filippis, Pennacchia, et al., 2013; Samet-Bali et al., 2013;

Morales et al., 2003; Rodarte et al., 2011; Chaves-Lopez et al., 2006). On the contrary, *B. thermosphacta* and lactic acid bacteria were mainly correlated to carbohydrates metabolism. Accordingly, none of the strains of *B. thermosphacta* and *Carnobacterium maltaromaticum* previously tested were found as proteolytic and lipolytic, while producing off-flavours arising from sugar catabolism (Casaburi et al., 2014; Casaburi et al., 2011). Moreover, lactic acid bacteria are reported as producers of polysaccharydic ropy slime (Pothakos, Stellato, et al., 2015; Lyhs et al., 2004; PM et al., 1992). However, spoilage related activities have to be considered as strain-specific and may be influenced by abiotic factors, such as pH, NaCl concentration or temperature, as well as interactions with other components of the microbial community (Doulgeraki et al., 2012; Casaburi et al., 2015; Pothakos, Stellato, et al., 2015).

2.3.5 Conclusion

Our results supported the importance of environmental microbiota in influencing the quality and the safety of meat and highlights the lack of difference between microbiota distribution in small vs large scale meat processing environments. Meat contamination is strongly dependent by that of the environment where it is handled and processed. The initial level of microbial contamination and the community composition will influence meat potential shelf-life, depending on storage conditions. Therefore, adequate choice of surface materials and extremely accurate cleaning procedures are necessary in order to avoid spreading of bacteria that can contaminate the meat and potentially cause spoilage.

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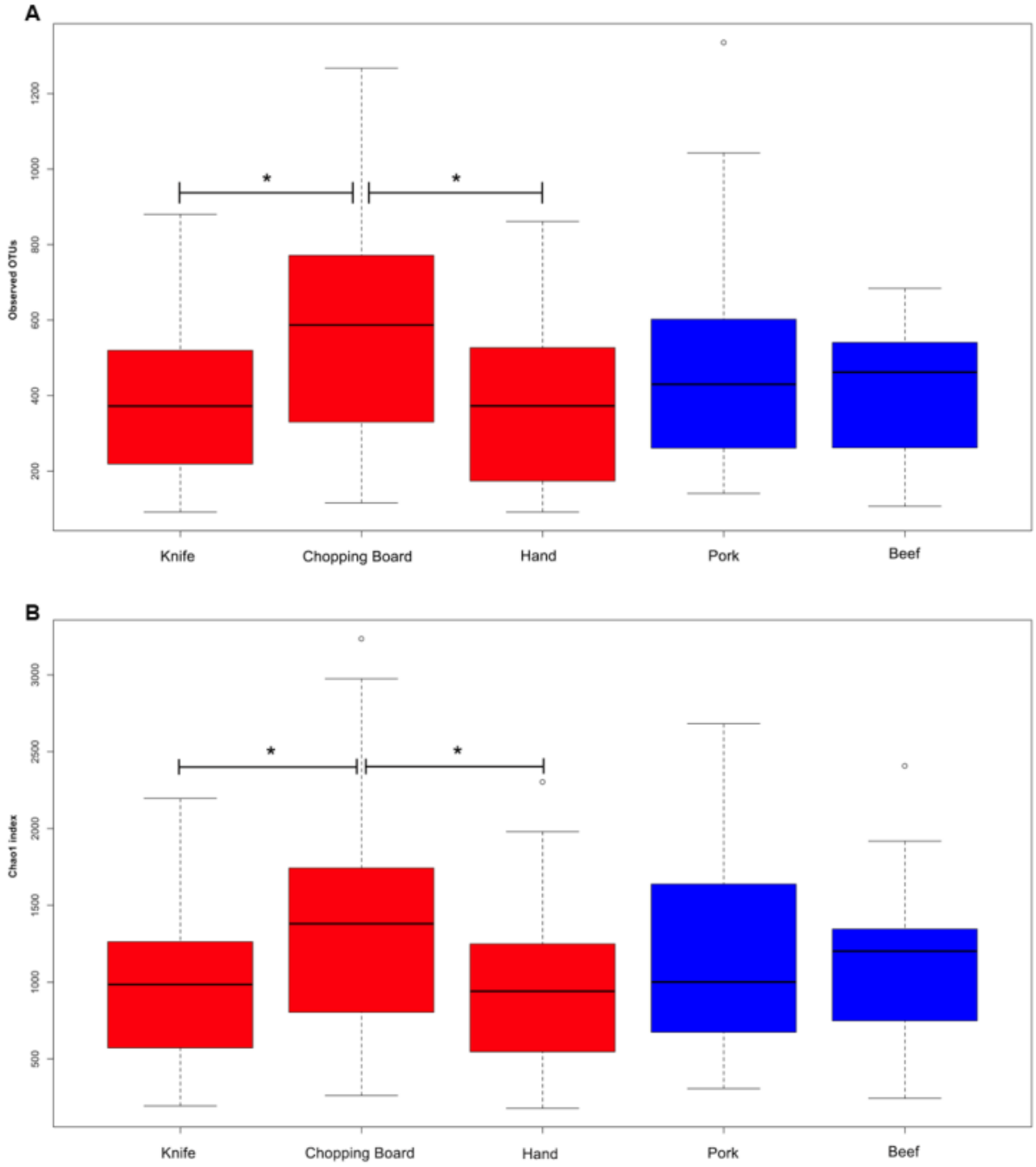


Figure 1 – Box plots showing number of observed OTUs (a) and Chao1 diversity index (b) in the environmental swabs (red) and meat samples (blue). Boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside represents the median (2nd quartile). Whiskers denote the lowest and the highest values within 1.5 x IQR from the first and third quartiles, respectively. Circles represent outliers beyond the whiskers. Asterisks indicate a significant difference as obtained by pairwise Wilcox test (FDR < 0.05).

a

Figure 2a - Abundance of bacterial species in meat samples from the SDs. Only OTUs showing a relative abundance > =1% and occurring in more than 5 samples are reported.

b

Figure 2 - Abundance of bacterial species in environmental (panel b) samples from the SDs. Only OTUs showing a relative abundance > =1% and occurring in more than 5 samples are reported.

c

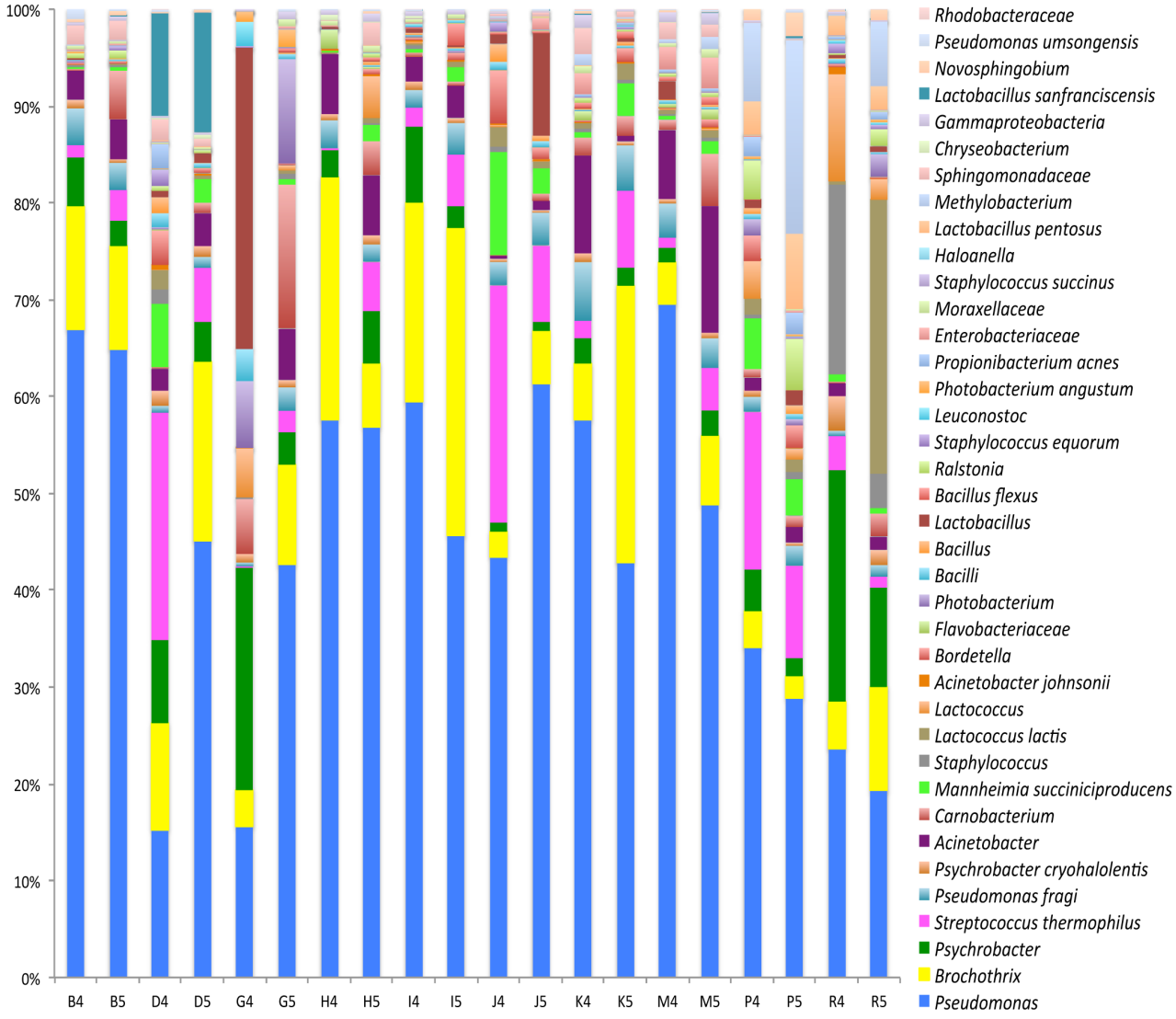


Figure 2 - Abundance of bacterial species in meat (panel c) samples from the LDs. Only OTUs showing a relative abundance > =1% and occurring in more than 5 samples are reported.

d

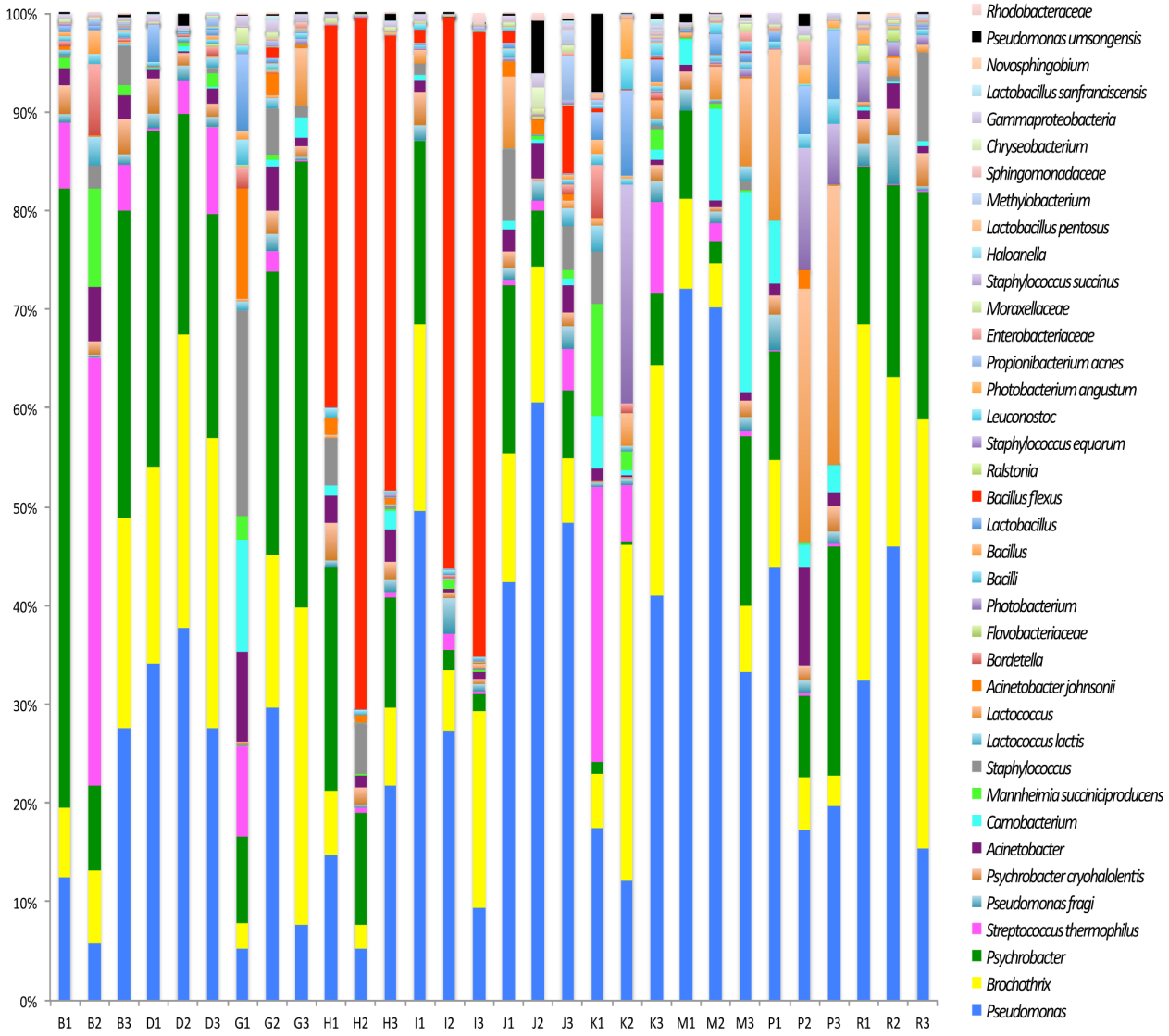
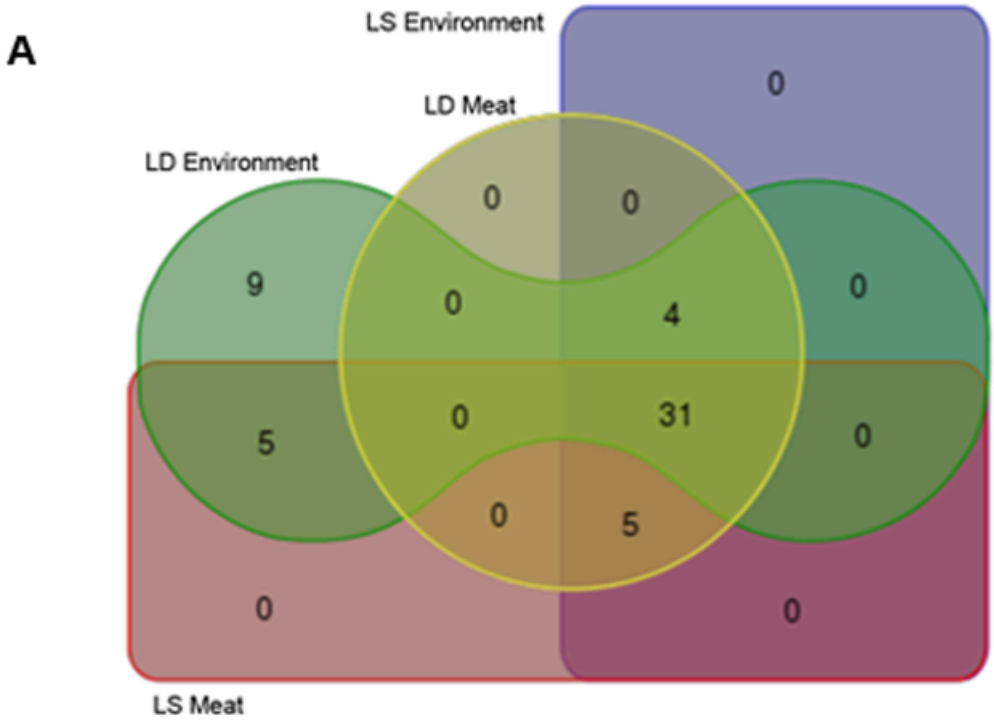


Figure 2 - Abundance of bacterial species in environmental (panel d) samples from the LDs. Only OTUs showing a relative abundance > =1% and occurring in more than 5 samples are reported.



