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THE MICROBIOME IN DAIRY PRODUCTS ASSESSED BY METAGENOMICS AND METATRANSCRIPTOMICS

Tutor:
Prof. Danilo Ercolini

PhD Student:
Francesca De Filippis

Co-ordinator:
Prof. Giancarlo Barbieri

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ABSTRACT

Cheese is a biologically and biochemically dynamic food containing microorganisms both deliberately added as starters and non-starter adventitious contaminants. The microbiota present in cheese is complex and its growth and activity represent the most important, but the least controllable steps. During manufacturing and ripening, the microbiota is in continuous evolution, driven by the changes in the environmental conditions. Studies of the cheese microbiota can address several questions that are important for the improvement of dairy production and the monitoring of microbial species during manufacture and ripening can give important insights to understand process dynamics and work out conditions that can assure a premium quality. However, 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 thesis was the study of microbial diversity and dynamics of microorganisms involved in the cheese manufacturing and ripening processes by using a new culture-independent high-throughput sequencing (HTS) approach. Thus, different ecosystems were investigated in order to comprehend the specific role played by microorganisms in each cheese manufacture and in each step of cheese production.

Very different cheese productions were taken into account: fresh and medium-ripened pasta-filata cheeses (Mozzarella and Caciocavallo Silano) and long-ripened cheeses (Grana Padano, Parmigiano Reggiano). Moreover, a novel approach for a sequencing-based strain monitoring of *Streptococcus thermophilus* was evaluated, through sequencing of the species-specific *lacS* gene amplicons. Finally, the application of shotgun metatranscriptome sequencing was firstly investigated for the monitoring of microbial gene expression during cheese manufacturing and ripening.

Overall, thanks to the different HTS approaches it was possible to obtain a complete picture of the microbiome involved in each dairy production. In most of the cheese manufactures, a naturally-selected core microbiome was found in both the fermentation and the ripening phases, including few species well-adapted to the dairy environment. Curd fermentation is mainly driven by few thermophilic lactic acid bacteria (LAB), while mesophilic non-starter LAB take over during the ripening. The evolution of the microbiota and its activities during ripening are strictly dependent on the environmental conditions and can be shaped through the modulation of the technological parameters applied. The application of shotgun metatranscriptome allowed the identification and quantification of microbial key genes involved in cheese ripening. Finally, the HTS-based strain-monitoring has been shown to be a promising application, if genes highly variable within a species are selected.

Understanding microbial behavior during cheese manufacturing is a pivotal step in order to ensure safety and quality in dairy productions. In this context, HTS allows an unprecedented in-depth analysis of the microbial *consortia* in dairy environments.

RIASSUNTO

I formaggi possono essere considerati come un alimento biologicamente e biochimicamente dinamico, contenente microrganismi aggiunti deliberatamente come starter o contaminanti ambientali. Il microbiota caseario è molto complesso e la sua crescita ed attività rappresentano le fasi più importanti, ma meno controllabili del processo di caseificazione. Durante la lavorazione e la stagionatura dei formaggi, la continua evoluzione del microbiota è guidata dai cambiamenti nelle condizioni ambientali. Gli studi riguardanti il microbiota dei formaggi sono utili per il miglioramento delle produzioni casearie ed il monitoraggio delle specie microbiche durante la manifattura e la stagionatura può dare importanti conoscenze utili per capire le dinamiche coinvolte e stabilire le condizioni che possono assicurare una elevata qualità. Gli approcci metodologici per lo studio del microbiota sono cambiati ed oggi è possibile identificare e monitorare specie batteriche e biotipi velocemente, con un'elevata sensibilità ed affidabilità.

L'obiettivo del presente lavoro di tesi è stato lo studio del microbiota caseario e delle sue attività durante la manifattura e la stagionatura, utilizzando il nuovo metodo coltura-indipendente basato sul sequenziamento ad alto rendimento (high-throughput sequencing, HTS). Pertanto, sono stati studiati diversi ecosistemi per comprendere il ruolo specifico dei microrganismi in ogni manifattura e nelle diverse fasi della produzione. Sono state considerate tipologie di formaggi molto diversi: formaggi a "pasta filata" freschi ed a media stagionatura (Mozzarella e Caciocavallo Silano) e formaggi a lunga stagionatura (Grana Padano e Parmigiano Reggiano). Inoltre, è stata valutata l'applicazione di un monitoraggio di biotipi di *Streptococcus thermophilus* basato sul sequenziamento di ampliconi del gene specie-specifico *lacS*. Infine, è stata investigata per la prima volta l'applicazione della metatrascrittomica per il monitoraggio dell'espressione genica durante la produzione e stagionatura dei formaggi.

Grazie ai differenti approcci di HTS è stato possibile ottenere un'immagine completa del microbioma coinvolto in ogni produzione casearia. Nelle manifatture studiate è stata riscontrata la presenza di un microbiota "core" selezionato, comprendente poche specie ben adattate all'ambiente caseario. La fermentazione della cagliata è guidata da poche specie di batteri lattici termofili, mentre i lattobacilli non-starter mesofili prendono si succedono durante la stagionatura. L'evoluzione del microbiota e delle sue attività durante la stagionatura sono strettamente dipendenti dalle condizioni ambientali e possono essere influenzate attraverso la modulazione deiparametri tecnologici applicati in questa fase. L'utilizzo della metrascrittomica "shotgun" ha permesso l'identificazione e la quantificazione di geni con un ruolo importante nella stagionatura del formaggio. Infine, l'utilizzo dell'HTS per il monitoraggio dei biotipi all'interno di una specie si è mostrata un'applicazione promettente, se vengono selezionati geni con elevata variabilità intra-specie.

Comprendere il comportamento microbico è un passo fondamentale per assicurate qualità e sicurezza nelle produzioni lattiero-casearie. In questo contesto, l'utilizzo dell'HTS permette un'analisi approfondita dei consorzi microbici negli ambienti caseari.

PREFACE

Food matrices host complex and dynamic microbial consortia where bacteria, yeasts and fungi can coexist. The study of the microbial ecology of foods has dramatically changed (O'Flaherty & Klaenhammer, 2011). Food microbial ecology has been based on the study of microbial isolates for decades. The total number of microbial cells on Earth is estimated to be 10^{30} (Turnbaugh et al., 2008). Prokaryotes represent the largest proportion of individual organisms, comprising 10^6 to 10^8 separate genospecies (Sleator et al., 2008), but ninety-nine per cent of all micro-organisms in almost every environment on earth remain, as yet, uncultured (Amann et al. 1990; Curtis 2002). Culture-independent analyses arose to overcome the limitations of the classical culture-based approach (Ercolini, 2013) and fingerprinting tools have been extensively used for the last 20 years in food microbiology. In particular the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) approach, after its introduction in microbial ecology (Muyzer et al., 1993), was extensively employed to monitor microbial populations during food production and spoilage dynamics (Cocolin et al., 2013; Ercolini, 2004). The technological advances in the analysis of food microbiota have completely revolutionized the way we study these microbial ecosystems, leading to a 'cultural' evolution: our mental approach to food microbiology has changed and we learnt to think of food microbes as consortia (Cocolin & Ercolini, 2015).

Recently, we have switched from fingerprinting tools to high-throughput sequencing (HTS) technologies. Overall, the sequencing-based culture-independent approach to food microbial ecology is not only faster and more reliable than culture-based microbiology, but it also offers a higher sensitivity than fingerprinting techniques, allowing to profile subdominant microbial populations into microbial consortia. Nevertheless, the unprecedented advantage of sequencing-based tools is having a quantitative monitoring of microbial taxa in food ecosystems. The use of ribosomal RNA (rRNA) amplicon sequencing to obtain the taxonomic composition and the relative abundance of the taxa (that is, the microbiota) is the most common HTS application in food microbial ecology. This approach, recently re-names meta-genetics (Esposito & Kirschberg, 2014), is based on the sequencing of amplicons arising from a complex mix of microbial genomes directly extracted from a food sample. The target genes are those of taxonomic interest, with the 16S rRNA gene being the most widely used for bacteria. rRNA amplicons are sequenced and sequences are compared to reference databases to identify the operational taxonomic units (OTUs). Moreover, it is considered quantitative as the number of sequence reads identified as the same OTU allows an estimation of the relative abundance of each microbial *taxa* in the food sample analyzed. Studying the changes in microbial populations can provide useful information to follow natural fermentation dynamics or the shifts in spoilage-associated populations (recently reviewed by Cocolin & Ercolini, 2015).

Furthermore, we do not only aim at defining the structure of the microbiota and addressing the question of "who is there", but another useful HTS application is the study of the expression of microbial activities directly in food. Such opportunity is given by metagenomics and metatranscriptomics that are intended to study the food microbiome, that is, the microbiota with all its functions. These approaches are based on the sequencing of the total DNA (metagenomics) or RNA (metatranscriptomics) directly extracted from the food matrix and allow identifying, besides the taxonomic composition, also the pool of microbial genes and therefore the potential activities (if the analysis is based on the DNA) or the activities actually expressed (if the analysis is based on the RNA) in the food matrix. The meta-omics offer tremendous chances to look at fermentation, ripening or spoilage dynamics in foods through the analysis of the genes expressed during such events and to understand how technological parameters (temperature, humidity, ingredients, packaging, etc.) employed by the food industry may affect, and may be changed to affect, the microbiome and its activities. Pioneer studies in food metagenomics have been carried out (Wolfe et al., 2014; Erkus et al., 2013), while only one study reported a cheese metatranscriptomic analysis (Lessard et al., 2014). However, shotgun DNA-seq and RNA-seq are still relatively expensive and the data are not easy to deal with in order to have a reliable idea of the distributions of genes and functions in food (Ercolini, 2013). Therefore, despite the great potential of these applications, their use in food microbial ecology is still underexploited.

HTS allows an unprecedented in-depth analysis of the microbial *consortia* in food environments, helping to understand microbial behavior during food manufacturing and spoilage.This opens the field to targeted innovation of the processes with the final aim of constantly improving food safety and quality.

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1 STATE OF THE ART

1.1 Cheese: a brief "overview"

1.1.1 Historical notes

Cheese is the generic name of a group of fermented dairy products, produced throughout the world in a great diversity of flavours, textures, and forms; there are more than 1000 varieties of cheese. Cheese is an ancient food whose origins predate recorded history. It is commonly believed that cheese evolved in the "Fertile Crescent" between the Tigris and Euphrates rivers, in what is now Iraq, about 8000 years ago, during the so-called "Agricultural Revolution", when certain plants and animals were domesticated as sources of food. There is no conclusive evidence indicating where cheese-making originated. The first fermented dairy foods were produced by a fortuitous combination of events, the ability of a group of bacteria, the lactic acid bacteria (LAB), to grow in milk and to produce enough acid to reduce the pH of milk to the isoelectric point of the caseins, at which these proteins coagulate. Neither the LAB nor the caseins were designed for this outcome. The ability of LAB to ferment lactose, a specific milk sugar, suggests that this characteristic was acquired relatively recently in the evolution of these bacteria. Their natural habitats are environment and/or the intestine, from which they presumably colonized the teats of dairy animals, contaminated with lactose; it is likely that through evolutionary pressure, these bacteria acquired the ability to ferment lactose. Like mainly fermentation-derived food products, cheese cannot be easily and succinctly defined. Cheese is essentially a microbial fermentation of milk. Milk is a rich source of nutrients for bacteria, which contaminate milk and grow well in the ambient conditions. Cheese manufacture accompanied the spread of civilization through Egypt, Greece, and Rome. Fermentation and/or salting, two of the classical principles for food preservation, were used to preserve meat, fish, vegetables and milk, and to produce beer, wine, fermented milks, butter, and cheese. Within large estates, individuals acquired special skills, which were passed on to succeeding generations. Traditionally, many cheese varieties were produced in limited geographical regions, especially in Italy. The localized production of certain varieties is now protected and encouraged through the European definition of Protected Denomination Origin (PDO), which legally defines the region and manufacturing technology for certain cheese varieties. In the past there were thousands of farm-scale cheese-makers and there must have been great variation within any one general type; even today, there is very considerable inter- and intra-factory variation in the quality and characteristics of well-defined varieties, in spite of the very considerable scientific and technological advances. The curds for many famous varieties of cheese, for example, Parmigiano Reggiano, Grana Padano, Emmental, and Roquefort, are produced in many farm-level dairies under the supervision of a producer consortium and the ripening of cheese and the marketing are organized by central facilities. Research on microbiology, chemistry, and technology of cheese started toward the end of the nineteenth century and continues today, as a result of which cheese science and technology are quite well understood. However, there are still large gaps in our knowledge, for example, the complete description of all cheeses flavour, how the flavour compounds are produced, the structure of some cheese, etc. With the gradual acquisition of knowledge on the chemistry and microbiology of milk and cheese, it becomes possible to study and control the changes involved in cheese-making.

1.1.2 Cheese manufacturing

The production of all varieties of cheese involves a generally similar protocol (**Figure 1.1.1**); various steps can be modified to give a product with the desired characteristics.

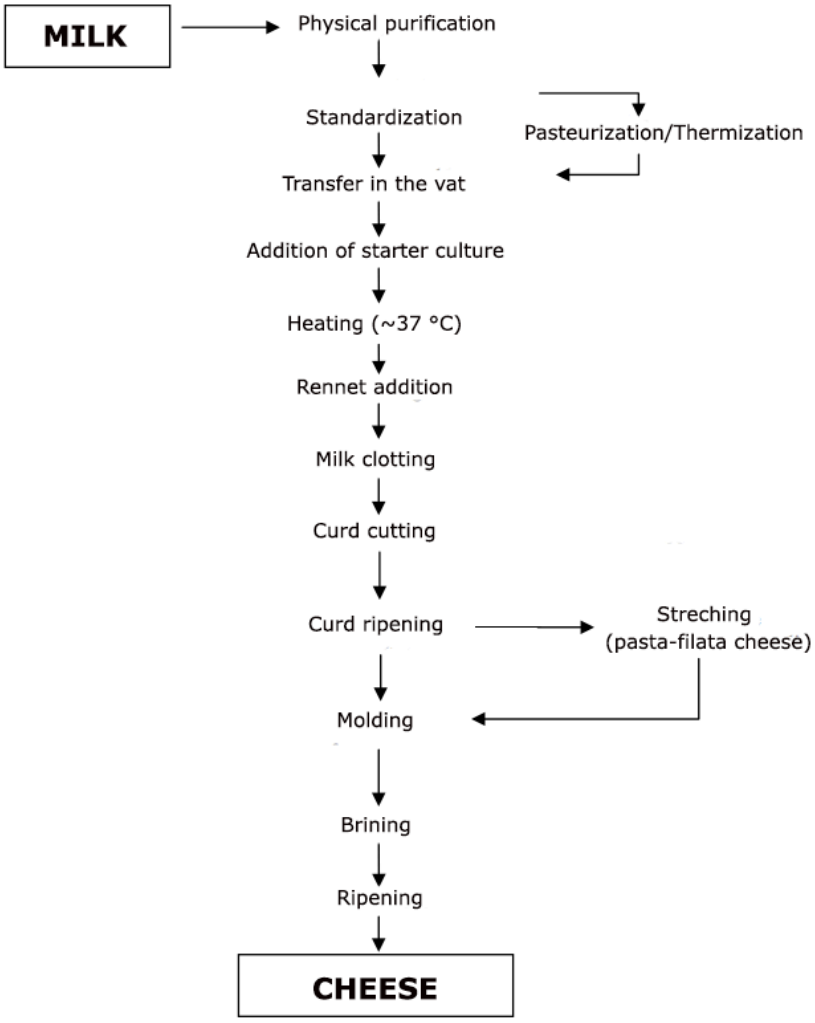

Figure 1.1.1 General cheese manufactuing process.

An essential 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 moulded curd may be consumed fresh (shortly after manufacture, for example within 1 week) or matured for periods of ~2 weeks to several years to form a ripened cheese. The gelation 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);
- 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;

The casein component constitutes around 80 g in 100 g of milk proteins. It is present as roughly spherical aggregates called casein micelles, consisting of several thousand of casein molecules. K-casein is predominantly located at the micelle surface with the hydrophobic para-k-casein part (residues 1–105) linked to the micelle, and the hydrophilic and negatively charged caseinmacropeptide (CMP) part (residues 106–169), rich in carbohydrates, protruding into the solution. The caseins generally have negative overall charge at fresh milk pH values (~6.5-6.7). Moreover, additional stabilisation comes from the fact that the CMP part of k-casein protrudes from the micelle surface, thus physically hindering contact between micelles through steric stabilization. Upon hydrolysis of k-casein by the chymosin, CMP is released, leaving para-k-casein attached to the micelle. The removal of CMP from the micelle surface leads to a decrease in electrostatic repulsion between micelles and they can start to aggregate. The rates of hydrolysis, aggregation and syneresis increases with increasing temperature, until the enzyme starts to be heat inactivated. The optimum temperature for curd formation at pH 6.5 is in the range of 34–38°C for

most commercial coagulants. In practice, coagulation is usually done at temperatures from 30 to 35°C to have adequate control over curd firmness at cutting, and to give the starter culture suitable conditions to start fermenting the milk. The pH has a large effect on coagulation and the properties of the curd, as a reduction in pH will speed up the rate of k-casein hydrolysis and the subsequent aggregation of casein micelles. Lowering the pH and increasing the temperature of the milk from normal values (~pH 6.6 and 31°C) allow the coagulation to occur at a lower degree of hydrolysis of k-casein (Guinee & Wilkinson, 1992). A moderate decrease in milk pH (e.g. to pH 6.4) results in modest solubilisation of the calcium from the casein micelles, which leads to a faster formation and a firmer curd. However, a higher degree of calcium solubilisation leads to extensive demineralisation of casein micelles, which results in weaker and more flexible curd gels (Choi et al., 2007). For some soft cheeses, a step of extensive demineralisation is required before coagulant addition to obtain the desired structure and body of the mature cheese. Following gel formation, 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. Following whey drainage, the curd particles knit together into a cohesive mass of curd, which is treated to enhance further whey expulsion and concentration to the desired dry matter content of the cheese variety being manufactured; these treatments differ according to variety but typically include further lactose fermentation and pH reduction, cutting the curd mass into pieces (slabs), moulding the pieces to the desired shape and weight of finished cheese and salt addition. Following manufacture, rennet-curd cheeses are usually ripened by holding under specific conditions of temperature and humidity for periods which range from ~2 to 4 weeks for soft cheeses (for Camembert-type cheeses) to ~2 years for some hard cheeses (for Parmesan-style cheeses). During this period, a host of physico-chemical changes take place which transform the 'rubbery/chewy'-textured fresh cheese curd to the finished cheese with the desired variety quality characteristics

1.1.3 Microbiota evolution during cheese manufacturing and its sources

Microorganisms present in dairy products may come from the milk (most of all for traditional cheese productions, where often raw or mildly thermized milk is used), the starter lactic acid bacteria (SLAB), the production environment and equipments. None of the classifications or categories of cheeses have considered the microbial diversity characterizing different types of cheese. Cheese is a microbiologically dynamic food, hosting diverse metabolically active bacteria, yeasts and moulds (Ndoye et al., 2011). In many cases, despite being made under standard manufacturing conditions, cheese from different days at the same dairy or in different dairies exhibit variations in the final characteristics. The composition and activity of the microbiota is the least controllable of all the parameters involved in cheese production (Fox et al., 2000a).

1.1.3.1 Raw milk microbiota

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 (Mucchetti & Neviani, 2006; Hassan & Frank, 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 (Fox, 2004). 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 (Hassan & Frank, 2011). 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 & Buchin, 2004). The loads of most microbial groups have remained stable in raw cow's milk since the mid 1990s (Mallet et al., 2012) 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 (**Table 1.1.1**). For all microbial groups, inter-farm variability is wide while intra-farm

variability is generally much lower except from season to season (Desmasures & Guéguen, 1997). Despite this low counts, raw milk still exhibits substantial microbial diversity. More than 100 genera and

400 microbial species have been detected in raw milk (Montel et al., 2014). 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. A literature survey of the bacterial genera commonly found in raw milk is reported in **Table 1.1.1**. Strain diversity in raw milk is also substantial but varies between species and between farms. In fact, up to 43 genotypes of *Lactococcus lactis* have been described in raw milk in France, with 1 to 11 genotypes per farm (Corroler et al., 1998; Dalmasso et al., 2008). 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 (Ercolini 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* such as *Hafnia alvei* (Ercolini 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 24 h (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 (Rasolofo et al., 2010). 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).

Table 1.1.1 Bacterial genera previously reported in raw milk (from Montel et al., 2014).

Phylum	Family	Genus
Actinobacteria	Actinomycetaceae	*Actinomyces*
	Actinomycetaceae	*Rothia*
	Bifidobacteriaceae	*Bifidobacterium*
	Brevibacteriaceae	*Brevibacterium*
	Corynebacteriaceae	*Corynebacterium*
	Intrasporangiaceae	*Ornithinicoccus*
	Microbacteriaceae	*Microbacterium*
	Micrococcaceae	*Arthrobacter*
	Micrococcaceae	*Kocuria*
	Nocardioidaceae	*Nocardioides*
	Propionibacteriaceae	*Propionibacterium*
Bacteroidetes	Bacteroidaceae	*Bacteroides*
	Cytophagaceae	*Adhaeribacter*
	Flavobacteriaceae	*Chryseobacterium*
	Flavobacteriaceae	*Empedobacter*
	Prevotellaceae	*Prevotella*
	Rikenellaceae	*Alistipes*
Firmicutes	Aerococcaceae	*Aerococcus*
	Aerococcaceae	*Facklamia*
	Bacillaceae	*Bacillus*
	Clostridiaceae	*Faecalibacterium*
	Enterococcaceae	*Enterococcus*
	Lachnospiraceae	*Catenibacterium*
	Lachnospiraceae	*Ruminococcus*
	Lactobacillaceae	*Lactobacillus*
	Lactobacillaceae	*Pediococcus*
	Leuconostocaceae	*Carnobacterium*
	Leuconostocaceae	*Leuconostoc*
	Leuconostocaceae	*Weissella*
	Peptostreptococcaceae	*Anaerococcus*
	Planococcaceae	*Kurthia*
	Staphylococcaceae	*Jeotgalicoccus*
	Staphylococcaceae	*Macrococcus*
	Staphylococcaceae	*Staphylococcus*

	Streptococcaceae	*Lactococcus*
	Streptococcaceae	*Streptococcus*
Fusobacteria	Leptotrichiaceae	*Leptotrichia*
Proteobacteria	Aeromonadaceae	*Aeromonas*
	Bradyrhizobiaceae	*Bosea*
	Burkholderiaceae	*Pandoraea*
	Caulobacteraceae	*Caulobacter*
	Enterobacteriaceae	*Enterobacter*
	Enterobacteriaceae	*Escherichia*
	Enterobacteriaceae	*Klebsiella*
	Methylobacteriaceae	*Methylobacterium*
	Moraxellaceae	*Acinetobacter*
	Moraxellaceae	*Psychrobacter*
	Oceanospirillaceae	*Marinomonas*
	Phyllobacteriaceae	*Phyllobacterium*
	Pseudoalteromonadaceae	*Pseudoalteromonas*
	Pseudomonadaceae	*Pseudomonas*
	Ralstoniaceae	*Ralstonia*
	Rhodobacteraceae	*Paracoccus*
	Rhodocyclaceae	*Thauera*
	Sphingomonadaceae	*Sphingomonas*
	Xanthomonadaceae	*Stenotrophomonas*

1.1.3.2 Microorganisms deliberately added during cheese manufacturing

Modern cheese manufacturing usually involves deliberate addition of one or more lactic acid bacteria (LAB) species to ensure a proper fermentation. Their primary role is to acidify the milk through converting lactose into lactic acid. Starter LAB (SLAB) possess an array of predominantly intracellular peptidases that degrade peptides formed by proteolytic agents to aminoacids, which then act as precursors for a range of volatile flavor compounds. When starter culture cells lyse in cheese, the intracellular peptidases are also available to act upon peptides in the cheese matrix itself. LAB starter culture metabolism can also directly affect cheese flavor development by forming various compounds from lactose and citrate (Powell et al., 2011). Either mesophilic or thermophilic starter cultures are used, depending on type of manufacture. Starter bacteria encountered most often are members of the genera *Lactococcus, Lactobacillus, Streptococcus, Leuconostoc* and *Enterococcus* (Beresford et al., 2001). Natural mesophilic cultures are mainly composed of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis.* Thermophilic natural whey starters are composed of undefined strains of lactobacilli such as *L. delbrueckii, L. helveticus, L. fermentum* and *S. thermophilus* (Ercolini et al., 2001; Ercolini et al., 2008; Gatti et al., 2003; Lazzi et al., 2004; Fornasari et al., 2006). They are produced by incubating cheese whey under conditions that favour the growth of thermophilic lactic acid bacteria (Mucchetti & Neviani, 2006). Starter cultures can be subdivided into natural cultures also referred as mixed (undefined) cultures, which are produced every day at the dairy and whose the number and the identity of the strains is unknown, and defined cultures, which are composed of a known number of strains (Beresford et al., 2001; Mucchetti & Neviani, 2006). The natural starter cultures are subjected to a great variability in terms of either microbial composition and performance. Industrial scale cheese production requires starters that give reproducible performance and are free of undesirable organisms. Since this is difficult to achieve

using traditional methods, defined starters are usually employed for industrial cheese production (Powell et al., 2011). Also the so-called "secondary cultures" may be added during cheese manufacturing. They are added mainly for their effect on flavor, colour, texture and eye formation in cheese, while their contribution to acid production is limited or absent. The principal secondary cultures used for the ripening include: *Propionibacterium freudenreichii* that are involved in flavor and eye formation in Swiss-type cheese, *Penicillium camemberti,* that are mainly involved in proteolysis in mould surface-ripened cheese as Camembert and Brie cheeses, *Penicillium roqueforti* involved in flavour, colour, lipolysis and some proteolysis in blue-veined cheese (e.g. Stilton, Roquefort, Gorgonzola), and *Brevibacterium linens* that are involved in flavour and colour in bacterial surface-ripened cheeses (e.g. Müster, Limburger, Tilster) (Ndoye et al., 2011; Rattray & Eppert, 2011).

1.1.3.3 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 (Lewis & Gilmour, 1987; Suarez et al., 1992) and non-starter lactic acid bacteria (NSLAB) (Somers et al., 2001; Broadbent et al., 2003; Agarwal et al., 2006; Steele et al., 2006) can form on equipment surfaces, acting as a source of contamination in successive batches of cheese. Wooden processing surfaces, including aging boards (Mariani et al., 2007; Feligini et al., 2012) 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., 2006). 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; Licitra et al., 2007). Wooden vats can increase microbial loads in the milk compared to those in milk before pouring into the vat (Didienne et al., 2012; Lortal et al., 2009; Settanni et al., 2012). 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 dominate in succession on the surface of Reblochon de Savoie, a raw milk PDO cheese, and also dominate the biofilms of shelves used for ripening (Mariani et al., 2007; Oulahal et al., 2009). 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, 2013).

1.1.4 Changes occurring during cheese ripening

Acid-coagulated cheeses are usually ready for consumption at the end of manufacture. Although rennet-coagulated cheese may be consumed as fresh, most of these cheeses are ripened for a period ranging from about 3 weeks to more than 2 years. For PDO (Protected Designation of Origin) cheeses the disciplinary imposes a specific time of ripening (e.g. Parmigiano Reggiano can be sold after at least 12 months, Caciocavallo Silano after 1 month). Although curds for different cheese varieties are recognizably different at the end of manufacture (mainly due to compositional and textural differences), the unique characteristics of each variety develop during ripening as a result of a complex set of biochemical reactions (Fox et al., 2004). The ripening process of cheese is very complex and involves microbiological and biochemical changes to the curd, leading to the specific flavour and texture of that cheese variety (McSweeney, 2004).

1.1.4.1 Microbiota evolution during cheese ripening

Microbiological changes in cheese during ripening include the death and lysis of starter cells (SLAB) and the growth of secondary microflora (particularly NSLAB). The microbiota associated to cheese ripening is extremely different; however, as mentioned above, it may be conveniently divided into two groups: the SLAB and the NSLAB. Some of the strains/species present in vat milk (indigenous and/or starter and/or

adjuncts strains) can grow, survive and even become dominant during the cheese manufacturing and ripening. This depends on the microorganisms' metabolic potential and the expression of that potential, which both depend on environmental conditions and are species- or even strain-specific. The environmental conditions encountered by the microbiota are first the biochemical composition of vat milks, then that of the curd matrix as modified by the acidifying starters and technological factors (e.g. rennet addition, temperature), and finally the technology applied during ripening (salting, smoking, temperature from 2 to 17 °C; relative humidity from 85% to 97%, gas composition) (Callon et al., 2011). This results in dramatic and continuous changes in the microbial balance during cheese making and ripening, but also in different microbial dynamics depending on the cheese technology applied. Microbial dynamics in the cores and on the surfaces of traditional cheeses have been extensively described. They vary between cheese varieties and, within a variety, between dairies and time periods. They are impacted by a complex and poorly understood network of interactions involving biotic factors (microbiota composition at species and strain levels, population counts and balances, etc.) and abiotic factors (physico-chemical composition and structure of cheese matrix, such as pH, a_w, redox potential, NaCl, CO_2, anaerobiosis, (un)dissociated acids, aminoacids, fatty acids and products of their catabolism, small peptides and carbon sources content, physicochemistry of cheese environment) (Callon et al., 2011; Charlet et al., 2009; Irlinger & Mounier, 2009; Pelaez & Requena, 2005).

Microbiota in cheese cores

In the cores of uncooked pressed and ripened cheeses, the balance of dominant species varies with time and between cheese varieties (Berthier et al., 2001; Bouton et al., 2002; Casey et al., 2006; Depouilly et al., 2004; Settanni et al., 2012). Microbial interactions occur as early as the pressing step, as shown in experimental cooked hard cheeses made from raw milk (Charlet et al., 2009), and during ripening (Fröhlich-Wyder et al., 2002). Usually, LAB diversity increases only in the core because LAB are well adapted to the conditions prevailing there: low pH, high NaCl, anaerobiosis, lack of fermentable carbohydrates (Montel et al., 2014). NSLAB are abundant in almost all ripened cheese varieties, whether traditional or not. The most widespread and most frequently dominant are *Lactococcus lactis*, *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Lb. casei*, *Enterococus faecalis* and *E. faecium*. In addition, intra-species genomic heterogeneity in non-starter LAB is wide within and between cheeses of different origins (Callon et al., 2004; Feutry et al., 2012a, 2012b) and several strains of *S. thermophilus*, *Lb. paracasei* and/or *Lb. delbruekii* can co-exist in Caciocavallo, Gruyère and Comté PDO cheeses (Montel et al., 2014). Mesophilic lactobacilli are among the most common non-starter LAB. Their relative abundance during the ripening of raw milk cheeses varies according to the type of technology (soft, semi-hard, hard) (Quigley et al., 2012) and the length of ripening (Depouilly et al., 2004; Gatti et al., 2008). In the cores of traditional raw milk cooked hard cheeses and pasta-filata cheeses (also made from raw milk), LAB largely dominate at all stages of the cheese ripening process, together with propionic acid bacteria in cheeses with eyes (Swiss-type cheeses). Mesophilic viable/cultivable NSLAB often become dominant, but they predominate over viable thermophilic lactobacilli only after 10 to 30 months of ripening (Gala et al., 2008; Gatti et al., 2008; Rossi et al., 2012). Microbial growth and the balance between cultivable LAB vary from just under the surface to the centre of the core in Grana Trentino cheese (Monfredini et al., 2012).