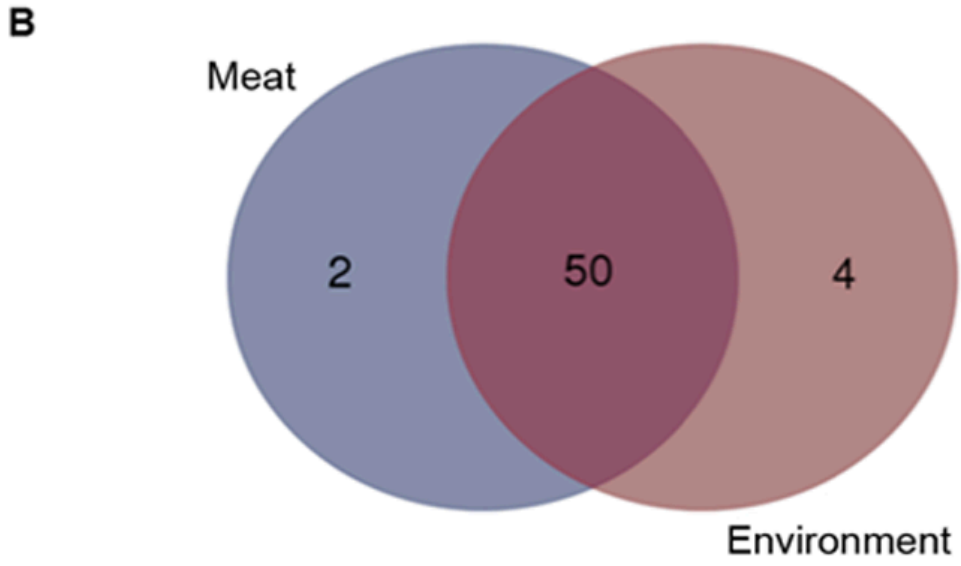


Figure 3 - Venn Diagram showing the number of shared OTUs between groups of samples obtained by 16S rRNA gene pyrosequencing analysis. Samples were grouped by meat and environmental samples for both LD and SD groups (panel a); and their combination separating environmental from meat samples (panel b).

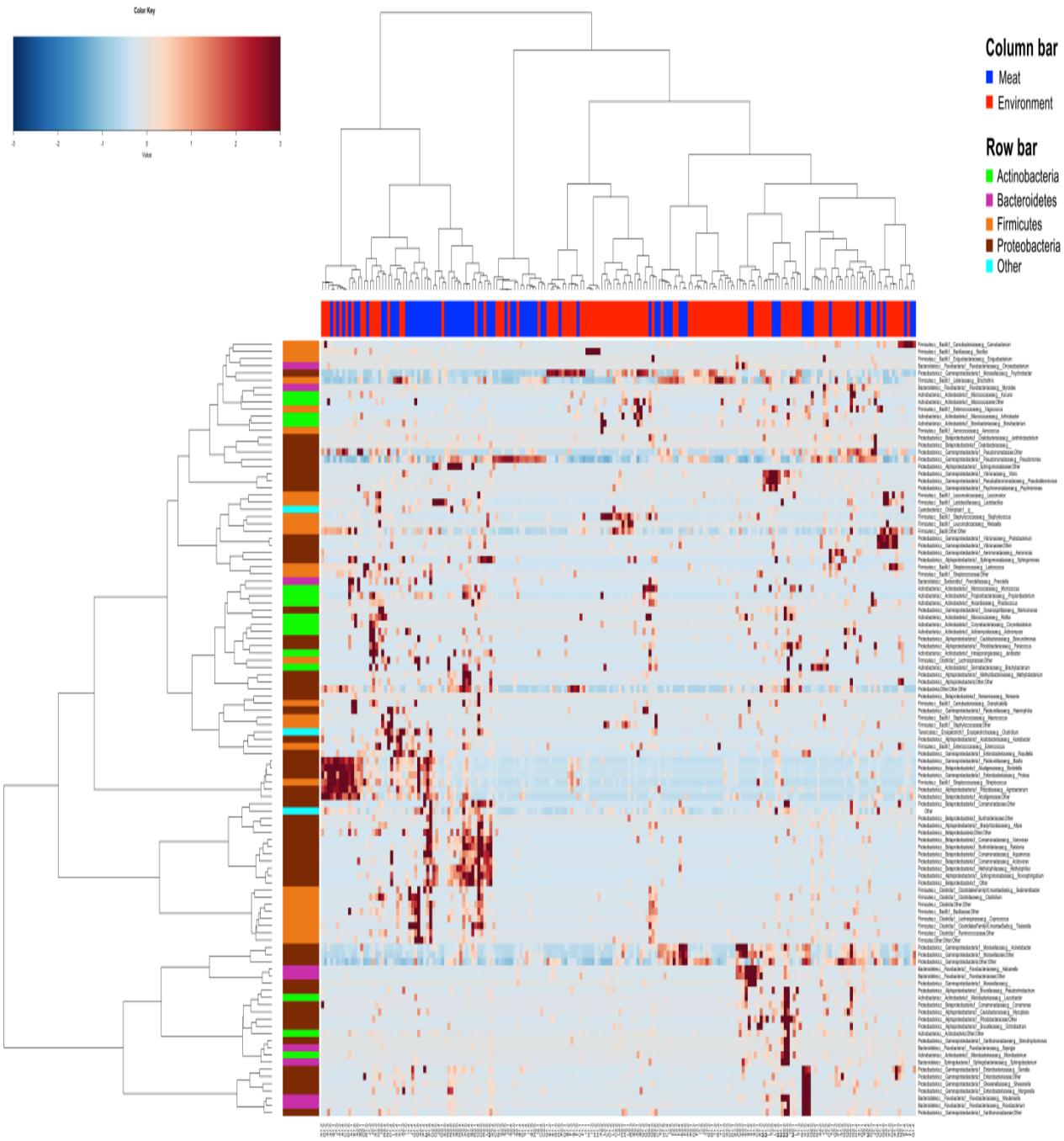


Figure 4 – Hierarchical average-linkage clustering of the samples based on the Pearson's correlation coefficient of the abundance of OTUs present at least in 20% of the samples. The color scale represents the scaled abundance of each variable, denoted as Z-score, with red indicating high abundance and blue indicating low abundance. Column bar is colored according to the type of sample (meat or environmental swab) and the row bar is colored according to the OTU classification at phylum level.

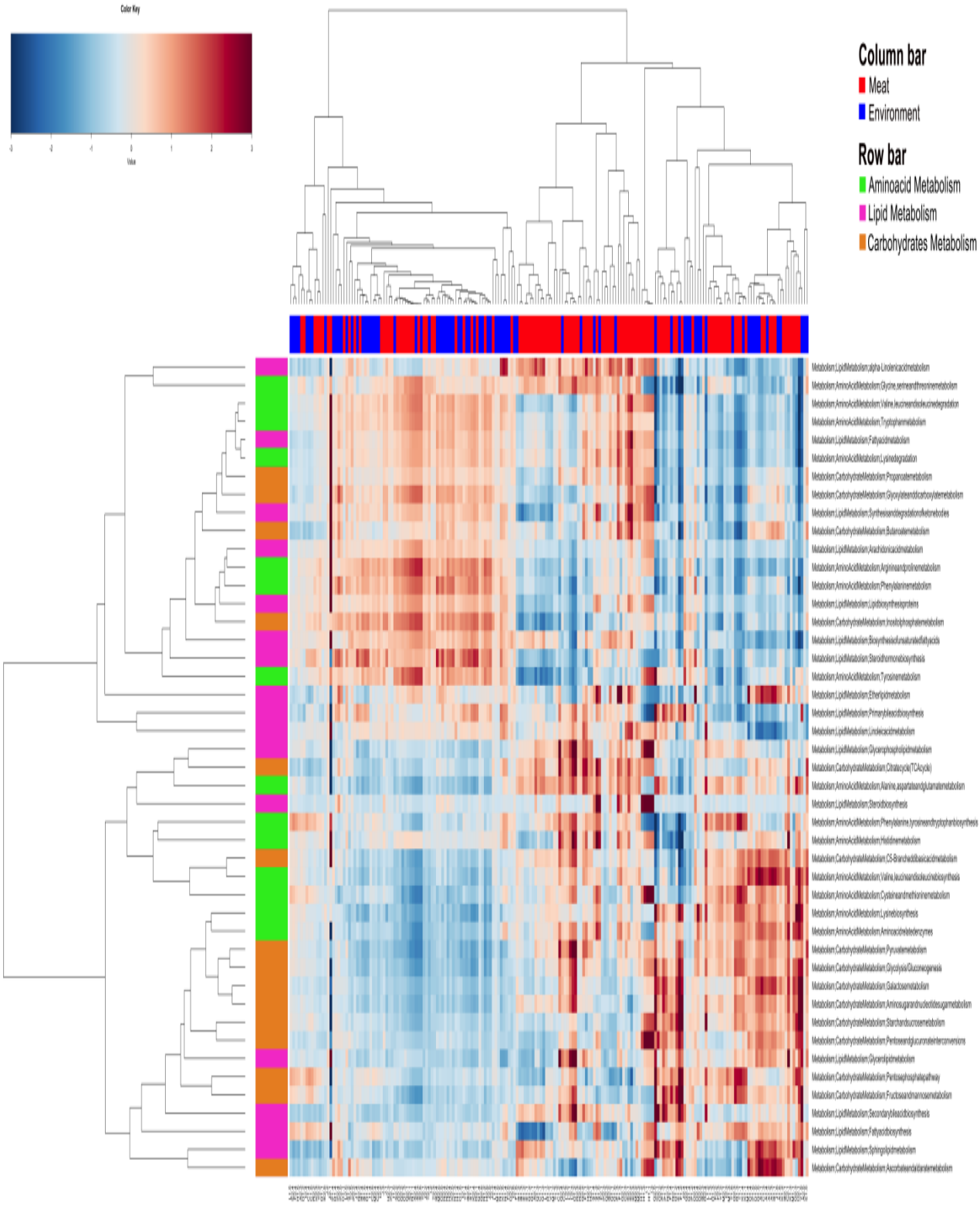


Figure 5 – Hierarchical average-linkage clustering of the samples based on the Pearson's correlation coefficient of the abundance of predicted KEGG Orthologs collapsed at level 3 of hierarchy, filtered for sample prevalence of at least 20%. The colour scale represents the scaled abundance of each variable, denoted as Z-score, with red indicating high abundance and blue indicating low abundance. Column bar is coloured according to the type of sample (meat or environmental swab). Row bar colour denotes the higher level of hierarchy in the KEGG classification. Only KEGG Orthologs related to carbohydrates, aminoacid and lipid metabolisms are reported.

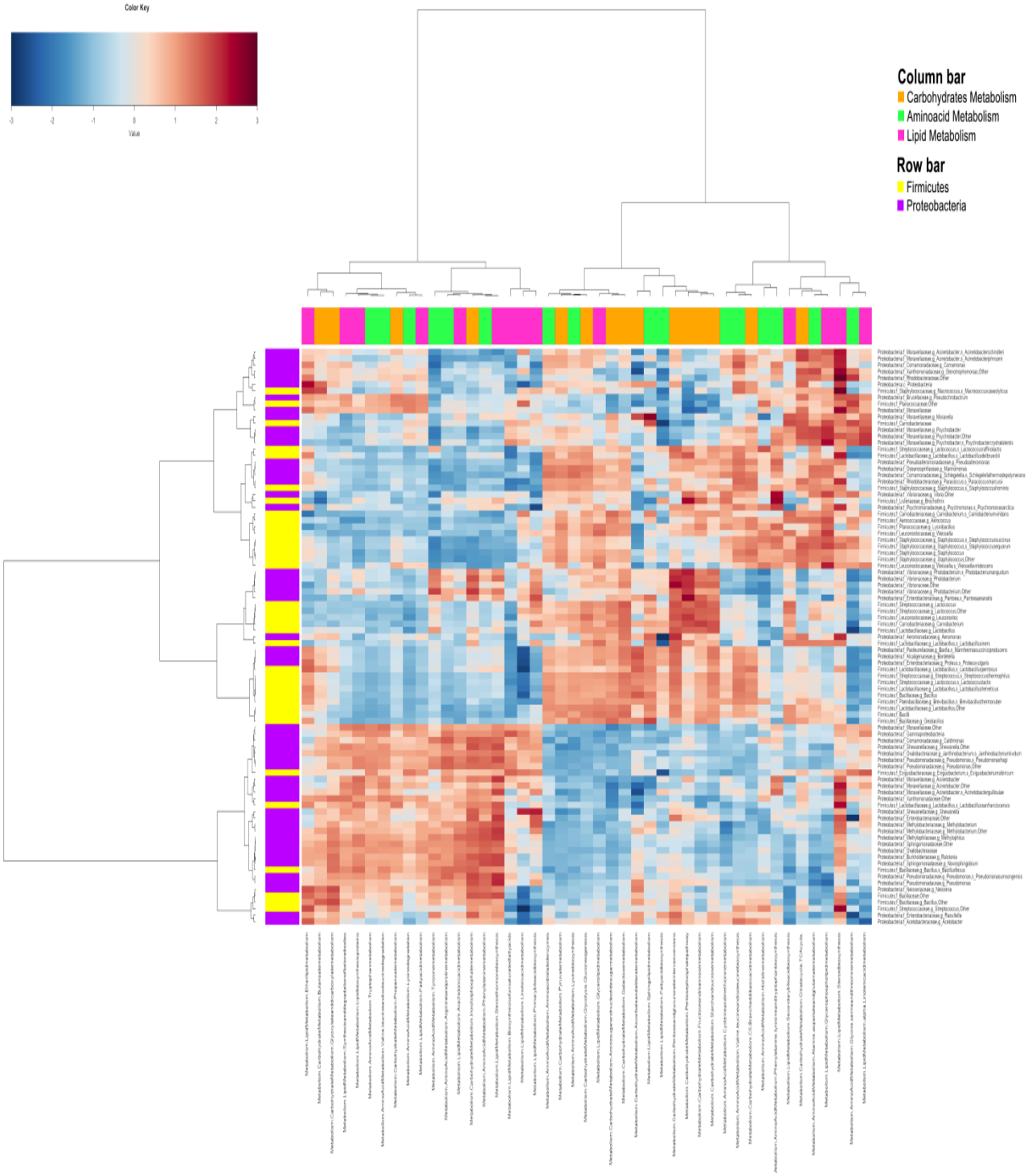


Figure 6 – Heatplot showing the correlations between Firmicutes and Proteobacteria OTUs and predicted KEGG Orthologs collapsed at level 3 of hierarchy, both filtered for sample prevalence of at least 20%. Rows and columns are clustered by Euclidean distance and Ward linkage hierarchical clustering. The intensity of the colours represents the degree of association between the OTUs and the KEGG Orthologs as measured by the Spearman's correlations. Row bar is coloured acorrding to the OTU classification at phylum level. Column bar colour denotes the higher level of hierarchy in the KEGG classification. Only KEGG Orthologs related to carbohydrates, aminoacid and lipid metabolisms and are reported.

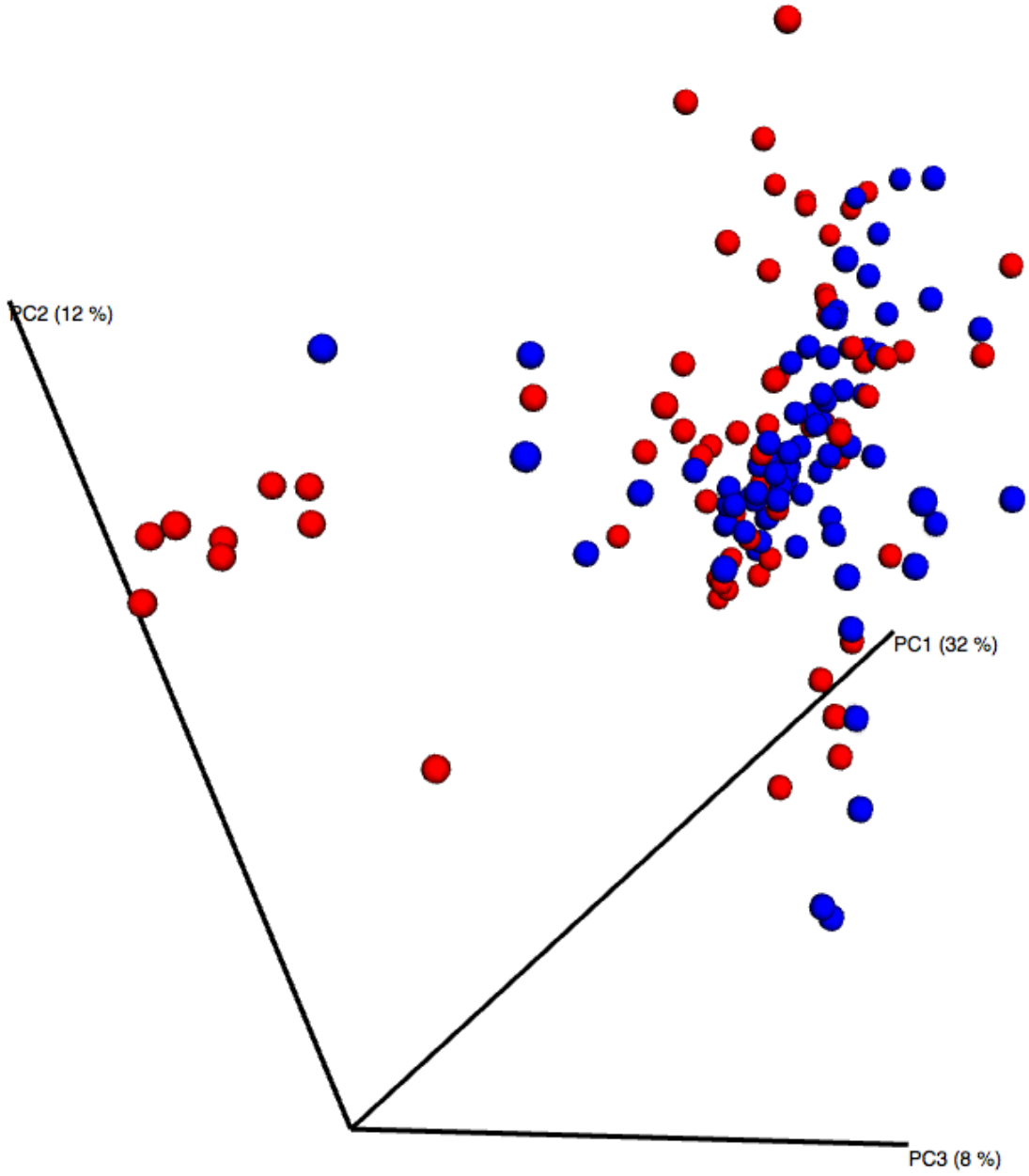


Figure S1 - Principal Coordinates Analysis (PCoA) based on weighted UniFrac distance matrix of the microbial composition. Samples are labeled with different colors: first sampling (red dots) and second sampling (blue dots).

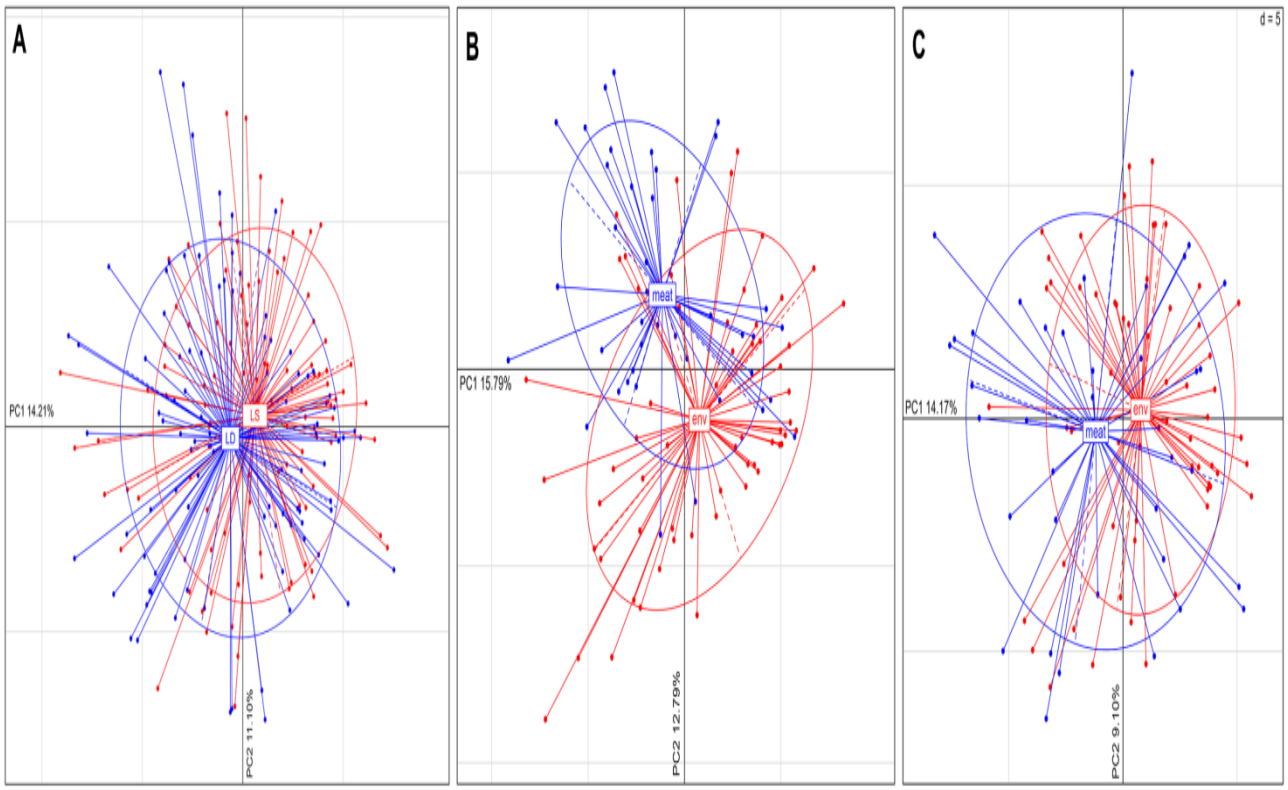


Figure S2 - Principal Component Analysis based on the microbiota composition. The two principal components were plotted using the *vegan* package in R. The center of gravity for each cluster is marked by a rectangle indicating the sample type (LD, large-scale retail distribution; LS, small-scale retail distribution). Only those OTUs which showed a loading score > = 0.7 are shown in the figure.

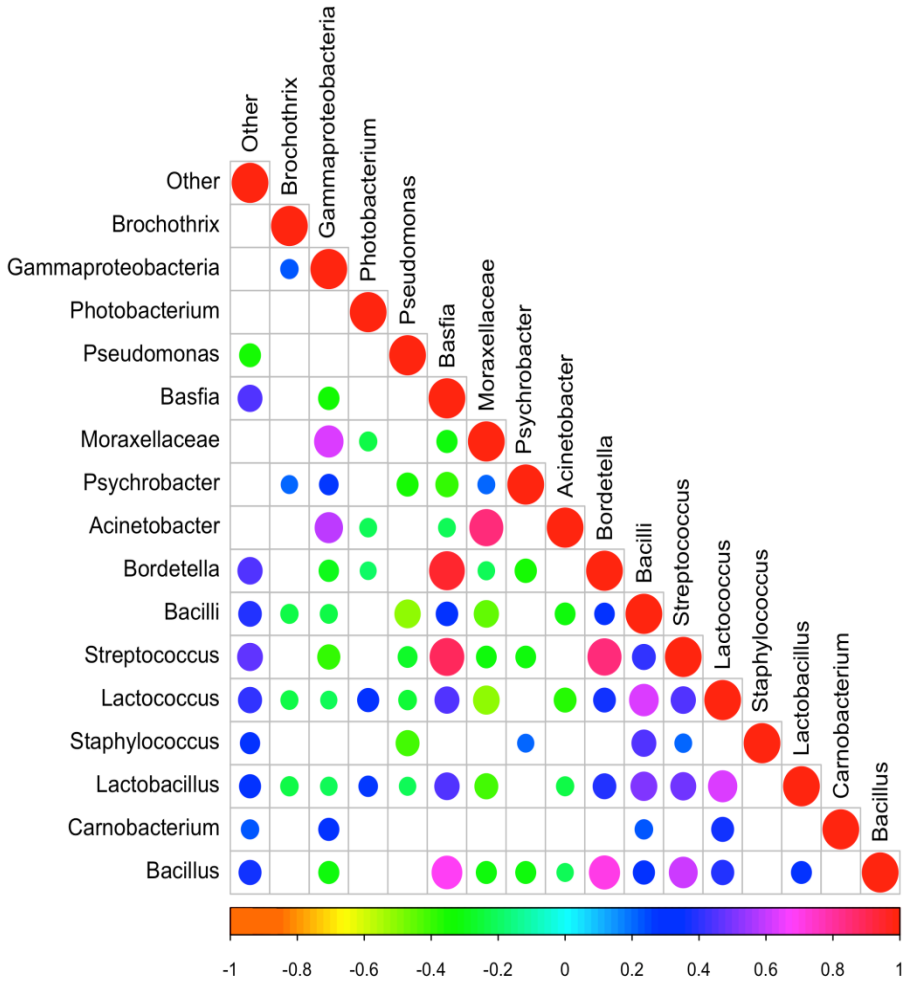


Figure S3 - Spearman's rank correlation matrix of bacterial OTUs with > = 0.1% abundance in at least 50% of the samples. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (dark blue) and −1 indicating a perfectly negative correlation (dark red) between two microbial genera. Only significant correlations (FDR<0.05) are shown**.**

Table 1 - Aerobic plate counts (APC) in meat and environmental samples analyzed in this study.

Sample	Mean ± SD (Log_{10} CFU/g)		Mean ± SD (Log_{10} CFU/cm^2)		
	Beef	Pork	Hand	Knife	Chopping board
A	3.27 ± 1.06	2.79 ± 1.86	1.27 ± 1.13	1.14 ± 0.28	0.96 ± 0.02
*B	5.93 ± 0.78	5.69 ± 0.31	1.95 ± 1.15	1.30 ± 1.02	1.13 ± 0.61
C	5.51 ± 0.49	5.69 ± 0.63	2.16 ± 0.47	1.60 ± 1.06	1.22 ± 0.82
*D	4.58 ± 0.97	4.29 ± 0.69	1.21 ± 0.76	1.38 ± 1.40	1.37 ± 0.00
E	5.18 ± 1.76	4.64 ± 1.32	1.28 ±1.80	1.42 ± 0.54	1.18 ± 1.06
F	4.94 ± 2.17	5.35 ± 1.59	2.00 ± 1.01	1.81 ± 0.34	1.37 ± 0.00
*G	6.45 ± 0.01	6.03 ± 1.87	2.31 ± 1.37	1.68 ± 0.88	1.39 ± 0.95
*H	6.33 ± 0.20	4.63 ± 0.54	1.15 ± 0.24	0.68 ± 0.58	0.83 ± 2.51
*I	5.21 ± 1.24	5.70 ± 1.11	1.28 ± 0.47	1.53 ± 0.63	0.80 ± 1.81
*J	5.23 ± 0.27	4.73 ± 1.06	1.20 ± 0.00	1.42 ± 1.73	1.02 ± 1.02
*K	4.45 ± 1.41	4.66 ± 0.69	1.37 ± 0.85	1.19 ± 0.53	0.92 ± 1.54
L	4.82 ± 0.49	4.31 ± 0.11	1.92 ± 1.24	1.44 ± 0.96	1.19 ± 0.99
*M	4.25 ± 0.15	4.85 ± 1.53	1.73 ± 0.19	1.52 ± 0.20	1.31 ± 0.20
N	4.15 ± 0.34	4.58 ± 0.88	1.71 ± 0.87	1.29 ± 0.83	1.03 ± 0.13
O	5.21 ± 1.17	3.60 ± 0.04	1.54 ± 0.81	1.40 ± 0.54	1.02 ± 0.17
*P	3.92 ± 0.90	4.11 ± 0.29	1.94 ± 0.96	1.59 ± 0.31	1.37 ± 0.00
Q	4.89 ± 0.82	4.08 ± 0.28	0.91 ± 0.55	0.90 ± 1.03	0.63 ± 0.03
*R	5.18 ± 0.83	4.35 ± 0.12	1.13 ± 0.38	1.16 ± 1.01	1.31 ± 0.31
S	4.92 ± 0.01	5.20 ± 0.57	2.30 ± 0.00	1.82 ± 0.31	1.37 ± 0.00
T	5.00 ± 0.10	6.10 ± 0.53	1.23 ± 1.34	1.80 ± 0.41	1.37 ± 0.00
Mean	4.97 ± 1.02[a]	4.77 ± 1.09[a]	1.58 ± 1.21[b]	1.40±1.03[ac]	1.14±1.17[ab]

SD = Standard Deviation. Different uppercase letters indicate significant differences ($P < 0.05$). * indicates samples from large-scale retail distribution.