Microbiota on cheese surfaces

Cheese surface is a more open ecosystem than the core and shows different population dynamics (Bockelmann et al., 2005; Law, 2010). Yeasts, moulds and aerobic bacteria such as *Corynebacteriaceae* and *Micrococcaceae* develop mainly on cheese surface, where O_2 is more readily available than in the core (Montel et al., 2014). In some type of cheeses, an artificial inoculation is done by spraying the surface with secondary cultures composed by suspensions of spores of desired microorganisms (i.e. *Penicillium camemberti)* in Camembert cheese (Addis et al., 2001), or by immersion in water and salt solutions containing desired microorganisms (i.e. secondary cultures of *Brevibacterium linens* in Gubbeen cheese) (Brennan et al. 2002). Cheeses that are periodically wipe down (Gubbeen, Limburger, Appenzeller) are often identified with the name of washed-rind cheeses and are also called smear or red smear cheeses because of the development of viscous, red-orange smears on their surfaces during ripening composed of bacteria and yeasts (Mounier et al., 2005). On the surfaces of smear-ripened and mould-ripened cheeses, bacterial populations (mainly starter LAB in the earliest stages) dominate numerically throughout ripening. Microbial growth is initially due to budding yeast populations followed closely by *Geotrichum candidum*, when present. Yeast counts range around 6–8 log CFU/cm^2 within 2–7 days; they then remain fairly constant until the end of ripening, with variations in species balance. The yeast growth leads to an increase in the pH values and then bacterial growth occurs. From day 10 (for Reblochon, Gubbeen) or days 14–20 (for Camembert, Limburger, Raclette-type cheese, Saint-Nectaire,

Tilsit) and until the end of the ripening process, various bacteria grow on the cheese surface, reaching counts of 8–11 log CFU/cm^2. On the surface of mould-ripened cheeses, yeast growth is followed by mould growth. For example, on Saint-Nectaire type cheese, *Mucor* sp. starts to grow on day 4 and *Fusarium domesticum* on day 20 of ripening, while on Camembert, *Penicillium camemberti* starts to grow on days 6–7 of ripening and covers the surface by days 10–12. However, in all type of cheeses, surfaces exhibit a high species and genus diversity of both eukaryotes and prokaryotes. At least 30 different yeast species, can be found (Montel et al., 2014). The main species are *Debaryomyces hansenii*, *G. candidum*, *Candida catenulata*, *Kluyveromyces lactis* and *Yarrowia lipolytica*. Proteobacteria can reach counts as high as those of Actinobacteria on the surfaces of some smear cheeses (Larpin-Laborde et al., 2011). They are mainly *Enterobacteriaceae* (*Hafnia* and *Proteus*), *Moraxellaceae* (*Psychrobacter*), *Halomonadaceae* (*Halomonas*), Alcaligenes (Coton et al., 2012; Larpin-Laborde et al., 2011; Maoz et al., 2003; Mounier et al., 2009; Quigley et al., 2012). Among yeasts, *D. hansenii* and *K. lactis* are particularly present at early ripening stage, while *G. candidum*, when present, is present throughout ripening and *Y. lipolytica* is mainly detected in ripened cheeses. Regarding moulds, Panelli et al. (2012) found that *Penicillium commune* dominated on the surface of PDO Taleggio cheese, but also detected *Cladosporium* sp. and *Aureobasidium pullulans*. Yeast–yeast and yeast–bacterium interactions condition the establishment of the cheese surface ecosystem (Lessard et al., 2012; Mounier et al., 2009). Yeasts and moulds when present, metabolize lactic acid and produce NH_3, so raising the surface pH (from 4.8–5.2 to up to 6–8.2) and allowing salt-tolerant and acid-sensitive bacteria to grow. *Penicillium* species are decisive for the flavour and texture of white mould-ripened cheeses owing to their lipolytic and oxidative activities, leading to high production of aromatic ketones and alcohols (Molimard & Spinnler, 1996). They are added as ripening cultures to both raw and pasteurized milks.

1.1.4.2 Biochemical events during cheese ripening

The biochemical reactions that occur during ripening are caused by one or more of the following agents:

- indigenous milk enzymes, especially proteinase, such as plasmin, and perhaps lipase (from the rennet paste) (Sousa et al., 2001);
- secondary activity of chimosin (Reid et al.,1997);
- SLAB and their enzymes,
- NSLAB and their enzymes.

The biochemical changes may be grouped into primary events that include the metabolism of residual lactose and of lactate and citrate, lipolysis and proteolysis. Following these primary events secondary biochemical events are very important for the development of many volatile flavour compounds and include the metabolism of fatty acids and the catabolism of aminoacids (McSweeney, 2004).

It is possible to divided these biochemical reactions into three principal groups:

- Catabolism of residues of lactose, lactic acid and, in some varieties, citric acid; this results in changes in flavour and texture.
- Lipolysis and the catabolism of fatty acids; in some varieties, for example, blue cheeses, these reactions dominate ripening, while in Parmigiano Reggiano the catabolism of fatty acids can be considered important for flavour production.
- Proteolysis and modification of aminoacids, which are the most complex, and perhaps the most important, reactions in cheese ripening, especially in internal bacterially ripened varieties; they affect flavour and texture (Fuquay et al., 2010).

Carbohydrates metabolism

As cheese is a fermented dairy product, a key feature of its manufacture is the metabolism of lactose to lactate by selected cultures of SLAB. Starter bacteria metabolize the majority of lactose; in some cheese a low amount of lactose can remain and it can be used by NSLAB (Fox et al., 2004). Lactate produced from lactose is an important substrate for a range of reactions that occur in cheese during ripening. D-Lactate may be formed directly from lactose by NSLAB or by racemisation of L-lactate (Fox et al., 2000b). The rate at which L-lactate is racemised depends on the composition of the NSLAB flora (Thomas & Crow, 1983). The pathway for lactate racemisation probably involves oxidation of L-lactate by L-lactate dehydrogenase to form pyruvate, which is then reduced to D-lactate by the action of D-lactate dehydrogenase. Racemisation of lactate is significant because the solubility of Ca-DL-lactate is lower than that of Ca-L-lactate (Dybing et al., 1988; Thomas & Crow, 1983) and thus racemisation favours the formation of Ca-DL-lactate crystals, which are manifested in cheese as white specks (McSweeney, 2004). Lactate can be oxidized by LAB in cheese to products including acetate, ethanol, formate and CO_2 (Fox et al., 2000b). However, the extent to which this pathway occurs in cheese depends on the NSLAB population and the availability of O_2 (Fox et al., 2004). Citrate is an important precursor for flavour compounds in some kind of cheese made using mesophilic starter cultures (Fox et al., 2000b). In some long ripened cheeses citrate can be used by NSLAB when the residual level of carbohydrates are limiting and lactose or glucose are absent (Diaz-Muniz et al., 2006). The products of NSLAB metabolism of citrate are acetoin, acetate and diacetyl (Palles et al., 1998).

Lipid metabolism

In cheese, oxidative changes are very limited due to the low oxidation/reduction potential (about 250 mV) (McSweeney & Sousa, 2000). However, triglycerides in all cheese varieties undergo hydrolysis by the action of indigenous, endogenous and/or exogenous lipases, which result in the liberation of fatty acids in cheese during ripening. The triglycerides of ruminant milk fat are rich in short-chain fatty acids that, when liberated, have low flavour thresholds that contribute significantly to the flavour of many cheese varieties. Although some lipolysis occurs in most or all cheeses, it is most extensive in some hard Italian varieties and in blue cheese (McSweeney, 2004). Lipolytic agents in cheese generally originate from the milk, the coagulant (in the case of rennet paste) and the cheese microflora (SLAB and NSLAB). LAB possess intracellular esterolytic enzymes. As SLAB and NSLAB are present in cheese in high numbers, enzymes from these organisms are responsible for the liberation of significant levels of fatty acids during the long ripening period of many internal bacterially ripened cheeses (Collins et al., 2004). Lipolytic enzymes from SLAB are intracellular (Fernandez et al., 2000), and hence are released into the cheese matrix on lysis.

Protein metabolism

Proteolysis is the most complex and, in most cheese varieties, is the most important of the primary biochemical events occurring during ripening. The pattern of proteolysis in many cheese varieties may be summarised as follows: the caseins are hydrolysed initially by residual coagulant activity retained in the curd and by plasmin (and perhaps other indigenous proteolytic enzymes) to a range of large and intermediate sized peptides that are hydrolysed by proteinases and peptidases from the starter LAB and NSLAB, to shorter peptides and amino acids (McSweeney, 2004). These biochemical reactions have a direct influence on flavour through the production of short peptides and aminoacids, some of which are flavoured (often bitter), by facilitating the release of sapid compounds from the cheese matrix and most of all by providing free amino acids that are substrates for a series of catabolic reactions (an important secondary biochemical event during cheese ripening) that generate many important flavour compounds. The proteinases and peptidases that catalyse proteolysis in cheese during ripening originate from four sources, namely, the coagulant, the milk, the SLAB and the NSLAB (Sousa et al., 2001). LAB require many aminoacids and thus have complex proteolytic systems to liberate the aminoacids necessary for growth from the proteins in their environment. The proteinases and peptidases of LAB have been the subject of several studies (Liu et al., 2010; Upadhyay et al., 2004; Christensen et al., 1999). LAB also contain intracellular peptidases that are very important for the final stages of proteolysis in cheese during ripening and the ultimate liberation of free aminoacids as substrates for catabolic reactions.

Production of flavouring compounds

Biochemichal pathways leading to the production of flavor compounds are summarized in **Figure 1.1.2**.

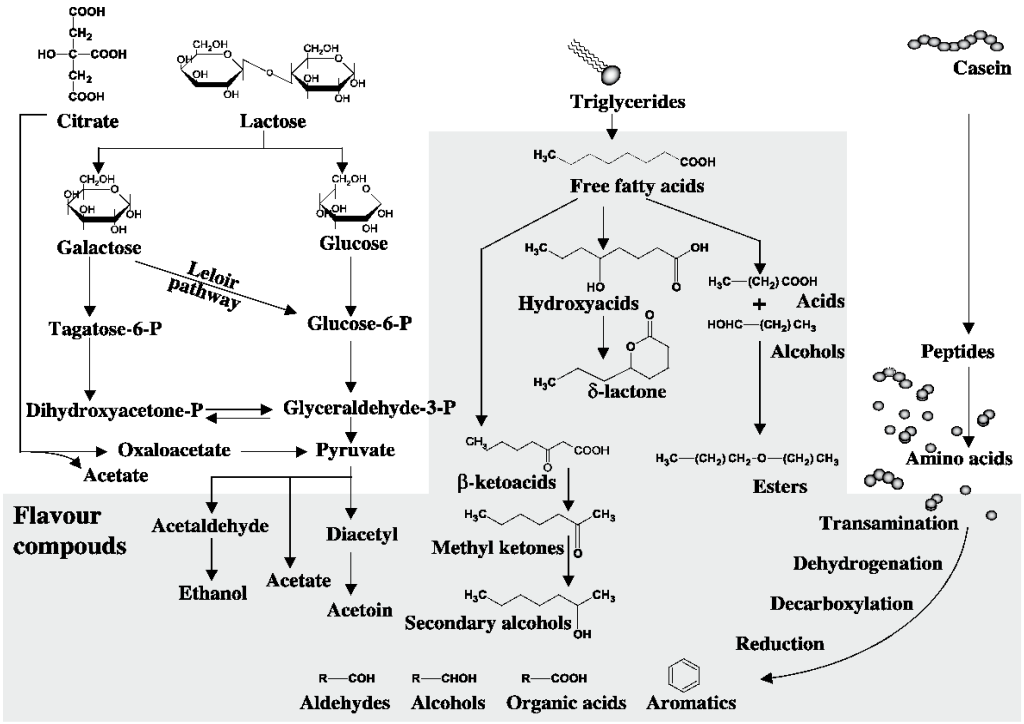

Figure 1.1.2 Biochemical pathways leading to the formation of flavour compounds (from Marillay & Casey, 2004)

Esters are found commonly in many cheese varieties and are produced by the reaction of a FFA with an alcohol. Ethanol is the limiting reactant in the production of esters; this alcohol is derived from the fermentation of lactose or from amino acid catabolism. Thioesters are compounds formed by the reaction of FFAs with sulphydryl compounds, usually methanethiol (McSweeney & Sousa, 2000). Also lactones were found in cheese, but during ripening their production is limited by the amount of precursors (hydroxyacids) (McSweeney & Sousa, 2000). The catabolism of aminoacids during ripening is another main secondary event that produce flavour compounds (Figure 3). The aminoacids catabolic pathways were reported in detail in a review of Fernandez & Zuniga (2006). For each aminoacid there is a catabolic pathway that leads to the formation of specific compounds. Aminoacids catabolism produces, in turn, a number of compounds, including ammonia, amines, aldehydes, phenols, indole and alcohols, which contribute as a whole to cheese flavour (Marilley & Casey, 2004). There are usually three recognisable steps in this complex process (Tavaria et al., 2002):

- reactions of decarboxylation, deamination, transamination, desulfuration and hydrolysis of side chain;
- conversion of the resulting compounds (amines and α-ketoacids);
- reduction of aldehydes to alcohols or their oxidation to carboxylic acids.

Several authors studies the flavour compounds formation by aminoacids catabolism pathways (Smit et al.,2005; Yvon & Rejien 2001; McSweeney & Sousa 2000). The flavours produced through these pathways and leading to the specific aroma of each cheeses have been studied in many research works (Sádecká et al., 2014; Randazzo et al., 2010; Randazzo et al., 2008; Ziino et al., 2005; Di Cagno et al., 2003; Qian & Reineccius, 2002; Mauriello et al., 2001; Moio et al., 2000; Moio & Addeo, 1998; Bosset & Gauch, 1993).

1.2 Metagenomics

1.2.1 From "traditional" metagenomics to high-throughput sequencing

The total number of microbial cells on Earth is estimated to be 10^{30} (Turnbaugh et al., 2008). Prokaryotes represent the largest proportion of individual organisms, comprising 10^6 to 10^8 separate genospecies (Sleator et al., 2008), but ninety-nine per cent of all micro-organisms in almost every environment on earth remain, as yet, uncultured (Amann et al. 1990; Curtis, 2002). Culture independent analyses arose to overcome the well-known limitations of the classical culture-based approach (Ercolini, 2013): since most of the microorganisms are not able to grow in the common laboratory media, when we use the classical cultivation methods we select only a part of the microbiota (those microbes which are able to grow in the medium used) and we can get only a partial picture of the microbial diversity of that environment. For this reason, in the past 20 years, we moved to the culture-independent approach, which allow to analyse the microbiota by using the DNA directly extracted from the matrix, without applying any selection. A new era of microbial ecology was initiated with the concept of cloning DNA directly from the environment, which is commonly attributed to Pace et al. (1986), and was applied by Schmidt et al. (1991) who characterized 16S rRNA sequences from a Pacific Ocean picoplankton population by cloning environmental DNA into a phage genome and screening for clones that contained 16S rRNA genes. By 1998, this technique of randomly cloning environmental DNA followed by elaborate screening methods became known as metagenomics (Handelsman et al. 1998), which can be translated as "beyond the genome" (Gilbert & Dupont 2011). This new label referred to the concept that researchers were now exploring the genomic DNA from all the genomes of all the organisms in an environmental community through cultivation-independent methods, and, therefore, going beyond the single genome. The advent of next-generation sequencing technologies in 2004 provided unprecedented sampling depth compared to traditional culture-independent approaches, such as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993; Ercolini, 2004), terminal restriction fragment length polymorphism (T-RFLP) analysis (Fierer & Jackson, 2006; Tiedje et al., 1999), or Sanger sequencing of 16S rRNA gene clone libraries (Sogin et al., 2006). In fact, one of the main advantage of HTS approaches is the higher sensitivity, which allow to detect even species present at very low abundance. Moreover, HTS is uniquely quantitative: it is possible to gather information on how many reads of different operational taxonomic units (OTUs) occur in a template and therefore have an estimate of the occurrence (%) of different OTUs.

1.2.2 Different high-throughput approaches for the study of food microbial ecology

HTS studies in microbial ecology can be grouped into two fields: amplicon metagenomics (sequencing of libraries of a PCR-amplified gene of interest), and shotgun metagenomics (sequencing of libraries of randomly isolated DNA fragments) (**Figure 1.2.1**). In the first 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 common HTS application in microbial ecology. This entails the so called "microbiota", that is the taxonomic composition of the microbial community present in the sample and the relative abundance of each OTU (Operational Taxonomic Unit). In addition, HTS of specific target genes can provide strain monitoring in food samples.

In the shotgun approach, no PCR selection is performed and total DNA is fragmented and directly sequenced. In this way, after a computational assembly and a comparison with databases, you can get the abundance of all the genes present in the environment, so the potential activities that the microbiota could carry on. In this case, we will refer to the "microbiome", the complex of the microorganisms and their genomes in the environment in question, with consequent identification of the microbial genes occurring in that specific environment and their relative abundances (Figure 1). If we want to know which genes are actually expressed, then we need to approach the RNA-seq (Mutz et al., 2013). After total RNA extraction, depletion of ribosomal RNA (rRNA) is necessary (since rRNA makes up more than 80% of total RNA). Then, the RNA enriched of messenger RNA (mRNA) is retrotrascribed and it is sequenced with the shotgun approach as described above.

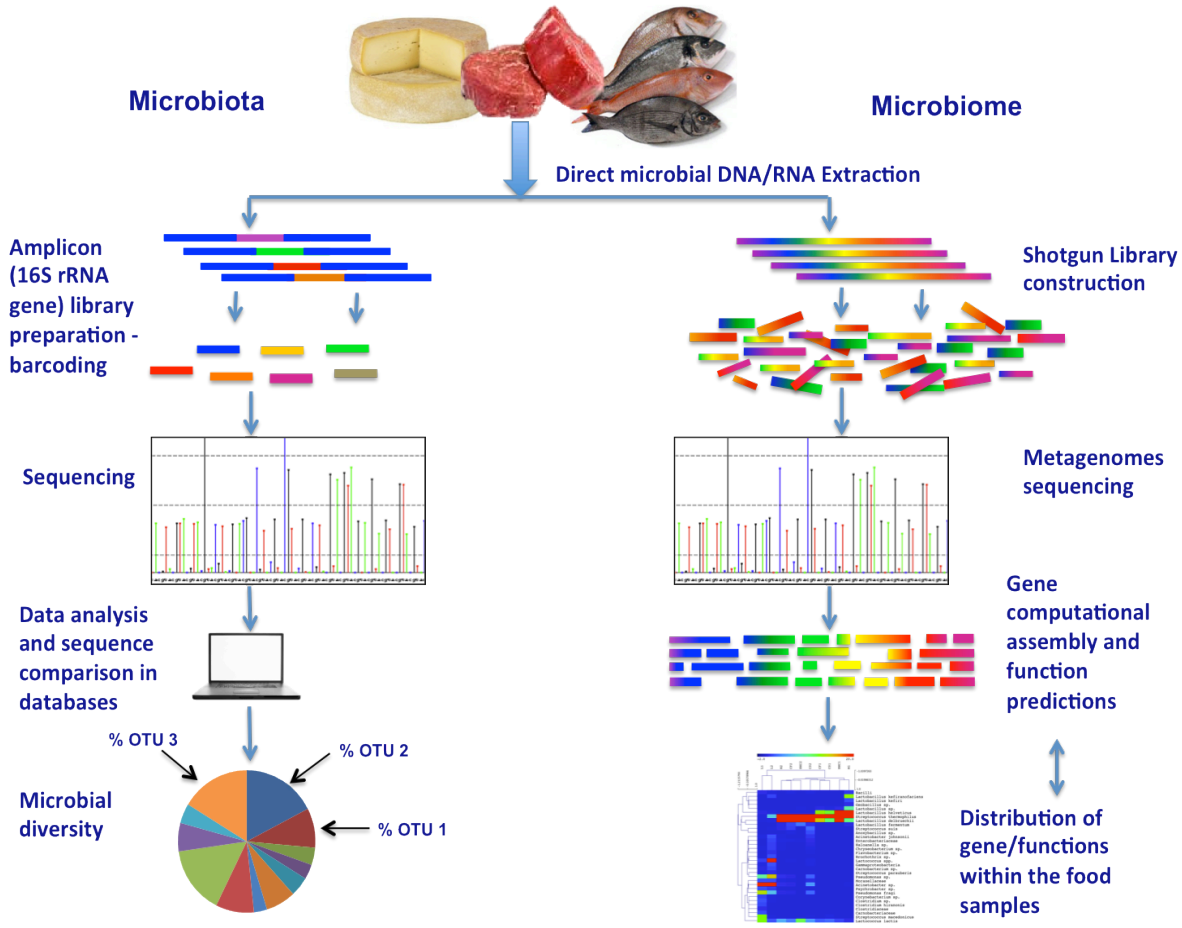

Figure 1.2.1 HTS workflow to study food microbiota and microbiome.

1.2.3 Next generation sequencing technology

Two platforms for massively parallel DNA sequencing read production are in reasonably widespread use at present: 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). Each platform embodies a complex interplay of enzymology, chemistry, high-resolution optics, hardware and software engineering. The libraries are obtained by annealing platform-specific linkers. Because the presence of adapter sequences means that the molecules then can be selectively amplified by PCR, no bacterial cloning step is required to amplify the genomic fragment in a bacterial intermediate as is done in traditional sequencing approaches. Another contrast between these instruments and capillary platforms is 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 has arrived up to 1000 bp and the yield in number of reads can vary from several hundred thousand reads (Roche/454) to tens of millions of reads (Illumina and Applied Biosystems SOLiD) (Mardis, 2008).

1.2.3.1 Roche/454 FLX Pyrosequencer

This next-generation sequencer was the first to achieve commercial introduction (in 2004) and uses an alternative sequencing technology known as pyrosequencing. In pyrosequencing, each incorporation of a nucleotide by DNA polymerase results in the release of pyrophosphate, which initiates a series of downstream reactions that ultimately produce 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 1.2.2**), the 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 which each sequencing reaction 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.

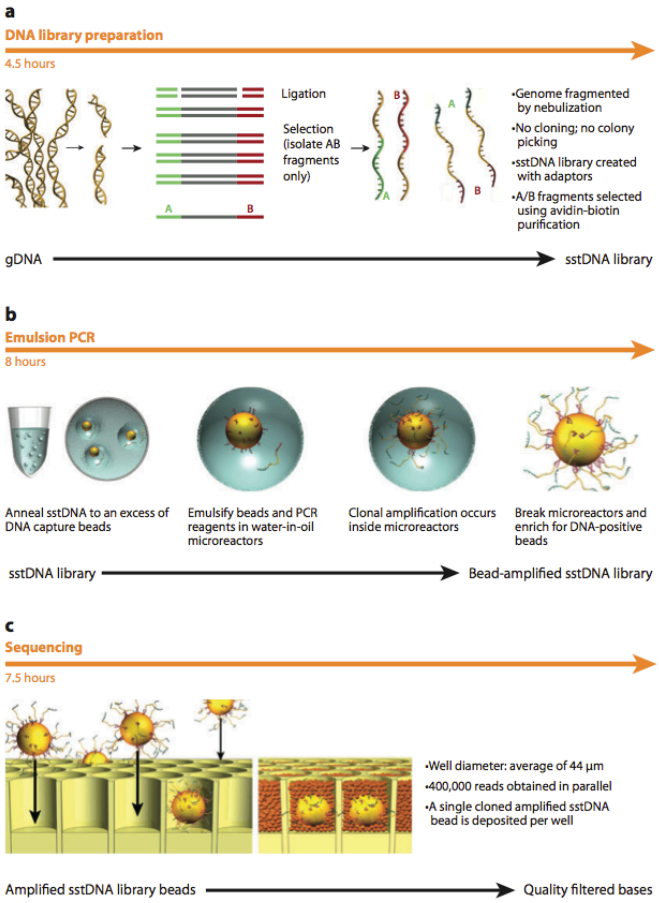

Figure 1.2.2 The method used by the Roche/454 sequencer to amplify single-stranded DNA copies from a fragment library on agarose beads. A mixture of DNA fragments with agarose beads containing complementary oligonucleotides to the adapters at the fragment ends are mixed in an approximately 1:1 ratio. The mixture is encapsulated by vigorous vortexing into aqueous micelles that contain PCR reactants surrounded by oil, and pipetted into a 96-well microtiter plate for PCR amplification. The resulting beads are decorated with approximately 1 million copies of the original single-stranded fragment, which provides sufficient signal strength during the pyrosequencing reaction that follows to detect and record nucleotide incorporation events. sstDNA, single-stranded template DNA (from Mardis, 2008).

1.2.3.2 Illumina Genome Analyzer

The amplification step for the Illumina Genome Analyzer starts with an Illumina-specific adapter library, takes place on the oligo-derivatized surface of a flow cell, and is performed by 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 utilizes a sequencing-by-synthesis approach in which all four nucleotides are added simultaneously to the flow cell channels, along with DNA polymerase, for incorporation into the oligo-primed cluster fragments (see **Figure 1.2.3** for details). Specifically, 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 specific 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.

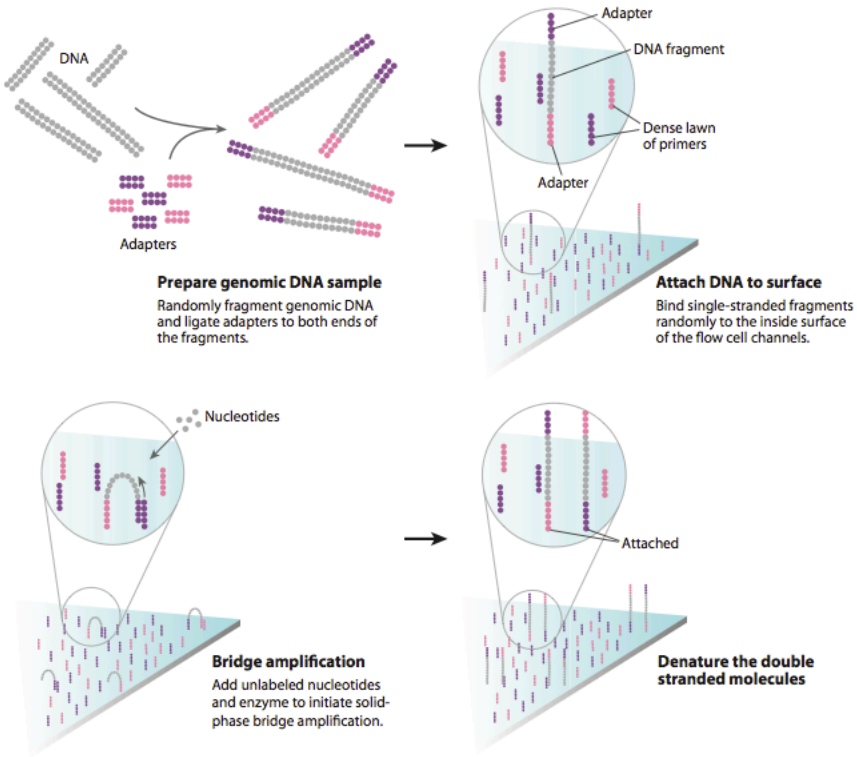

Figure 1.2.3 The Illumina sequencing-by-synthesis approach. Cluster strands created by bridge amplification are primed and all four fluorescently labeled, 3′-OH blocked nucleotides are added to the flow cell with DNA polymerase. The cluster strands are extended by one nucleotide. Following the incorporation 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).

1.2.4 Food microbology in high-throughput sequencing era

Food quality and safety are heavily influenced by the microorganisms contaminating food and by their possible development during production, handling, storage and distribution. Since different microbes

have different genotypes and metabolic potential, it is hardly surprising that the study of the structure of food microbiota and its role in achieving desired food quality has been the focus of food microbiologists for decades. The study of the microbial ecology of foods has dramatically changed in the last years. Following the trends in environmental microbiology, other disciplines, including food microbiology, have benefited from the advances in molecular biology and adopted novel strategies to detect, identify and monitor microbes. The science and approach to studying microorganisms in food has radically changed. The current age is that of functional genomics, transcriptomics, proteomics and metabolomics, all applied to determine the overall role of microorganisms in food (O'Flaherty & Klaenhammer, 2001). Nevertheless, the "detectomics" era is still with us: all possible efforts are devoted to developing and optimizing molecular methods for the detection, reliable identification and monitoring of food-associated microorganisms (Ercolini & Cocolin, 2008). Culture-independent analyses are now routinely applied in many food microbiology laboratories; such analyses arose to overcome the limitations of the classical culture-based approach and have so far been extensively used so far to study food microbiota. They comprise mostly PCR-dependent electrophoretic methods such as PCR-DGGE and other techniques whose technical details and applications to foods have been reviewed elsewhere (Giraffa & Neviani, 2001; Ercolini, 2004; Quigley et al., 2011; Cocolin et al., 2011). The actual analysis of food microbial ecology is now performed by targeting microbial DNA or RNA directly from food rather than pursuing traditional isolation and biochemical characterization of microbes from food.

Food microbiologists aim to study the diversity and dynamics of microbial populations in foods. The scope of their analysis can depend on the specific food and on the types of microbes that can be (i) pathogens, (ii) spoilage-associated or (iii) technologically relevant microorganisms. Such microbial populations have to be monitored because of their role in food contamination, decay or fermentation/production. When culture-independent approaches are employed for the study of ecology and biodiversity in food fermentations, the target molecules considered are DNA and RNA. The significance of the results that can be obtained using one or other nucleic acid needs to be properly evaluated since these two molecules have different properties and can lead to different results. DNA is very stable and is long present even after the cell has died. By contrast, RNA, and especially messenger RNA (mRNA), can have a very short life. Hence studying the DNA of a microbial ecosystem will allow definition of the microbial ecology and diversity, while RNA analysis will highlight the microbial populations that are metabolically active, thereby contributing to the transformation in progress in food at the time of analysis.

1.2.4.1 Sample coverage and taxonomic resolution: how deep do we want to go?

With the HTS approach based on rRNA gene sequencing the structure of a microbial community from food or any other environment can be identified by determining a large number of sequences for each sample analyzed. As stated above, this can be achieved through different technologies that do not give exactly the same result in terms of sample coverage and taxonomic resolution. Sequence coverage is given by the number of sequences retrieved for a particular sample. In theory, the higher the better: analyzing tens of thousands of sequences for a single sample ensures thorough determination of the structure of the microbial community, with the possibility of highlighting the presence of very minor OTUs. However, determining a very large number of sequences per sample can be fairly expensive and is sometimes redundant. Rarefaction analyses of sequencing data (Gotelli & Colwell, 2001) can lend a very strong support to decide a numerical threshold of sequences that can be informative for the type of sample under study. Examples of rarefaction plots are depicted in **Figure 1.2.4**.

These can be retrieved by most of the bioinformatics tools that can be used to analyze rRNA-based HTS results. Typically, a rarefaction curve shows the variation in the number of OTUs identified at a given % of identity (mostly 97%) as a function of the number of sequence reads obtained per sample. Ideally, an optimal number of reads to describe the microbiota is identified by the plateau of the curve, which indicates that increasing the number of reads does not change the number of OTUs that can be determined, and therefore the optimal coverage of the microbial diversity within the sample is reached (**Figure 1.2.4**). In other words, when the curve becomes flatter to the right, a reasonable number of sequences have been considered and more intensive sampling is likely to yield only few additional species. These trends depend very much upon the level of the diversity of each particular sample. In the specific case of food, samples with a complex microbiota such as raw milks or any other "raw" material are likely to be characterized by a large number of OTUs and will need more reads to be properly characterized; in such cases, the plateau is only reached at a high sequence number (**Figure 1.2.4**). On the other hand, for pasteurized foods, fermented foods or when starter cultures are added, and where the microbiota is less complex because a selection of OTUs has taken place and a limited number of species occurs, even a low number of sequences can be enough to properly define the structure of the community

(**Figure 1.2.4**). The data on food products reported in the literature include a very variable number of reads per sample, ranging between 100 and more than 10,000 reads depending on the specific study and sample analyzed. However, not all the studies report a rarefaction analysis and it is not always possible to understand how deep the sample coverage has been. For example, a coverage obtained by about 1,000 reads was satisfactory to determine the microbiota of some fermented soybean products and kefir grains (Nam et al., 2012a; Nam et al., 2012b; Leite et al., 2012); by contrast, more than 5,000 reads were required for the analysis of a different soybean product (Kim et al., 2011). In another study, most samples of soft, semi-hard and hard Irish cheeses were adequately covered with slightly more than 1,000 reads, while cheese rinds, which have a more complex microbiota, required more sequences to be adequately studied (Quigley et al., 2012). However, when group-specific primers are used to target specifically certain OTUs, even very few sequences per sample will suffice (Oakley et al., 2012). The decision is left to the researcher: a large number of sequence reads is very useful for an in-depth assessment of the community structure while a lower number of sequences can still be useful to compare different samples or to look at population dynamics during food fermentation or storage.

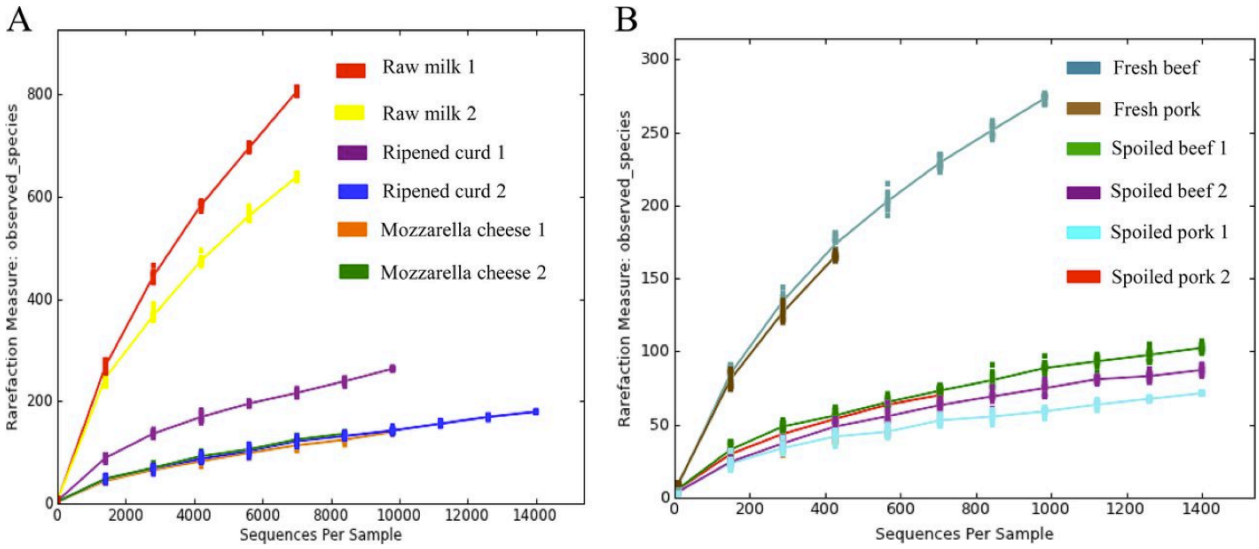

Figure 1.2.4 Examples of rarefaction curves reporting the number of observed OTUs as a function of the number of sequence reads. Data were retrieved from HTS analyses of samples of mozzarella cheese and production intermediates (A) and fresh and aerobically spoiled pork and beef (B) (from Ercolini, 2013).

Taxonomic identification is another issue to be taken care of when approaching the study of food microbiota by HTS. Foods are microbiologically complex matrices but are not as rich in taxa as other environmental samples such as soil, wastes, feces etc. where microbial diversity can be fruitfully investigated at genus level or even larger hierarchical taxonomic ascriptions such as family, order, class or phylum. Monitoring population dynamics in food can be performed at any taxonomic level; however, the taxonomic resolution required can vary depending on the purpose of the study. For example, genus-level monitoring of microbial diversity in a food during fermentation or storage can be used when dramatic changes in the structure of the community are expected. However, in many cases species-level identification is needed to have useful information in food. In a typical example, in population changes during cheese ripening there is often a succession of *Lactobacillus* species where thermophilic species are responsible for the initial fermentation while mesophilic lactobacilli take over during cheese ripening. Similar cases are those of other fermented products such as sourdough or fermented meats where many different species of the same genus can occur and take turns during fermentation. In such cases an HTS study at genus level is not informative and species assignment should be the target of the analysis. To this purpose, long sequence reads including more variable regions of the 16S rRNA gene are required for accurate assignment. However, even with long reads, 16S rRNA gene is not always heterogeneous enough for species identification of food-related bacteria, which is proven in several cases such as that of *Pseudomonas* spp. (Moore et al., 1996; Anzai et al., 2000). In addition, in the cases where heterogeneity allows species identification, this must be carefully checked in more than one database analyzing the specific read quality and nucleotide sequence and considering the possibility of occurrence of other taxonomically close species in the same environment. Fragments of 16S rRNA from 150 to 500 bp

including one to three different variable regions have been employed and often the longer the amplicons sequenced, the deeper are the taxonomic assignments that can be obtained (Ercolini, 2013).

In conclusion, the choice of amplicon length and sample coverage is entirely left to the microbial ecologist and will depend on the specific food and on the scope of the project. A good number of reads is advisable for sample coverage and a satisfactory fragment length is desirable for in-depth, reliable taxonomic identification.

1.2.5 HTS applications

1.2.5.1 Microbiota involved in food fermentation.

16S rRNA gene-based high-throughput sequencing can be used for the assessment of the microbiota in a specific fermented food, intended as the final product at the end of production, or for monitoring the microbial populations occurring during fermentation from raw materials to the end of the process.

The first use of high-throughput sequencing for the detection of food microbiota was to study the microbiota of fermented pearl millet slurries from Burkina Faso (Humblot & Guyot, 2009). The members of the microbiota occurring at the beginning and after 24 h of pearl millet fermentation obtained by three different procedures were identified at genus level by pyrosequencing of the V3 region of the 16S rRNA gene. *Firmicutes* including *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Weissella* were found with different abundances from the beginning to the end of three fermentations while genera belonging to *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were only found in traditional fermentation processes.

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 describe the microbial ecology of *oscypek*, a traditional Polish Protected Designation of Origin (PDO) smoked cheese made from sheep's milk without starter cultures (Alegria et al., 2012). Samples of curd, fresh and smoked cheese were mainly populated by *Lactococcus*, unclassified *Lactobacillales* and *Leuconostoc*; interestingly, *Bifidobacteriaceae* and *Moraxellaceae* (mostly *Enhydrobacter*) were also found, albeit at much lower percentages. The microbiota of Danish raw milk cheeses was investigated by HTS of the V3-V4 16S rRNA region by targeting both DNA and cDNA extracted from different intermediates of production of different cheese batches (Masoud et al., 2011). It was possible to demonstrate that the populations found (part of the starter cultures, and belonging to the genera *Lactobacillus*, *Streptococcus* and *Lactococcus*) dominated the raw milk cheese system and, based on the overall agreement between DNA and cDNA data, the microorganisms found were metabolically active (Masoud et al., 2011). The same authors demonstrated that *E. coli* deliberately added to milk could be found to be metabolically active (by cDNA pyrosequencing) up to seven days of ripening of Danish cheeses while it decreased in subsequent stages. By contrast, *Listeria innocua* and *S. aureus* were only detected by DNA and not cDNA analysis, indicating an unviable state (Masoud et al., 2012). The above are very good examples of HTS-based monitoring of both starter cultures and unwanted bacteria during complex dairy productions.

Analysis of the microbiota found in the final products is another common HTS application. A comprehensive survey of about 60 Irish soft, semi-hard or hard cheeses was recently performed by HTS to screen for differences in bacterial diversity according to type of cheese, milk and production technology (Quigley et al., 2012). *Lactococcus* was the dominant OTU in all the cheeses, although in hard cheeses its abundance decreased with increasing *Lactobacillus* reads. In addition, minor contaminants found mainly in cheese rinds including *Faecalibacterium*, *Prevotella* and *Helcococcus* (Quigley et al., 2012). In *nukadoko*, a Japanese fermented bran mash, it was shown that *Lb. namurensis* and *Lb. acetotolerans* were the predominant species that could influence the microbial dynamics during fermentation (Sakamoto et al., 2011).

Beverage fermentation can also be monitored by culture-independent methods. The microbiota involved in brewing American coolship ale was investigated by a combination of molecular approaches including HTS of the V4 region of the 16S rRNA gene. The authors demonstrated that *Enterobacteriaceae* dominated at the initial fermentation stages while *Lactobacillales* and yeasts took over in the subsequent phases (Bokulich et al., 2012a). This was a clear example of how to demonstrate the stability of autochthonous bacteria in long processes and highlight the role of resident microbiota (that of the brewhouse in the specific case) in fermentation. The same authors assessed the microbiota of botrytized wines by using the same approach, demonstrating that V4 sequencing yielded a better taxonomic resolution than region V5 of 16S (Bokulich et al., 2012b). Moreover, combining the HTS results with 16S-TFRLP of LAB communities, it was found that in botrytized wines acetic acid bacteria are more

abundant than in unaffected wines, and the succession of LAB genera during different vintage fermentations was described (Bokulich et al., 2012b). By using the internal transcribed spacer region 1 (ITS1) of fungi as PCR target, Li et al. (2011) showed how *Saccharomycetaceae* and *Saccharomycopsidaceae* represented about 60% and 30% of the fungal population during Chinese *fen* liquor fermentation. In addition, the ITS2 region was used to target the fungi during Korean rice beer fermentation: it was found that the fungal diversity did not differ significantly between commercial and traditional fermentations (Jung et al., 2012).

The microbiota of traditional Korean fermented foods was analyzed by culture-independent HTS of the V1-V2 regions of the 16S rRNA gene of local as well as commercial fermented foods (Nam et al., 2012a; 2012b; 2012c). *Bacillus* spp. was the dominant OTU in commercial as well as local *cheonggukjang*, a traditional soybean food. However, a much higher diversity of both other genera and within the *Bacillus* OTU was found in local compared to commercial products (Nam et al., 2012c). In the soybean paste *doenjang*, Nam et al. (2012a) showed that species of *Bacillus* previously thought to be dominant in the product were in fact more abundant only in samples from central Korea, while LAB dominated in other samples. In addition, compared to the more complex local brands, commercial brands of *doenjang* showed a simple structure of the community including *Tetragenococcus* and *Staphylococcus*, therefore suggesting the use of bacterial inocula for process standardization (Nam et al., 2012a). A common diversity pattern was found in the fermented soybean *meju*, where 26% of the OTUs were shared by the different samples analyzed and included mostly *Bacillus sonorensis* and *Enterococcus durans* (Kim et al., 2011). A region-specific distribution of microbiota was also found in *kocujang*, another fermented soybean product, where *Bacillus subtilis* and *B. licheniformis* were the main OTUs found (Nam et al., 2012a).

In a few kefir grains analyzed by HTS, more than 90% of the OTUs were ascribable to the genus *Lactobacillus* while other LAB only constituted minor populations (Leite et a., 2012). In addition, *Lactobacillaceae* dominated the microbial population of an Irish kefir grain, while *Streptococcaceae* were mainly found in the kefir-fermented milk (Dobson et al., 2011).

The microbiota of *narezushi,* an archetype of modern Japanese sushi obtained by fermentation of salted fish with rice, was assessed in the final product (Koyanagi et al., 2011) and during fermentation (Kiyohara et al., 2012) by HTS of V1-V2 regions of the 16S. The product was mainly populated by *Lactobacillus* and *Pediococcus* (Koyanagi et al., 2011) while *Lactobacillus* spp. increased during fermentation (Kiyohara et al., 2012). A survey of Korean fermented seafoods was also performed by HTS targeting the 16S rRNA gene of both Archea and Bacteria. Within the *Halobacteriaceae*, members of the genera *Halorubum* and *Halalkalicoccus* were the most frequently found *Archea*, and cuttlefish had 99.6% of uncultured *Crenarchaeota*. Within Bacteria, most of the fermented seafood samples were populated by LAB of the genera *Lactobacillus* and *Weissella* (Roh et al., 2010).

1.2.5.2 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. Comparison of the microbial community occurring in marinated and unmarinated broiler meat after chill storage in modified atmosphere packaging showed that both consortia were mainly populated by Gram positives; in addition, *Carnobacteriaceae* and *Streptococcaceae* were the only dominant bacteria in unmarinated meat, while *Lactobacillaceae* and *Leuconostocaceae* also occurred in marinated samples (Nieminem et al., 2012a). The approach was also useful to establish that marination reduced the proportion of minor taxa associated with meat spoilage, and the results were confirmed in a further study involving broiler fillet strips (Nieminem et al., 2012b). 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 et al., 2011). The initial meat prior to packaging was found to be 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.

1.2.5.3 Analysis of viromes in food

A very interesting HTS application was performed for the investigation of the bacteriophage diversity in fermented shrimp, kimchi and sauerkraut (Park et al., 2011). Since little is known about the ecological role of viruses in the ecosystem of fermented foods, the study aimed to define the viral communities in the above fermented foods. Technically, viral DNA was extracted from the foods, a genomic library was prepared by linker-amplified shotgun protocol and pyrosequenced on a 454 FLX platform; this procedure only targeted dsDNA. Despite the large number of unknown genes and the difficulty in correctly identifying the viral sequences due to viral genomes being poorly represented in databases, the authors demonstrated that the selected viruses belonged to the order *Caudovirales* and that the family *Siphoviridae* was abundant in shrimp (53%) and sauerkraut (60%) while *Podoviridae* abounded in kimchi (53%). Overall, the authors found less complex viral communities in food than those reported in other environments and underlined the importance of metagenomic approaches to ascertain the ecology and role of viruses in food as well other complex ecosystems (Park et al., 2011).

1.2.5.4 HTS-based validation of specific PCR assays

Foodborne pathogens give cause for concern among food scientists and are very often the selected targets of projects to define their fate in food processing, fermentation or storage. To this aim, many different group-, genus- and species-specific PCR assays have been developed and used for molecular detection of pathogens in food (Postollec et al., 2011; Dwivedi & Jaykus, 2011). In all cases, once a set of specific primers has been designed, the PCR assay must be checked for cross-reaction and false positive occurrence with non-target microorganisms. The HTS approach has been successfully used to validate a *Campylobacter*-specific PCR assay: using the specific primers to amplify DNA from a chicken fluff sample, it was possible to check that 100% of the sequences were *Campylobacter* while employing broad-range primers 178 different genera were detected in the same sample (Oakley et al., 2012). This clearly shows how specific primers can be easily and quickly checked for cross reaction in complex samples including hundreds of non-target bacteria avoiding long and often incomplete screening of reference strains.

1.2.5.5 Potential impact of "proper" metagenomics

The application of "proper" metagenomics or metatranscriptomics in food environment is still in delay compared to applications in other environments. The first study reporting metagenome sequencing from food is that by Nieminen et al. (2012a) investigating the microbiota of marinated and unmarinated broiler meat. Pairwise statistical comparisons of the annotated functions indicated 32 SEED subsystems out of 703 that significantly differed between the two types of meat storage. Bacteria in marinated meat had more genes involved in metabolism of carbohydrates such as xylose, sucrose, L-arabinose and fructooligosaccharides that are found in plants and not in meat. This difference reflected the phylogenetic composition between marinated and unmarinated meat (Nieminem et al., 2012a). Jung et al (2011) studied the metagenome of kimki and found a prevalence of genes involved in heterotrophic lactic acid fermentation of carbohydrates, which was supported by the detection of mannitol, lactate, acetate, and ethanol as fermentation products. Erkus et al. (2013) applied the metagenomic approach to the study of a defined starter culture, highlighting that the degree of biodiversity at strain level is probably maintained by a density-dependent phage sensitivity of the fittest strains that prevents the complete eradication of some genetic lineages from the starter culture during propagation. Recently, a metagenomic study indicated that cold-adapted proteolytic and lipolytic enzymes can contribute to cheese rind ripening (Wolfe et al., 2014).

To the author's knowledge, the only metatranscriptome application in food microbial ecology is the one by Lessard et al. (2014), who studied the gene expression of *Penicillium camemberti* and *Geotrichum candidum* during the ripening of a Camembert-type cheese.

Although the studies of metagenomes from food are of great potential, a current limitation is the database availability due to the many genomes of food-related bacteria still to be sequenced. In addition, metagenomic data often include a large proportion of genes encoding for basic cell functions that are not always related to specific activities that may be of interest for a particular food microbiota such as key enzymes for flavor compound production, toxin synthesis or specific aminoacid degradation. Detection by sequencing of such activities will probably be quenched by the detection of sequences of genes encoding for basic functions. It is thus desirable that in the near future enrichment protocols can be studied and developed for *ad hoc* sequencing of discrete parts of the metagenomes in order to allow monitoring of changes in abundance not only in species diversity but also in activities.

1.2.6 Critical issues

Since culture-independent HTS analysis of microbiota is considered quantitative, all the possible issues that can lead to an alteration of the original proportion of microbial cells (or DNA extracted therefrom) in a specific food sample must be avoided because it may lead to unreliable pictures of the microbiota. In theory, the approach is quantitative because there will be proportion between abundance of a specific microorganism in the food, quantity of nucleic acid extracted, quantity of amplicons obtained and the number of sequences gained belonging to that specific microorganism. Therefore, the number of sequences obtained is ultimately proportional to the abundance of the microorganism in question. All possible efforts thus need to be made to keep the above proportion unaltered.

Sampling and sample handling are frequent problem sources regardless of the analytical approach used (Brehm-Stecher et al., 2009). Once the sample is collected, altering the proportion of the microorganisms both before and after nucleic acid extraction must be avoided. Any such alterations would result in appreciable changes in the ratios between sequence numbers and OTU abundance, with doubtless oversights in the estimation of the proportions of microbial populations in the original food sample. As far as sample handling is concerned, aerobic or anaerobic storage, transport, freezing or chilling may affect the development of the microorganisms in the food by altering the number and species to be detected.

A further source of variability can be the nucleic acid extraction. Not all microbial species have the same sensitivity to lytic agents, the differences being mainly due to the organization of the cell wall. This affects the analyses based on *in situ* nucleic acid extraction since a high yield in pure DNA/RNA is desired as well as the detection of all the species occurring in that environment. The more complex the matrix, the more difficult it is to obtain good extraction and to get rid of all the impurities that can negatively affect the PCR amplification step. The case of food matrices is particularly awkward; the presence of natural constituents such as lipids, proteins, carbohydrates and salts may render extraction very hard and some of these molecules can persist until the end of the extraction and be found in the extract where they might act as PCR inhibitors (Wilson, 1997). It is thus very important to choose an extraction procedure that is most efficient and provides templates from all the microbial entities occurring in the original sample. Some examples of optimization of DNA extraction from food matrices can be found in the literature (Pirondini et al., 2010).

The PCR itself may be a source of bias in culture-independent analysis of food samples. Differential or preferential amplification of rRNA genes by PCR is an acknowledged problem (Reysenbach, 1992; Varadaraj & Skinner, 1994). Preferential amplification would determine that the abundance of the OTUs detected may not exclusively reflect the proportion of the microorganisms in the original sample.

The HTS approach is greatly influenced, indeed driven, by analysis of sequences (Scholz et al., 2012). Several open-source programs are available for processing 16S amplicon HTS data (Caporaso et al., 2010; Schloss et al., 2009; Meyer et al., 2008). The use of such bioinformatics tools in HTS-based microbial ecology has been reviewed elsewhere (Zaneveld et al., 2011; Kuczynski et al., 2012). The accuracy and reliability of the final determined structure of the food microbiota depends very much on the quality of the reference database used to assign the taxonomy (Nilsson et al., 2006; Tedersoo et al., 2011; McDonald et al., 2012). Various databases are available for prokaryotes (McDonald et al., 2012; De Santis et al., 2006; Pruesse et al., 2007; Cole et al., 2009) all containing reliable-quality 16S rRNA gene sequences.

There is currently less in-depth coverage of fungi: although they can be identified on the basis of internal transcribed spacer (ITS), small and large ribosomal subunits, the public databases often have poor quality sequences and curated databases have limited coverage (Nilsson et al., 2006; Tedersoo et al., 2011). In addition, in the specific case of ITS, amplicon length unevenness can promote preferential amplification

of shorter sequences, making it necessary to optimize the target regions to be analyzed (Bokulich & Mills, 2013b). This is particularly inconvenient for food analysis by HTS, which would benefit from application to fungi given the extreme importance of yeasts and moulds in fermentation, ripening and spoilage of food products.

1.2.7 Space for further exploitation and future perspectives.

A promising application of HTS in food microbiology is the possibility of strain typing and monitoring. Strain typing is of great importance for in-depth investigation of microbial dynamics in foods. Indeed, strain monitoring can address many important questions. For example, some microbial species play a major role in triggering food spoilage and subsequent dynamics (Doulgeraki et al., 2012). However, is there a dominant strain population that drives spoilage? The same question applies to species of starter cultures used for food fermentation. Does a starter "strain" actually dominate the ecosystem during fermentation? In addition, foods contaminated by pathogens could benefit from strain-specific investigation of the microbiota that could reveal the occurrence of more than one pathotype in the case of correlated episodes of infection/intoxication and could contribute to perform concomitant molecular risk analysis. For these purposes, culture-independent strain-typing by HTS could be performed by studying key genes that have significant intra-species heterogeneity.

Another interesting application of HTS could be the study of microbiomes from foods, determining the proportion of all the microbial genes in a given sample. As stated above, this application, common for other environment, is still not exploited enough in food microbiology.

1.2.8 Pros and cons: can we take HTS beyond research purposes?

HTS was conceived and is currently employed for research laboratories. To evaluate the possibility of scaling up the analysis for the benefit of food industry requirements, strengths and weaknesses need to be analyzed (**Figure 1.2.5**). 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 (**Figure 1.2.5**). 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.

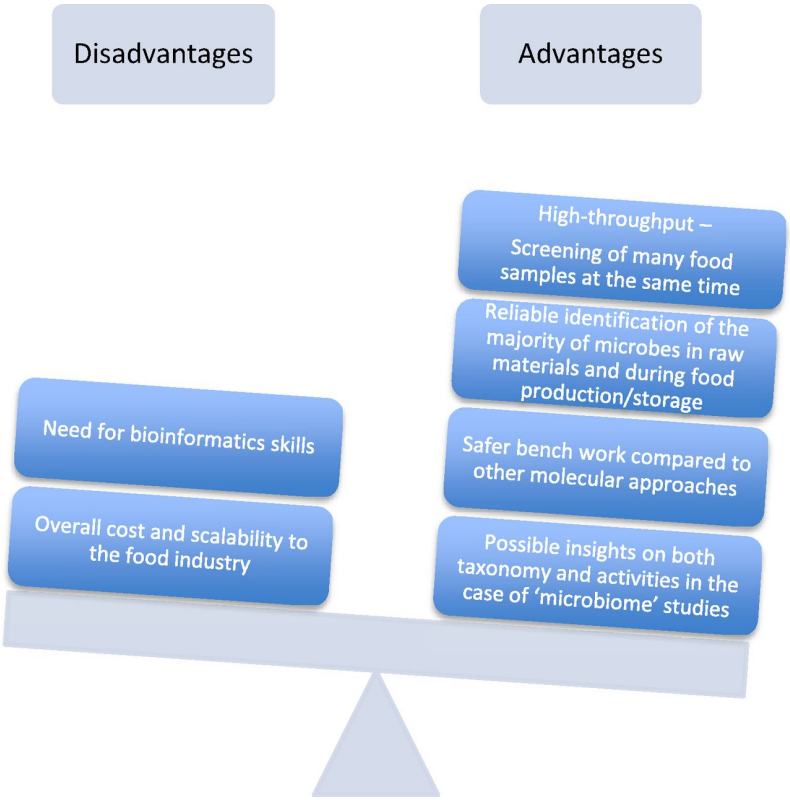

Figure 1.2.5 Advantages and disadvantages of the use of HTS to study food-associated microbial ecology (from Ercolini, 2013)

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2 RESULTS AND DISCUSSION

2.1 Evolution of the microbiota during Mozzarella cheese making

2.1.1 Introduction

Mozzarella is perhaps the most popular non-ripened cheese. The traditional Mozzarella is mainly produced in Southern Italy from water buffalo's milk even though it is widely exported and also industrially produced in other countries. The technology of manufacture has been described in detail in previous works (Coppola et al., 1990; Ercolini et al., 2004). The cheese is made from whole raw water buffalo's milk by adding a natural whey culture (NWC, from the manufacture of the previous day) as starter. After a curd ripening phase (4-4.5 h at 35-37 °C), which occurs under whey, the optimal pH (4.9-5.1) is reached and the drained curd is stretched in hot water (90-95 °C). The elastic product formed is then moulded (traditionally hand-moulded) in order to get the final typical round shape. The specific characteristics of the final product mainly arise from the raw materials employed, the agro-ecosystem of the area of production and the traditional technology of manufacture. The use of raw buffalo's milk and the NWC in the manufacture have been so far recognized as strong points for the traditional Mozzarella production because premium quality products arise as result of fermentation by the specific microbiota of raw milk and NWC. Due to the use of microbiologically complex raw materials, the traditional cheese manufactures are the most difficult to control and it is of interest to develop reliable methods to monitor the fermentation in order to support a standardization of the process for good quality products, while preserving their typical traits. The microbiota of mozzarella cheese has been studied in the past by culture-independent fingerprinting without identification of microbial taxa (Coppola et al., 2001; Ercolini et al., 2004). However, the microbiota involved in the buffalo's mozzarella production has never been thoroughly assessed by microbial species identification, although the complexity of the microbiota is recognized on the basis of culture-based microbiological determinations (Coppola et al., 1988; Coppola et al., 1990; Morea et al., 1999; Parente et al., 1997). Tracing In this study, intermediates of production of two manufactures of traditional mozzarella cheese were analyzed by culture-independent pyrosequencing in order to provide insights in the microbiota responsible for the production of such appreciated dairy product.

2.1.2 Materials and methods

2.1.2.1 Sampling

The samples were collected in May 2012 from 2 different dairies producing top quality traditional water buffalo Mozzarella cheese, located in Campania region (Southern Italy) in the provinces of Salerno (Manufacture 1) and Caserta (Manufacture 2), respectively. Samples of raw milk (L), Natural whey cultures (NWC), curd at the beginning (C0) and at end (CF) of the ripening, and final Mozzarella cheese (M) were aseptically collected, cooled at 4 °C, and analyzed within six hours. Duplicate samples were collected from 2 different manufactures within the same day of work, equal amounts of samples were pooled prior to DNA extraction. Raw milk was taken from the vat before the manufacture started; curd at the beginning and end of ripening were taken after 20 min and 5 h after NWC addition, respectively; Mozzarella cheese samples were collected from their governing liquid 20 min after molding.

2.1.2.2 DNA extraction, library preparation and pyrosequencing

Total DNA extraction from the dairy samples was carried out by using the Biostic™ Bacteremia DNA isolation kit (MO BIO Laboratories, Inc. Carlsbad, CA). The dairy samples were two-fold diluted in quarter strength Ringer's solution and the protocol was applied to the pellet (12,000 g) of 1 ml of suspension. The microbial diversity was studied by pyrosequencing of the amplified V1-V3 region of the 16S rRNA gene by using primers Gray27F 5'-TTTGATCNTGGCTCAG and Gray519r 5'-GTNTTACNGCGGCKGCTG amplifying a fragment of 520 bp (Andreotti et al., 2011). 454-adaptors were included in the forward primer followed by a 10 bp sample-specific Multiplex Identifier (MID). Each PCR mixture (final volume, 50 μl) contained 50 ng of template DNA, 0.4 μM of each primer, 0.50 mmol l^{-1} of each deoxynucleoside triphosphate, 2.5 mmol l^{-1} $MgCl_2$, 5 μl of 10 X 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 first purified by QIAquick gel extraction kit

(Qiagen, Milano, Italy) and then by Agencourt AMPure kit (Beckman Coulter, Milano, Italy) prior to further processing. The amplicons were used as a template for pyrosequencing on a GS Junior platform (454 Life Sciences, Roche Diagnostics, Italy) according to the manufacturer's instructions by using a Titanium chemistry. All the sequencing data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information (SRP014821).

2.1.2.3 Bioinformatics and data analysis

A first filtering of the results was performed by using the 454 Amplicon signal processing, then sequences were analyzed by using QIIME 1.5.0 software (Caporaso et al., 2010a). In order to guarantee a higher level of accuracy in terms of OTU detection, after the split library script performed by QIIME, the reads were excluded from the analysis if they had an average quality score lower than 25, if they were shorter than 200 bp and if there were ambiguous base calls. Sequences that passed the quality filter were denoised (Reeder & Knight, 2010) and singletons were excluded. Operational Taxonomic Units (OTUs) defined by a 97% of similarity were picked using the uclust method (Edgar, 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 using the Greengenes 16S rRNA gene database (McDonald et al., 2012). Representative sequences for OTUs showing an incidence above 5% in at least one sample were double checked with the Blast (Blastn) search program (http://www.ncbi.nlm.nih.gov/blast/) to confirm the taxonomy assignment made by QIIME. Sequences PyNAST aligned by QIIME (Caporaso et al., 2010b) were used as input in Mothur 1.26.0 software (Schloss et al., 2009) to generate Good's coverage, Chao1 richness (Chao & Bunge, 2002) and Shannon diversity indices (Shannon & Weaver, 1949). The OTU taxonomy table generated by QIIME was used to produce a heat map by using the clustering software TMeV v 4.8 (Saeed et al., 2003).

2.1.3 Results

The run produced 90,500 reads after 454 amplicon signal quality control; after the further filtering protocols, 77,277 reads were obtained with an average length of 492 bp; the reads were distributed among the samples as reported in Table 1. The filtering of the sequences eliminated no more than 15% of the reads per sample on average. A total of 511 OTUs were obtained. The rarefaction analysis and the diversity indexes indicated that there was a satisfactory coverage of the diversity for the all intermediates of production within the 2 manufactures (**Table 2.1.1**). The highest diversity was associated to raw milk samples that had also lower estimated sample coverage. Overall, despite the diversity of sequencing depth between samples, the rarefaction analysis indicated that a number of reads above 2,000 per sample was sufficient to obtain a good coverage.

The application of 16S rRNA gene pyrosequencing allowed the determination of the microbial diversity in the intermediates of production of Mozzarella cheese and also provided the relative abundance of the taxonomic levels of bacteria detected. The distribution of the OTUs with an incidence above 3% is reported in **Figure 2.1.1** where the evolution of the microbial diversity during the 2 different manufactures of Mozzarella cheese is shown. The raw water buffalo milk used for both manufactures displayed a complex microbiota composed of 192 (L1) and 97 (L2) total OTUs, respectively. The most abundant OTUs in both milks were the psychrotrophic *Acinetobacter* sp. and *Pseudomonas* sp. with an incidence of about 21% and 20%, respectively (**Figure 2.1.1**). *Lactococcus lactis* and *Streptococcus macedonicus* were also found with an incidence above 10% each in L1, whereas *Lactococcus* sp. represented almost 30% of the OTUs in L2 (**Figure 2.1.1**). The most abundant OTUs in the natural starter NWC1 were *Strep. thermophilus* (47%), *Lactobacillus delbrueckii* (19%) and *Lb. helveticus* (31%) while *Strep. thermophilus* (44%) and *Lb. delbrueckii* (41%) were the main OTUs in NWC2. The above OTUs were the most abundant even in the remaining samples collected during each specific manufacture (**Figure 2.1.1**).

Considering the minimum incidence of the 0.5% in at least 1 sample we considered 33 OTUs and used the percentage of abundance in each sample to generate the hierarchical clustering reported in **Figure 2.1.2**. Raw milk samples with the most complex microbiota formed a cluster separated from the other samples, while the intermediates of production formed minor clusters, according to the specific dairy (**Figure 2.1.2**). Most of the OTUs occurred in raw milks and not in the samples within each manufacture (**Figure 2.1.2**). In addition, the OTUs associated to each fermentation, and determining the shown degree of similarity between samples are easily detected in the heat map (**Figure 2.1.2**). Abundant groups of Gram-negative OTUs such as *Pseudomonas* sp. and *Acinetobacter* sp., as well as other contaminants such as clostridia, carnobacteria, enterobacteria and some other streptococci characterized the microbiota of

raw milks (**Figure 2.1.1**). It was also possible to note that *Lb. helveticus* specifically occurred in manufacture 1, while *Lb. fermentum* was only found in manufacture 2 (**Figure 2.1.1**). Some accessory OTUs also occurred; *L. lactis* represented 12% of the OTUs in raw milk L1 but was never found beyond 2% during manufacture 1. By contrast, in production 2, *L. lactis* originated from the NWC2 (11%) and occurred with incidence up to 5% in the other samples up to final product. *Strep. macedonicus* or *L. lactis* occurring as dominant taxa in the raw milk L1 only represented minor populations during the corresponding manufacture, similar case is that of *Lactococcus* sp. in L2 (**Figure 2.1.1**). *Pseudomonas* sp. coming from the raw milk contaminated also intermediates of production of manufacture 2, while some mesophilic lactobacilli occurred in the final mozzarella of the dairy 1 including *Lb. kefiri* and *Lb. kefirofaciens* (**Figure 2.1.2**).

2.1.4 Discussion

The deep sequencing approach was useful to identify the sources of bacteria in the manufacture and to ascertain whether they originated from the milk or were carried from the NWC starter or from other sources. The quantitative distribution of the OTUs within the samples suggests that the microbiota involved in the fermentation is carried by the NWC and that the microorganisms of raw milk do not develop during fermentation. Moreover, samples from the two manufactures clearly separated in the heatmap according to the microbiota. The two manufactures studied here were from two different provinces of the main area of production. Accordingly, the microbiota associated to the production of buffalo mozzarella cheese and also the aroma profiles of mozzarella have been shown to be dependent on the geographical origin (Bonizzi et al., 2007; Mauriello et al., 2003; Mazzei et al., 2012).

In previous studies the NWCs as natural starters have been characterized (Coppola et al., 1988; Coppola et al., 1990; Ercolini et al., 2001; Mauriello et al., 2003) and regarded as complex consortia of microorganisms of great importance to drive the fermentation and for the quality of the traditional product (Ercolini et al., 2004; Mauriello et al., 2003). In this study we assessed the microbiota of two premium quality mozzarella manufactures covering the principal geographical area of production. Overall, we demonstrated that the microbiota associated to this dairy production is not as complex as previously thought and that a few thermophilic LAB drive the fermentation, while mesophilic LAB such as *L. lactis* are quantitatively less abundant during manufacture. Therefore, although diversity at strain level can also play an important role, the complexity of the aroma profiles of mozzarella cheese do not likely arise exclusively from microbial fermentation. Other environmental factors including farming, specific feeding and raw milk quality can impact the aroma of mozzarella cheese (Brescia et al., 2005; Cifuni et al., 2007; Moio et al., 1993), which together with fermentation give the typical traits to the end product.

A small sample size was analyzed in this study; in spite of the good sample coverage, the analysis of a wider number of samples from the same and even other dairies of the two provinces would further support the results obtained. However, the sensitivity of high-throughput sequencing can reveal minor OTUs occurring during cheese making. This can represent an advantage for research purposes in some food manufactures, when low quality products arise and reliable monitoring of the microbiological quality is needed in order to detect and identify microbial contaminants and their sources in intermediates of production or final products.