Table 2 – Counts of *Enterobacteriaceae* in meat and environmental samples analyzed in this study

Sample	Mean ± SD (Log_{10} CFU/g)		Mean ± SD (Log_{10} CFU/cm^2)		
	Beef	Pork	Hand	Knife	Chopping board
A	1.13 ± 0.49	0.89 ± 0.16	0.00 ± 0.00	0.06 ± 0.21	0.29 ± 1.00
*B	2.96 ± 0.96	2.80 ± 0.04	0.49 ± 0.98	0.06 ± 0.21	0.79 ± 0.34
C	1.31 ± 0.75	1.30 ± 0.00	0.68 ± 1.62	0.41 ± 1.38	0.74 ± 1.22
*D	0.89 ± 0.16	0.78 ± 0.00	0.00 ± 0.00	0.24 ± 0.83	0.65 ± 0.12
E	0.89 ± 0.16	1.66 ± 0.93	0.14 ± 0.49	0.76 ± 1.28	0.81 ± 0.90
F	2.98 ± 1.39	1.84 ± 1.19	0.77 ± 2.60	0.64 ± 1.19	1.69 ± 2.04
*G	2.82 ± 0.26	2.61 ± 1.61	0.71 ± 0.88	0.76 ± 2.14	1.00 ± 0.66
*H	2.77 ± 0.84	1.28 ± 0.71	0.41 ± 0.28	0.27 ± 0.92	0.78 ± 0.09
*I	1.37 ± 0.83	1.99 ± 1.40	0.00 ± 0.00	0.39 ± 0.49	0.30 ± 1.01
*J	1.39 ± 0.12	2.05 ± 1.06	0.06 ± 0.21	0.66 ± 2.25	0.18 ± 0.21
*K	1.54 ± 1.08	0.89 ± 0.16	0.00 ± 0.00	0.20 ± 0.67	0.20 ± 0.67
L	1.24 ± 0.34	0.81 ± 0.05	0.34 ± 0.74	0.00 ± 0.00	0.21 ± 0.71
*M	0.92 ± 0.11	1.22 ± 0.54	0.06 ± 0.21	0.20 ± 0.93	0.33 ± 0.71
N	0.92 ± 0.11	1.48 ± 0.00	0.47 ± 0.74	0.30 ± 0.60	0.36 ± 0.82
O	1.87 ± 0.80	2.01 ± 0.75	0.00 ± 0.00	0.06 ± 0.21	0.22 ± 0.76
*P	0.70 ± 0.70	1.61 ± 1.18	0.42 ± 0.43	0.98 ± 0.38	1.04 ± 0.18
Q	1.15 ± 0.64	0.74 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
*R	0.89 ± 0.16	1.44 ± 1.04	0.00 ± 0.00	0.16 ± 0.55	1.60 ± 1.28
S	1.82 ± 1.38	2.68 ± 0.14	0.21 ± 0.71	1.00 ± 0.18	0.94 ± 0.72
T	2.93 ± 0.58	3.18 ± 0.06	0.18 ± 0.64	0.98 ± 0.69	1.33 ± 0.43
Mean	1.62 ± 0.94[a]	1.66 ± 0.90[a]	0.24 ± 0.86[b]	0.41±1.07[b,c]	0.67 ± 1.30[a,c]

SD = Standard Deviation. Different uppercase letters indicate significant differences ($P < 0.05$). * indicates samples from large-scale retail distribution.

Table 3 - Counts of lactic acid bacteria in meat and environmental samples analyzed in this study

Sample	Mean ± SD (Log_{10} CFU/g)		Mean ± SD (Log_{10} CFU/cm^2)		
	Beef	Pork	Hand	Knife	Chopping board
A	2.56 ± 0.12	2.45 ± 1.20	0.99 ± 0.11	0.92 ± 1.23	0.73 ± 0.50
*B	4.55 ± 0.54	4.09 ± 0.38	1.57 ± 0.40	1.31 ± 1.48	1.07 ± 1.02
C	4.11 ± 0.32	4.90 ± 1.31	1.69 ± 0.28	1.42 ± 1.11	1.27 ± 0.54
*D	3.70 ± 0.98	4.25 ± 0.39	0.92 ± 0.37	1.14 ± 1.04	1.21 ± 0.31
E	4.56 ± 1.50	4.15 ± 0.98	1.12 ± 0.52	0.86 ± 0.59	1.04 ± 1.84
F	4.11 ± 0.83	4.68 ± 0.37	1.90 ± 1.33	1.00. ± 1.33	1.24 ± 0.71
*G	5.13 ± 1.39	5.04 ± 0.31	2.13 ± 1.10	1.61 ± 0.26	1.30 ± 0.19
*H	4.08 ± 0.45	3.57 ± 0.65	1.29 ± 1.10	1.22 ± 0.21	1.14± 1.26
*I	4.11 ± 0.34	4.32 ± 1.89	0.50 ±1 .69	1.25 ± 0.62	1.27± 0.44
*J	4.02 ± 0.26	3.67 ± 1.12	0.87 ± 0.06	1.23 ± 1.12	1.00± 1.01
*K	3.55 ± 0.89	3.14 ± 1.32	0.83 ± 0.5	1.10 ± 1.76	1.01± 0.79
L	4.79 ± 0.95	4.16 ± 1.86	1.94 ± 1.18	1.28 ± 0.93	1.16± 0.26
*M	2.84 ± 0.90	2.83 ± 0.75	1.12 ± 1.38	1.00 ± 2.13	0.75± 1.56
N	3.02 ± 0.23	3.99 ± 0.86	1.52 ± 0.24	1.05 ± 0.31	0.83± 0.26
O	4.26 ± 1.29	4.50 ± 0.74	1.64 ± 0.18	1.44 ± 0.91	1.32± 0.26
*P	2.70 ± 0.32	3.43 ± 0.39	1.47 ± 0.68	1.20 ± 0.01	1.13± 0.71
Q	3.82 ± 0.35	3.30 ± 0.33	1.22 ± 0.00	0.83 ± 0.04	0.62± 0.29
*R	4.65 ± 0.61	3.89 ± 0.71	1.33 ± 0.29	1.31 ± 0.49	1.31± 0.19
S	4.92 ± 0.05	4.25 ± 0.04	1.28 ± 2.26	1.67 ± 0.58	1.27± 0.54
T	4.25 ± 0.74	4.68 ± 1.34	0.94 ± 0.31	1.27 ± 0.36	1.22± 0.05
Mean	3.99 ± 0.92$^{a.b}$	3.96 ± 0.98$^{a.b}$	1.31 ± 1.19^{c}	1.23±0.96$^{a.b}$	1.0975±1.09^{c}

SD = Standard Deviation. Different uppercase letters indicate significant differences ($P < 0.05$). * indicates samples from large-scale retail distribution.

Table 4- Counts of *Pseudomonas* spp. in meat and environmental samples analyzed in this study

Sample	Mean ± SD (Log_{10} CFU/g)		Mean ± SD (Log_{10} CFU/cm^2)		
	Beef	Pork	Hand	Knife	Chopping board
A	2.39 ± 2.28	1.86 ± 1.53	0.44 ± 2.06	0.36 ± 0.14	0.26 ± 0.72
*B	6.05 ± 0.60	5.65 ± 0.10	0.49 ± 1.08	0.39 ± 0.84	0.29 ± 0.15
C	5.41 ± 0.54	5.58 ± 0.62	0.49 ± 0.39	0.37 ± 3.30	0.29 ± 0.11
*D	4.13 ± 0.48	4.05 ± 0.74	0.45 ± 1.82	0.39 ± 1.32	0.30 ± 0.26
E	4.61 ± 2.17	4.63 ± 0.86	0.46 ± 2.08	0.37 ± 0.16	0.29 ± 0.81
F	4.90 ± 2.23	5.04 ± 2.03	0.49 ± 1.84	0.40 ± 0.57	0.30 ± 0.27
*G	5.44 ± 1.23	5.60 ± 1.88	0.50 ± 2.45	0.41 ± 0.79	0.29 ± 1.92
*H	6.46 ± 0.02	4.84 ± 0.00	0.47 ± 1.20	0.39 ± 1.17	0.29 ± 0.32
*I	3.71 ± 0.05	6.49 ± 0.02	0.46 ± 0.45	0.40 ± 0.72	0.29 ± 0.28
*J	5.27 ± 0.60	5.07 ± 1.21	0.47 ± 0.31	0.39 ± 2.16	0.29 ± 1.43
*K	4.15 ± 0.44	4.53 ± 1.00	0.47 ± 1.66	0.37 ± 0.87	0.28 ± 1.17
L	4.57 ± 0.52	4.15 ± 0.15	0.49 ± 1.02	0.40 ± 0.80	0.29 ± 1.23
*M	4.36 ± 0.09	5.12 ± 1.52	0.48 ± 0.38	0.40 ± 0.07	0.30 ± 0.27
N	4.19 ± 0.74	4.44 ± 1.25	0.48 ± 0.32	0.39 ± 0.45	0.29 ± 1.16
O	5.58 ± 1.06	3.21 ± 0.59	0.46 ± 1.98	0.39 ± 1.27	0.28 ± 0.00
*P	2.81 ± 0.86	4.57 ± 2.27	0.47 ± 0.83	0.40 ± 0.73	0.30 ± 0.00
Q	5.01 ± 0.56	4.03 ± 1.11	0.45 ± 0.75	0.38 ± 0.82	0.27 ± 0.10
*R	4.92 ± 1.27	4.18 ± 0.24	0.46 ± 0.10	0.39 ± 0.87	0.30 ± 0.42
S	4.79 ± 0.05	5.28 ± 0.43	0.51 ± 0.02	0.41 ± 0.47	0.30 ± 0.00
T	4.91 ± 0.16	6.09 ± 0.54	0.46 ± 1.67	0.41 ± 0.29	0.30 ± 0.00
Mean	4.68 ± 1.23[a]	4.72 ± 1.30[a]	0.47 ± 1.43[b]	0.39 ±1.31[b]	0.29 ±1.12[a]

SD = Standard Deviation. Different uppercase letters indicate significant differences ($P < 0.05$). * indicates samples from large-scale retail distribution.

Table 5- Counts of *Brochothrix thermosphacta* in meat and environmental samples analyzed in this study

Sample	Mean ± SD (Log_{10} CFU/g)		Mean ± SD (Log_{10} CFU/cm^2)		
	Beef	Pork	Hand	Knife	Chopping board
A	1.95 ± 1.65	1.58 ± 1.13	0.17 ± 0.60	0.47 ± 1.92	0.33 ± 0.78
*B	5.39 ± 0.84	4.79 ± 0.07	1.68 ± 0.62	1.13 ± 0.87	1.00± 0.39
C	5.56 ± 0.99	5.24 ± 0.31	1.76 ± 0.76	1.10 ± 2.73	1.10 ± 0.11
*D	3.76 ± 0.53	3.57 ± 0.67	0.36 ±1.25	1.09 ± 2.06	1.31 ± 0.2
E	4.77 ± 2.41	4.37 ± 0.72	0.78 ± 1.78	0.46 ± 1.49	0.71 ± 0.27
F	4.69 ± 2.53	4.87 ± 2.27	1.94 ± 1.22	1.47 ± 0.81	1.30 ± 0.38
*G	5.77 ± 1.66	5.64 ± 2.10	2.14 ± 1.60	1.29 ± 2.45	1.09 ± 1.67
*H	5.67 ± 0.20	4.04 ± 0.51	1.23 ± 1.57	0.68 ± 0.59	1.30 ± 0.20
*I	4.25 ± 1.19	5.61 ± 1.15	1.05 ± 1.22	0.90 ± 2.09	1.09 ± 0.56
*J	4.37 ± 0.29	3.96 ± 0.57	0.68 ± 0.20	0.84 ± 2.58	0.72± 1.55
*K	4.21 ± 1.74	4.29 ± 0.09	1.12 ± 0.63	0.95 ± 0.35	0.50 ± 0.08
L	4.69 ± 0.62	3.92 ± 0.09	1.85 ± 0.80	1.41 ± 1.39	1.19 ± 1.00
*M	3.06 ± 0.87	4.02 ± 2.32	1.22 ± 0.16	1.07 ± 0.71	0.94 ± 0.11
N	3.27 ± 0.73	4.41 ± 0.94	1.76 ± 0.40	1.19 ± 0.00	0.98 ± 1.39
O	4.10 ± 0.53	2.81 ± 0.47	0.98 ± 1.36	0.92 ± 1.18	0.75 ± 1.29
*P	2.11 ± 0.47	2.37 ± 0.83	1.23 ± 0.34	1.03 ± 0.71	1.11 ± 1.01
Q	4.39 ± 0.22	3.09 ± 0.83	0.82 ± 0.70	0.52 ± 0.06	0.42 ± 0.04
*R	4.34 ± 1.18	3.69 ± 0.12	1.04 ± 0.65	0.97 ± 1.44	1.29 ± 0.43
S	4.61 ± 0.20	4.69 ± 0.32	1.38 ± 2.58	1.72 ± 0.70	1.22 ± 0.70
T	4.65 ± 0.63	5.44 ± 1.46	1.05 ± 0.90	1.63 ± 0.99	1.37 ± 0.00
Mean	4.28 ± 1.34[a]	4.10 ± 1.37[a]	1.21 ±1.47[b]	1.04 ±1.47[b]	0.98±1.37[a]

SD = Standard Deviation. Different uppercase letters indicate significant differences ($P < 0.05$). * indicated butcher counter in large scale retail distribution.

Table 6- Microbial counts in small and large-scale retails

Butchery type	Mean ± SD (Log_{10} CFU/g)		Mean ± SD (Log_{10} CFU/cm^2)		
	Beef	Pork	Hand	Knife	Chopping board
Aerobic plate counts					
Traditional	1.56 ± 1.24	1.68 ± 1.13	1.89 ± 1.07	1.56 ± 1.24	1.68 ± 1.13
Modern	1.63 ± 1.18	1.75 ± 0.77	1.99 ±1.25	1.63 ± 1.18	1.75 ± 0.77
Enterobacteriaceae counts					
Traditional	0.26 ± 0.92	0.39 ± 1.08	0.64 ± 1.27	0.26 ± 0.92	0.39 ± 1.08
Modern	0.20 ± 0.74	0.46 ± 1.08	0.74 ± 1.41	0.20 ± 0.74	0.46 ± 1.08
Lactic acid bacteria counts					
Traditional	1.35 ± 1.05	1.48 ± 0.94	1.82 ± 1.02	1.35 ±1.05	1.48 ± 0.94
Modern	1.23 ± 1.50	1.51 ± 1.04	1.90 ± 1.02	1.23 ± 1.5	1.51 ± 1.04
***Pseudomonas spp.* counts**					
Traditional	1.43 ± 1.50	1.42 ± 1.40	1.81 ± 1.17	1.43 ± 1.50	1.42 ± 1.40
Modern	1.42 ± 1.34	1.58 ± 1.06	1.98 ± 0.97	1.42 ± 1.34	1.58 ± 1.06
***Brochothrix thermosphacta* counts**					
Traditional	1.18 ± 1.60	1.27 ± 1.60	1.64 ± 1.45	1.18 ± 1.60	1.27 ± 1.60
Modern	1.30 ± 1.16	1.26 ± 1.18	1.69 ± 1.21	1.30 ± 1.16	1.26 ± 1.18

SD = Standard Deviation. No significance difference was found between the two retail types ($P > 0.05$).