2.1.5 References

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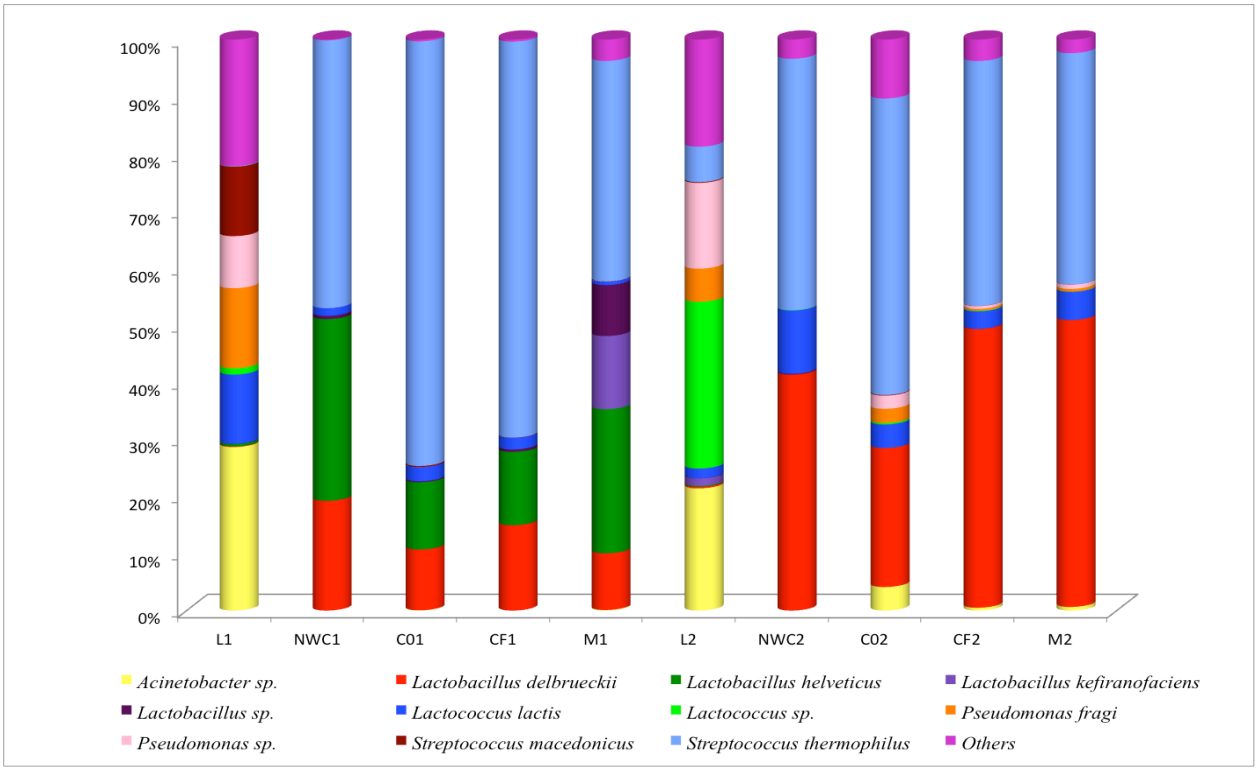

Figure 2.1.1 Incidence of OTUs based on 16S rRNA gene pyrosequencing analysis of all the DNA samples directly extracted from intermediates of production of two Mozzarella cheese manufactures. Only OTUs with an incidence above 3% in at least one sample are shown.

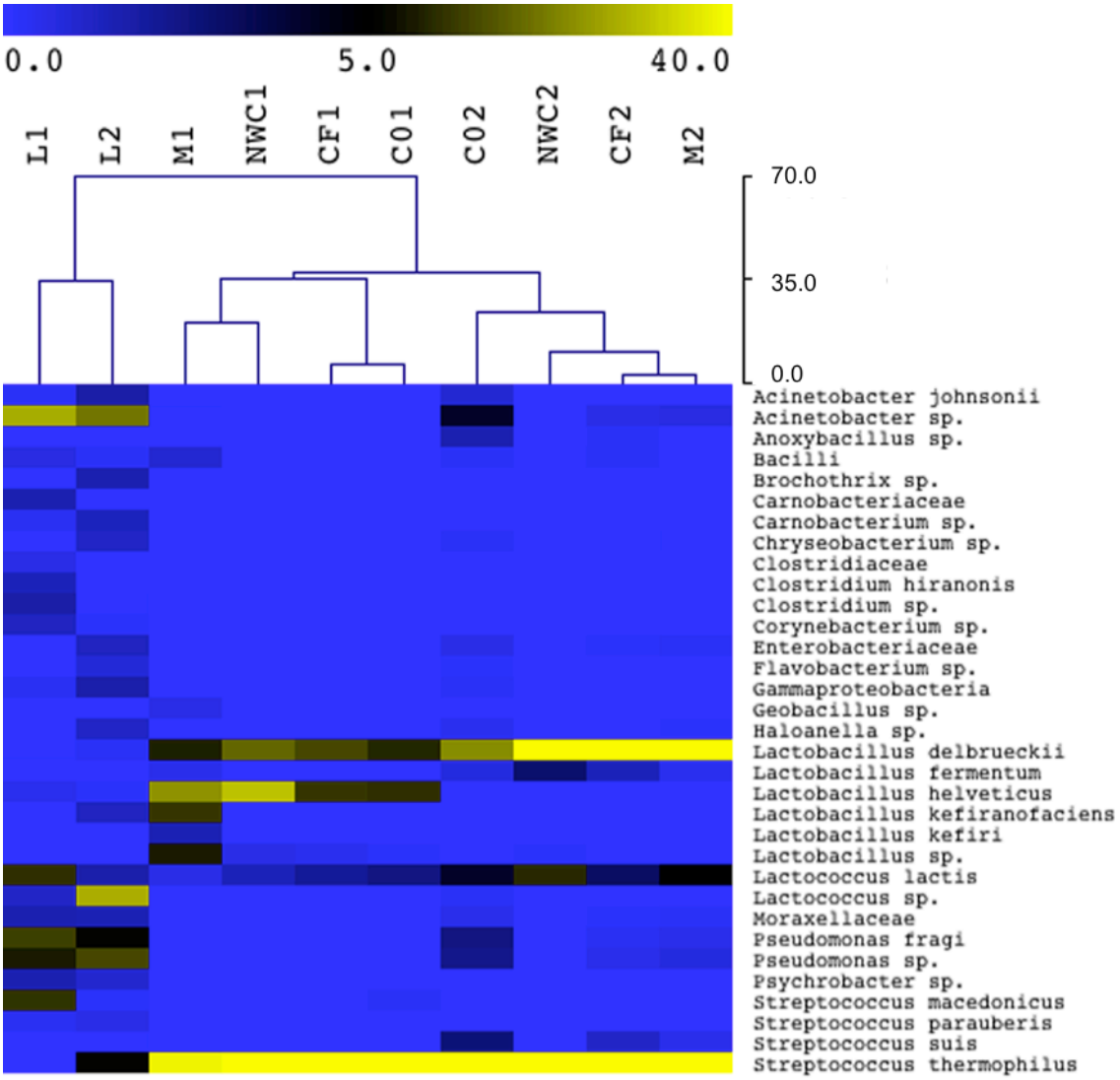

Figure 2.1.2 Heat map depicting bacterial diversity and relative abundance in intermediates of production of two Mozzarella cheese manufactures. Hierarchical dendrogram shows different bacteria distribution based on average linkage clustering and Euclidean distance. Legend and scale shown in the upper part of the figure represent colors in the heat map associated with the relative percentage of each OTU within the samples.

Table 2.1.1 Number of sequences analyzed, observed diversity and estimated sample coverage for 16S rRNA amplicons from Mozzarella cheese manufactures.

Sample ID	Reads	OTUs	Chao1	Shannon	ESC
L1	7860	192	1963.52 (1684.77; 2326,65)	3.45 (3.40; 3.51)	93.7%
NWC1	14066	11	790.98 (500.60; 1336,95)	1.46 (1.44; 1.48)	99.1%
C01	10459	26	332.40 (245.90; 490.22)	1.07 (1.04; 1.10)	99.2%
CF1	8920	19	297.12 (229.89; 416.97)	1.21 (1.18; 1.24)	99.0%
M1	10219	35	693.18 (535.76; 941.45)	2.05 (2.02; 2.09)	98.3%
L2	7579	97	945.28 (782.52; 1181.72)	3.49 (3.45; 3.53)	97.0%
NWC2	2947	13	196.50 (152.98; 282.11)	1.82 (1.76; 1.88)	98.0%
C02	4510	55	457.78 (371.12; 598.41)	2.24 (2.17; 2.30)	97.2%
CF2	5661	33	402.12 (310.92; 558.83)	1.72 (1.67; 1.77)	98.1%
M2	5056	30	304.29 (236.74; 425.68)	1.73 (1.68; 1.78)	98.3%

Abbreviations: OTU, operational taxonomic unit; ESC, estimated sample coverage. Chao1, Shannon and ESC were calculated with Mothur at the 3% distance level. Values in brackets represent 95% confidence intervals. 1 and 2 indicate the manufactures from the province of Salerno and Caserta, respectively. L, raw milk; NWC, Natural whey culture; C0, curd at the beginning of ripening; CF, curd at end of the ripening; M, final Mozzarella cheese.

2.2 Microbiological quality of industrial mozzarella cheeses produced with different acidification methods

2.2.1 Introduction

High-moisture Mozzarella cheese is a soft, unripened pasta filata cheese manufactured from cow's milk using a variety of acidification methods, including direct acidification by addition of citric acid, or natural acidification by addition of thermophilic defined or undefined strain starters, including natural whey or milk cultures (De Angelis & Gobbetti, 2011). In the direct acid addition process citric acid (or more rarely lactic acid) is added to pasteurized milk before rennet addition, and the curd is ready for stretching soon after coagulation (Faccia et al., 2009). A variety of starter cultures are used for cultured high-moisture Mozzarella cheese. Complex undefined whey cultures or milk cultures are required by the standards of identity of Protected Designation of Origin Water-Buffalo Mozzarella cheese (Ercolini et al., 2004; Ercolini et al., 2012; De Filippis et al., 2014) or Traditional Specialty Guaranteed cow's milk Mozzarella cheese (De Angelis & Gobbetti, 2011) and are used for traditional mozzarella cheese production (Parente et al., 1997; Coppola et al., 2006; De Candia et al., 2007). These cultures are dominated by S. thermophilus, but other thermophilic (*Lactobacillus delbrueckii*, *Lb. helveticus*) and mesophilic (*Lactococcus lactis*, *Leuconostoc*, other lactobacilli) lactic acid bacteria (LAB) and enterococci are often present as subdominant organisms (Parente et al., 1997; Ercolini et al., 2004; Coppola et al., 2006; De Candia et al., 2007; Ercolini et al., 2012; De Filippis et al., 2014). Defined starter cultures for cultured Mozzarella cheese usually include *S. thermophilus* alone, or in combination with *L. delbrueckii* subsp. bulgaricus or *L. helveticus* (De Angelis & Gobbetti, 2011), although more complex starters have been proposed (De Angelis et al., 2008). After production, high moisture mozzarella cheese is packaged in liquid (water, whey, stretching water, brine) (Faccia et al., 2013) and stored under refrigerated conditions. Because of its high moisture content (50-60%) and relatively high pH (>5.5), Mozzarella cheese has a short shelf-life, which usually does not exceed 5 days at refrigeration temperature. Addition of coatings or of preservatives has been proposed to increase the shelf-life of Mozzarella (Sinigaglia et al., 2008; Del Nobile et al., 2009; Baruzzi et al., 2012; Lucera et al., 2014). Spoilage is often caused by proteolytic psychrotrophic microorganisms (Baruzzi et al., 2012); or by discoloration (Nogarol et al., 2013; Andreani et al., 2014), and members of the genus *Pseudomonas* have been found to dominate the spoilage association (Baruzzi et al., 2012). Pyrosequencing of rRNA genes is being increasingly used in the study of microbial communities in cheese (Ercolini et al., 2012; De Filippis et al., 2014; De Pasquale et al., 2014a, 2014b; Schornsteiner et al., 2014; Dolci et al., 2014; Delcenserie et al., 2014; Riquelme et al., 2015). Its unprecedented depth of analysis compared to other molecular methods (Ercolini, 2013) is extremely appealing and its use is significantly improving the understanding of the role of microorganisms in cheese. The objective of this work was to analyze the composition of the microbiota of commercial samples of high-moisture Mozzarella cheese after refrigerated storage in order to evaluate the diversity of starter and spoilage organisms in cheeses produced with different acidification methods. Moreover, analyses on replicate samples for the same lot and on different lots for three different products were used to evaluate the repeatability and reproducibility of the analysis and of the effect of dairy and lot on the occurrence of starter and spoilage organisms.

2.2.2 Materials and methods

2.2.2.1 Sampling

Samples (20 samples, named from A to N) of high moisture Mozzarella cheese belonging to 14 different commercial brands were purchased in local supermarkets over one month and were stored for 5 days at 10°C before analysis. The shelf-life duration indicated on the packages varied between 5 and 20 days, and 5 days was the most frequent consume by date. Of the 14 brands, five were produced by industrial cheese plants and nine by artisanal cheese plants. For three brands (one industrial, two artisanal) cheeses were purchased in three different days during the sampling period. The cheeses were produced using different acidification systems: five brands declared the use of citric acid, four the use of starter cultures, while for the remaining brands no indication was provided on the labels. All samples were produced from cow's milk.

2.2.2.2 DNA extraction

Two individual cheeses (125 g) obtained from two packages were used for each sample. For the three brands for which three replicate samplings were carried out, cheeses from two packages were used

separately for DNA extraction, while for all the other cheeses the two cheeses were pooled before extraction. Cheese samples (10-20 g) were aseptically homogenized (1:3) in 2% sterile sodium citrate using a Stomacher 400 Lab Blender (International PBI, Milan, Italy), 40°C. The suspension was centrifuged (13,000 x g, 3 min) and the pellet was washed in the same solution to remove precipitated casein. MoBio PowerFood Bacterial DNA Extraction kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) was used as described in the manufacturer's instructions and a FastPrep®-24 Instrument (MP BIOMEDICALS) was used for the lysis step (speed 4, 3x20 sec). The purified DNA was stored at -80°C until used.

2.2.2.3 16S rRNA gene amplicon library preparation and pyrosequencing

The microbial diversity was studied by pyrosequencing of the amplified V1-V3 region of the 16S rRNA gene by using primers Gray27F 5'-TTTGATCNTGGCTCAG and Gray519r 5'-GTNTTACNGCGGCKGCTG amplifying a fragment of 520 bp (Andreotti et al., 2011). 454-adaptors were included in the forward primer followed by a 10 bp sample-specific Multiplex Identifier (MID). Each PCR mixture (final volume, 50 μl) contained 50 ng of template DNA, 0.4 μM of each primer, 0.50 mmol l^{-1} of each deoxynucleoside triphosphate, 2.5 mmol l^{-1} $MgCl_2$, 5 μl of 10 X 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 first purified by QIAquick gel extraction kit (Qiagen, Milano, Italy) and then by Agencourt AMPure kit (Beckman Coulter, Milano, Italy) prior to further processing. The amplicons were used as a template for pyrosequencing on a GS Junior platform (454 Life Sciences, Roche Diagnostics, Italy) according to the manufacturer's instructions by using a Titanium chemistry. All the sequencing data were deposited at the Sequence Read Archive of the National Center for Biotechnology Information (SRP052240).

2.2.2.4 Bioinformatics and data analysis

Raw reads were first filtered according to the 454 processing pipeline. The sequences were then demultiplexed and further filtered using QIIME 1.8.0 software (Caporaso et al., 2010). In order to guarantee a higher level of accuracy in terms of OTU detection, after the split library script performed by QIIME, the reads were excluded from the analysis if they had an average quality score lower than 25, if they were shorter than 200 bp and if there were ambiguous base calls. Sequences that passed the quality filter were denoised (Reeder & Knight, 2010) and singletons were excluded. Operational Taxonomic Units (OTUs) defined by a 97% of similarity were picked using the uclust method (Edgar, 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 using the Greengenes 16S rRNA gene database (McDonald et al., 2012). Alpha and beta diversity were carried out in QIIME, as reported elsewhere (De Filippis et al., 2013). Pseudo-heat maps were generated using Permutmatrix 1.9.3 (Caraux & Pinloche, 2005) on the matrices of log-transformed OTU frequencies. Dissimilarities were calculated as Euclidean distance, seriation was performed using multiple fragment heuristic, and clustering using Weighted Pair-Group Method with Averages (WPGMA). OTU networks were generated using Gephi 0.8.2-beta (https://gephi.github.io/). Further statistical analysis (nonparametric tests, mixed linear models) and graphics were performed using Systat 13 (Systat Software Inc., San Jose, CA, USA).

2.2.3 Results

2.2.3.1 The microbiota in high-moisture Mozzarella cheese

A total of 169,637 raw reads were obtained after the 454 processing; 133,331 reads passed the filters applied through QIIME, with an average value of 4,597 reads/sample and an average length of 504 bp. From 20 to 145 OTUs were found for each sample. Shannon and Simpson diversity indices ranged from 1.2 to 4.7 and from 0.09 to 0.91, respectively. Chao1 ranged from 27.9 to 161. Coverage was always >0.99 and therefore satisfactory for all samples. Diversity was significantly higher ($p<0.001$, Kolmogorov-Smirnov two-sample test) for samples produced by artisanal cheesemaking plants. No significant effect of the acidification mode was found. A total of 166 OTUs were identified by pyrosequencing. Identification at the species (41.8%) or genus (46.8%) was possible for the majority of OTUs while the others were identified at the family level (8.9% or above). The distribution of major OTUs (OTUs appearing with an frequency of at least 1% in at least one sample) is shown as a pseudo-heat map in **Figure 2.2.1**. Since the number of sequences per sample ranged from 2,502 to 8,580, 0.01%

was used as a dummy value for OTUs whose frequency was 0 for a given sample to allow the calculation of the logarithm of frequencies. Cheeses clustered in different groups that included samples produced with similar acidification systems and, cheeses obtained from the same cheese plant (same initial letter, i.e. A, C and F) and from the same lot (same initial letter and number, i.e. A1A and A1B) clustered together. Cheeses for which the addition of starter was declared on the label were close together and were always dominated by *S. thermophilus*. Many other lactic acid bacteria belonging to the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Carnobacterium*, *Streptococcus*, *Weissella* and enterococci were generally found at low frequencies (median value 0.01-3%). In order to evaluate if any effect of the mode of acidification or of the producing plant on the spoilage microbiota was detectable, the % of the OTUs associated with spoilage was recalculated after subtracting starter species, other lactic acid bacteria and other Firmicutes which are associated with animal niches. The results are shown in **Figure 2.2.2**. Four groups of OTUs (*Acinetobacter*, *Enterobacteriaceae*, *Pseudomonas* and, to a lesser degree *Raoultella*) have relatively high frequencies in most samples, while two (*Moraxellaceae* and *Brochothrix*) are associated to a more limited number of samples. No clear association with the producer or with the mode of acidification is evident. The distribution of different groups of OTUs (starter, other lactic acid bacteria, *Pseudomonas* spp., other psychrotrophs) in the three groups of Mozzarella cheeses is shown as box plots in **Figure 2.2.3**. Although cheeses produced by direct acid addition had the lowest proportion of starter microorganisms, these were still between 6 and 40% of the microbiota and, together with other lactic acid bacteria they were a dominant part of the microbiota. Pshychrotrophic bacteria and *Pseudomonas* had the highest median frequency in these samples. Cheeses produced by addition of defined starter cultures had the lowest contamination with psychrotrophs and contaminants and the lowest diversity in the composition of LAB, with *S. thermophilus* always as the dominant microorganisms. The third group of samples, for which no indication of the mode of acidification was provided on the label, were all obtained from artisanal cheese plants and had the highest variability for all groups, although the presence of psychrotropic spoilage microorganisms was not necessarily higher than that of cheeses produced in industrial plants. As a further tool for representing the microbiota of high-moisture Mozzarella samples, an OTU network was built using GEPHI (**Figure 2.2.4**). The network shows only OTUs for which the sum of frequencies in different samples was ≥5%. Nodes for weakly connected OTUs (which occur at low frequencies) are pushed on the periphery of the graphs while nodes for OTUs which are shared by many samples tend to be at the center of groups of samples in which they appear. Several groups of samples are evident. One group, including all samples produced by addition of starter cultures, most of which are from industrial plants, and samples from artisanal plant C, for which an artisanal undefined starter was probably used, are close to *S. thermophilus* and *Lb. delbrueckii* nodes. A second group including all samples from artisanal cheese plant F, which again were likely produced with a natural starter dominated by *Lb. helveticus*, cluster around the node for this species and, because they have a very high diversity in lactic acid bacteria, close to the nodes for *Leuconostoc*, *Enterococcus* and, to a lesser extent *Lactococcus*. The other samples, most of which were produced by direct acid addition, are scattered around the center of the graph (these reflects the occurrence of a diverse microbiota), while sample G is clearly dominated by *Pseudomonas* and it is positioned accordingly. As to OTUs, the size of their nodes clearly reflects their importance in the whole data set, allowing to quickly visualize important species and genera.

2.2.3.2 Repeatability and reproducibility

A secondary objective of this work was to evaluate the repeatability of the combined procedure of DNA extraction, amplification and sequencing and the lot-to-lot variability. Therefore, for three brands (one produced by an industrial plant using a defined starter, A; and two artisanal probably produced using an undefined artisanal starter, C and F), three lots of cheese were purchased and for each two different cheeses from two different packages were used for independent extractions of DNA. As shown in **Figure 2.2.1**, **Figure 2.2.2**, **Figure 2.2.4**, the cheeses produced from each given plant have a similar community structure. However, neither cluster analysis nor ordination methods allow to make inferences on the effect of brand, lot or technical factors affecting extraction amplification and sequencing. The correlation between log transformed OTU% between technical replicates was high ($r=0.925$, $p<0.00001$). Correlation between OTU frequencies was highest for frequencies ≥1% ($r=0.991$) but decreased greatly for OTUs appearing at frequencies between 1 and 0.1% ($r=0.588$, $p<0.0001$) and was not significantly different from 0 for rare OTUs (<0.1%). In order to evaluate the effect of the different factors on the occurrence of different OTUs, a linear mixed model with cheesemaking plant (Dairy) as a fixed factor and replicate lot (sample, within dairy) as a random factor was tested on square root transformed OTU percentages and variance components were estimated sequentially as Type I Sum of Squares (SS). Variance proportions for each factor (the error included all unattributed factors, including technical variability) were calculated and the significance of fixed and random factors was assesses for all OTUs which were present at >1% in

any of the samples. The results are presented in **Figure 2.2.5**. Only for 9 OTUs, three of which were starter organisms (*S. thermophilus*, *L. delbrueckii* and *L. helveticus*) and six other lactic acid bacteria, the effect of the cheese making plant was significant. For some of these the effect of the replicate lot was also significant, while for all spoilage organisms for which a significant difference was found, this was due to the effect of replicate lots rather than to the cheesemaking plant. On the other hand, no significant effect of dairy or lot was found for *Pseudomonas* sp., which was present at high average frequencies (3.97%).

2.2.4 Discussion

In this study, an in-depth analysis of the microbiota involved in the spoilage of high-moisture mozzarella cheese was firstly provided. Samples of mozzarella cheese produced by different acidification modes and by artisanal/industrial dairies were collected. The ESC shows a generally high coverage of the microbial diversity for all the samples (> 0.99%) Nevertheless, an effect of the sequencing depth was detected. In fact, the coverage clearly improved with the number of valid sequences (ranging from 2,502 to 8,580) and this was evident for the technical replicates (duplicate samples obtained from the same lot) such as samples A2A and A2B. Direct comparisons with published work is difficult. Only a few studies based on pyrosequencing of Mozzarella cheese are available. The diversity in high-moisture Mozzarella microbiota was lower than that reported for two samples of fresh water-buffalo Mozzarella cheese immediately after production (Ercolini et al., 2012) and was of the same order of magnitude of that reported for water-buffalo Mozzarella cheese curds (De Filippis et al., 2014). In both cases, the cheese had been produced with natural whey starters. On the other hand a much lower diversity was found in Caciocavallo Pugliese (a semi hard pasta filata cheese) curd and cheese at 1-7 d of ripening in a study based on cDNA pyrosequencing (De Pasquale et al., 2014a), with Chao1 richness between 18 and 22: although the cheese was produced using raw milk, a defined starter culture was used at high concentration (10^7 CFU/ml). Nevertheless, in our study no attempt was made to target the active microbial community and, due to the lethal effect of stretching of the curd in hot water (De Angelis & Gobbetti, 2011; Minervini et al., 2012), the frequency of viable OTUs from the starter might be overestimated. The abundance of major OTUs is shown in **Figure 2.2.1**. With the exception of cheeses produced by direct addition of citric acid, the microbiota was dominated by species commonly used as starters (*S. thermophilus*, *Lb. delbrueckii*, *Lb. helveticus*) in mozzarella and other pasta filata cheeses (Parente & Cogan, 2004; De Angelis & Gobbetti, 2011; Ercolini et al., 2012). In fact, a clustering of samples according to the acidification systems was observed. Moreover, cheeses obtained from the same dairy (same initial letter, i.e. A, C and F) and from the same lot (same initial letter and number, i.e. A1A and A1B) clustered together. Cheeses for which the addition of starter was declared on the label were close together and were always dominated by *S. thermophilus*. Even for the samples from dairy C and D, for which no indication of the acidification mode was provided on the label, the use of starters, possibly natural starters (Parente, 2006), is likely. In fact, they show a microbiota composition similar to samples from dairy A, L, M, H, for which the use of starters is ascertained (**Figure 2.2.1**). In **Figure 2.2.4**, the lack of a core microbiota common to all samples is evident. Nodes for weakly connected OTUs (which occur at low frequencies) are pushed on the periphery of the graphs while nodes for OTUs which are shared by many samples tend to be at the center of groups of samples in which they appear. Several groups of samples are evident. In particular, samples produced by addition of starter clustered clearly together and *S. thermophilus* and *Lb. delbrueckii* were the most abundant OTUs in samples from dairies C, I and N, for which the acidification mode was not declared, but where a starter was likely used. On the contrary, a higher diversity was detected in samples where citric acid was used. *S. thermophilus* is invariably the dominating species in both natural starters, curd and cheese of traditional Mozzarella cheese as shown by PCR-DGGE (Coppola et al., 2001; Ercolini et al., 2001), and pyrosequencing (Ercolini et al., 2012; De Filippis et al., 2014). However, both *Lb. helveticus* and *Lb. delbrueckii* have frequently been found as subdominant species (Coppola et al., 2001; Ercolini et a., 2001; De Candia et al., 2007; De Angelis et al., 2008; Ercolini et al., 2012; De Filippis et al., 2014). In fact, *Lb. helveticus* and *Lb. delbrueckii* were found in all samples produced by artisanal cheese making plants and, in one case (dairy F) *Lb. helveticus* was the dominating species in the cheese. *Lactococcus* sp., *Lc. lactis*, *Lc. garviae* and *Lc. raffinolactis* were also found, with higher abundance in artisanal cheeses. *Lactococcus lactis* is a mesophilic starter culture which has frequently been found as a dominating or minor member of traditional starter cultures and Mozzarella cheese (Coppola et al., 2001; Ercolini et al., 2001; De Candia et al., 2007; Ercolini et al., 2012; De Filippis et al., 2014); the significance of this species as a subdominant member of the starter microbiota is unclear, because it is unlikely that it would grow significantly at the temperatures most frequently used for Mozzarella cheese manufacture (39-42°C). Its presence may indicate the use of lower temperatures or poor temperature control during cheese making or curd ripening. Many other lactic acid bacteria belonging to the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Carnobacterium*, *Streptococcus*, *Weissella* and enterococci were generally found at low frequencies (median value 0.01-3%) but were

sometimes quite abundant (see for example members of the genera *Enterococcus*, *Leuconostoc* and *Lactococcus* which were not identified at the species level). While the source of many of these species may be the raw milk (Quigley et al., 2013) or the cheesemaking environment, their role is uncertain and members of the genus *Lactobacillus*, *Carnobacterium* and *Leuconostoc* have been involved in the spoilage of fresh products (Remenant et al., 2015). This may be especially true for cheese produced by direct acid addition (G, B, E, K, I, J) in which several starter and non-starter LAB occurred at relatively high frequencies. A variety of psychrotrophic Bacteroidetes (*Flavobacteriaceae* such as *Chryseobacterium*, *Flavobacterium*, *Haloanella*), Proteobacteria (*Aeromonadaceae* such as *Aeromonas*, *Enterobacteriaceae* including *Pantoea*, *Raoultella*, *Serratia*; *Moraxellaceae* including *Acinetobacter*; *Pseudomonaceae*; *Shewanellaceae*) and Firmicutes (*Listeriaceae* such as *Brochothrix*), which have been associated to the spoilage of fresh products and with environmental sources, including water (Remenant et al., 2015) and with raw milk (Quigley et al., 2013), were found in all samples, although their abundance was higher in cheeses produced by direct acid addition. With a few exceptions (*Pseudomonas*, *Enterobacteriaceae*) their frequency was low. High-moisture Mozzarella is a rather non-selective substrate for microbial growth: the pH is relatively high (in the cheeses used in this study pH ranged between 4.8 and 6.4, with most samples >5.6, data not shown) and the water activity is not limiting (the moisture of the cheese is 50-60% and the cheese is stored in liquid, Lucera et al., 2014), therefore the growth of psychrotrophs is possible and these species have been associated with the spoilage of Mozzarella (Baruzzi et al., 2012; Nogarol et al., 2013). Genera including animal pathogens such as *Arcanobacterium* (Machado et al., 2012), *Arcobacter* (Yesilmen et al., 2014), or *Fusobacterium* (Tadepalli et al., 2009) or human and animal pathogens such as *Staphylococcus aures* were found at very low frequencies in some samples, (0.04-0.09%) and their origin is most likely the raw milk. In **Figure 2.2.2**, only OTUs associated to spoilage is shown. No clear association with the producer or with the mode of acidification is evident. Although the data may be affected by the relatively low proportions of spoilage organisms in most samples, this may suggest that the contamination with these species is more affected by random factors and/or that a clear spoilage association has not developed yet, since all samples were analyzed at a fixed time rather than at the onset of spoilage. Moreover, the mixed linear model (**Figure 2.2.5**) shows that an higher proportion of the total variance is explained by the replicate lot, rather than the dairy, when considering the abundance of spoilage microorganisms. In this work, we also dealed with the issue of reproducibility and quantitative nature of pyrosequencing data. An early work on soil microbial communities (Zhou et al., 2011) claims that amplicon based pyrosequencing is not quantitative and has poor technical reproducibility. Several strategies were proposed to improve the value of comparisons: removing singletons and rare OTUs, adding sequences from different technical replicates, increasing biological replicates, using sequences from both forward and reverse primers. Pinto et al. (2012) using mock archeal and bacterial communities found that PCR biases affect community structure; deeper sequencing did necessarily alleviate this but reduced variability among replicates. Different measures of a and b diversity were affected in different ways by biases and estimates for low frequency OTUs had the highest variability. In a recent study on sausage spoilage (Benson et al., 2014) a correlation coefficient of 0.805 was found among technical replicates using the 20 most abundant OTUs; this includes all steps extraction amplification and sequencing; although the authors claimed that most dispersion is found for relative abundances <0.001, significant dispersion is evident even for taxa appearing at higher relative abundances. On the other hand in a study on polluted aquifer communities (Pilloni et al., 2012) highly reproducible abundance estimates were obtained for dominating taxa and no significant bias for low abundance taxa. Recently, in a study on the spoilage microbiota of seafood and meat samples (Chaillou et al., 2014), lack of significant PCR and of pyrosequencing biases was demonstrated. Correlation between sequencing replicates was higher (r=0.95 for replicates in different runs; 0.96-0.97 between technical replicates obtained using forward and reverse primers in the same sequencing run) compared to our results. Poor correlation was found for low frequency OTUs. However, taxonomic resolution was at the genus level (we used the lowest order taxa available) and effects related to extraction or amplification were not included in the analysis. Overall, our study confirms that, although estimates of OTU frequencies for dominant and subdominant species may be reliable, the abundance figures for rare OTUs should be taken with caution and used carefully in making inferences on community structure. The need for biological and technical replicates would mainly depend on the purpose of the study and, whenever possible biological replicates should be used to assess the significance of differences among treatments.

In conclusion, the microbiota of high-moisture Mozzarella cheese at the end of storage was affected mainly by the mode of acidification. The use of defined starters (as declared on the label) or of undefined starters (as inferred from the composition of the microbiota) shaped the dominant microbiota and clearly separated cheeses produced by different dairies. The occurrence of psychrotrophic spoilage microorganisms was more related to random contamination: these species dominated, together with mesophilic lactic acid bacteria, the microbiota of cheeses produced by direct addition of citric acid but

their pattern of occurrence seemed to be more related to lot to lot variability rather than to plant or product-specific spoilage associations. While reproducibility was satisfactory for OTUs appearing at >1%, it was poor for rare OTUs. However, the use of biological and, to a lesser extent, technical replicates allowed to make inferences on the effect of dairy and lot on OTU frequencies.

2.2.5 References

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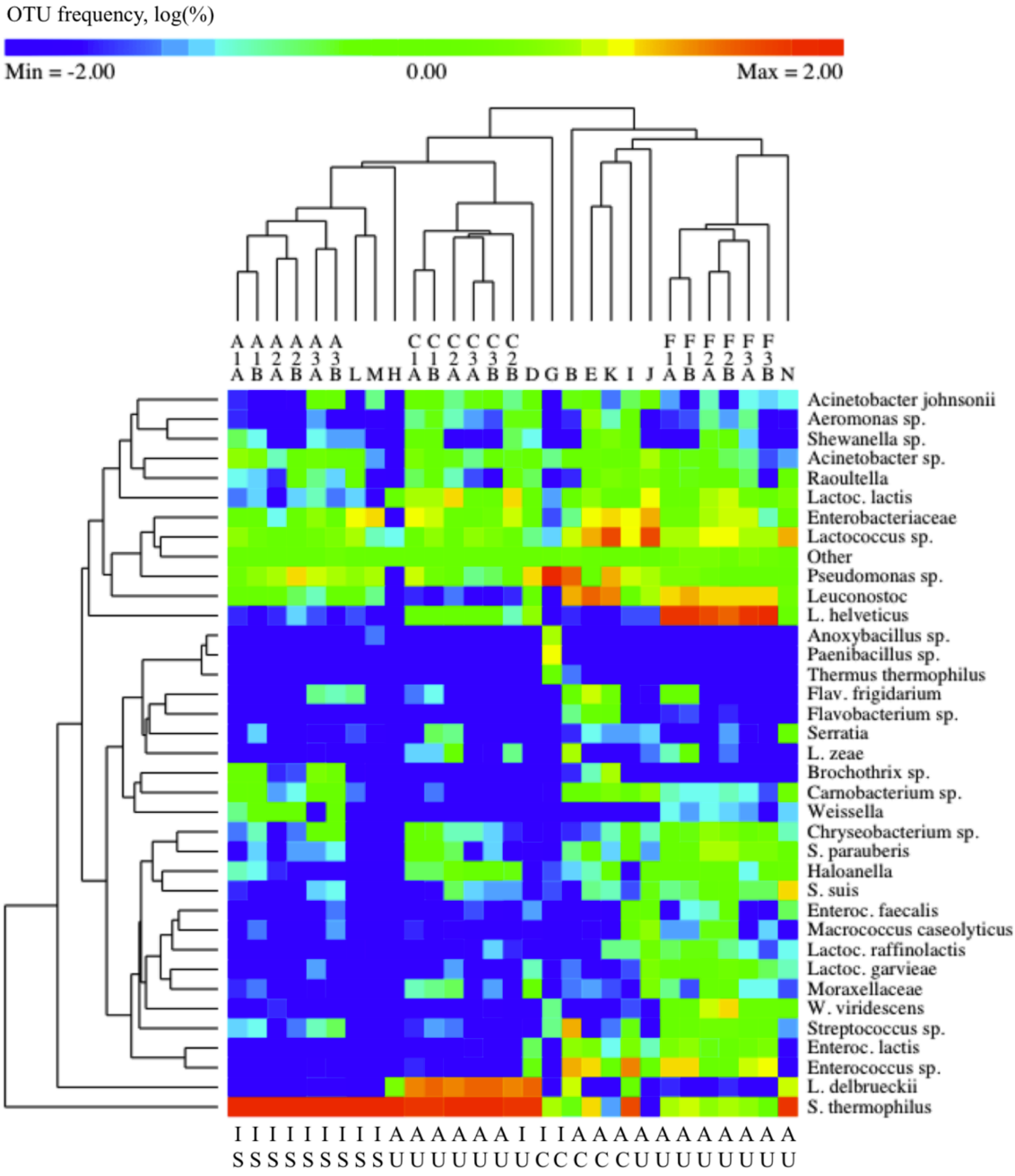

Figure 2.2.1 Pseudo-heat map of the major OTUs (occurring at >1% in at least one sample) retrieved by 16S pyrosequencing of samples of high-moisture Mozzarella cheese. Frequencies were calculated as log(%) to improve the visibility of rare OTUs. The label of the samples is nindicated on top. Samples beginning with the same letter were produced by the same cheesemaking plant. The type of cheese plant (industrial, I, versus artisanal, A) and the mode of acidification (direct addition of citric acid, C; addition of starter, S; unknown, U) are indicated at the bottom of the heat map.

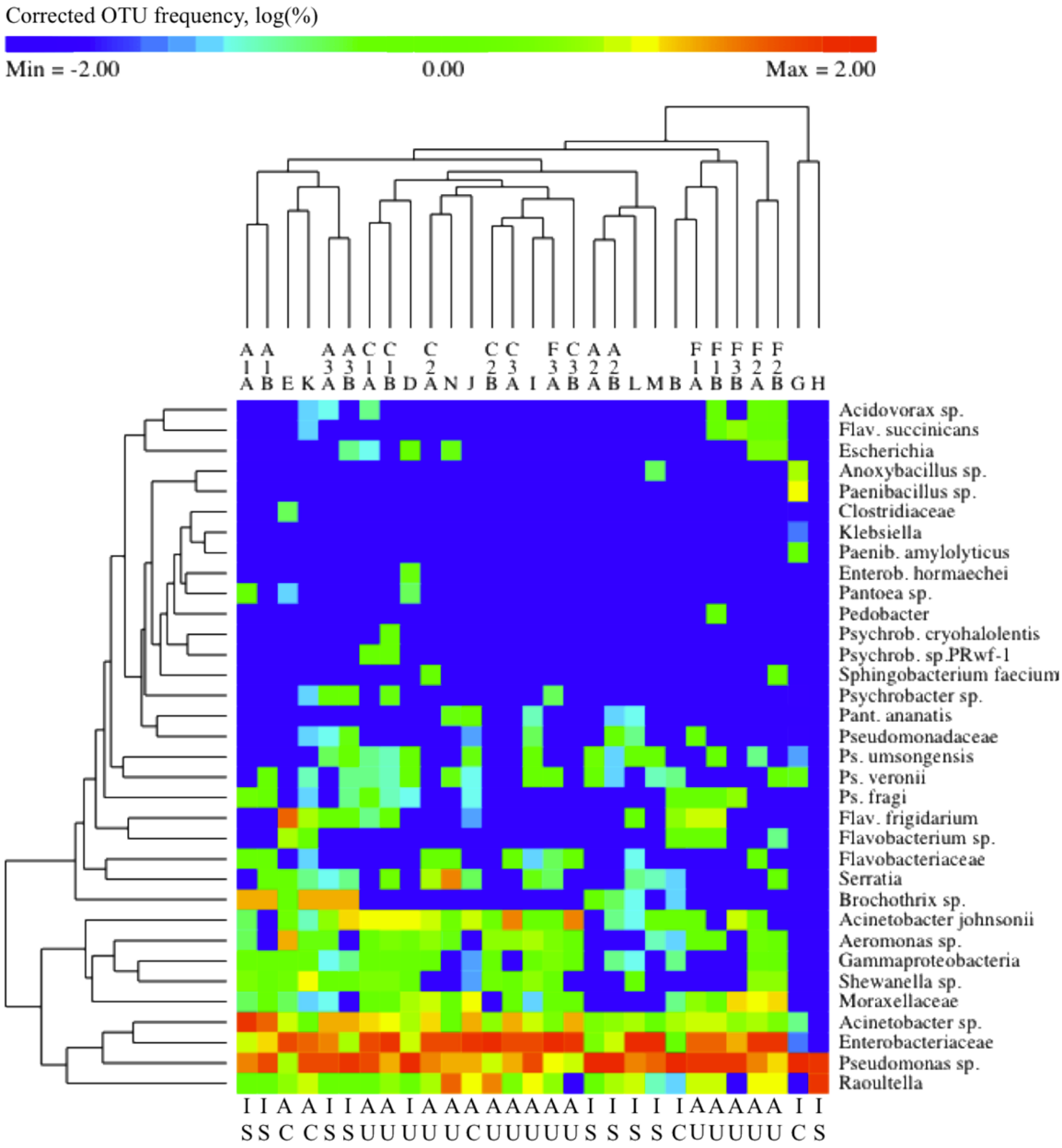

Figure 2.2.2 Pseudo-heat map of the spoilage microorganisms retrieved by 16S pyrosequencing of samples of high-moisture Mozzarella cheese. Frequencies were recalculated by substracting the frequencies of starter organisms and log-transformed before analysis to give more emphasis to rare OTUs. The label of the samples is indicated on top. Samples beginning with the same letter were produced by the same cheesemaking plant. The type of cheese plant (industrial, I, versus artisanal, A) and the mode of acidification (direct addition of citric acid, C; addition of starter, S; unknown, U) are indicated at the bottom of the heat map.

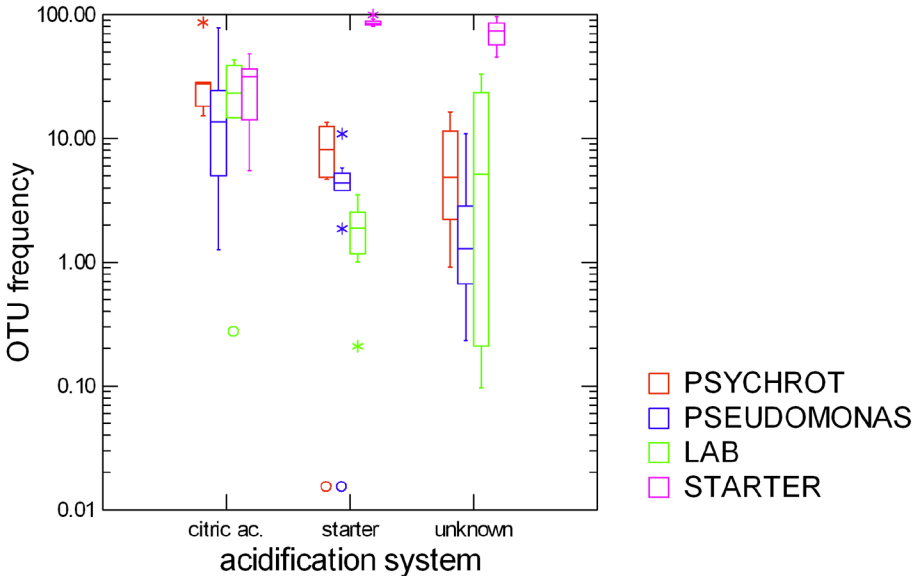

Figure 2.2.3 Box plots showing the distribution of different functional groups of OTUs in high-moisture Mozzarella cheese produced with three different acidification systems.

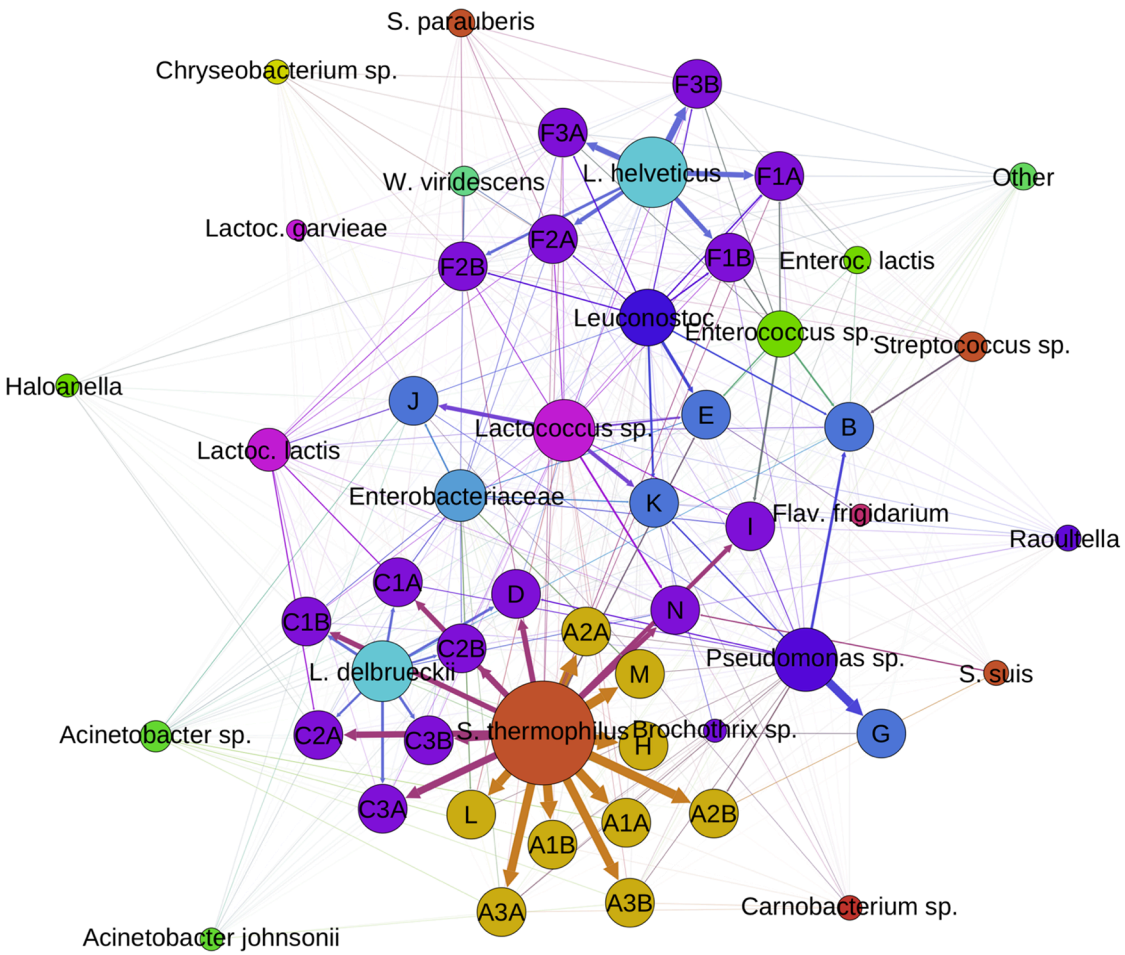

Figure 2.2.4 OTU network summarizing the relationships between taxa and samples of high-moisture Mozzarella cheese. The size of the nodes is made proportional to weighted degree (i.e. for OTUs this measures the total occurrence of an OTU in the dataset) using a power spline, while the size of the edges is made proportional to the weighted degree (thus reflecting the occurrence of any given species in a given sample). The colour of the nodes is on the basis of the mode of acidification system (samples) or genus (OTUs).

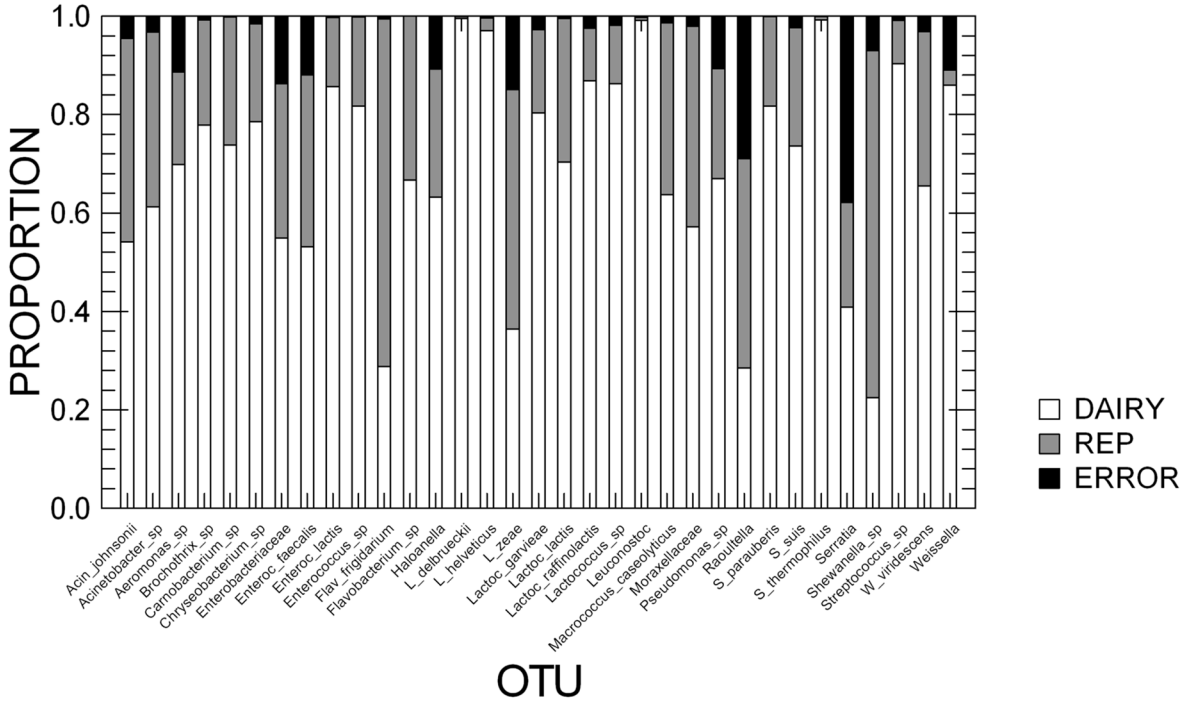

Figure 2.2.5 Variance components for OTUs found by pyrosequencing in high-moisture Mozzarella cheese produced by three different cheese-making plants from which lots of cheese were obtained in three different sampling. Each sample was analyzed in duplicate. Estimates for variance components were transformed in proportions of total variance and the significance of different factors is shown on the bottom of the bar plot (white ns, black $p<0.01$, grey 0.05-0.01).

2.3 A selected core microbiome drives the early stages of curd fermentation in cheese making

2.3.1 Introduction

Mozzarella (M), Grana Padano (GP) and Parmigiano Reggiano (PR) are three of the most important traditional Italian cheeses. They are all protected designation of origin (PDO) cheeses and the technology of manufacture, as well as the microbiota involved, have been described in previous works (Coppola et al., 2000; Ercolini et al., 2004; Ercolini et al., 2012; Neviani et al., 2013; Rossetti et al., 2008; Santarelli et al., 2008). Mozzarella is a "pasta filata" cheese traditionally produced in Southern Italy. The cheese is made from whole raw water buffalo's milk by adding natural whey culture (NWC) as starter in a 5-h curd fermentation. PR and GP are hard, cooked cheeses made from raw, partly skimmed cow's milk supplemented with NWC. Although completely different production technologies are employed, all these cheeses share the use of the NWC from the production of the previous day as starter for the curd acidification, according to the traditional back-slopping procedure. The microbiota of the natural starters has been characterized using both traditional and molecular procedures and defined as a consortium of micro-organisms of great importance for the quality of the traditional products. The concomitant pressure of both temperature and low pH leads to the selection of a characteristic microbiota, consisting of thermophilic, aciduric, and moderately heat resistant lactic acid bacteria (LAB), that play an important role in the achievement of the typical and appreciated sensory characteristics of cheese (Mauriello et al., 2003). NWCs are generally characterized by a LAB community including both thermophilic and mesophilic bacteria (Coppola et al., 2000; Ercolini et al., 2001; Ercolini et al., 2004; Ercolini et al., 2012; Bottari et al., 2010; Lazzi et al., 2004).

In this study, we used culture-independent high-throughput sequencing (HTS) of 16S rRNA gene amplicons to study in depth the microbial diversity of NWCs from three Italian traditional cheeses and its evolution during curd fermentation.

2.3.2 Materials and methods

2.3.2.1 Sampling

Samples from M cheese manufactures were collected from twelve dairies producing top-quality traditional water buffalo mozzarella PDO cheese, located in the Campania region (Southern Italy) in the provinces of Salerno and Caserta. Samples from GP and PR manufactures were collected from six and seven dairies located in different places within the GP and PR area of production (Northern Italy). Samples of NWCs and curds at end of the ripening were aseptically collected, cooled at 4°C, and analyzed within 6 h. NWC samples were from the manufacture of the previous day and used for the production of the corresponding curds according to the traditional back-slopping procedure. Curd samples were collected after 5 h from the adding of the NWC for M cheese and after 24 h for GP and PR cheese.

2.3.2.2 DNA extraction

Total DNA extraction from the dairy samples was carried out by using the 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 NWC or from 2 ml of a homogenized 2-fold dilution of the curd in one-quarter-strength Ringer's solution (Oxoid, Milano, Italy).

2.3.2.3 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 by using primers Gray27f 5'-TTTGATCNTGGCTCAG and Gray519r 5'-GTNTTACNGCGGCKGCTG amplifying a fragment of 520 bp (Andreotti et al., 2011). 454-adaptors were included in the forward primer followed by a 10 bp samplespecific Multiplex Identifier (MID). Each PCR mixture (final volume, 50 µL) contained 50 ng of template cDNA, 0.4 µM of each primer, 0.50 mmol/L of each deoxynucleoside triphosphate, 2.5 mmol/L MgCl 2 , 5 µL 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 QuantiFluor™ (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, Monza, Italy) according to the manufacturer's instructions by using a Titanium chemistry.

2.3.2.4 Bioinformatics and data analysis

Raw reads were first filtered according to the 454 amplicon processing pipeline. Sequences were then analyzed by using QIIME 1.6.0 software (Caporaso et al., 2010). Raw reads were demultiplexed and further filtered through the split_library.py script of QIIME. In order to guarantee a higher level of accuracy, 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. Sequences that passed the quality filter were denoised (Reeder & Knight, 2010) and singletons were excluded. OTUs defined by a 97% of similarity were picked using the uclust method (Edgar, 2010) and the representative sequences, chosen as the most abundant in each cluster, 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). Alpha and beta diversity were evaluated through QIIME as recently described (De Filippis et al., 2013). The OTU table filtered at 0.1% abundance was used to generate an OTU network by QIIME and a bipartite graph was constructed in which each node represented either a sample or a bacterial OTU. Connections were drawn between samples and OTUs, with edge weights defined as the number of sequences from each OTU that occurred in each sample. Network was visualized using Cytoscape 2.5.2 (Shannon et al., 2003). Moreover, OTUs tables generated through QIIME were used to draw a pseudo-heatmap in R environment (http://www.r-project.org) using gplots package. Representative sequences belonging to clusters identified as *Lactobacillus* spp. were double-checked using the BLAST (BLASTN) search program (http://www.ncbi.nlm.nih.gov/blast/). Weighted UniFrac distance matrix were used to perform Adonis and Anosim statistical tests through the compare_category.py script of QIIME, in order to verify if there were differences among the three types of cheese. Moreover, the otu_category_significance.py script was run in order to test whether the presence/abundance of any OTUs was significantly associated to a specific cheese.

2.3.3 Results

A total of 296,385 raw reads were obtained after the 454 processing; 221,903 reads passed the filters applied through QIIME, with an average value of 4,191 reads/sample and an average length of 469 bp. The number of reads obtained for each sample, the number of OTUs, the Good's estimated sample coverage (ESC), the Chao1 and the Shannon indices are reported in **Table 2.3.1**. Rarefaction analysis showed that there was a satisfactory coverage for all the samples (ESC above 99% for all the samples).

After QIIME analysis, 82 OTUs were identified, but only 6 had a relative abundance higher than 1% in at least two samples (**Figure 2.3.1**). *Lb. delbrueckii* and *Lb. helveticus* were the major OTUs in GP and PR samples (reaching a relative abundance of 59 and 93 % of the total OTUs, respectively). *S. thermophilus* was also always present, but its abundance was never above 24 %, with a higher abundance in GP samples. *Lb. fermentum* occurred only in some samples, but at very low percentage. On the contrary, samples from M manufactures were characterized by abundance of *S. thermophilus* (up to 70 % of the total OTUs), while *Lb. delbruekii* and *Lb. helveticus* were present at lower extent compared to GP and PR samples (**Figure 2.3.1**). *Lactococcus lactis* and *Lb. fermentum* were also among the most represented OTUs in M samples, with a maximum abundance of about 13 and 12 %, respectively (**Figure 2.3.1**). Many samples contained a low percentage of *Lactobacillus* sp. that was not possible to identify at species level. Representative sequences belonging to this cluster were double-checked using the BLAST (BLASTN) search program (http://www.ncbi.nlm.nih.gov/blast/). Although the identity scores were quite low, they were identified as *Lb. acidophilus* (87-95 %), *Lb. johnsonii* (90-93 %), *Lb. gasseri* (92-94 %), *Lb. crispatus* (86-95 %).

Moreover, sub-dominant populations were also identified and 25 OTUs occurred with an abundance higher than 0.01 % in at least 2 samples (**Figure 2.3.2**). M samples showed a higher complexity and many sub-dominant species reached abundandances higher than GP/PR samples. Many of them belonged to *Enterobacteriaceae* family or to the LAB group (*Lactococcus* sp. and *Leuconostoc* sp.). *Acinetobacter johnsonii* and *Acinetobacter* sp. reached 0.5% in some curd samples from all the three different manufactures. *Propionibacterium acnes* was found only in GP and PR samples (0.02-0.07%), while *S.*

suis was present only in M samples (0.02-0.2 %). *Bifidobacterium longum* occurred only in one PR NWC and in the relative curd.

The OTU network in **Figure 2.3.3** showed clearly that samples from M manufactures clustered separately from GP and PR samples. However, a core microbiota of few OTUs was shared among the samples (*S. thermophilus*, *Lb. delbrueckii*, *Lb. helveticus*, *Lb. fermentum*, *Lactobacillus* sp., and some sub-dominant OTUs previously discussed) while some M or GP/PR specific OTUs can be also identified. The same conclusion was drawn from β-diversity analysis (data not shown). The statistical Adonis and Anosim tests run by QIIME showed that the samples significantly differed ($P<0.001$) according to cheese type. Moreover, the ANOVA and g test run through the otu_category_significance.py script of QIIME showed that *S. thermophilus*, *Lc. lactis* and *Lb. helveticus* abundance was significantly different in the three cheeses ($P<0.001$). On the contrary, no significant difference was found between M samples from Salerno (MS) and Caserta (MC) area ($P>0.05$).

2.3.4 Discussion

In this study, the microbiota of NWC and curds from manufactures of three traditional Italian cheeses was analyzed. Culture independent HTS was used for an in depth quantitative determination of the structure of the microbial populations. NWCs are generally characterized by a relatively simple LAB microbiota. This LAB community is generally thermophilic and well adapted to the peculiar physico-chemical conditions (e.g. low pH and redox) of the whey substrate (Neviani et al., 1995; Giraffa et al., 1996). Therefore, methods allowing direct DNA analysis from these environments are valuable in order to avoid biases of the culture-dependent approach. 16S rRNA sequencing revealed a very simple microbial community in all the types of cheese. GP and PR cheeses were characterized by a very similar microbiota and curd fermentation seemed to be driven by *Lb. delbrueckii* and *Lb. helveticus*. Accordingly, *Lb. helveticus* and *Lb. delbruekii* were reported as dominant and *Lb. fermentum* and *S. thermophilus* as sub-dominant species in NWCs for GP manufactures (Lazzi et al., 2004). *S. thermophilus* was generally present at very low concentration in NWCs for PR, reaching the 12% only in one sample. Moreover, it did not increase in abundance during curd fermentations, suggesting a minor contribution to acidification. On the contrary, GP curd ripening conditions seemed to be more suitable to the development of this microorganism, since a higher amount of *S. thermophilus* was found in all the curds, compared to the corresponding NWCs. This was more likely related to the curd ripening conditions, rather than to the abundance of this OTU in the NWC. In fact, a higher abundance of *S. thermophilus* in the NWC, did not lead to a higher amount of this OTU in the relative curd (**Figure 2.3.1**). In agreement with our results, *S. thermophilus* was found more frequently in GP compared to PR NWCs (Gatti et al., 2014). Higher abundance of *S. thermophilus* in GP curds could be due to a lower cooking temperature (51-48 °C vs 53-54 °C). A thermal gradient that starts with a lower temperature can cause a reduction of the heat stress within the molded curd, favoring the presence of *S. thermophilus* (Gatti et al., 2014). For the same reason, the mesophilic *Lb. fermentum* was more often found in GP than PR. Accordingly, *Lb. fermentum* was even more abundant in M since no curd cooking is employed in mozzarella cheese production.

As shown in Figure 1, many samples contained a low percentage of *Lactobacillus* sp., that was not possible to identify at species level, and that possibly belonged to non-starter LAB (NSLAB) group. NSLAB are often isolated from whey cultures (Coppola et al., 2000; Neviani et al., 2009); they do not contribute to acid production during manufacture, but can play a significant role during ripening (Neviani et al., 2013; Gatti et al., 2014). Mozzarella whey starters were characterized by a higher abundance of *S. thermophilus*, that often increased in abundance during curd ripening, together with thermophilic lactobacilli. *Lc. lactis* and *Lb. fermentum* were present at lower concentration and not in all the samples. In particular, *Lc. lactis* was present only in M samples, even if not in all the manufactures (**Figure 2.3.1**) and its presence was significantly correlated to M samples, as confirmed by the g test ($P<0.001$). This microorganism was previously suggested to be related to the lower level of industrialization of the manufacture, correlating the occurrence of this species in traditional dairy products obtained from unselected microbiota and non-pasteurized milk (Coppola et al., 2001). However, another possible reason could be the lower level of heat stress occurring during M manufacturing compared to GP and PR. Even if raw milk used in these manufactures was not analysed, the same OTUs were found both in the NWC and in the relative curd, indicating that the fermentation is driven by the NWC and that the microorganisms present in the milk do not play a key role, as previously suggested (Ercolini et al., 2012). The OTU network in **Figure 2.3.3** clearly showed a separation between M and PR/GP samples. A few abundant OTUs constituted a shared core microbiota between the three cheeses. Although they are completely different cheeses, the fermentation process is most probably entirely relying on those common species. Also 25 sub-dominant OTUs were identified (**Figure 2.3.2**). M samples showed a higher complexity, likely due to a less industrialized manufacture or to the lower selective pressure. Many sub-

dominant OTUs are clearly environmental contaminants, like *Escherichia* sp., *Enterobacter cowanii* and other OTUs belonging to *Enterobacteriaceae* family. *Agrobacterium* sp. and *Alicyclobacillus* sp. probably arose from soil and agricultural environment (Groenwald et al., 2008; Pitzskchke, 2013). On the contrary, *Propionibacterium acnes*, found only in GP and PR samples, was likely of human origin (Brook & Frazier, 1991). *Pseudomonas fragi* was often associated to milk and dairy products (Ercolini et al., 2012; Quigley et al., 2013), where is able to produce volatile esters (Morales et al., 2005). *S. suis*, an emerging zoonotic pathogen that can be transmitted to human (Lun et al., 2007), was found only in M samples. It was previously found in Mozzarella (Ercolini et al., 2012), as well as in another pasta-filata cheese (Coppola et al., 2006). However, the distribution of these low-abundance OTUs was really variable among the samples, suggesting that their presence is associated to sporadical contaminations. As previously suggested (Quigley et al., 2012; Ercolini et al., 2013), a RNA-based approach would be useful to understand which of these OTUs are metabolically active.

This study provided an in-depth description of the microbiota involved in curd fermentation in three popular Italian cheese productions. The results showed a high degree of homogeneity in the microbiota involved in the early stages of the three dairy manufactures, highlighting a naturally-selected core microbiome that is fundamental for the fermentation in these dairy preparations.

2.3.5 References

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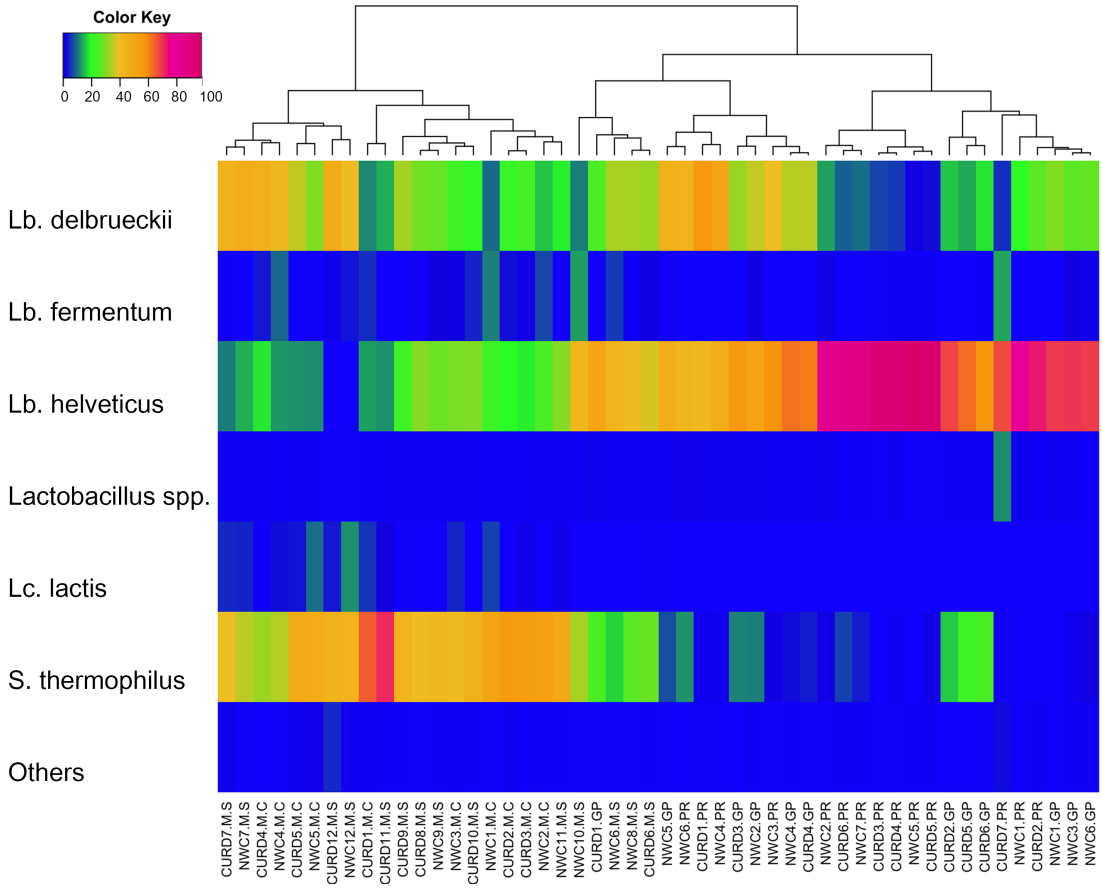

Figure 2.3.1 Pseudo-heatmap depicting distribution (%) of bacterial genera and species in NWC and curd samples from Grana Padano (GP), Parmigiano Reggiano (PR) and Mozzarella manufactures from Caserta (MC) and Salerno (MS) area of production. Only OTUs occurring at >1% abundance in at least 2 samples were included. Clustering of samples was obtained using Euclidean distance mesure and the average linkage method.