Table 7- Relative abundance of the core OTUs shared by meats and surface samples.

genera	Min (%)	Max (%)	Average (%)
Acetobacter	0.01	0.00	0.00
*Acinetobacter**	0.33	0.00	0.16
Actinobacteria	0.02	0.00	0.01
Aeromonas	0.02	0.00	0.01
Agrobacterium	0.00	0.00	0.00
Alcaligenaceae	0.01	0.00	0.00
Alphaproteobacteria	0.01	0.00	0.00
Arthrobacter	0.04	0.00	0.02
Bacillaceae	0.01	0.00	0.00
*Bacilli**	0.03	0.00	0.02
Bacillus	0.69	0.00	0.35
Basfia	0.10	0.00	0.05
Bordetella	0.08	0.00	0.04
Brevibacterium	0.01	0.00	0.01
*Brochothrix**	0.42	0.00	0.21
*Carnobacterium**	0.22	0.00	0.11
Chryseobacterium	0.25	0.00	0.12
Comamonadaceae	0.00	0.00	0.00
Comamonas	0.03	0.00	0.02
Corynebacterium	0.05	0.00	0.02
*Enterobacteriaceae**	0.07	0.00	0.04
Enterococcus	0.01	0.00	0.00
Flavobacteriaceae	0.23	0.00	0.12
Flavobacterium	0.10	0.00	0.05
*Gammaproteobacteria**	0.01	0.00	0.01
Haloanella	0.04	0.00	0.02
Janthinobacterium	0.03	0.00	0.01
Kocuria	0.06	0.00	0.03
*Lactobacillus**	0.31	0.00	0.15
*Lactococcus**	0.33	0.00	0.16
Leuconostoc	0.07	0.00	0.03
Marinomonas	0.02	0.00	0.01
Methylobacterium	0.18	0.00	0.09
Methylophilus	0.02	0.00	0.01
*Moraxellaceae**	0.07	0.00	0.04
Moraxellaceae	0.02	0.00	0.01
Novosphingobium	0.04	0.00	0.02
Paracoccus	0.01	0.00	0.01
Photobacterium	0.25	0.00	0.12
Propionibacterium	0.04	0.00	0.02
*Proteobacteria**	0.01	0.00	0.00
Proteus	0.01	0.00	0.01
*Pseudomonas**	0.85	0.03	0.44
*Psychrobacter**	0.64	0.00	0.32
Ralstonia	0.09	0.00	0.05

2.4 Distribution of *Pseudomonas* sp. oligotypes describes contamination routes in food processing environment

2.4.1 Introduction

Processing environment can be a fundamental source of food contamination across the food chains. This is particularly challenging especially for fresh foods or for those types of products that are not subjected to heat treatments or other sanitization during their preparation. The level of contamination at manufacturers is assured by the application of internal control procedures and adequate environmental hygiene and personnel training. The spread of potential food spoilers or pathogens from environment to food is an ancient major concern in the food industry and recently, several studies have focused on the mapping of microbial contamination in food processing environments with the final aim to assess the types of microbes that can colonize the food processing environment and their abundance on surfaces and tools (Stellato, De Filippis, et al., 2015; Stellato et al., 2015; Hultman et al., 2015; Calasso et al., 2016; Bokulich et al., 2013; Bokulich & Mills 2013). Organic residues from food processing can create microenvironments for growth and accumulation of microorganisms that can be a relevant source of cross-contamination (Brooks & Flint 2008; Hood & Zottola 1997; McLandsborough et al., 2006).

Pseudomonas spp. are recognized as major food spoilers (Doulgeraki et al., 2012; Ercolini, Casaburi, et al., 2010; Martin et al., 2011; Marchand et al., 2012; Franzetti & Scrpellini 2007), they are psychrotrophic bacteria that easily develop in foods stored aerobically and at low temperatures such as meat, fish, milk and dairy products (Remenant et al., 2015; Nychas et al., 2008; De Jonghe et al., 2011). Species such as *Pseudomonas fragi*, *P. fluorescens*, *P. putida, P. jessardii, P. ludensis* (Ercolini et al., 2006; Ercolini, Casaburi, et al., 2010; De Jonghe et al., 2011) have been often isolated from spoiled foods and are currently recognized among the most threatening food spoilers. Once they colonize the food matrix, they can be responsible of slime and off odour production that finally compromise food quality and consumer's acceptability of the product (Casaburi et al., 2015; Pothakos, Devlieghere, et al., 2015; Doulgeraki et al., 2012; Nychas et al., 2008; Martin et al., 2011; Andreani et al., 2015). However, food spoiling potential is more than a species-specific trait. Studies on fresh meat have demonstrated that diverse strains of the same species can behave differently in exactly the same food matrix and storage conditions; accordingly, we have demonstrated that different biotypes can have different metabolic behaviours that will drive or not the spoilage-related activities and therefore determine whether the food spoilage will occur (Casaburi et al., 2014; Casaburi et al., 2011; Ercolini, Casaburi, et al., 2010). Such biodiversity of *Pseudomonas* beyond the species is surely reflected in colonization capability of the food-processing environment, which is the major source of contamination. In fact, different *Pseudomonas* species and biotypes are characterized by resistance to routine cleaning of surfaces and tools and capability for biofilm formation (Giaouris et al., 2014; Grobe et al., 2001; Wirtanen et al., 2001) that make them ideal candidates to become resilient microbiota of the food processing environment. The advances in technology and analysis tools for studies of microbial ecology have provided the possibility for in depth studies of microbial diversity in food and food-related environments and 16S rRNA gene high-throughput sequencing of microbial communities is routinely applied in many food science laboratories (Ercolini 2013). *Pseudomonas* has been found as an abundant member of milk (De Jonghe et al., 2011; Marchand et al., 2012) beef (Ercolini et al., 2009; Ercolini et al., 2006), pork (Bruckner et al., 2012), chicken (Mellor et al., 2011), fish (Reynisson et al., 2008) and as a major contaminant of different surfaces (Bagge-Ravn et al., 2003; Brightwell et al., 2006; Licitra et al., 2007; Stellato, La Storia, et al., 2015). However, identification at the genus is often unsure and identification even beyond the genus level cannot be achieved by 16S rRNA gene-based metagenomics. In fact, such approaches rely on taxonomic assignments using reference database to identify operational taxonomic units (OTUs). These methods of microbial identification fail to resolve ecologically meaningful differences between closely related organisms in complex environments (Eren et al., 2013). Alternative methods for rRNA gene sequencing data analyses have proved much more powerful to draw ecological information from culture-independent analysis of microbial communities and olygotyping can satisfy excellently this request.

Given the above discussed ecological relevance of the biodiversity within *Pseudomonas* in the food environment, in this study we used oligotyping to investigate the diversity of *Pseudomonas* populations in meat and dairy processing environments in order to investigate the overlap between food and food production environment, the existence of food- and environment-specific *Pseudomonas* types that can be possibly linked to resiliency in the food-processing environment and to possible food spoilage occurrence.

2.4.2 Matherials and Method

Sample collection and processing

A selection of samples from larger studies was used in order to better assess the ecological factors that contribute to the distribution of total *Pseudomonas* and individual types within and between food and environmental samples. Two previous large datasets one including samples from dairy plants (Stellato et al., 2015; Calasso et al., 2016) and one with

samples from meat processing plants (De Filippis et al., 2013; Chapter 3) were used, including both food and environmental samples. The dairy samples were collected from the two different dairy plants including cheese samples and environmental swab samples; they were both sampled twice (Stellato et al., 2015; Calasso et al., 2016). Meat samples were collected from twenty butcheries (belonging to large and small retail) including fresh beef and pork cuts and environmental swab samples; they were all sampled twice (De Filippis et al., 2013; Chapter 3). After collection, samples were cooled at 4^{o}C and analyzed within 3h.

Sequencing

The bacterial diversity was studied by pyrosequencing of the amplified V1–V3 region of the 16S rRNA gene, amplifying a fragment of 520 bp (Ercolini et al., 2012). 454-adaptors were included in the forward primer followed by a 10 bp sample-specific Multiplex Identifier (MID). PCR conditions were previously described (De Filippis, La Storia, et al., 2013). After agarose gel electrophoresis, PCR products were purified twice by Agencourt AMPure kit (Beckman Coulter, Milano, Italy), quantified using the PlateReader AF2200 (Eppendorf, Milano, Italy) and equimolar pools were obtained prior to further processing. Amplicons were prepared and sequenced using a GS Junior platform (454 Life Sciences, Roche, Italy) according to the Roche standard protocols.

Oligotyping analysis

For oligotyping analysis we used 308,842 quality-controlled V1-V3 reads from 197 samples that The Global Assignment of Sequence Taxonomy (GAST Huse *et al.*,, 2008) identified as "*Pseudomonas*". The PyNAST algorithm (Caporaso et al., 2010) aligned the 454 reads against the Greengenes (McDonald et al., 2012) multiple sequence alignment template (97% OTUs, 6 October 2010 release). Following the entropy analysis oligotyping was performed using the version 2.1 of the oligotyping pipeline (available from https://meren.github.io/projects/oligotyping/) using 14 components following the initial entropy analysis. To reduce noise in the results, we imposed that each oligotype must: appear in at least one sample, occur in more than 1% of the reads for at least one sample and have a most abundant unique sequence to occur at a minimum of 0 reads and and have a most abundant unique sequence with a minimum abundance of 25. After removal of oligotypes that did not meet these criteria, the analysis retained 299,055 reads (88.765% of the original reads). Oligotyping analysis identified 15 oligotypes, which had at least one perfect match for their representative sequences in rRNA entries in NCBI non-redundant (nr) database.

Cross-correlation, auto-correlation analysis and heatmap

Correlation and co-occurrence tests within oligotypes were carried out using R (version 3.2.2) considering the counts matrix for each oligotype (the number of reads assigned to each oligotype in each sample). Significance (*p* value) was calculated using the "corr" function, which employs a Student's t distribution for a transformation of the correlation. We used the Bonferroni correction for multiple tests by multiplying significance estimates by $315^2 \sim= 10^5$.

Two distinct indices (binary Jaccard: presence–absence and Morisita-Horn: relative abundance) estimated dissimilarity in pairwise comparisons of oligotypes sequences.

Moreover, the pairwise sequence identity was calculated by o-sequence-distances and o-visualize-distance pipeline to generate the distance matrix (i.e. percent identity matrix). Morisita-Horn was used for calculating the distance between each matrix cell (to get a "score" that represents row-wise similarity) and then clustered according to the Ward metric. The dendrogram represents the Morisita-Horn distances between each row/column, so the distance between each pair of oligotype sequence. For all visualizations we used the ggplot2 package in R.

All the oligotypes were chosen for construction of a heatmap where samples and oligotypes were clustered based on Horn distances and Ward clustering.

2.4.3 Results

Oligotypes community composition

The two datasets included 299,055 reads over the V1-V3 region of the 16S rRNA gene. Although reads from this region is quite short after trimming, oligotyping analysis focused on *Pseudomonas* provides information that allows taxonomic resolution beyond genus level.

We analyzed the distribution of each oligotype across the sampled environmental sites and among food products, and we characterized the diversity of the manufactures within each processing-plant. We detected differences within

Pseudomonas population between dairy- and meat-associated samples, which were previously undetectable by standard OTU-clustering based approaches for classification. Interestingly, some oligotypes occurred both in cheese and meat samples and related processing environment. Comparing individual oligotype sequences with reference sequences in nr database we tried to associate oligotypes to known species.

A total of 15 oligotypes were identified across the samples and were associated to meat and cheese datasets (Table 1). Moreover, many oligotypes showed remarkable differencial abundance in food and related environment, resulting in a distinctive community composition between cheese and meat dataset.

Oligotyping of Pseudomonas in cheese

In the cheese-dataset 8 oligotypes (oligo_1 - oligo_6 and oligo_15) were identified. The distribution of the oligotypes across the samples is shown in Figure 1, where the relative abundance of *Pseudomonas* genus compared to the other genera found in each dataset is also shown.

Pseudomonas was more abundant in the environmental samples compared to cheese. The most abundant oligotypes were oligo_1 to oligo_3 with a different distribution among the samples (Figure 1). Nevertheless, high variability in oligotype composition was found and oligo_5 prevailed in some samples (Figure 1). A clear site-specific distribution appeared as some oligotypes occurred mainly and abundantly only in some of the samples, e.g. oligo_2 in the cheese samples, or oligo_4 on tool surfaces (Figure 2). Interestingly, none of the oligotypes were typical of cheese samples but they all shared with the environment (Figure 3). Correlation tests indicated that the most abundant oligotypes (oligo_1 and oligo_2) were strongly positively correlated, as well as oligotypes 4 and 6 that appear together with a major abundance in the environmental samples (Figure 4). Moreover, the abundance of oligo_1 was negatively correlated to that of oligo_4 (Figure 4). All the most abundant oligotypes occurring in the cheese dataset (1-6) generally co-occurred; in particular, oligo_1 co-occurred with the other abundant oligotypes (oligo_2, 3, 4), while oligo_15 mainly excluded the others (is the least abundant) (Figure 5).

Oligotyping of the genus Pseudomonas in meat

Nine oligotypes (oligo_1, 2, 3, 5, and oligo_7-14) were identified in the meat dataset. The oligotypes 1-3 were the most abundant in both meat and environmental samples, while oligo_5 dominated on the carcasses (Figure 6). Interestingly, carcasses samples had the lowest *Pseudomonas* incidence. *Pseudomonas* was particularly abundant in spoiled meat and in some specific tool surfaces, such as knife and chopping boards (Figure 6). Considering the most abundant oligotypes, oligotypes 2 decreased and 1 increased from the environment to meat samples (Figure 7). As for the cheese dataset, also in this case the oligotypes occurring in meat were all also present in the environment (Figure 8). The most abundant oligotypes (oligo_1 and 2) were negatively correlated (Figure 9). Meanwhile, oligotypes that showed a remarkable abundance only in some samples (oligo_3 and 5) were positively correlated (Figure 9). Some sub-abundant oligotypes were positively correlated: e.g. oligo_8 and 9; oligo_11 and 12; oligo_10 and 14 and they co-excluded all the others (Figure 10).

Sequence distance matrix

Sequence distance matrix representation (Figure 11) shows the percent nucleotide identity between each pair of oligotypes.

Overlap of oligotypes between dairy and meat environment

The results obtained showed that both dataset shared some oligotypes, although with differences in abundance. Considering the most abundant oligotypes (oligo_1- 5), they all occurred in meat, cheese and related environmental samples, except oligo_4 that was specific of Cheese dataset. Some comments have to be made regarding the relative abundance of the mentioned oligotypes among the samples: oligo_1 occurred with high abundance in both dataset, oligo_2 was more abundant in the meat dataset, where the relative abundance of *Pseudomonas* genus was remarkable; on the contrary the oligo_3 showed higher levels in the cheese dataset but, in this case, the relative abundance of *Pseudomonas* genus was lower. Oligo_5 occurred in both dataset, but had a remarkable bundance only in some samples.

Meanwhile, it was evident that all the less abundant oligotypes were more specific for one or other dataset; particularly oligo_6 and oligo_15 occurred only in cheese dataset and oligo_7 to 14 occurred only in meat dataset.

2.4.4 Discussion

Bacterial spoilage causes significant economic losses for the food industry. Product contamination with psychrotrophic microorganisms is a particular concern for fresh foods or for those types of products that are not subjected to any treatment during their preparation. Dairy and meat products are a clear example of bacterial spoilage since they are processed and distributed at refrigerated temperature allowing the growth of these organisms. Microorganisms that may be defined as psychrotrophics are ubiquitous in nature and can be isolated from soil, water, and vegetation (Dogan & Boor 2003). Moreover, psychrotrophic bacteria colonize meat and dairy products (Gram et al., 2002; Pennacchia et al., 2009; Viljoen 2001) but generally constitute only a small amount of the initial flora in unprocessed food. Bacterial spoilage occurs when conditions during storage favour the growth of psychrotrophic microorganisms and they become the dominant microbiota (Stellato et al., 2015).