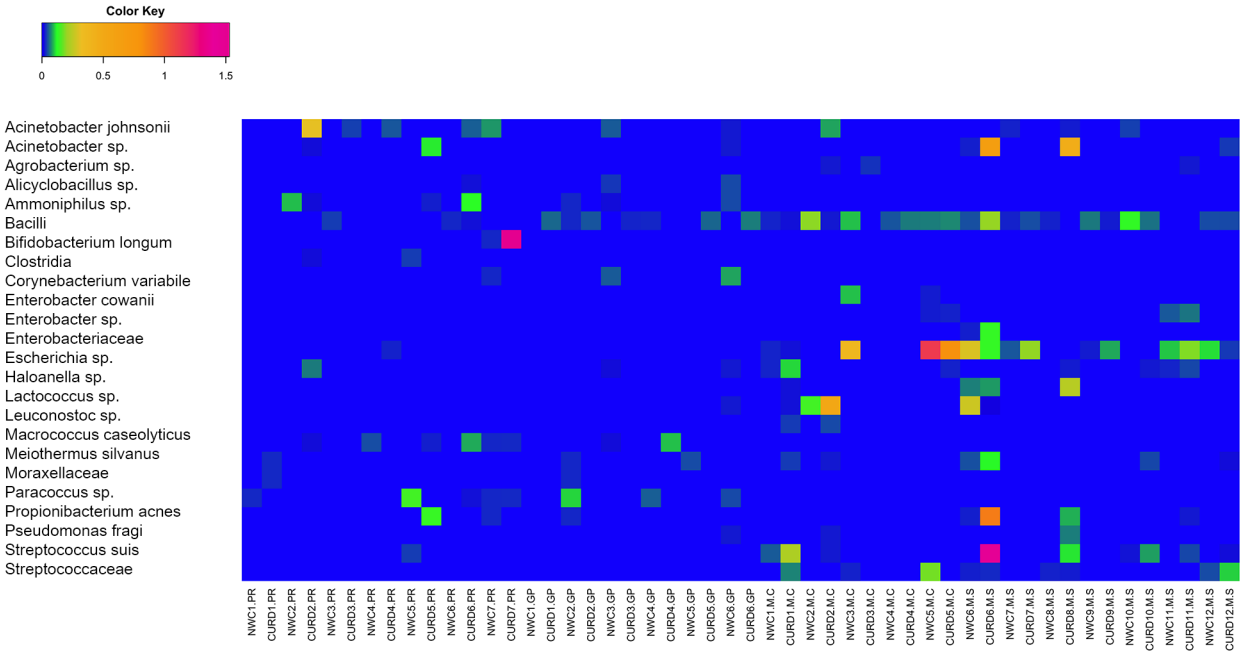

Figure 2.3.2 Pseudo-heatmap depicting distribution (%) of bacterial genera and species in NWC and curd samples from Grana Padano (GP), Parmigiano Reggiano (PR) and Mozzarella manufactures from Caserta (MC) and Salerno (MS) area of production. Only OTUs (except those reported in Fig. 2.3.1) occurring at >0.01% abundance in at least 2 samples were included.

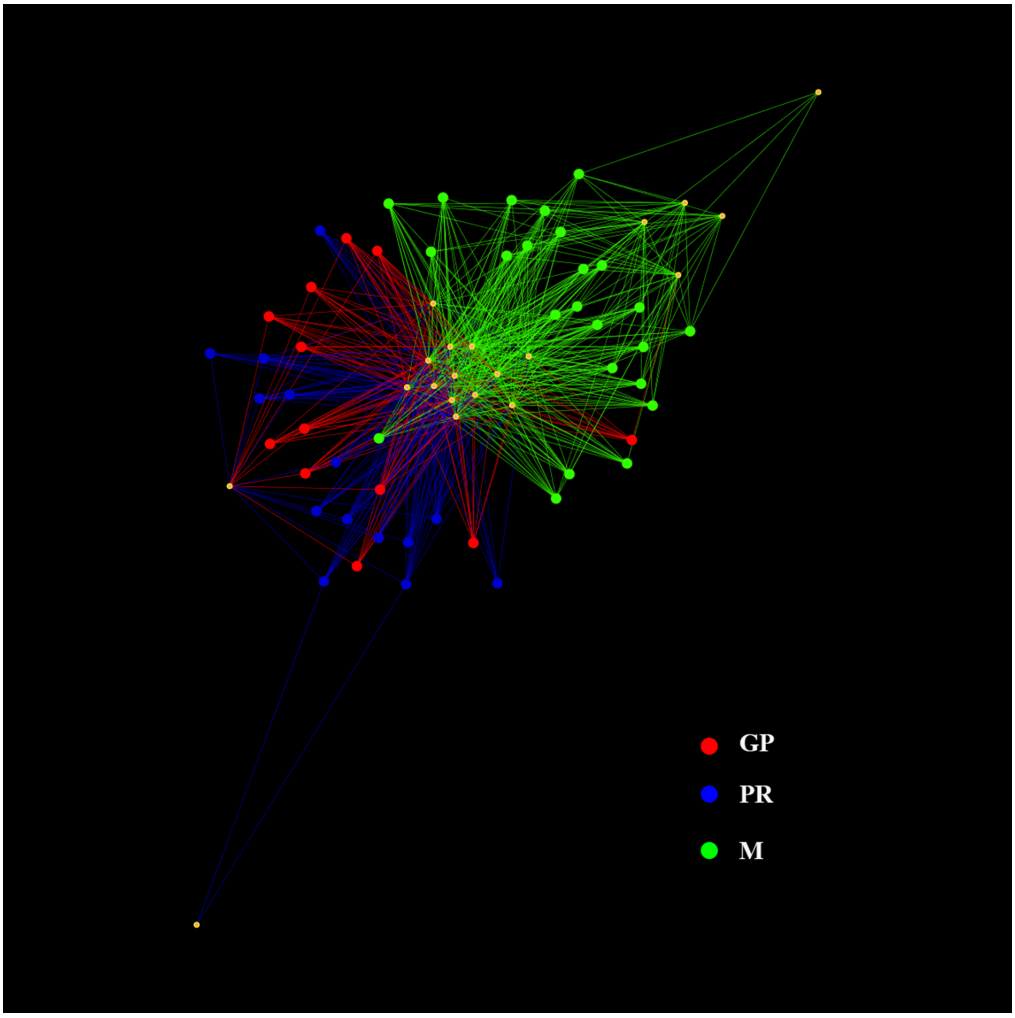

Figure 2.3.3 Simplified illustration of possible cheese - microbe networks. Network nodes are color coded by cheese type. Only OTUs with abundance > 0.1% were considered.

Table 2.3.1 Number of sequences analyzed, observed diversity and estimated sample coverage for 16S rRNA amplicons analyzed in this study.

Sample	Reads	OTUs	Chao1	Shannon	ESC (%)
NWC1-PR	3789	38	62.00	1.16	99.58
CURD1-PR	3747	48	69.00	1.53	99.44
NWC2-PR	5627	36	51.00	1.35	99.72
CURD2-PR	2726	19	26.50	0.47	99.63
NWC3-PR	3172	40	68.50	1.26	99.40
CURD3-PR	6132	40	66.25	1.04	99.66
NWC4-PR	4513	47	68.38	1.56	99.58
CURD4-PR	4197	49	91.17	0.80	99.45
NWC5-PR	3889	53	86.33	1.05	99.36
CURD5-PR	3750	73	124.67	2.12	99.17
NWC6-PR	3877	47	98.00	2.14	99.54
CURD6-PR	5766	63	92.55	1.21	99.55
NWC7-PR	2782	28	39.00	0.50	99.60
CURD7-PR	4444	45	73.50	0.69	99.57
NWC1-GP	2980	34	40.60	1.31	99.60
CURD1-GP	3587	43	63.00	2.17	99.55
NWC2-GP	3863	58	145.00	2.07	99.22
CURD2-GP	4240	48	52.13	1.81	99.72
NWC3-GP	6052	64	83.25	1.59	99.64
CURD3-GP	4084	48	61.91	1.96	99.56
NWC4-GP	3857	59	122.00	1.67	99.27
CURD4-GP	3156	40	85.33	1.69	99.46
NWC5-GP	2350	37	56.50	1.93	99.45
CURD5-GP	3612	40	55.00	1.87	99.58
NWC6-GP	4787	41	54.91	1.30	99.62
CURD6-GP	4539	39	44.50	1.88	99.74
NWC1-M-C	4041	53	74.11	2.51	99.51
CURD1-M-C	5760	69	101.50	2.23	99.55
NWC2-M-C	2079	31	46.60	2.10	99.37
CURD2-M-C	4806	54	84.00	2.09	99.56
NWC3-M-C	4211	51	60.10	2.53	99.67
CURD3-M-C	3241	40	80.00	2.14	99.51
NWC4-M-C	4307	56	96.63	2.42	99.40
CURD4-M-C	4581	50	125.00	2.20	99.45
NWC5-M-C	4523	61	100.43	2.60	99.47
CURD5-M-C	4211	49	99.60	2.17	99.45
NWC6-M-C	4450	46	55.00	2.36	99.80
CURD6-M-C	7600	82	117.10	2.06	99.64
NWC7-M-S	4198	42	99.75	2.11	99.48
CURD7-M-S	4598	55	109.38	2.17	99.35
NWC8-M-S	4295	45	66.11	2.17	99.53
CURD8-M-S	4544	55	136.25	2.25	99.43
NWC9-M-S	4651	44	90.00	2.04	99.48
CURD9-M-S	4678	45	95.00	2.07	99.47
NWC10-M-S	5424	55	69.62	2.22	99.63
CURD10-M-S	4913	55	91.91	2.04	99.41
NWC11-M-S	4079	50	71.38	2.43	99.53

CURD11-M-S	4850	58	95.80	2.19	99.42
NWC12-M-S	18679	63	76.33	2.12	99.91
CURD12-M-S	11888	70	82.67	1.81	99.83

Abbreviations: OTU, operational taxonomic unit; ESC, estimated sample coverage. Chao1, Shannon and ESC were calculated with Qiime at the 3% distance level.

2.4 Possible use of culture-independent strain monitoring by HTS targeting species-specific genes

2.4.1 Introduction

The strain diversity of bacteria involved in cheese manufacture is considered a technologically important aspect (Bottari et al., 2010; Gatti et al., 2003; Coppola et al., 2000) and many efforts have been done to implement reliable methods for strain discrimination and monitoring (Gelsomino et al., 2001; Gatti et al., 2004; Miller et al., 2012; Seseña et al., 2005; Giraffa & Rossetti, 2004; Rahman et al., 2014; Cai et al., 2007; Passerini et al., 2010; Rademaker et al., 2007). *Streptococcus thermophilus* is an important species in many dairy technologies. It is commonly used as starter for the manufacturing of yogurt and cheeses and it is also often found in naturally fermented cheeses (Neviani et al., 2013; Rossetti et al., 2008; Bottari et al., 2010; Ercolini et al., 2008; Pogačić et al., 2013). The principal role of *S. thermophilus* in dairy preparation is the production of lactic acid from lactose, which is achieved through a well-studied metabolic pathway (Poolman et al., 1989; Poolman et al., 1990; de Vos & Vaughan, 1994). Monitoring of this species at biotype level is an important target of the dairy industry and several molecular methods have been proposed (Moschetti et al., 1998; Andrighetto et al., 2002; Mora et al., 2003; El-Sharoud et al., 2012; Lazzi et al., 2009). Recently, the diffusion of high-throughput sequencing technologies highlighted the possibility of strain monitoring through sequencing of genes with high intra-species variability (Ercolini, 2013), but this application has not been exploited yet. Previous studies suggested the presence of high polymorphism in nucleotide sequence of *lacS* gene in *S. thermophilus* strains (Ercolini et al., 2005). The *lac* operon in *S. thermophilus* contains the *LacZ* gene encoding for the ß-galactosidase enzyme located downstream from the *LacS* gene encoding for the lactose permease LacS (de Vos & Vaughan, 1994; Vaillancourt et al., 2002; van den Bogaard et al., 2000; van den Bogaard et al., 2004). The LacS protein catalyzes both a lactose/H^+ symport and a lactose/galactose antiport and its overall action is to provide lactose for the ß-galactosidase and to eliminate the excess of galactose that is not used within the cell (Poolman et al., 1992; Foucaud & Poolman, 1992).

The high level of polymorphism found in previous studies made the *lacS* gene a good candidate for sequencing-based biotype monitoring of *S. thermophilus*. In the present study, an HTS approach was firstly applied for the study of the variability within *S. thermophilus* species. The *lacS* gene was sequenced from samples of natural whey culture (NWC) and curd of Mozzarella (M), Parmigiano Reggiano (PR) and Grana Padano (GP) manufactures.

2.4.2 Material and mathods

2.4.2.1 Sampling

Samples from M cheese manufactures were collected from twelve dairies producing top-quality traditional water buffalo mozzarella PDO cheese, located in the Campania region (Southern Italy) in the provinces of Salerno (MS) and Caserta (MC). Samples from GP and PR manufactures were collected from six and seven dairies located in different places within the GP and PR area of production (Northern Italy). Samples of NWCs and curds at end of the ripening were aseptically collected, cooled at 4°C, and analyzed within 6 h. NWC samples were from the manufacture of the previous day and used for the production of the corresponding curds according to the traditional back-slopping procedure. Curd samples were collected after 5 h from the addition of the NWC for M cheese and after 24 h for GP and PR cheese.

2.4.2.2 DNA extraction

Total DNA extraction from the dairy 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 NWC or from 2 ml of a homogenized 2-fold dilution of the curd in one-quarter-strength Ringer's solution (Oxoid, Milano, Italy).

2.4.2.3 lacS gene amplicon library preparation and sequencing

The 454 Universal Tailed Amplicon protocol was used with a double PCR step (454 Sequencing System – Guidelines for Amplicon Experimental Design). The variable region of 250 bp upstream from the *lacS* gene was amplified using the primers LCS62f 5'-GGCTTCCAATACTTTAATT and LCS312r 5'-

AAGTGAGTTGTCACAAACAT (Ercolini et al., 2005). The universal primers M13f 5'-TGTAAAACGACGGCCAGT and M13r 5'-CAGGAAACAGCTATGAC were included at 5' and 3' ends of the LCS primers (Daigle, 2011). Each PCR mixture (final volume, 50 µl) contained 100 ng of template DNA, 0.1 µM of each primer, 0.50 mmol l^{-1} of each deoxynucleoside triphosphate, 2.5 mmol l^{-1} $MgCl_2$, 5 µl of 10 X PCR buffer and 2.5 U of native Taq polymerase (Invitrogen, Milano, Italy). The following PCR conditions were used for *lacS* gene amplification: 94 °C for 5 min, followed by 20 cycles at 94 °C for 1 min, 45 °C for 1 min, 72 °C for 2 min. A final extension was carried out at 72 °C for 7 min. After agarose gel electrophoresis, PCR products were purified with a QIAquick gel extraction kit (Qiagen, Milano, Italy) and 20 ng of the purified product were used as template in a second PCR where primers M13f and M13r were used, with the addition of 454-adaptors and a 10 bp sample-specific Multiplex Identifier (MID). The PCR mixture was prepared as above described and the PCR conditions were the following: 94 °C for 5 min, followed by 20 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 7 min. PCR products were purified twice by Agencourt AMPure kit (Beckman Coulter, Milano, Italy) and then quantified using the QuantiFluor™ (Promega, Milano, Italy). An equimolar pool of amplicons was prepared and it 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 and a bidirectional sequencing.

2.4.2.4 Bioinformatics and data analysis

Raw reads were first filtered according to the 454 amplicon processing pipeline. Sequences were then analyzed by using QIIME 1.7.0 software (Caporaso et al., 2010). Raw reads were demultiplexed and further filtered through the split_library.py script of QIIME. The script was carried out twice, in order to demultiplex both forward and reverse reads, after obtaining the reverse complement. In order to guarantee a higher level of accuracy, 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 200 bp. The analysis pipeline carried out was the following: forward and reverse-complemented demultiplexed sequences that passed the quality filters were denoised (Reeder & Knight, 2010), using a sequence similarity threshold of 99 %. After primer truncation, singletons were excluded and *lacS* gene sequence types defined by a 100 % of similarity were picked using the uclust method (Edgar, 2010). The longest sequence of each cluster was picked as representative sequence. The representative sequences were trimmed to a fixed length and aligned to the *lacS* sequence of the strain A147 (accession no. M23009) by using MEGA 5.2.2 software (Tamura et al., 2011), manually checked in order to confirm mutations detected by QIIME and mismatches likely due to sequencing errors were corrected. After alignment, a phylogenetic tree was built using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method. Those clusters represented by sequences characterized by a 100 % of similarity were merged and *lacS* sequence types were defined as having at least one point of mutation compared to the reference sequence. The abundance of each sequence type in the samples was also determined.

2.4.3 Results

S. thermophilus variability at biotype level was firstly investigated through pyrosequencing of *lacS* gene amplicons. A total of 226,008 raw reads were obtained from *lacS* gene pyrosequencing; 195,315 passed the filters applied through QIIME, with an average value of 3,488 reads/sample and an average length of 250 bp. Clustering at 100% of similarity allowed identification of 28 different sequence types, but only 13 of them had a relative abundance higher than 1 % in at least one sample. The average percentage of the 6 most abundant *lacS* types in the three manufactures is reported in **Table 2.4.1**. The highest diversity in sequence types was found in M samples. In particular, MC samples showed the highest number of different *lacS* types, while GP the lowest. The mutations identified are reported in **Figure 2.4.1**, while the abundance of the *lacS* types is shown in **Figure 2.4.2**. One *lacS* type (*lacS* type 1) occurred very often, with abundance ranging 87-99 % in almost all the samples. A total of 60 mutation points were detected that allowed the differentiation of 28 *lacS* gene sequences. Most of the differences were found in the promoter region upstream from the *lacS* gene. Within the promoter region, -10 regions did not show sequence variability, whereas region -35 turned from TTGACT to TTGACA in 9 out of 28 reference sequences. Twenty-seven points of mutations were found in the protein coding sequence of the gene (**Figure 2.4.1**), but only some of them led to amino acid changes in the primary structure of the protein. In particular, only 9 amino acid changes were found in the putative protein.

2.4.4 Discussion

S. thermophilus is one of the most important species in dairy environment and it was often found in the NWC of several traditional cheeses (Bottari et al., 2010; Ercolini et al., 2008; Neviani et al., 2013; Rossetti et al., 2008). Its genome is shaped by its domestication to the dairy environment, with gene features that conferred rapid growth in milk, stress response mechanisms and host defense systems that are relevant to its industrial applications (Goh et al., 2011). Since many studies highlighted the presence of strain-specific phenotypic traits, such as exopolysaccharide production (Vaningelgem et al., 2004), urease activity (Mora et al., 2002; Zotta et al., 2008), galactose fermentation (De Vin et al., 2005; Giraffa et al., 2001; Vaughan et al., 2001) and nitrogen metabolization (Broadbent & Steele, 2005), monitoring of this species at biotype level can be very interesting in order to trace biotypes with specific traits during cheese manufactures. Several strain-monitoring methods have been proposed, based both on phenotypic and genotypic approaches (El-Sharoud et al., 2012; Giraffa et al., 2001; Lazzi et al., 2009; Mora et al., 2002; Mora et al., 2003;Moschetti et al., 1998). All these methods are based on cultivation of strains prior to molecular analysis, so they are subject to the well-known biases of culture-dependent methods. In fact, the presence of a high unculturable fraction of *S. thermophilus* in NWCs for GP production has been reported (Fornasari et al., 2006). The occurrence of species-specific genes with substantial sequence heterogeneity can be a valid premise to apply high-throughput sequencing strategies to achieve strain specific monitoring without cultivation (Ercolini, 2013). In this study, high-throughput sequencing of *S. thermophilus lacS* gene was carried out, in order to explore the biotype diversity in the samples studied and to evaluate the possibility of using this technique to monitor *S. thermophilus* biotypes without cultivation. Overall, the sensitivity of pyrosequencing allowed identifying some low-frequency mutations representing less than 1 % of the total *lacS* sequences. Although we have no mean to be 100% sure that when only one mutation occurs this is not due to pyrosequencing error, we decided to define a sequence type as having at least one mutation point compared to the reference sequence of the strain A147 for *lacS* gene. Our results showed that only few *lacS* types of *S. thermophilus* were abundant in all the samples (**Figure 2.4.2** and **Table 2.4.1**). In particular, one of them dominated in all GP and PR samples and in all M samples from Salerno area (MS). Such *lacS* type was also present in M samples from Caserta dairies (MC), which had the highest variability in *lacS* types, indicating a higher diversity of *S. thermophilus lacS* types in this area of production. In fact, *lacS* type 2 was present only in MC, while the type 3, although present also in some MS at very low concentration, was a dominant *lacS* type in MC. Sequence types 2 and 3 represented 14 and 15% of the total *lacS* sequences of MC samples, respectively (**Table 2.4.1**). The highest diversity in *lacS* sequence types was found in M samples, that also showed a higher abundance of *S. thermophilus*, compared to GP and PR. This could indicate that the higher the abundance of a species, the higher the diversity in term of different biotypes that can coexist. In all the cases, the same *lacS* types were found both in the NWC and in the relative curd (data not shown). The dominance of *lacS* type 1 (**Figure 2.4.2** and **Table 2.4.1**) that was the most abundant sequence type in all the samples is probably due to the insufficient level of heterogeneity of the *lacS* gene within *S. thermophilus* biotypes. Pyrosequencing allowed identification of a total of 60 mutation points, most of all in the region upstream from the *lacS* gene, in agreement with the previous study (Ercolini et al., 2005). In particular, in 9 out of 28 sequences a mutation in the -35 box was found. This hexamer, beside to the -10 box, plays as a binding site for the δ subunit of RNA polymerase and allows the transcription to start (Burgess et al., 1969). Since the transcription level was not investigated, we do not know if this could be considered a down mutation, possibly affecting the transcription efficiency. The presence of a putative catabolite responsive element (*cre*) overlapping the -10 box was previously highlighted (Ercolini et al., 2005), indicating a possible role for regulation by a catabolite control protein A (CcpA) (van den Bogaard et al., 2000). Our results confirmed that this is a conserved region, except for three points of mutation: a T and two A turning all to G at position 3, 4 and 6 of the *cre* site (underlined in **Figure 2.4.1**), respectively. The mutations in position 3 and 6 were already pointed out by Ercolini et al. (2005), while the one in position 4 was identified in this study, even if this *lacS* type occurred at low abundance and only in few samples. However, only the substitution in position 3 creates a mismatch in the *cre* sequence.

This study provided the first example of the application of an HTS approach for culture-independent typing of microbiota beyond the species in food. Although *lacS* gene did not prove enough variable within *S. thermophilus* species to be used for quantitative strain monitoring, we highlighted the possibility of using non rRNA genomic amplicons for a culture-independent identification of biotypes within a species in food matrices.

2.4.5 References

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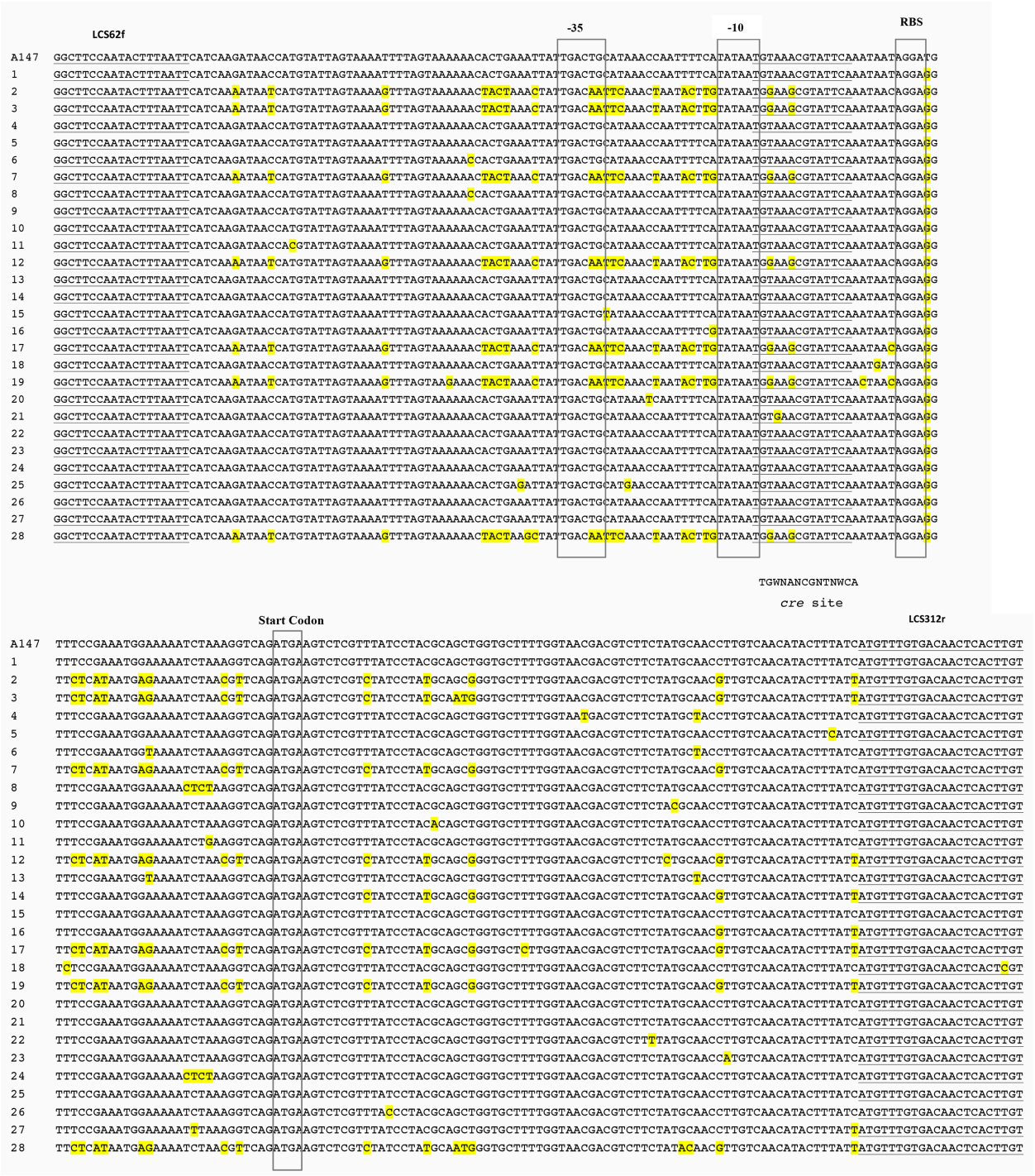

Figure 2.4.1 Nucleotide sequence alignment of the 28 *lacS* gene sequence types identified in this study. Sequences are aligned to the reference sequence of strain A147 (accession no. M23009). The ribosome binding site (RBS), the -35 and -10 sequences and the start codon are boxed. The putative *cre* site is underlined and aligned with the consensus sequence. The primer sequences are underlined.

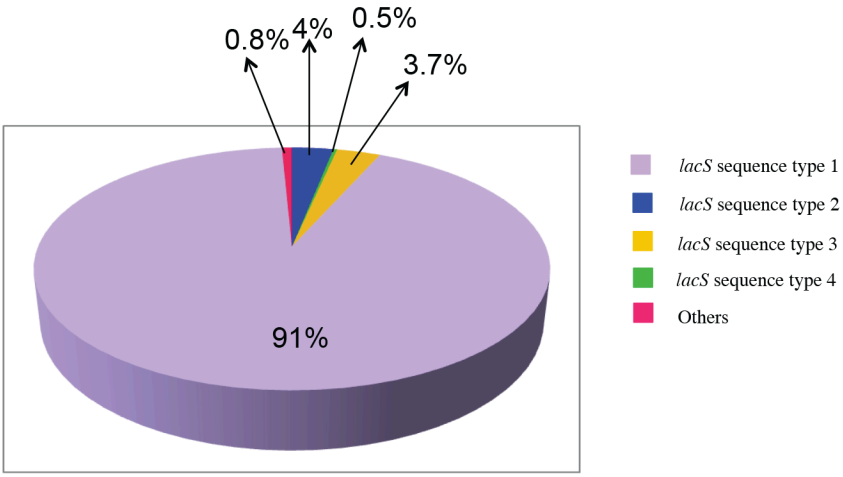

Figure 2.4.2 Abundance (%) of the *lacS* sequence types identified in this study. The abundance values are averaged for the three cheeses and only sequence types with an occurrence of at least 0.5% are showed in the color legend.

Table 2.4.1 Total number and average relative abundance (%) of the prevalent *lacS* sequence types identified in NWC and curd samples from Grana Padano (GP), Parmigiano Reggiano (PR) and Mozzarella manufactures from Caserta (MC) and Salerno (MS) area of production. Only types occurring at >0.5% abundance were included.

Sequence type	GP	PR	MC	MS
1	98	98.7	69	99.5
2	-	-	14	-
3	-	<0.5	15	<0.5
4	1	<0.5	-	-
5	<0.5	0.5	-	-
6	<0.5	0.5	-	-
Total n° of sequence types identified	9	16	19	10

2.5 rRNA-based monitoring of the microbiota involved in Fontina PDO cheese production in relation to different stages of cow lactation

2.5.1 Introduction

Fontina Protected Designation of Origin (PDO) is a full-fat semicooked washed-rind cheese that is traditionally made in Aosta Valley (Northwest Italy), according to PDO production specifications (Reg. UE 1107/1996, revised in 2004). According to the Fontina PDO Regulation, the addition to the milk of an autochthonous starter cultures is allowed. They are composed of three strains belonging to the species *Streptococcus thermophilus*, *Lactobacillus delbrueckii* and *Lactococcus lactis*, selected and stored at the Institut Agricole Régionale of Aosta (Italy) and added to the milk with an initial load of 10^6 CFU/mL. After the addition of starter cultures, the milk, obtained from a single milking and treated within 2 h after milking, is coagulated with calf rennet at 36°C and the curd is cut finely while the temperature is gradually raised to 46–48 °C. The curd, after a brief rest in whey, is collected in molds and pressed to eliminate any residual whey. Rounds are traditionally matured for at least 80 days in natural caves with a temperature that varies from 5 to 12°C and UR > 90%. During the first month cheeses are alternatively dry salted and brine washed to allow the spontaneous development of the characteristic red-brown rind. No specific microflora is intentionally inoculated on the cheese surface in this phase. The final cheese has cylindrical shape (30–45 cm diameter, 7.5–12 kg weight), with typical elastic body texture and pale-yellow colour. The rind surface microbiota of Fontina cheese has been studied previously through culture-dependent and -independent approaches (Dolci et al., 2009). It is charachterized by yeasts and bacteria. In particular, *Debaryomyces hansenii* and *Candida sake* were the yeast species present throughout the whole ripening process. As early as after 1 day since manufacture, *Lactococcus lactis* subsp. lactis and *Streptococcus thermophilus* were detected on cheese rinds, while *Arthrobacter nicotianae*, *Brevibacterium casei* and *Corynebacterium glutamicum* were found after 7–28 days. Another study focusing on the milk and curd microbiota found *Streptococcus thermophilus, Enterococcus faecium*, *Enterococcus faecalis*, *Lactococcus lactis* and *Leuconostoc lactis* as the most important species in milk, besides to adventitious bacteria (*Macrococcus caseolyticus*, *Rothia* spp.) and psychrotrophic bacteria (*Chryseobacterium* spp., *Pseudomonas* spp.), that were found in almost all samples, but disappeared after the warming up at 47–48 °C of coagulated milk, while *E. faecium*, *E. faecalis* and *S. thermophilus* were found as predominant in the curd (Giannino et al., 2009). The management of cattle farms calls for seasonal migration to high pastures, during the summer, to altitude higher than 2000 m in order to follow the vegetative cycle of grassland. Here, cattle is fed on grass, differently from the winter season when the use of hay or concentrated feed is allowed according to the regulation. The mountain pasture custom determines a concentration of calving during the autumn and the beginning of the winter. This organization of animal husbandry, common to many mountain dairy cattle farms, essentially results in three phases of lactation: post-partum (January to February), oestrus (February to March) and early gestation (March to April). It is hypothesized that the different cow physiological states might have an impact on average milk composition and, consequently, on its cheese making aptitude and on the final quality of the cheese. Remarkably, Fontina cheese manufactured from milk produced during cow oestrus stage is generally known to be of minor quality, in terms of organoleptic characteristics and typicity traits, and this aspect needs appropriate investigation. The aim of this study was to investigate the dynamics of bacterial populations during Fontina PDO cheese manufacture and ripening, and to evaluate possible correlations between microbiota and different lactation stages. In particular, the performance of the starters used for Fontina PDO production and selected from autochthonous lactic acid bacteria (LAB) was followed together with the activity of non-starter LAB (NSLAB) using a culture-independent high-throughput sequencing approach. In particular, pyrosequencing of amplified V1-V3 region of 16S ribosomal RNA (rRNA) was chosen to follow metabolically active populations from milk to curd and cheese matrices. Moreover, the role of non-dairy (ND) microbiota was considered in order to evaluate, eventually, their interference with starter culture activity. High-throughput sequencing is emerging as a new culture-independent tool for a quantitative investigation of the structure of microbial communities, beside being much more sensitive to detect sub-dominant populations (Ercolini, 2013). So far this technique was successfully used for an in-depth analysis of the bacterial diversity in a number of dairy foods (Alegria et al., 2012; De Filippis et al., 2014; Ercolini et al., 2012; Masoud et al., 2011; Quigley et al., 2012), but to the best of our knowledge, this is the first time this aproach is used for the study of Fontina PDO cheese microbiota.