Not all *Pseudomonas* strains have the same abilities to produce defects in food (Dogan & Boor 2003; O'Sullivan & O'Gara 1992; Iulietto et al., 2015). Therefore, methods for discriminating strains with high food spoilage potential are necessary in order to identify and reduce or eliminate the environmental contamination sources of the critical strains. Traditional microbiological methods for bacterial identification do not have the necessary discriminatory power and are biased by the inability of many microorganisms to grow on laboratory media. Moreover, results can be not always reproducible. 16S rRNA high-throughput sequencing technologies provided the possibility for in depth studies of microbial diversity in food and food-related environments but it fails in resolving ecologically meaningful differences between closely related organisms in complex environments (Eren et al., 2013). Oligotyping has been demonstrated to be able to provide beyond-genus level resolution starting from 16S rRNA amplicon sequencing (Eren et al., 2013).

The molecular identification of *Pseudomonas* is often difficult and controversial, since 16S rRNA gene is not satisfactorily discriminating between the different species, as highlighted in phylogenetic studies that inferred phylogenies based on 16S rRNA gene and demonstrated that it lacks resolution at intrageneric level because of its low rate of evolution (Anzai et al., 2000; Moore et al., 1996; Yamamoto et al., 2000).

In the present study, a total of 15 *Pseudomonas* oligotypes were identified across dairy and meat samples as well as in related environmental samples from the processing plants. The most abundant oligotypes were identified as *Pseudomonas fragi*, *Pseudomonas fluorescens* and *Pseudomonas putida,* already found in previous studies as inhabitant of dairy and meat environment (Ercolini et al., 2006; De Jonghe et al., 2011; Martin et al., 2011; Dogan & Boor 2003). Ercolini et al., 2007.

Since many activities related to food spoiling potential are strain-specific, an accurate analysis of the processing environment is necessary in order to focus on those surfaces that may be source of specific strains, and subsequently contaminate the food (Licitra et al., 2007; Brightwell et al., 2006).

Environmental samples from different areas both belonging to dairy and meat-processing plant were studied. The oligotypes distribution in the environmental samples differed from the related food; although the same oligotypes were identified, their relative abundance was different. The results suggested that different oligotypes might show adaptive selective mechanisms and might preferentially grow in some environmental conditions. Moreover, co-occurrence/exclusion mechanisms between oligotypes were highlighted. In carcass swabs, where *Pseudomonas* genus never reached levels < 5%, Oligo_5 dominated. Oligo_1 and Oligo_2 overgrew in the beef samples, where the abundance of *Pseudomonas* increased up to 60%, suggesting the inability of Oligo_5 to compete with the others. The increase in abundance of Oligo_1 and Oligo_2 during meat refrigerate storage suggested the intriguing possibility of a higher spoilage potential of these oligotypes. Moreover, Oligo_5 occurred in the butcher's swabs at very low abundance, highlighting that this oligotype might originate from slaughtering environment, operators' skin, transport circumstances, all previously identified as contamination sources for raw meat (De Filippis et al.,, 2013; Perez M.P. et al.,, 2002). Studies on fresh meat have demonstrated that diverse strains of the same species can behave differently in exactly the same food matrix and storage conditions and different biotypes can have different metabolic behaviours and spoilage potential (Casaburi et al., 2014; Casaburi et al., 2011; Ercolini et al., 2010). Therefore, the biotype-level composition may determine whether the food spoilage will occur (Casaburi et al., 2015).

In the cheese dataset a higher diversity among the samples was observed. The higher variability might be caused by the material used for the tools employed during cheese manufacturing (such as porous plastic gaskets and wooden vats); in fact, these materials may be more difficult to clean from organic residuals (Montel et al., 2014; Settanni et al., 2012) (Montel et al., 2014; Settanni et al., 2012). On the contrary, the homogenous distribution of two dominant oligotypes in steel tools from butcher's environment might be ascribed to the possibility of a through cleaning. Previous studies

showed that protein and fat residuals on food processing surfaces may represent a source of nutrients for attached and/or transient microorganisms (Brightwell et al., 2006; Møretrø et al., 2013; Bagge-Ravn et al., 2003). In addition, the diversity of resident bacteria depends on selective ecological factors such as physical and chemical cleaning regimes, temperature and water availability. Other studies showed that the microbiological quality of surfaces was an useful indicator of the efficiency of cleaning procedures and disinfection (Legnani et al., 2004; de Oliveira et al., 2014).

2.4.5 Conclusion

Applying oligotyping to the food matrices and environmental samples from food-processing plant made possible a more accurate characterization of the microbial communities inhabiting this environment compared to traditional OTU-level analysis. Our results showed a predominance of certain oligotypes within specific samples and correlations between food and related environmental samples. It revealed the selective distribution in meat during refrigerated storage of specific oligotype that may have higher spoilage potential. The results obtained allowed speculating on a potential ecological and functional biodiversity within *Pseudomonas* genus previously unexplored. The ability to extract maximum information from sequencing data opens up new possibilities for the analysis of the dynamics of the food microbial composition.

2.4.6 References

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Figures and Table

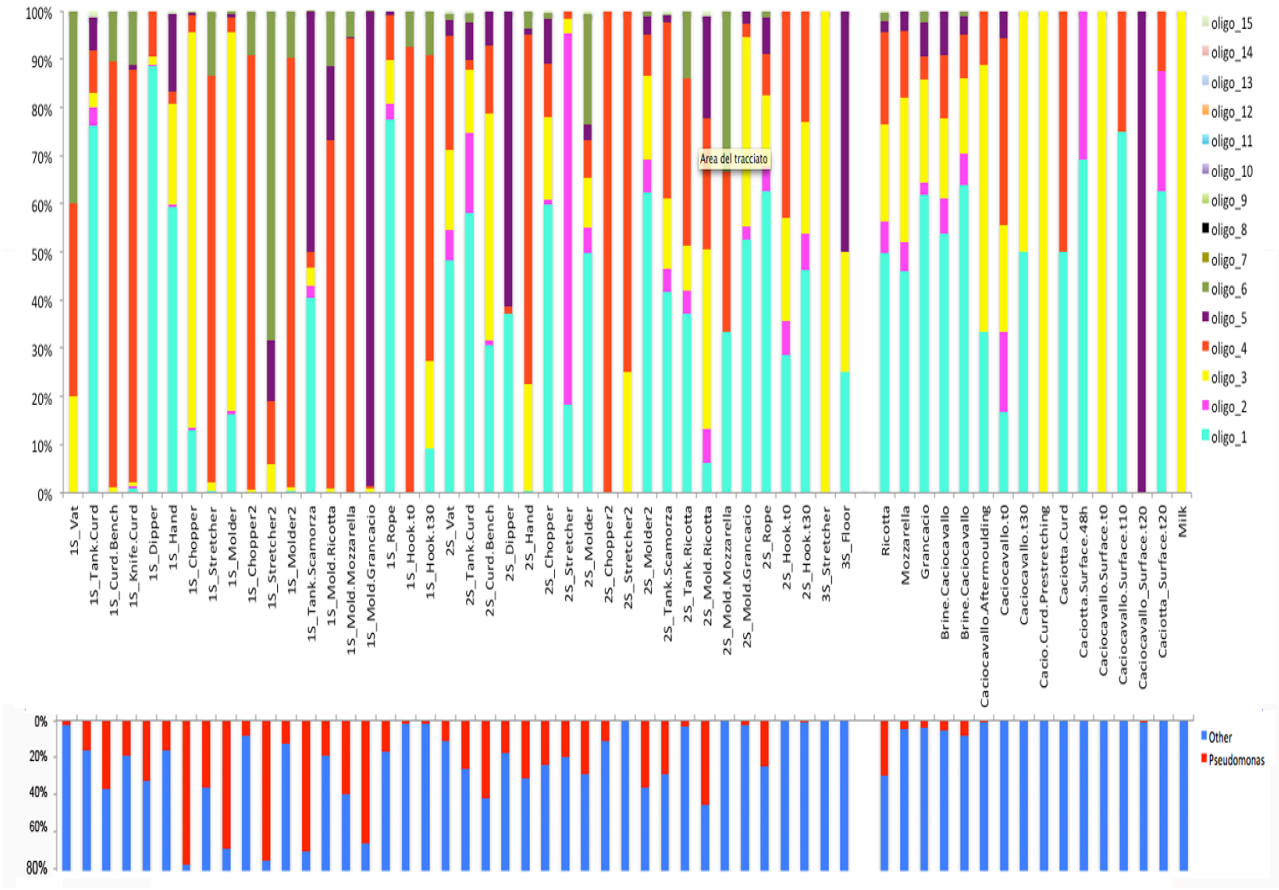
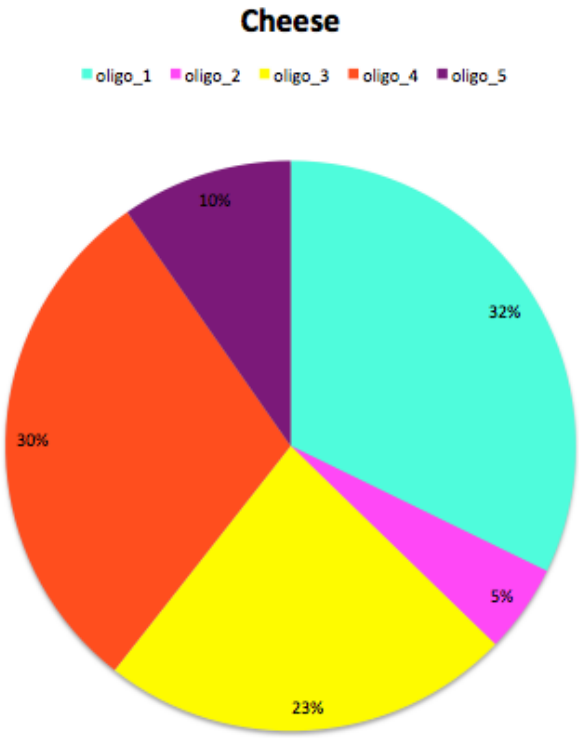


Figure 1. Relative abundance of the *Pseudomonas* genus in the single samples is showed (lower) and a zoom of the distribuition of the corresponding oligotypes (upper)



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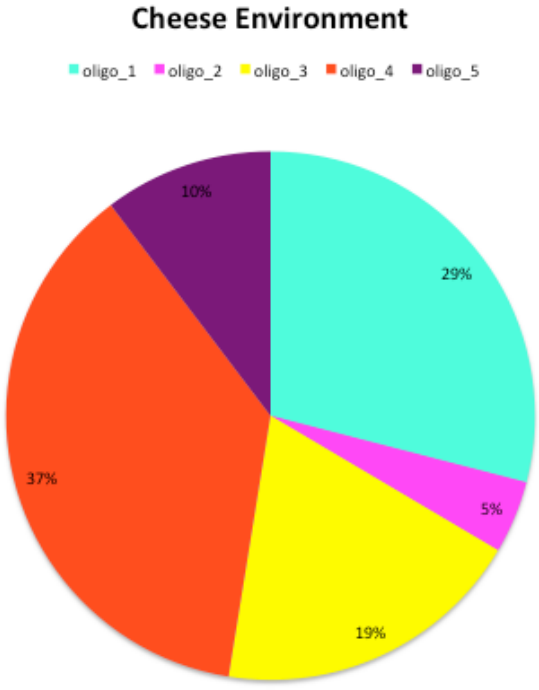

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Figure 2. Distribution of the most abundant oligotypes in Cheese samples (panel a) and Environmental cheese samples (panel b).

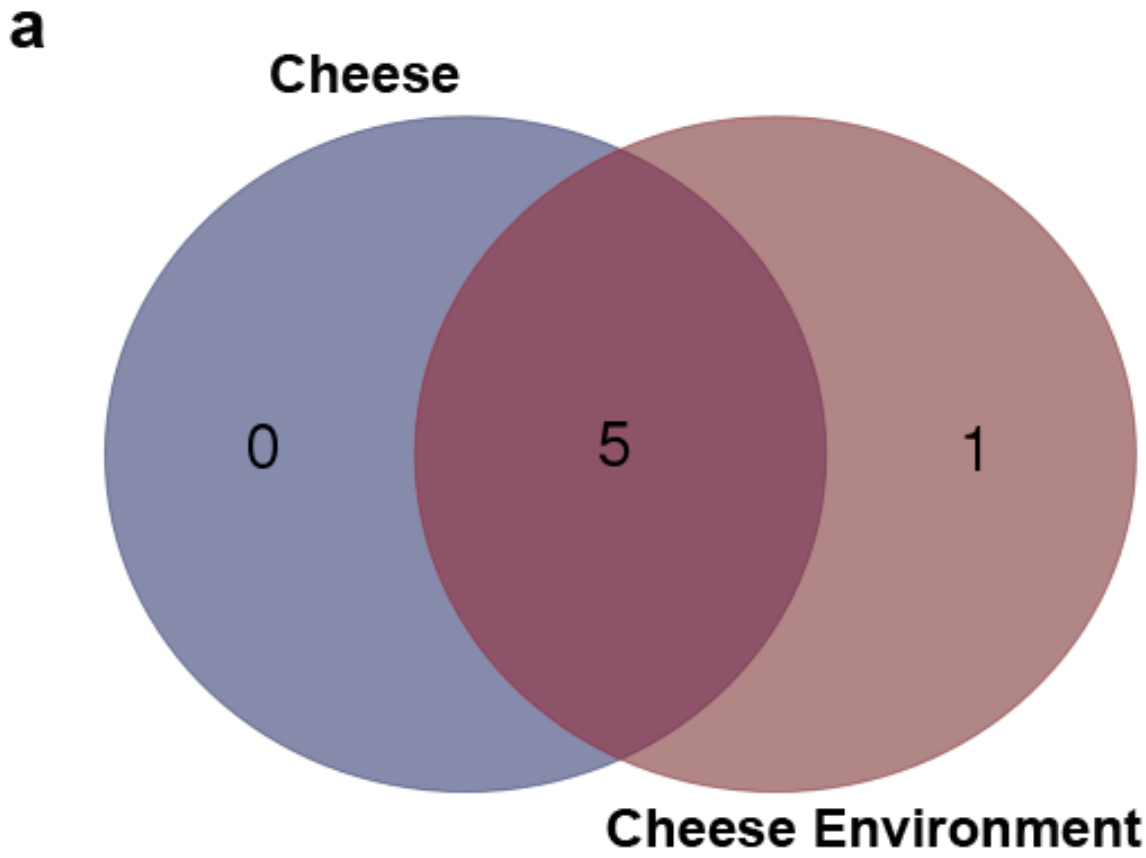


Figure 3. Venn Diagram showing the number of shared Oligotypes between Cheese and Cheese environment groups.