2.5.2 Materials and methods

2.5.2.1 Fontina PDO manufacturing and sampling

Fontina PDO cheese production was monitored in three different dairies named, in this paper, A, B and C, and placed at altitudes varying from 600 to 1200 m in Aosta Valley. Cheese-making was followed during three different cow lactation stages, precisely, post-partum (phase 1), oestrus (phase 2) and early gestation (phase 3), and, for each production, two replicates were investigated. The productions were manufactured in the middle period of each phase and the replicates were carried out with not more of a four day interval. Samples of raw milk before addition of starter cultures, curd after 24 h and cheese at 84 days of ripening (a 4-cm-thick section from the core of the product) were collected. Replicate RNA extractions were carried out and pooled before complimentary DNA synthesis.

2.5.2.2 Chemical analyses

The pH measurements were carried out on milk, after the inoculation of the starter cultures, and on 24 h curd samples by using a pH-meter (Sial-micros pH trend 10). All analyses were performed in triplicate. Acidification curves, after starter culture inoculation, were also followed and the time required for the beginning of milk acidification process (D_t) determined as the inflection point of the curves. The urea content of milk was evaluated by Milko-Scan FT 6000 (Foss, Hillerod, Denmark). T-tests were performed to compare data sets of D_t and urea concentration in relation to lactation stage and factory.

2.5.2.3 RNA extraction, cDNA synthesis and pyrosequencing

Metabolically active population was followed by pyrosequencing of RNA directly extracted from milk, curd and cheese samples. Sample preparation and RNA extraction were performed according to the protocol reported by Rantsiou et al. (2008). Two grams of each cheese was mixed with 20 ml of 2% (wt/vol) sodium citrate and incubated for 30 min at 45 °C. The mix was vortexed for 5 min and centrifuged at 6000 ×g for 10 min and the pelleted material was re-suspended in 1 mL of 10 mM Tris–5 mM EDTA, pH 8 (TE). After centrifugation at maximum speed (14,000 ×g), the pellet was re-suspended in 300 µL of TE and centrifuged for 10 min at maximum speed. The pellet was re-suspended in 120 µL of proteinase K buffer containing 50 mM Tris–HCl, 10 mM EDTA, 0.5% (wt/vol) sodium dodecyl sulfate (SDS), pH 7.5, and the suspension was transferred to a 1.5 ml screw cap tube containing 0.3 g of glass beads with a diameter of 0.5 mm. Twenty microliters of proteinase K (25 mg/ml; Sigma) and lysozyme (50 mg/ml; Sigma) were added and the mixture was incubated at 50 °C for 1 h. Then, 150 µL of 2x breaking buffer, composed of 4% (vol/vol) Triton X-100, 2% (wt/vol) SDS, 200 mM NaCl, 20 mM Tris, pH 8 and 2 mM EDTA, pH 8 were added. Three hundred microliters of phenol–chloroform, 5:1, pH 4.7 (Sigma) was added and three 30-s bead beater (Fast Prep; Bio 101, Vista, CA, USA) treatments were performed, at maximum speed, with an interval of 10 s between each treatment. Three hundred microliters of TE was added and the tubes were centrifuged for 10 min at 12,000xg at 4 °C. The aqueous phase was collected and nucleic acids were precipitated by the addition of 1 mL of absolute ethanol. The RNA was pelleted by centrifugation at 14,000xg for 10 min at 4 °C. Each pellet was air dried and 50 µL of sterile water was added. Three microliters of TURBO-DNase (Ambion, Milan, Italy) was added to digest the DNA in the RNA samples, with an incubation of 3 h at 37 °C. The presence of residual DNA in the RNA samples was checked by PCR and the treatment repeated if necessary. The cDNA was obtained as previously described (Alessandria et al., 2010): one µg of RNA was mixed with 1 µL of 10 µM 519r primer and sterile water to a final volume of 10 µl and incubated at 75°C for 5 min. The mix was placed on ice and a mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 2 mM of each dNTP, 1 µL of 200 U/µL M-MLV reverse transcriptase (Promega) and 0.96 units of RNasin ribonuclease inhibitor (Ambion) was transferred to the reaction tube. The reverse transcription was carried out at 42°C for 1 h. One µL of RT reaction was used to study the microbial diversity by pyrosequencing of the amplified V1–V3 region of the 16S rRNA gene by using primers Gray27f 5'-TTTGATCNTGGCTCAG and Gray519r 5'-GTNTTACNGCGGCKGCTG amplifying a fragment of 520 bp (Andreotti et al., 2011). 454-adaptors were included in the forward primer followed by a 10 bp samplespecific Multiplex Identifier (MID). Each PCR mixture (final volume, 50 µL) contained 50 ng of template cDNA, 0.4 µM of each primer, 0.50 mmol/L of each deoxynucleoside triphosphate, 2.5 mmol/L $MgCl_2$, 5 µL 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 QuantiFluor™ (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, Monza, Italy) according to the manufacturer's instructions by using a Titanium chemistry.

2.5.2.4 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 1.7.0 software (Caporaso et al., 2010). In order to guarantee a higher level of accuracy in terms of Operational Taxonomic Unit (OTU) detection, after the split library script performed by QIIME, the reads were excluded from the analysis if they had an average quality score lower than 25, if they were shorter than 300 bp and if there were ambiguous base calls. Sequences that passed the quality filter were denoised (Reeder and Knight, 2010) and singletons were excluded. OTUs defined by a 97% of similarity were picked using the uclust method (Edgar, 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 using the Greengenes 16S rRNA gene database (McDonald et al., 2012). Alpha and beta diversity were evaluated through QIIME as recently described (De Filippis et al., 2013). Weighted UniFrac distance matrices (Lozupone & Knight, 2005) and OTU tables were used to perform Adonis, Anosim, and ANOVA statistical tests through the compare_category.py and the otu_category_significance.py scripts of QIIME, in order to verify the influence of the lactation phase on the microbial population and whether the abundance of any OTUs was significantly associated to a specific lactation phase. An OTU network was generated by QIIME and a bipartite graph was constructed in which each node represented either a sample or a bacterial OTU. Connections were drawn between samples and OTUs, with edge weights defined as the number of sequences from each OTU that occurred in each sample. Networks were visualized using Cytoscape 3.0.2 (Shannon et al., 2003).

2.5.3 Results

2.5.3.1 Acidification process and urea concentration

The pH values measured in milk samples (pH_i) before the addition of the starter cultures ranged from 6.58 to 6.72 and reached values from 5.33 to 5.61 in the curd after 24 h (pH_{24h}) (**Table 2.5.1**). The acidification process showed a different trend related to the different lactation phases. Precisely, the time required for the acidification process to begin (D_t) was, on average, of 444, 456 and 420 min referred, respectively, to phase 1 (milk samples A1, B1, C1), phase 2 (milk samples A2, B2, C2) and phase 3 (milk samples A3, B3, C3). Milk urea concentration was also determined. The values are shown in and varied from 19.1 mg/dL to 25.2 mg/dL. However, no significant differences were detected by t-test in acidification and urea concentration according to different lactation stages and dairies.

2.5.3.2 Microbial diversity

A total of 144,098 raw sequences were obtained and analyzed; 134,171 reads passed the filters applied through the QIIME split_library.py script, with an average value of 4969 reads/sample and an average length of 490 bp. The number of OTUs, the Good's estimated sample coverage (ESC), the Chao1 (Chao & Bunge, 2002) and Shannon (Shannon & Weaver, 1949) indices obtained for all the samples are reported in **Table 2.5.2**. The rarefaction analysis and the estimated sample coverage indicated that there was satisfactory coverage for all the samples (ESC > 97%). Interestingly, milk samples from lactation phase 1 always showed a lower level of complexity, compared to those from lactation phases 2 and 3. In **Figure 2.5.1**, only OTUs with a relative abundance > 0.05% in at least one sample are shown. The milk sampled in the post-partum lactation phase (phase 1) was unexpectedly characterized by the predominance of a unique species belonging to *L. casei* group, which survived throughout the ripening and appeared in most of the cheeses at 84 days. A major biodiversity was highlighted in milk samples during the oestrus (phase 2) and early gestation (phase 3). In these seasons, the microbiota was dominated by *P. acnes*, the genera *Staphylococcus* and *Pseudomonas*, and the *Enterobacteriaceae* family. Microbiota usually colonizing soil and grass habitats such as *Acinetobacter*, *Acidovorax*, *Hymenobacter*, *Brochothrix*, *Actinobacteria* and *Cyanobacteria* were also found (**Figure 2.5.1**). Accordingly, Adonis and Anosim statistical tests showed that milk samples were significantly different according to the lactation phase ($P < 0.001$). Moreover, *Lactobacillaceae* family abundance, and particularly *L. casei* group, were found to be significantly higher in milk samples from the lactation phase 1 by ANOVA ($P < 0.001$). On the contrary, no significant difference was found among samples from different dairies ($P > 0.05$). Regardless of the initial quality of the milk, the establishment of two of the starter species, *S. thermophilus* and *L. delbrueckii*, was evident in all the curds analyzed after 24 h from the beginning of

the production. *Pseudomonas* and *Enterobacteriaceae* were also strongly present, together with starter cultures, in curd samples produced in dairy farm C during the phases 1 and 3. Moreover, *S. thermophilus* and *L. delbrueckii* showed high adaptation throughout the ripening and they were always found in cheeses after 84 days of ripening, except for the cheese manufactured in dairy farm C (phase 2) where the maturing was almost completely carried out by *S. thermophilus* only. On the contrary, the starter species *L. lactis* performed well only in the cheeses produced in dairy farm B during the post-partum lactation (phase 1) and early gestation (phase 3) stages (**Figure 2.5.1**). Despite the prevalence of the starters inoculated, a few autochthonous microorganisms were also found metabolically active, in some cheeses, at the end of ripening. They belonged to *L. casei* group and prevailed, particularly, in the products sampled during the phases 1 and 3. Pyrosequencing allowed also the detection of low, but constant incidence of *Pseudomonas*, *Staphylococcus* and *Enterobacteriaceae* in most of the cheeses at 84 days of ripening (**Figure 2.5.1**). The OTU network in **Figure 2.5.2** clearly shows a separation between the milk and the curd/cheese samples. A high number of OTUs was shared among curds and cheeses after 84 days of ripening highlighting a core microbiota, while milk samples showed a higher number of unique OTUs. Moreover, the milk samples clustered according to the lactation phases.

2.5.4 Discussion

Overall, starter cultures were able to outcompete the autochthonous microbiota since the first hours of the fermentation and their performance seemed to be not affected by the different lactation stages. In fact, *S. thermophilus* and *L. delbrueckii* were found throughout manufacturing and ripening of Fontina PDO cheese, regardless of farm location and lactation period. Pyrosequencing was able to highlight the presence and the activity of these two starter species, while it underlined the low ability of *L. lactis* in establishing in Fontina curd and cheese samples. Uniquely, *L. lactis* had a good performance in the cheese manufacturing in the dairy farm B, where it was also detected after three months of ripening. The sensitivity of pyrosequencing allowed highlighting the presence of *L. lactis* also in curds and cheeses produced at dairy farms A and C, but with a very low incidence compared to *S. thermophilus* and *L. delbrueckii*. In Fontina cheese making the milk is coagulated at 36 °C and the curd is cut while the temperature is gradually raised to 46–48 °C. The temperature of the process could explain the low frequency of *L. lactis*, whose contribution could be limited only to the first hours of the acidification process. As known, cooking temperature can affect the viability of starter and non-starter cultures in hard and semi-hard cheeses (Sheehan et al., 2007). Moreover, Taïbi et al. (2011) identified, in *L. lactis*, a specific core of genes differentially expressed in response to heat stress. These genes are related to the coding of chaperones and proteases and linked to cell division and metabolism. The predominance, in Fontina curd samples, of *S. thermophilus* on *L. lactis* population was already highlighted in previous studies (Giannino et al., 2009; Senini et al., 1997), confirming the different attitudes of the two microorganisms to Fontina cooking temperature. In general, the establishment of the starter cultures limited the development of NSLAB, the only exception being the *L. casei* group. It was found in almost all the samples of curd and cheese, and, remarkably, was dominant in milk from the first phase of lactation. Thus, this species has to be considered as significant part of the autochthonous microbiota of Fontina cheese. In some cases, *L. casei* was able to compete with the starter cultures, since it was found with similar relative abundance. Also *Enterococcus faecalis*, considered typical in Fontina cheese and related to flavor formation (Giannino et al., 2009; Senini et al., 1997), was detected. The raw milk analyzed and transformed in the second and third phase of lactation was rich in *P. acnes*, *Pseudomonas*, *Staphylococcus*, and *Enterobacteriaceae*, generally associated to low quality milk (Quigley et al., 2013), and *Psychrobacter*, *Brochothrix*, *Acinetobacter* and *Cyanobacteria*, already found associated to food and dairy products in other studies (Afzal et al., 2013; Ercolini et al., 2006; Franciosi et al., 2011; Hayes et al. 2002; Meile et al., 2008). As known, raw milk microbiota contributes greatly to the sensory characteristics of raw milk cheeses in terms of the particular flavors and aromas they generate but, at the same time, spoilage flora and potential pathogens can negatively affect the final quality of the product (Mallet et al., 2012). However, in this study, the predominance of the starter cultures over ND microorganisms was already evident in the curd. In fact, starter activity limited the development of these microbial populations throughout Fontina manufacturing and ripening, with the exception of few cheese samples where *Enterobacteriaceae*, *Pseudomonas* and *P. acnes* were detected with moderate incidence. Remarkably, the presence of contaminant microbiota in the milk of the second and third stage of lactation and, occasionally, in curd and cheese samples of all the lactation phases, cannot explain the minor quality of Fontina, in terms of organolepitc quality and typicity, as detected by sensory analysis. This is probably due to the high performance of the starters, which were dominant in all the productions studied. Accordingly, the OTU network clearly showed that a high number of OTUs was shared among curds and cheeses after 84 days of ripening without a correlation with the lactation phases. In any case, the high incidence of starter cultures proves that the final quality of Fontina cheese cannot be correlated to

autochthonous microbiota. The metabolic microbial activity, which was supposed to affect the final quality of Fontina PDO cheese, was strictly associated to the presence of the starter, which did not show any difference in its performance according to both lactation stage and dairy farm. Milk urea concentration, which is an indicator of the health and nutrition status of dairy cows, could influence starter behavior due to the antimicrobial activity (Podhorsky & Cvak, 1989; Vega-Pérez et al., 2012). Some authors showed that an increase in milk urea content influenced milk acidification and led to a decrease of milk clotting ability (Mariani et al., 1992). Actually, in this study, correlation between these aspects was not found. No significant differences were detected in acidification and urea concentration according to different lactation stages. The fact remains that, empirically, in milk from cows in oestrus phase of lactation, a slower coagulation process was observed and this can result in a delay of the fermentation process. Actually, in this study, the average D_t calculated for each lactation season was higher in the oestrus lactation stage, confirming the difficulty in milk coagulation. Nevertheless, the results of this work lead to deny any type of correlation between the microbial dynamics and the quality of Fontina cheese in relation to the different stages of lactation.

2.5.5 References

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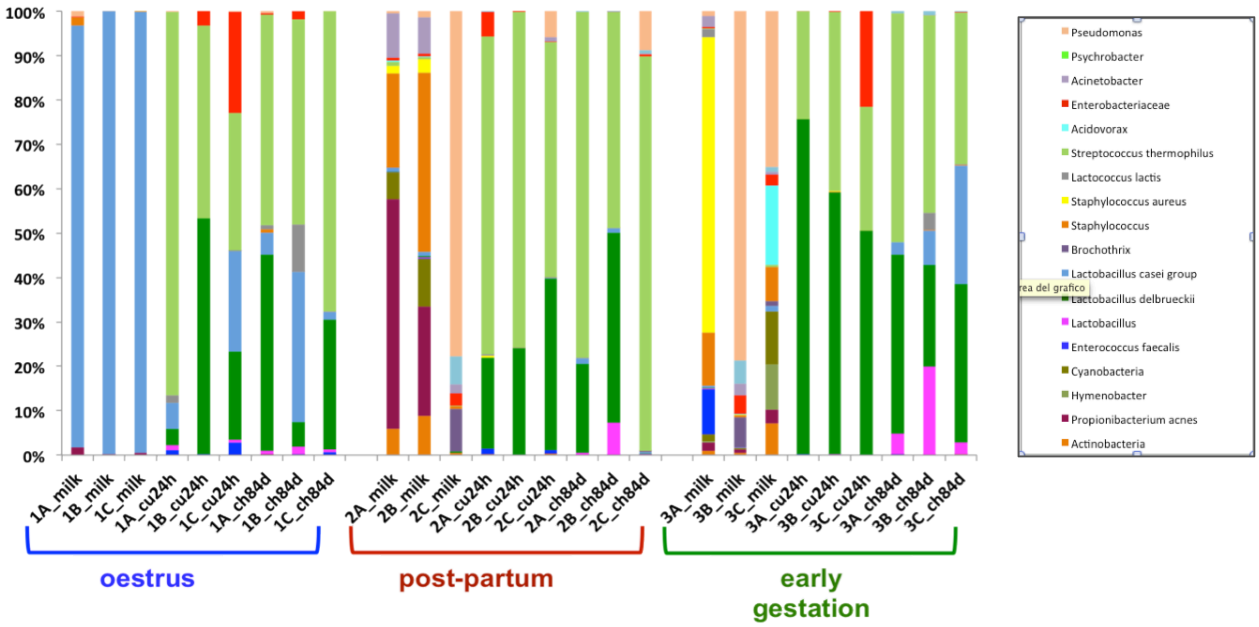

Figure 2.5.1 Incidence of the major taxonomic groups detected by pyrosequencing in samples of milk, curd (cu24h) and ripened Fontina PDO cheese (ch84d) analysed. Only OTUs with an incidence above 0.05% in at least one sample are shown. Samples are coded according to the lactation phase: 1, oestrus; 2, post-partum; 3, early gestation; A, B, C indicate the three different dairies.

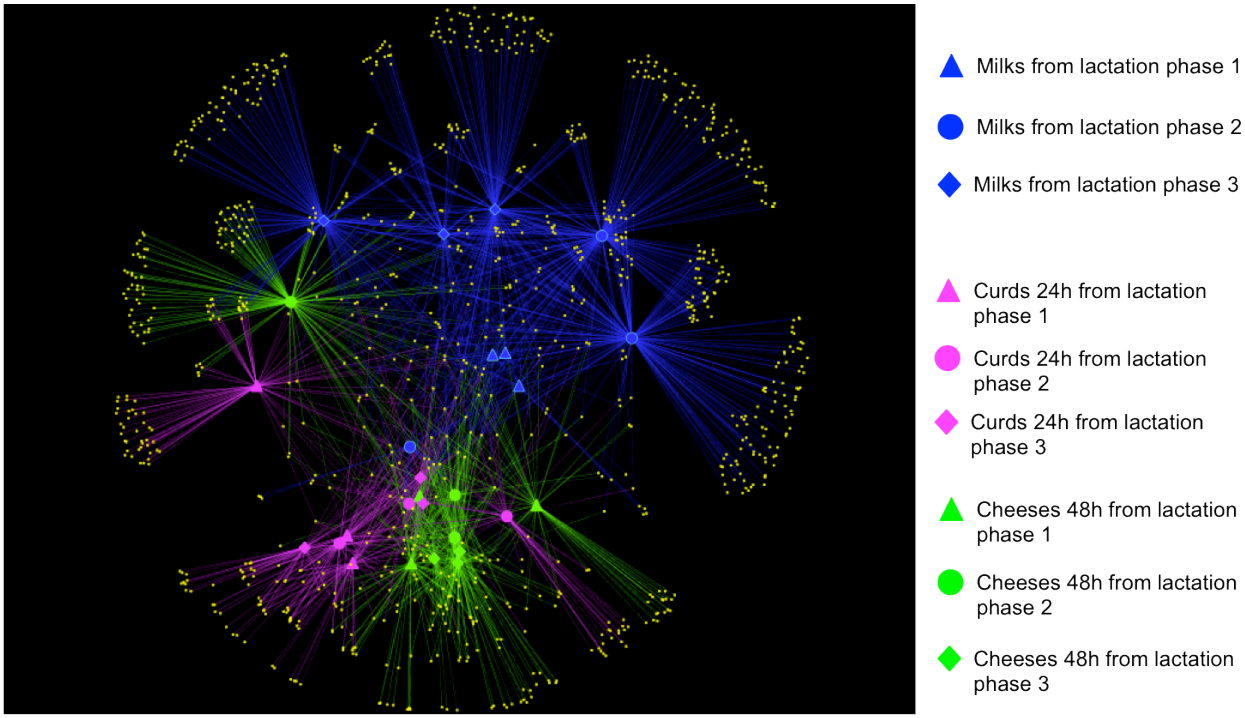

Figure 2.5.2 Simplified illustration of possible cheese - microbe networks. Network diagrams are color- and symbol-coded by sample type and lactation phase.

Table 2.5.1 Analysis of the milk used for the production of Fontina PDO cheese in the dairy farms A, B and C during the lactation phases 1, 2 and 3: pH measurements of milk (pH_i) and after 24 h (pH_{24h}), time required for the beginning of the acidification process (D_t) and milk urea concentration.

Dairy farm and lactation phase	**pH_i**		**pH_{24h}**		**D_t (min)**		**Urea (mg/dL)**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
A1	6.61	0.04	5.60	0.19	402	21	23.4	1.6
A2	6.66	0.03	5.41	0.06	443	69	22.2	1.4
A3	6.68	0.02	5.36	0.02	395	34	19.1	1.7
B1	6.62	0.01	5.61	0.28	402	29	21.1	2.8
B2	6.65	0.01	5.35	0.11	451	54	21.3	1.8
B3	6.72	0.05	5.45	0.05	400	38	22.4	3.2
C1	6.58	0.03	5.44	0.07	528	35	21.6	1.1
C2	6.62	0.02	5.33	0.09	474	65	25.2	0.8
C3	6.69	0.03	5.41	0.03	465	36	20.2	2.9

Table 2.5.2 Observed diversity and estimated sample coverage for 16S rRNA amplicons analyzed in this study.

Sample	OTUs	Chao1	Shannon	ESC
1A_milk	25	47.75	0.94	97.37
1A_cu24h	25	29.67	1.35	99.32
1A_ch84d	69	74.53	2.54	99.51
2A_milk	290	295.88	4.55	99.83
2A_cu24h	72	120.75	1.67	98.45
2A_ch84d	54	75.08	1.52	99.21
3A_milk	240	251.89	3.38	99.73
3A_cu24h	28	61.00	1.26	99.75
3A_ch84d	72	112.63	2.39	99.16
1B_milk	23	42.50	0.16	99.80
1B_cu24h	40	65.67	1.51	99.34
1B_ch84d	96	135.38	3.09	99.16
2B_milk	283	293.16	5.22	99.59
2B_cu24h	23	30.86	1.21	99.63
2B_ch84d	61	73.67	2.21	99.37
3B_milk	228	250.52	3.50	98.99
3B_cu24h	33	55.67	1.64	99.43
3B_ch84d	98	133.77	3.70	98.77
1C_milk	30	30.55	0.23	99.93
1C_cu24h	78	133.50	3.04	98.27
1C_ch84d	41	71.60	1.61	99.40
2C_milk	105	110.28	2.63	99.49
2C_cu24h	64	73.55	2.59	99.46
2C_ch84d	181	201.07	1.26	99.80
3C_milk	125	132.89	5.49	98.11
3C_cu24h	56	71.33	2.30	98.96
3C_ch84d	70	93.00	2.86	99.30

Abbreviations: OTU, operational taxonomic unit;
ESC,estimated sample coverage; Chao1, Shannon and
ESC were calculated with Qiime at the 3% distance level.

2.6 Microbiome involved in Caciocavallo Silano cheese ripening and effect of technological intervention

2.6.1 Introduction

Caciocavallo Silano is a semi-hard "pasta-filata" cheese that is granted a Protected Designation of Origin (PDO) label (European Regulation 1236/96) and is produced in five different regions of southern Italy (Campania, Basilicata, Calabria, Puglia and Molise). It is produced from raw or mildly thermally treated (58 °C for 30 sec) whole cow's milk with addition of kid rennet at 36-38°C. The fermentation is carried out for 4-10 h by addition of Natural Whey Cultures (NWCs) arising from the previous manufacture according to the traditional back-slopping procedure. The fermentation is stopped empirically when the curd is ready to be stretched in hot water. After stretching, the cheese is flask-like shaped in 1-2.5 kg size, cooled in water, salted in brine for at least 6 h, hanged, air dried and ripened. Ripening has to be at least 1 month long according to the PDO, but it can be longer. NWC from Caciocavallo Silano cheese manufactures were object of an extensive study (Ercolini et al., 2008) and were characterized by *Streptococcus thermophilus*, *Lactobacillus delbrueckii* and *Lb. helveticus*, while *Lactococcus lactis* occurred rarely. Other studies have focused on the microbiology and/or biochemistry of intermediates of production and final products, describing caciocavallo cheeses different from Silano (Coppola et al., 2003; Corsetti et al., 2001; De Pasquale et al., 2014; Gobbetti et al., 2002; Piraino et al., 2005). The final products were found to be dominated by mesophilic non-starter lactic acid bacteria (NSLAB) such as *Lb. plantarum*, *Lb. casei/paracasei*, *Lb. fermentum*, *Lb. buchneri/parabuchneri* (Coppola et al., 2003; Corsetti et al., 2001; Gobbetti et al., 2002; De Pasquale et al., 2014; Piraino et al., 2005). During the manufacture of pasta filata cheeses, the main role of starter cultures is to synthesize enough lactic acid to demineralize and transform the curd into the state that undergoes stretching in hot water at the target pH. Primary starters (mainly thermophilic LAB) provide the most significant contribution to the curd acidification, typically attaining densities higher than 10^8 CFU/g and declining throughout ripening (Gobbetti et al., 2007). This decline marks the beginning of a microbial succession, which should involve the appearance of adventitious microorganisms, mainly represented by NSLAB (Gobbetti et al., 2002; Gobbetti et al., 2007; Quigley et al., 2011), mesophilic homo- and facultatively hetero-fermentative lactobacilli (Gobbetti et al., 2002; Quigley et al., 2011). They often derive from raw milk (Berthier et al., 2001) or from the dairy environment and equipment surfaces (Somers et al., 2001) and play a pivotal role during the ripening of raw milk cheeses, thanks to their proteolytic and lipolitic activity (Di Cagno et al., 2006; Fröhlich-Wyder et al., 2013; Morea et al., 2007; Quigley et al., 2011). Understanding microbial behavior during cheese ripening is a pivotal step in order to ensure safety and quality (Montel et al., 2014); this goal now can be achieved through the study of the expression of the entire pool of microbial genes directly *in situ* in the cheese matrix. However, the application of the metatranscriptome approach in food microbiology is still underexploited.

In this study, an RNA-based approach was used to study the microbiota and the metatranscriptome of Caciocavallo Silano cheese during the different stages of manufacture and ripening and to evaluate how the microbiota and its activities can be modified through the manipulation of the ripening conditions. An exploratory experiment was carried out in order to evaluate how to intervene on the technological parameters in order to speed up the ripening, analyzing intermediate of production and cheese samples up to 60 days of ripening. Furthermore, a second experiment was carried out, modifying the relative humidity (RH) and temperature and monitoring the microbiome up to 30 days.

2.6.2 Materials and methods

2.6.2.1 Cheese manufacturing and sampling

The cheese manufacturing and ripening was carried out at the Campolongo dairy, Montesano S.M. (SA), Italy, following the PDO regulation. In a first experiment, raw and thermized cow milk, NWC, curd after 5 h of incubation and before streching (when the pH reached ca. 5.25), cheeses after molding, after brining and during the ripening (at 10, 20, 30 and 60 days) were collected and analysed through 16S rRNA sequencing and RNA-seq (**Table 2.6.1**). In a second experiment, in order to evaluate the effect of ripening parameters on the microbiome, we decided to ripe caciocavallo cheeses from the same manufacturing day in three different conditions: a control ripening (A) by using the standard conditions of the dairy (16 °C and 75% RH), increasing the temperature to 20 °C (B) or decreasing the RH to 65% (C). In this second experiment, the ripened cheeses were collected up to 30 days. 16S rRNA pyrosquencing was carried out on all the samples (raw and thermized milk, NWC, curd before stretching, cheese after

molding, cheese after brining and after 10, 20 and 30 days of ripening), while the metatranscriptome was analysed only on selected samples (**Table 2.6.1**). All samples were transported to the laboratory in thermal plastic bags under refrigerated conditions (ca. 4°C), after the addition of RNA later (Ambion) in a ratio 1:6. Then, they were stored at -80 °C til the RNA extraction. For the cheeses, samples of core (the inner part, collected in the middle of the cheese) and crust (the outer part, 1-2 cm thick, after peeling the most external layer) were cut in sterile conditions and analysed separately. Water activity (a_w, water readily available for microbial metabolic activities) was measured on samples of cheese core and crusts by using a HygroPalm23-AW (Rotronic AG, Basserdorf).

2.6.2.2 Samples preparation and nucleic acids extraction

Samples stored in RNA later were homogenized and 10 ml of the mixture was centrifuged for 10 min at 4 °C (12,000 x g). Two aliquots were centrifuged for each sample. The pellet was washed twice in PBS (Phosphate-Buffered Saline) solution, the two aliquots were merged and mixed and both DNA and RNA extractions were carried out.

DNA extraction was carried out by using the Biostic Bacteremia DNA isolation kit (Mo Bio Laboratories), while total RNA was extracted using the RNA Microbiome kit (Mo Bio Laboratories), according to the manufacturer's instructions. Both DNA and RNA extractions were carried out in duplicate and the samples were pooled. For RNA samples, DNA was removed by a treatment with TURBO-DNase (Ambion) for 3 h at 37 °C. The absence of DNA was checked by PCR and the treatment repeated if necessary. The quality of the RNA was checked by agarose gel electrophoresis and by the 2100 Bioanalyzer (Agilent Technologies). The Qubit and the Qubit RNA Assay or the Qubit dsDNA BR Assay Kits (Life Technologies) were used to quantify RNA and DNA, respectively.

2.6.2.3 DNA and cDNA library preparation for 16S rRNA pyrosequencing

Complimentary DNA (cDNA) was synthetized by using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems), starting from 200 ng of total RNA. The microbial diversity was studied by pyrosequencing of the amplified V1–V3 region of the 16S rRNA by using primers Gray27f 5'-TTTGATCNTGGCTCAG and Gray519r 5'-GTNTTACNGCGGCKGCTG amplifying a fragment of 520 bp. 454-adaptors were included in the forward primer followed by a 10 bp sample specific Multiplex Identifier (MID). Each PCR mixture (final volume, 50 µL) contained 50 ng of template DNA or cDNA, 0.4 µM of each primer, 0.50 mmol/L of each deoxynucleoside triphosphate, 2.5 mmol/L $MgCl_2$, 5 µL of 10X PCR buffer and 2.5 U of native Taq polymerase (Invitrogen). 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), quantified using the QuantiFluor™ (Promega) 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) according to the manufacturer's instructions by using a Titanium chemistry.

2.6.2.4 Ribosomal RNA depletion, cDNA synthesis and library preparation for whole metatranscriptome sequencing

Metatranscriptome was studied for all the samples from the first experiment and for selected samples for the second experiment (**Table 2.6.1**). Based on the results of the first experiment, the samples selected for RNA-seq in the second experiment were: core and crust of the cheese after brining (t0), at 10 and 30 days for the condition A (control ripening) and at 10, 20 and 30 days for the condition B (ripening at higher temperature). Two biological replicates were sequenced for all samples. Ribosomal RNA (rRNA) was depleted by using the Ribo-Zero Magnetic kit (Epicentre) and purified by Agencourt RNAClean XP (Beckman Coulter) following the manufacturer's instruction. Then, library preparation and sample multiplexing were carried out by using the ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre), following the manufacturer's instruction (insert size around 300 bp). cDNA sequencing was carried out on a NextSeq 500 Sequencer (Illumina) with the Mid Output Kit (Illumina), yielding to 150 bp single end reads.

2.6.2.5 Bioinformatics and data analysis

The pipeline applied for the 16S rRNA amplicons data analysis was the following: 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). In order to guarantee a higher level of accuracy in terms of Operational Taxonomic Unit (OTU) detection, after the split library script performed by QIIME, the reads were excluded from the analysis if they had an average quality score lower than 25, if they were shorter than 300 bp and if there were ambiguous base calls. Sequences that passed the quality filter were denoised (Reeder and Knight, 2010) and singletons were excluded. OTUs defined by a 99% of similarity were picked using the uclust pipeline (Edgar, 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 using the Greengenes 16S rRNA gene database (McDonald et al., 2012).