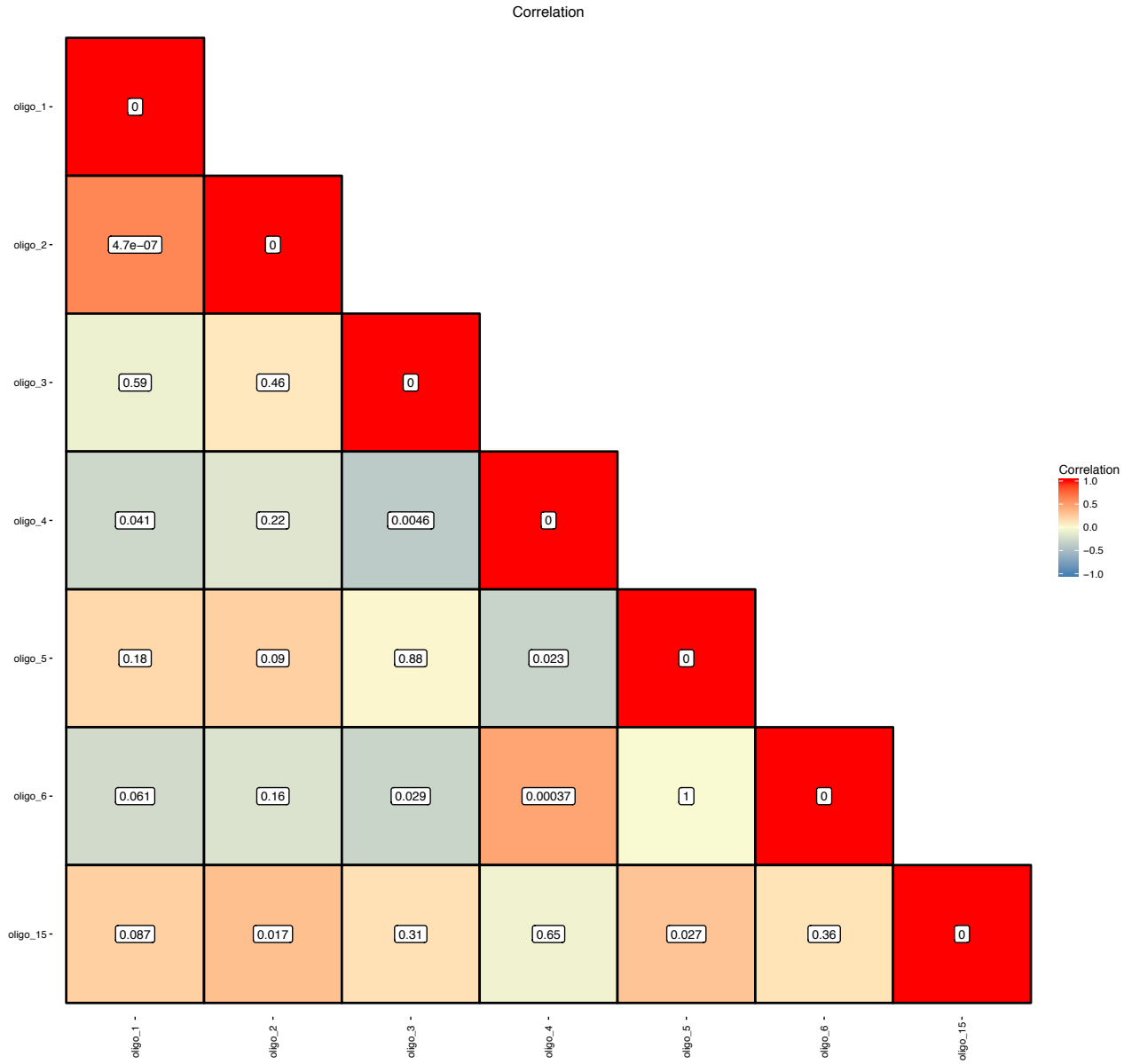


Figure 4. Correlation test within oligotypes occurring in Cheese dataset was carried out using R (version 3.2.2) considering the counts matrix for each oligotype. Significance (*p* value) was calculated using the "corr" function, which employs a Student's t distribution for a transformation of the correlation. Bonferroni correction was used for multiple tests by multiplying significance estimates by $315^2 \sim= 10^5$.

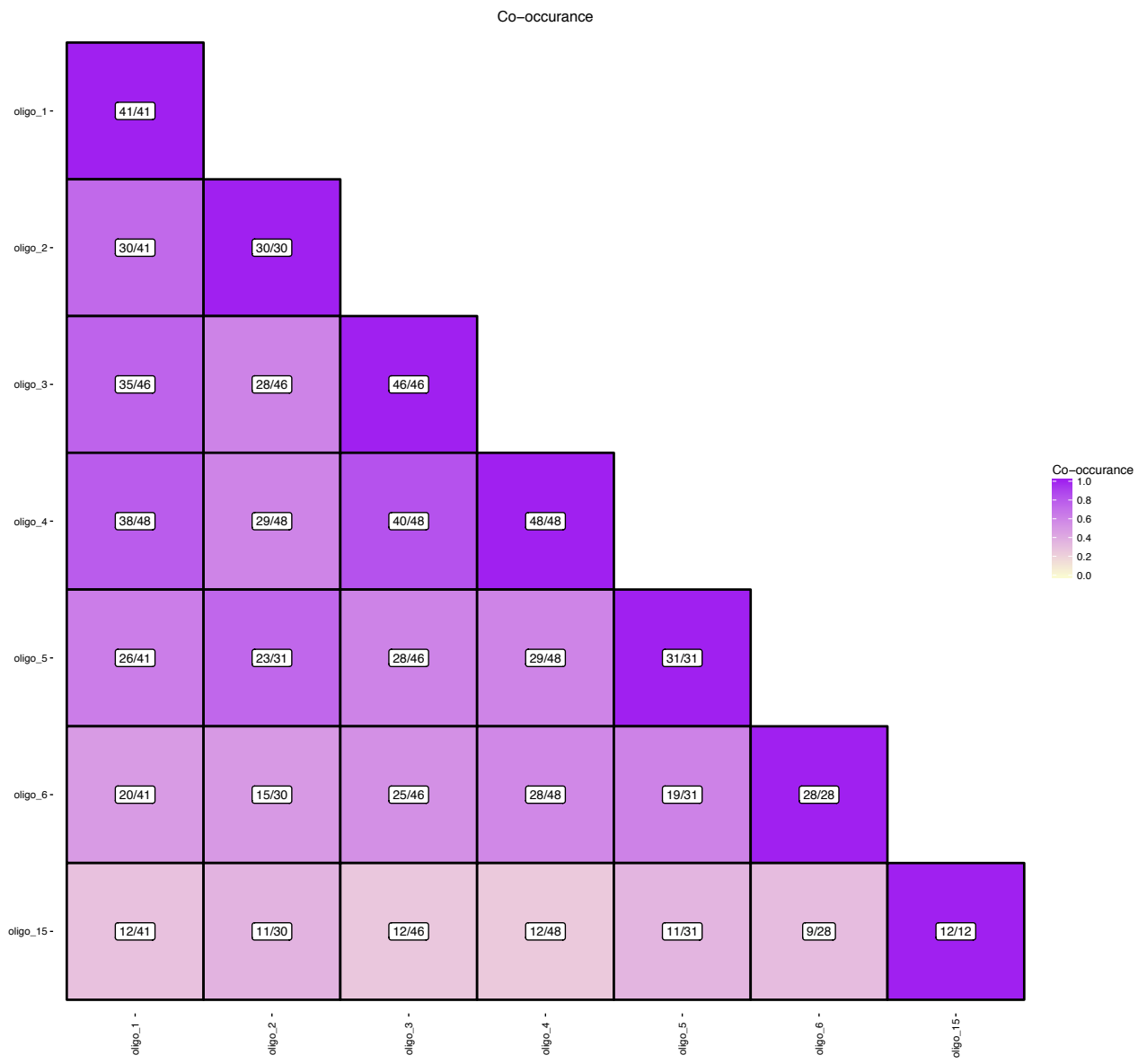
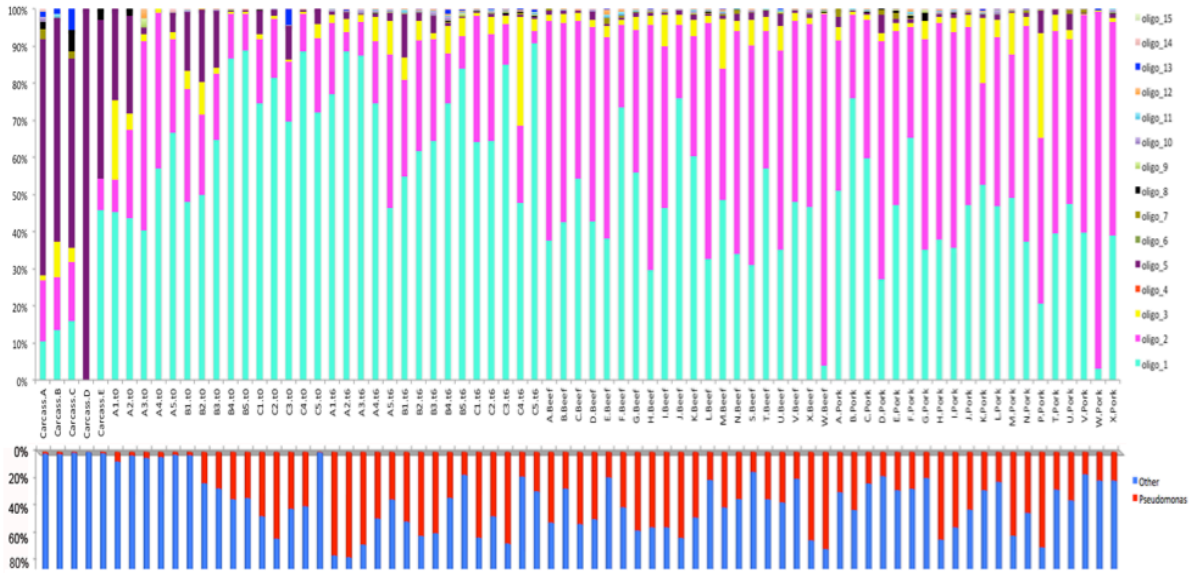


Figure 5. Co-occurrence test within oligotypes identified for the Cheese dataset was carried out using R (version 3.2.2) considering the counts matrix for each oligotype. Two distinct indices (binary Jaccard: presence–absence and Morisita-Horn: relative abundance) estimated dissimilarity in pairwise comparisons of oligotypes sequences.



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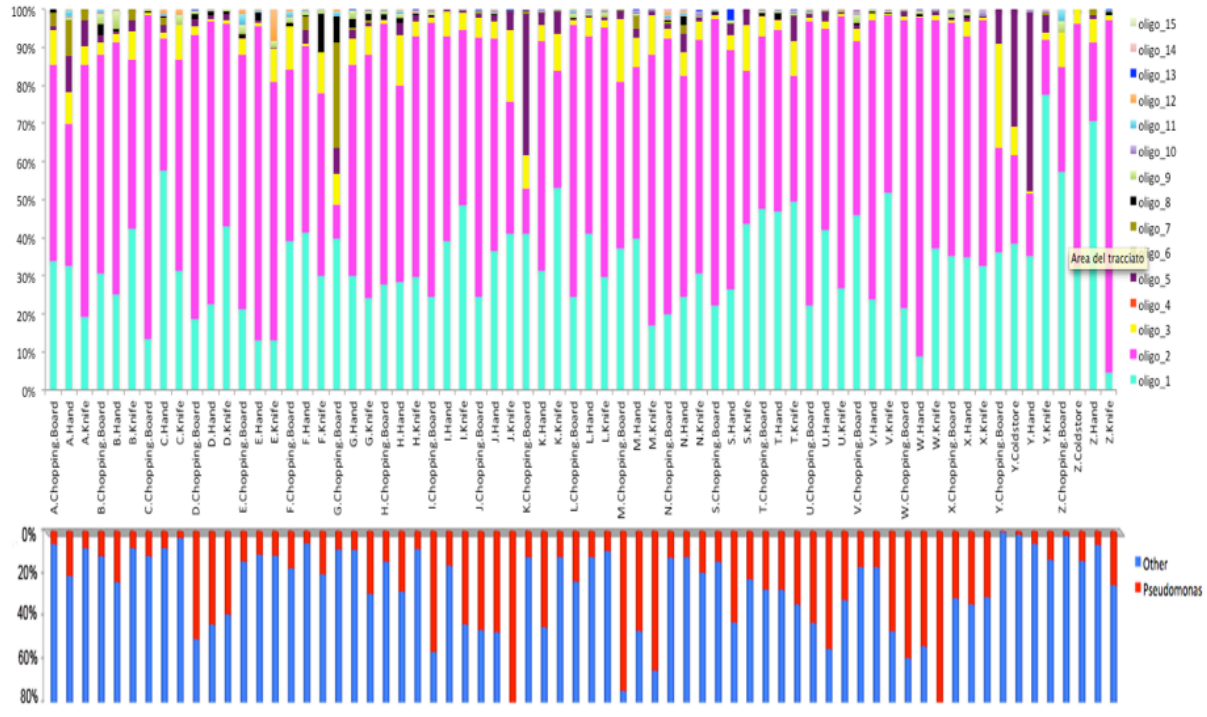

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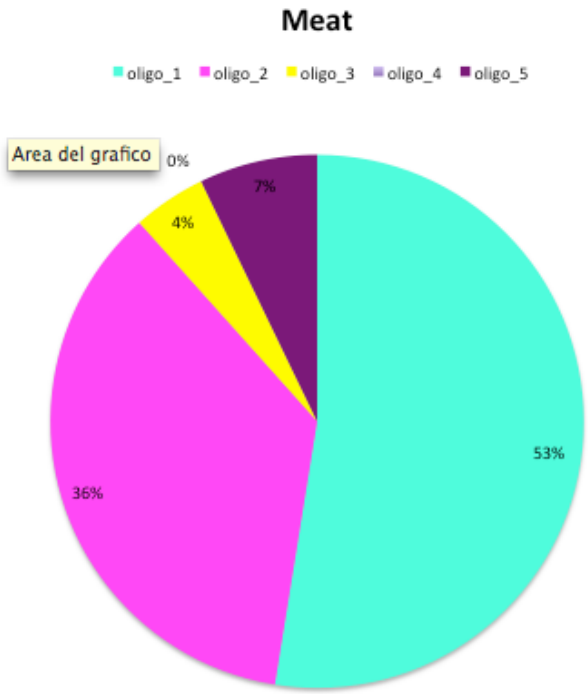
Figure 6. Relative abundance of the *Pseudomonas* genus in the meat samples (panel a) and in environmental samples (panel b) is showed (lower) and a zoom of the distribuition of the corresponding oligotypes (upper).



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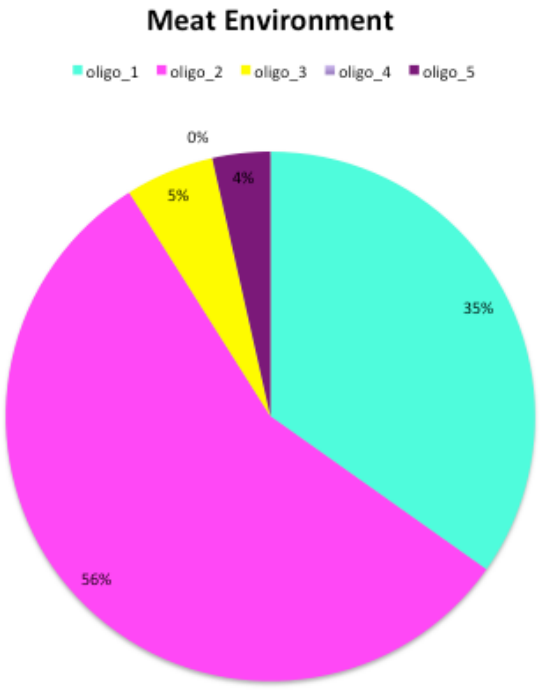

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Figure 7. Distribution of the most abundant oligotypes in Meat samples (panel a) and Environmental meat samples (panel b).

Figure 8. Venn Diagram showing the number of shared Oligotypes between Meat and Meat environment groups.

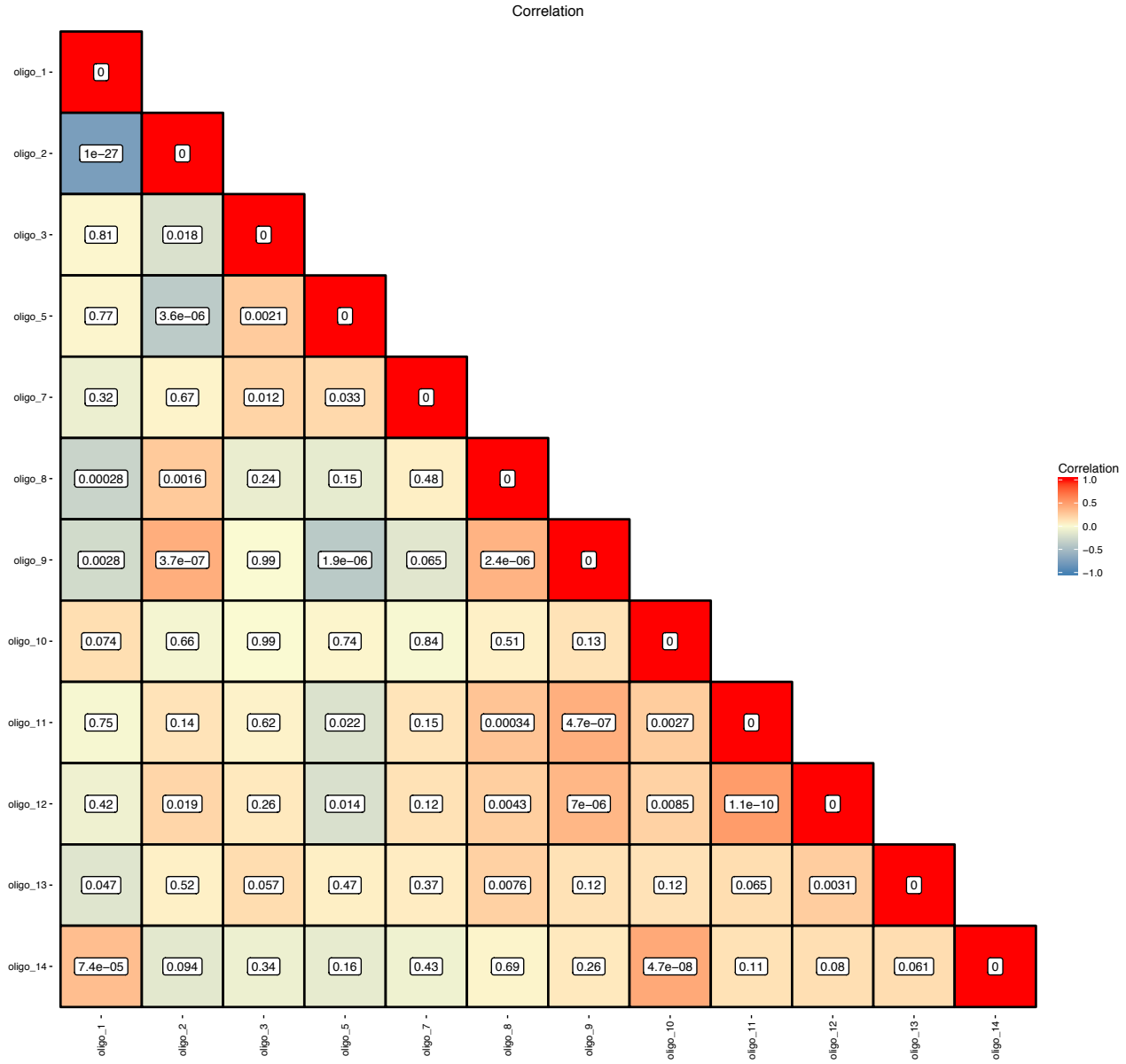

Figure 9. Correlation test within oligotypes occurring in Meat dataset was carried out using R (version 3.2.2) considering the counts matrix for each oligotype. Significance (*p* value) was calculated using the "corr" function, which employs a Student's t distribution for a transformation of the correlation. Bonferroni correction was used for multiple tests by multiplying significance estimates by $315^2 \sim= 10^5$.

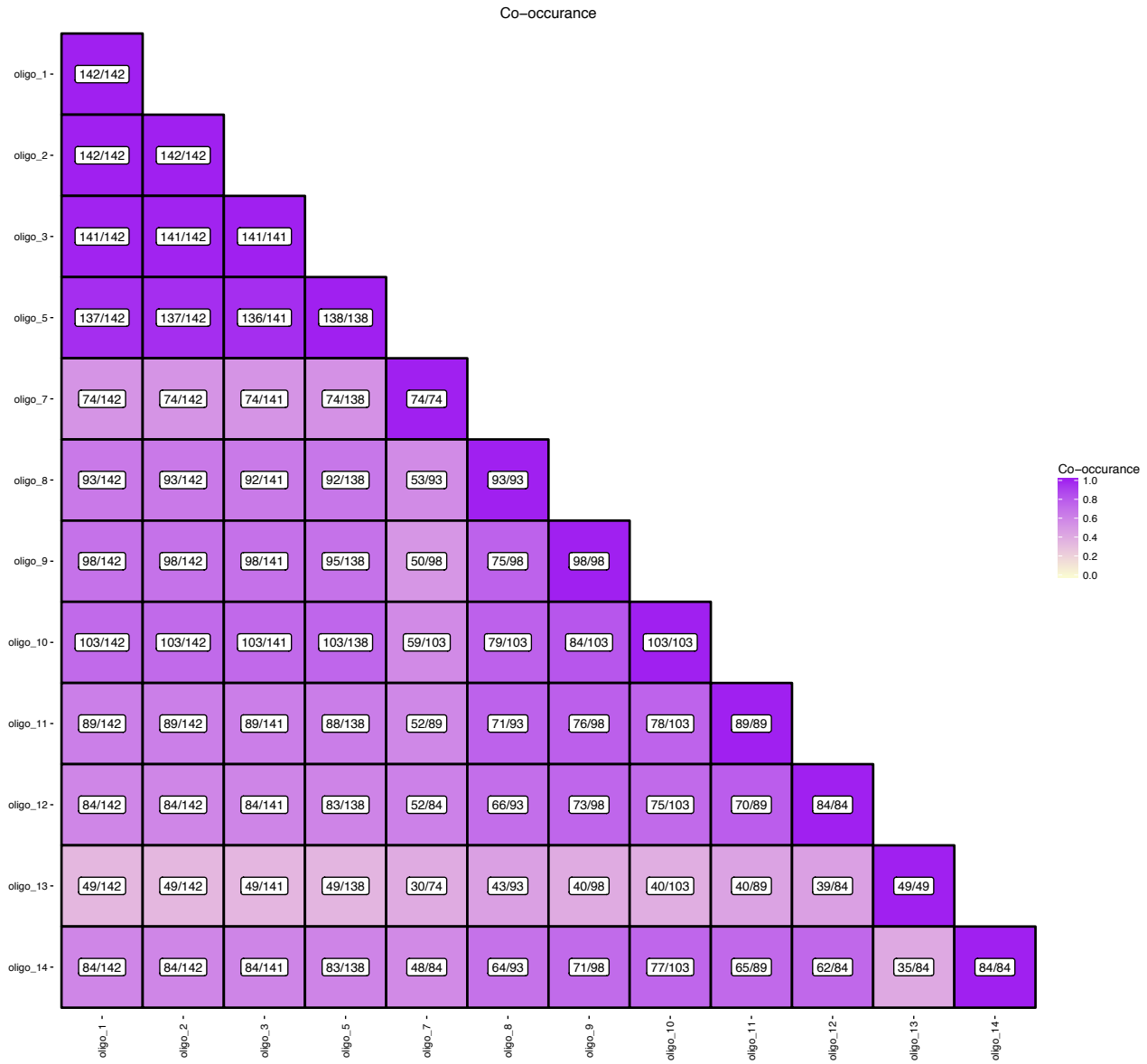

Figure 10. Co-occurrence test within oligotypes identified for the Meat dataset was carried out using R (version 3.2.2) considering the counts matrix for each oligotype. Two distinct indices (binary Jaccard: presence–absence and Morisita-Horn: relative abundance) estimated dissimilarity in pairwise comparisons of oligotypes sequences.

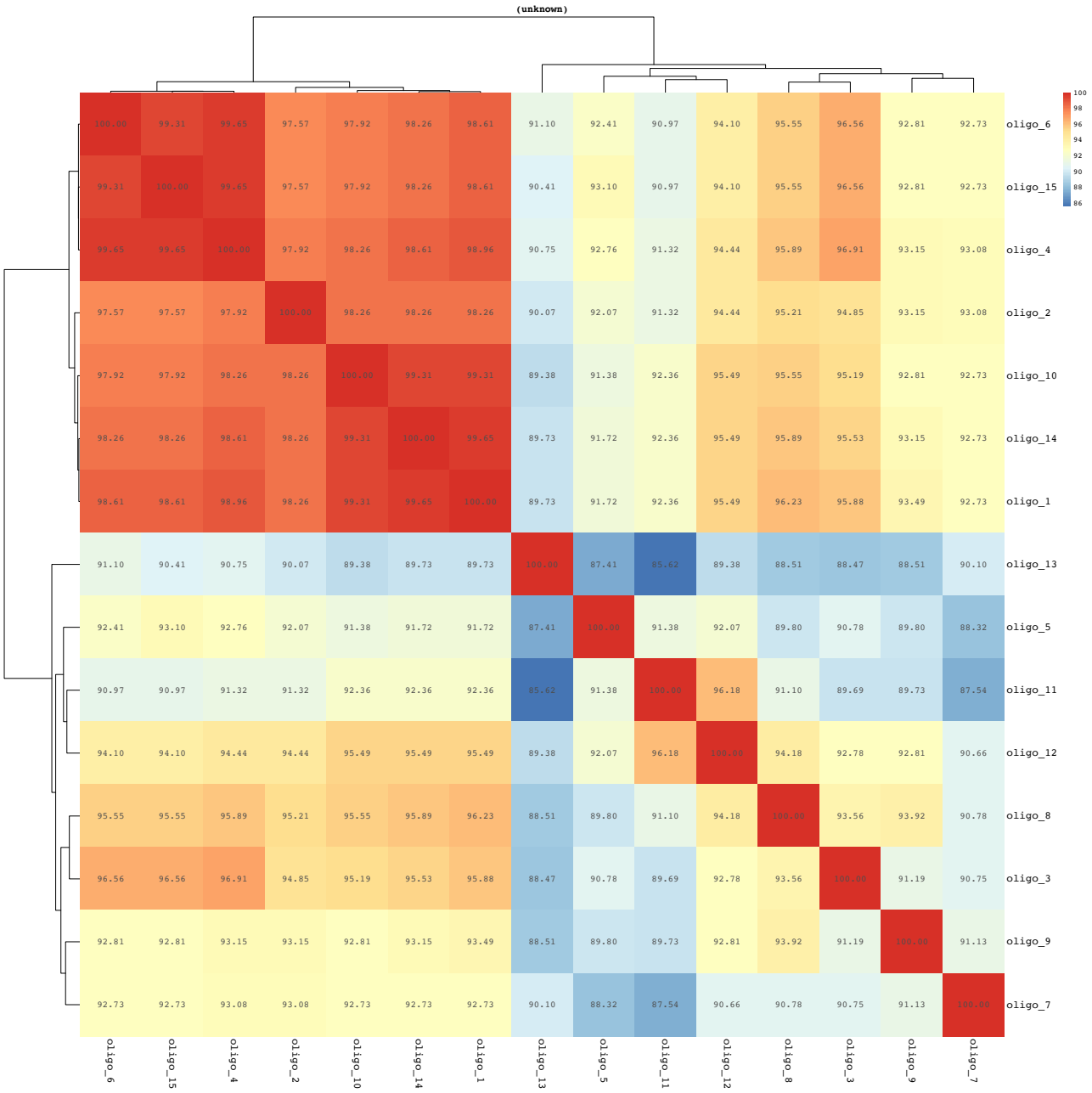

Figure 11. Pairwase sequence identity for the oligotypes identified among cheese and meat samples was calculated. Morisita-Horn was used for calculating the distance between each pairwase distance and then clustered according to the Ward metric.

Table 1. Different Pseudomonas Oligotypes identified by BLASTn search against the NCBI nr database.

Oligotype	Identification
oligo_1	*P. fragi strain F4*
oligo_2	*P. fragi strain P121*
oligo_3	*P. fluorescens strain LBUM223/UK4*
oligo_4	*P. fluorescens* strain A506
oligo_5	*P. putida*
oligo_6	*P. fluorescens* strain A506
oligo_7	*P. resinovorans* strain ATCC 14235
oligo_8	*P. fluorescens* strain A506
oligo_9	*P. fluorescens* strain A506
oligo_10	*P. frag*i strain JCM 5396
oligo_11	*P. putida* H8234
oligo_12	*P. frag*i strain F4
oligo_13	*P. syringae* UMAF0158
oligo_14	*P. fragi* strain F4
oligo_15	*P. fluorescens* strain PICF7

APPENDIX I – LIST OF PUBLICATIONS INCLUDED IN THE THESIS

1. **Stellato, G.,** De Filippis, F., La Storia, A., Ercolini, D. 2015. Coexistence of lactic acid bacteria and potential spoilage microbiota ina dairy-processing environment. Appl Environm Microbiol. doi:10.228/AEM.02294-15

2. **Stellato G.,** La Storia A., Cirillo T., Ercolini D. 2015. Bacterial biogeographical pattern in a cooking centre for hospital foodservice. Int J Food Microbiol 193:99-108.

3. **Stellato, G.,** La Storia, A., De Filippis, F., Ercolini, D. 2016. Overlap of spoilage microbiota between meat and meat processing environment in traditional vs large-scale retail distribution**.** Appl Environm Microbiol. *In press.*

4. **Stellato, G.,** Eren, A. Murat Ercolini, D. 2016. Different *Pseudomonas* sp. oligotypes reflect contamination routes in food processing environment. *Submitted.*

APPENDIX II – LIST OF PUBLICATIONS NOT INCLUDED IN THE THESIS

1. Calasso, M., Ercolini, D., Mancini, L., **Stellato, G.,** Minervini, F., Di Cagno, R., De Angelis, M., Gobbetti, M. 2015. Relationship among house, rind and core microbiotas during manufacture of traditional Italian cheeses at the same dairy plant. Food Microbiol. Doi:10.1016/j.fm.2015.10.008.

2. Pothakos, V., **Stellato, G**., Ercolini D., Devlieghere, F. 2015. Processing environment and ingredients are both sources of Leuconoston gelidum, with emerge sas major spoiler in ready-to-eat meals. Appl Environm microbiol. 81:3529-3541. Doi:10.1128/AEM.03941-14.

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