The whole metatranscriptome data analysis was carried out as follows: raw reads quality was evaluated by using the FastQC toolkit (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adaptor and primer contamination was elminated with CutAdapt (Martin, 2011). Then, low quality bases were trimmed and reads shorter than 60 bp were discarded with the SolexaQA++ software (Cox et al., 2010). Reads were aligned to a reference database by using Bowtie2 (Langmead & Salzberg, 2012) in end-to-end, sensitive mode. The database used was built downloading the protein coding portions of the genomes (.ffn files) from the NCBI RefSeq database (ftp://ftp.ncbi.nlm.nih.gov/genomes/ASSEMBLY_BACTERIA/) and from http://patricbrc.org/portal/. The species included were chosen according to the 16S sequencing results and picking species commonly found in dairy environment or common contaminant of raw materials (reported in **Table 2.6.3**). The concatenated .ffn files were aligned against the the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2014) version April 2011 by using mblastx (Davis et al., 2013) in order to obtain the functional annotation and the gene taxonomy. The number of reads mapped to each gene in the database was extracted by using SAMtools (Li et al., 2009) and normalized according to the library size using custom scripts built under R environment (www.r-project.org). Statistical analysis and plotting were carried out in R environment. Differential gene expression analysis was done by using the Bioconductor package DESeq (Love et al., 2014) and raw P-values were adjusted for multiple testing using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995).

2.6.3 Results

2.6.3.1 16S rRNA pyrosequencing

The microbiota composition during manufacturing and ripening was studied through pyrosequencing of the amplified V1-V3 region of the 16S rRNA, using both DNA and cDNA as template. The overall correlation between DNA and cDNA matrices was high (Spearman's rho = 0.63, P<0.00001) and the results of both templates showed an overall simplicity of the microbiota in all the intermediates of production and in the final cheese. The main difference between DNA/cDNA was found in samples of raw and thermized milk: after the thermic treatment, a clear decrease in the abundance of Proteobacteria and Bacteroidetes *phyla* can be observed when using cDNA as template (**Figure 2.6.1**). In particular, the abundance of psychrotrophic bacteria showed the major differences highlighted in clear trends: for example, *Pseudomonas* spp. decreased from 30.6 to 27.6% in DNA and from 39.7 to 16.8 % in cDNA. Moreover, *Lb. delbrueckii* abundance in ripened cheeses was lower in cDNA compared to DNA matrix. Considering the metabolically active population, a clear separation between cheese core and crusts can be seen in the heatplot in **Figure 2.6.2**. The sample clustering was driven by the different abundance of Firmicutes species, in particular mesophilic lactobacilli, more abundant in the cheese core. Zooming in the population of lactobacilli (**Figure 2.6.3**), NSLAB, mainly *Lb. casei* and *Lb. buchneri* groups were found after 10 days of ripening, their abundance increased during the ripening and it was higher in the cheese core compared to the crust. Higher abundance of NSLAB was found changing the ripening conditions (15.3% in condition B and 17.2% in C vs 8.3% in the control ripening A at 10 days). Cheeses ripened at lower RH showed a higher abundance of NSLAB after 10 days, but then their abundance decreased. *Lb. fermentum* (not detected in the control up to 1 month) was found in condition B already at 10 days.

2.6.3.2 Metatranscriptome sequencing

The results of the first experiment showed that the curd and the sample of cheese after molding and brining were characterized by the prevalence of genes involved in carbohydrates metabolism (data not

shown). During the ripening, an opposite trend was observed in cheese core and crust: the crusts showed an up-regulation of carbohydrates metabolism and activities related to genetic information processing and cellular processes (**Figure 2.6.4**). On the contrary, pathways related to aminoacid metabolisms were overexpressed in the cheese core, increasing in abundance during the ripening.

Based on the results of the first experiment and of the 16S rRNA sequencing, we decided to study the metatranscriptome of cheese core and crust ripened in condition A (control) and B (higher temperature) from t0 up to 30 days. A separate clustering of core and crust driven by amino acid vs carbohydrates metabolisms was observed also in the second experiment (**Figure 2.6.5**). Pathways related to energy production from carbohydrates (penthose-phosphate pathway, glycolysis) had higher expression on the crust, while aminoacid and lipid metabolisms prevailed in the core and increased during ripening (**Figure 2.6.5**). Considering the annotation at gene level, we selected activities related to protein and carbohydrates catabolism, particularly important for cheese ripening. Genes related to Leloir pathway of galactose degradation were over-expressed compared to the tagatose-6-phosphate pathway (**Figure 2.6.6**). Enzymes responsible for lactose break-down (*lacZ*, EC 3.2.1.23 and *lacG*, EC 3.2.1.85) reached maximum expression in the cheese core at t0, while decreasing during ripening. In particular, they had higher expression in the core of the cheese ripened in condition A compared to B after 10 days (**Figure 2.6.6**). Enzymes leading to acetoin and diacetyl production were over-expressed on the crust and reached higher values in condition B vs A (**Figure 2.6.7**). Moreover, a number of peptidases, amino acid and peptide permeases and genes involved in amino acid catabolic pathways were over-expressed in the core of cheeses ripened at higher temperature (**Figure 2.6.8**). The DESeq analysis identified 649 genes differentially expressed ($P < 0.05$) between the cheese cores ripened in condition A and B, regardless the ripening time (**Table 2.6.4**). Among them, the protease *degP*, the peptidases *pepA*, *pepB*, *pepN*, *pepD*, the dipeptide transporters *dppB* and *dppD*, the aminoacid permeases *livM* and *proW* and some aminotransferases, dehydrogenases, decarboxylases and lyases were over-expressed in the core of samples B compared to A (**Table 2.6.4**). The taxonomic assignment of the genes belonging to aminoacid metabolisms revealed that most of them were assigned to *Lb. casei* in the core samples (**Figure 2.6.9**). In addition, genes assigned to NSLAB (*Lb. buchneri, Lb. plantarum, Lb. gasseri, Lb. fermentum, Lb. rhamnosus*) and *Leuconostoc kimchii*, *Leuc. mesenteroides*, *Leuc. citreum*, *Pediococcus pentosaceus* clearly increased in condition B compared to A.

2.6.4 Discussion

The ripening process of cheese is very complex and involves microbiological and biochemical changes to the curd resulting in the flavour and texture characteristic of the particular variety. Moreover, different dynamics occur in the cheese core and crust, due to the different environmental conditions (a_w, NaCl concentration, O_2 availability) (Montel et al., 2014). In fact, a_w values found on the cheese crust were significantly lower than the core, at all ripening times (**Table 2.6.2**). The different dynamics occurring in the core and on the crust were extremely evident from both the taxonomic and the metatranscriptomic profiles of both the experiments carried out in this study, where cheese core and crust samples clearly showed different microbiome structure and functionality. Microbiological changes in the cheese during ripening include the death and lysis of the starter cells and the growth of NSLAB (Beresford & Williams, 2004), which are generally responsible of secondary proteolysis during cheese ripening. Residual lactose is metabolized quickly to L-lactate during the early stages of ripening at a rate largely determined by temperature and the salt-in-moisture (S/M) levels (Turner & Thomas 1980, Thomas & Pearce, 1981). At low S/M, lactose disappears after one week, while at average S/M levels (4-5), residual lactose is found after 4 weeks (Thomas & Pearce, 1981). This is in agreement with the transcriptome results; in fact, we found higher levels of enzymes involved in lactose break-down on the crust at 10 days compared to the core (**Figure 2.6.6**), where a lower a_w was found. In the cheese core, in more suitable conditions, the lactose break-down was probably quicker and mainly took place before 10 days. Accordingly, comparing the cheese core in condition A (standard) and B (higher temperature) at 10 days, we found a higher β-galactosidase (*lacZ*, EC 3.2.1.23) expression in condition A (**Figure 2.6.6**). Also in this case, it can be hypothesized that the lactose break-down took place before 10 days of ripening in condition B. Moreover, the lactate dehydrogenase (*ldh*, EC 1.1.1.27), responsible of the conversion of pyruvate to L-lactate, showed a similar expression pattern. Lactose is hydrolysed by starter cultures which produce glucose and galactose. Glucose is then oxidised to pyruvate by the Emden-Meyerhof pathway of glycolysis. Galactose is converted through the Leloir pathway to glucose-6-P and through the tagatose 6P pathway to glyceraldehyde-3-P (Marillay & Casey, 2004). We found higher levels of genes involved in Leloir compared to the tagatose pathway (**Figure 2.6.6**). Accordingly, the first one is known as the main way for galactose utilization in lactobacilli (Hickey et al., 1986; Turner & Martley, 1983), while *S. thermophilus* strains were often reported as Gal negative, even if Gal positive strains exists (de Vin et al., 2005).

Pyruvate, coming from glycolysis or penthose-phosphate pathway, is the starting compound for the formation of short-chain flavour molecules such as diacetyl, acetoin, acetate, acetaldehyde and ethanol (Henriksen & Nilsson, 2001; Syu, 2001; Melchiorsen et al., 2002). Our results suggest that the genes involved in acetoin production from pyruvate were over-expressed on the cheese crust and were enhanced by higher temperature (**Figure 2.6.7**; **Table 2.6.4**), while the butanediol dehydrogenase (EC 1.1.1.4), that convert acetoin in 2,3-butanediol, was not detected.

During the ripening, NSLAB (*Lb. casei* group, *Lb. buchneri*/*parabuchneri, Lb. fermentum*) increased in abundance from t0 to 30 days, reaching higher values in the cheese core, where higher a_w values and lower O_2 concentration created a more suitable environment for lactobacilli growth. NSLAB adapt to the lack of fermentable carbohydrates, low pH and a_w, and to the presence of bacteriocins, which altogether make hostile the environmental conditions during cheese ripening (De Pasquale et al., 2014). Moreover, higher NSLAB concentrations were found in the core of the cheeses ripened in condition B (**Figure 2.6.3**), showing that the higher temperature had an important effect in promoting their growth. The higher abundance of NSLAB in the cheese core compared to the crust and in the cheese ripened at higher temperature compared to the standard condition, was associated to an increased expression of genes related to proteolysis and amino acid catabolism (**Figure 2.6.8**). Moreover, the higher sensibility of the shotgun sequencing allowed the detection of genes belonging to NSLAB species not detected through 16S rRNA gene sequencing (**Figure 2.6.9**). Higher temperature caused a switch in the protein degradation and amino acid catabolism (**Figure 2.6.9**), enhancing *Lb. casei* metabolism, besides to other NSLAB. Their role during secondary proteolysis of cheeses was largely described. Usually, the use of NSLAB as adjunct cultures increased the level of peptides and FAA, which enhanced flavor intensity and accelerated cheese ripening (Fox et al., 2000; Gobbetti et al., 2007; Lynch et al., 1996; Lynch et al., 1999; Courtin et al., 2002). Moreover, the use of attenuated adjunt cultures of *Lb. casei*, *Lb. paracasei* and *Lb. plantarum* was shown to accelerate the ripening in Caciocavallo Pugliese cheese (Di Cagno et al., 2012). Gobbetti et al. (2002) reported an increase in NSLAB (*Lb. paracasei* and *Lb. parabuchneri*) in Caciocavallo Pugliese cheese during ripening, associated to higher levels of proteolysis, in particular in the inner part of the cheese. *Lb. casei* group and *Lb. parabuchneri* were also reported by De Pasquale et al. (2014) as correlated to free amino acid concentration and volatile organic compounds produced from amino acid catabolism in Caciocavallo cheese. Proteolysis is undoubtedly the most important biochemical process for flavour and texture formation in hardtype and semi-hard-type cheeses (van Kranenburg et al., 2002). Degradation of caseins by the activities of rennet enzymes, and the cell-envelope proteinase and peptidases from LAB yields small peptides and free aminoacids. LAB also possess peptide and amino-acid transport systems. Various aminoacid transport systems have been identified with a high specificity for structurally similar aminoacids, e.g. Glu/Gln, Ser/Thr, Ala/Gly, Lys/Arg/Orn, branched chain (Ile/Leu/Val), and aromatic (Phe/Tyr/Trp) residues (Konings et al., 1989). In this study, the peptidases *pepN* (EC 3.4.11.2), *pepA* (EC 3.4.11.1), *pepB* (EC 1.5.1.38), *pepD* (EC 3.4.11.23) and *degP* (EC 3.4.21.107) increased in the cheese core during the ripening and showed significantly higher levels of expression in condition B (**Table 2.6.4**), as well as *livM* (EC 2.6.1.42), *dppD* (EC 3.6.3.-), *dppB* (EC 3.6.3.-) and *proW* (EC 2.3.3.8), involved in aminoacid and dipeptide transport (**Figure 2.6.8**, **Table 2.6.4**). Free amino acids are the substrate for enzymatic reactions leading to several compounds important for cheese flavor. They can be converted in many different ways by enzymes such as deaminases, decarboxylases, transaminases (aminotransferases), and lyases (van Kranenburg et al., 2002; Marilley & Casey, 2004). Transamination of aminoacids results in the formation of α-keto acids that can be converted into aldehydes by decarboxylation and, subsequently, into alcohols or carboxylic acids by dehydrogenation. Many of these compounds are major aroma components. Direct dehydrogenation of α-keto acids results in the formation of hydroxy-acids, which do not contribute to the flavour of the product. Although analysis of volatile compounds was not carried out in this study, many aminotransferases, lyases, decarboxylases, dehydrogenases encoding genes have been found, with higher abundance in cheese ripened in condition B (**Figure 2.6.8**), suggesting that the higher temperature possibly enhance the producton of flavoring compounds thanks to the promoting effect on NSLAB growth, as suggested by Lynch et al. (1996; 1999), reporting that the addition of NSLAB in cheddar cheese caused a flavour improvement, due to the increased formation of amino acids.

In this study, we used an RNA-seq approach in order to obtain a complete picture of bacterial activities during ripening of a typical pasta-filata cheese. Moreover, results obtained suggest that the manipulation of temperature during cheese ripening can be a key factor in order to manipulate the microbiota and its activities, possibly accelerating the ripening.

2.6.5 References

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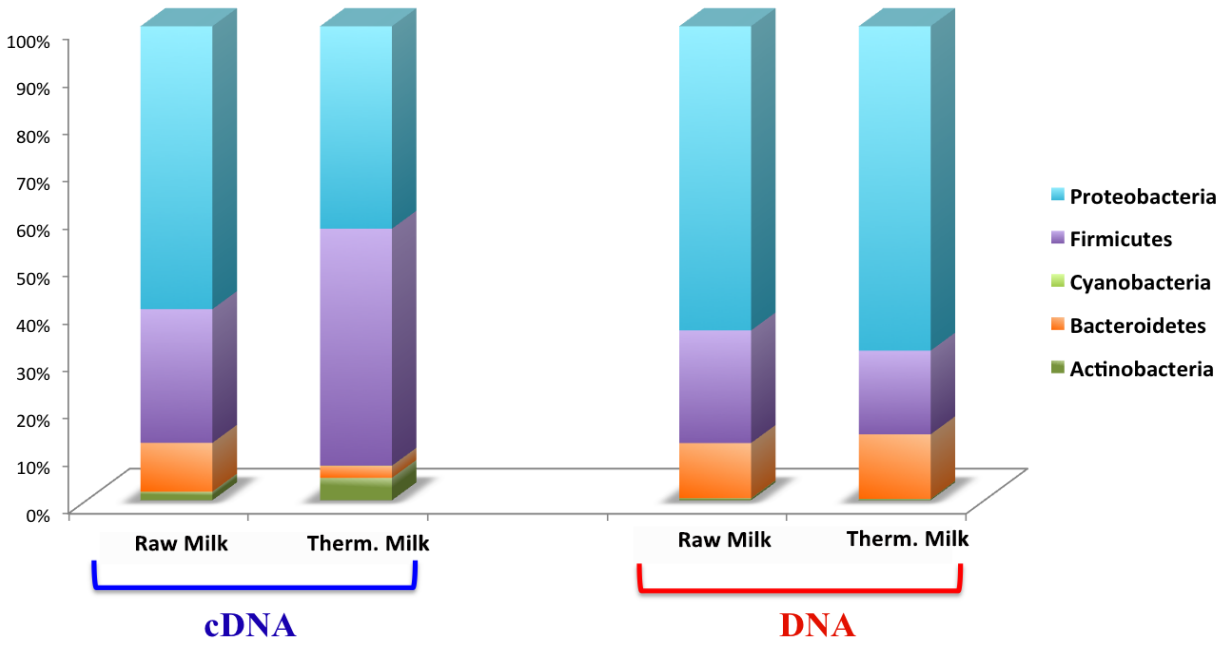

Figure 2.6.1 Abundance of baterial *phyla* identified in raw and thermized milk through pyrosequencing of cDNA or DNA.

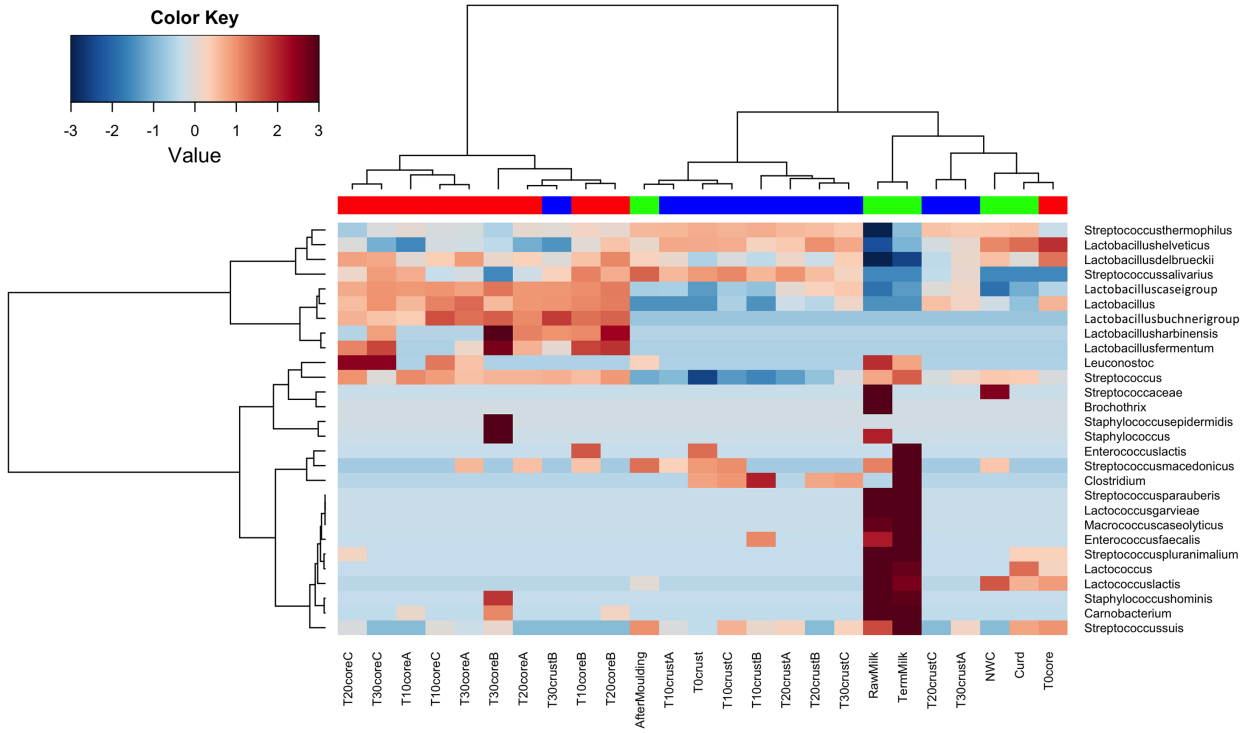

Figure 2.6.2 Hierarchical Ward-linkage clustering based on the Spearman correlation coefficients of the proportion of OTUs belonging to the Firmicutes *phylum*. Only OTUs with abundancr > 0.1% in at least 1 sample were included. The color scale represents the scaled abundance of each variable, denoted as Z-score, with red indicating high abundance and blue indicating low abundance. Column bar is coloured according to sample type: red, cheese core; blue, cheese crust; green, intermediates of production.

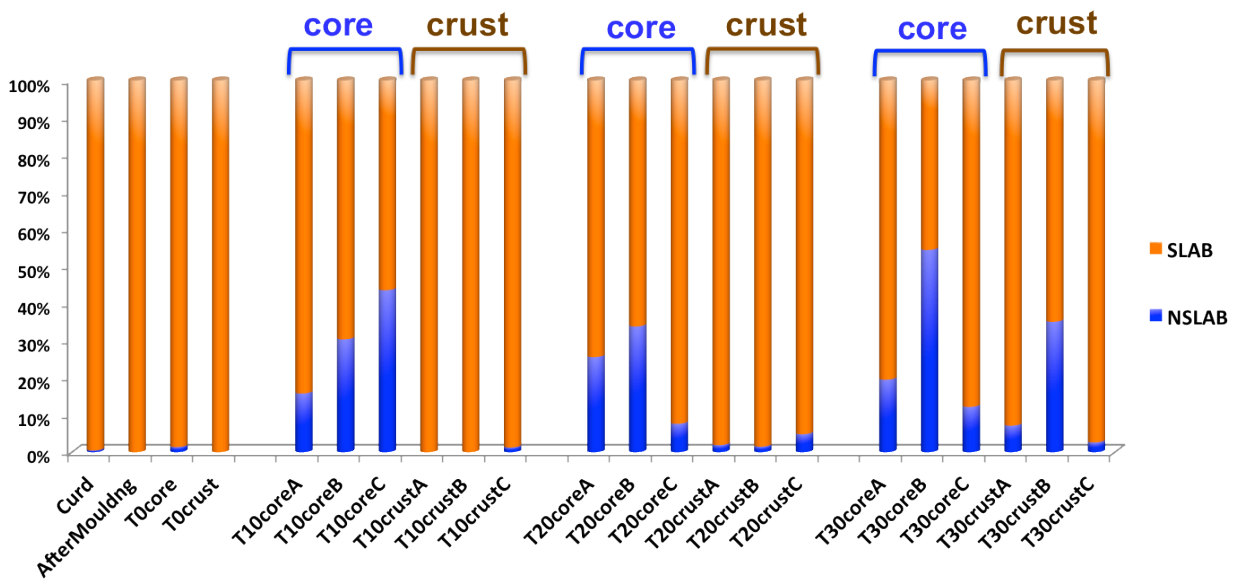

Figure 2.6.3 Abundance of starter and non-starter lactobacilli in the intermediates of production and in the ripened cheeses analysed in this study. Abundance was recalculated after subtracting reads of non-*Lactobacillus* species.

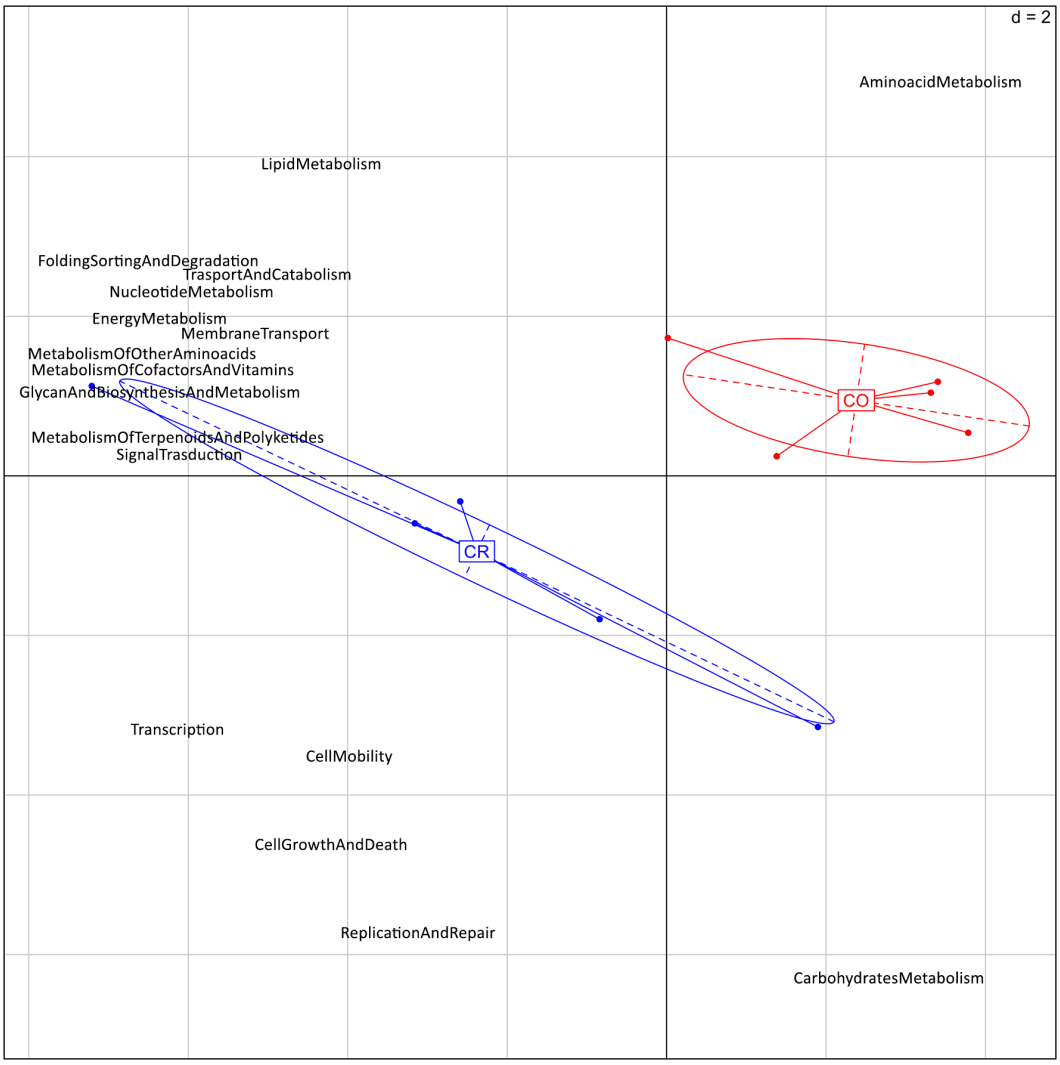

Figure 2.6.4 PCA of the abundance of the KEGG annotations at level 2 of hierachy of the samples of cheese core and crust from the first experiment carried out in this study. The first component (horizontal) accounts for the 65.7 % of the variance and the second component (vertical) accounts for the 12.2%.

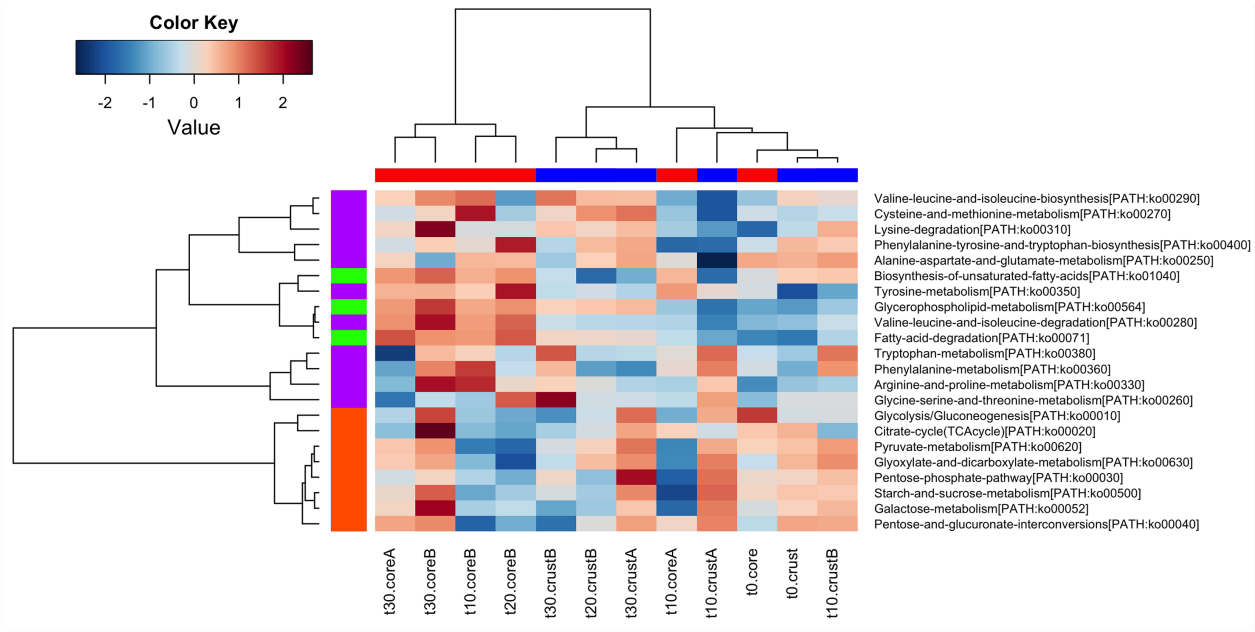

Figure 2.6.5 Hierarchical Ward-linkage clustering based on the Spearman correlation coefficients of the proportion of the KEGG pathways belonging to carbohydrates (orange), amino acids (violet) and lipids (green) metabolisms. Column bar is color-coded as follows: red, samples of cheese cores; blue, samples of cheese crusts. The color scale represents the scaled abundance of each variable, denoted as Z-score, with red indicating high abundance and blue indicating low abundance.

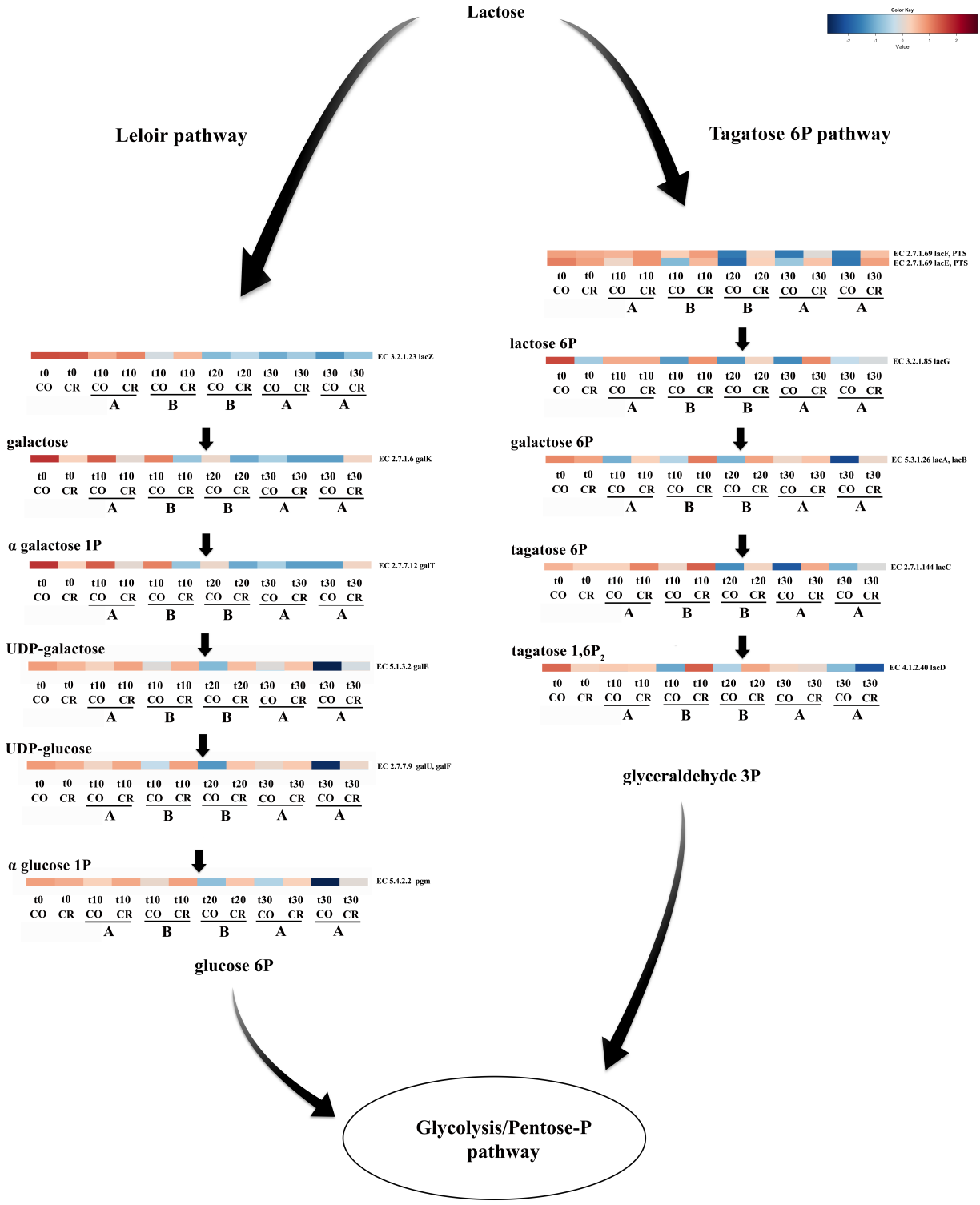

Figure 2.6.6 Galactose catabolism through Leloir and tagatose 6P pathways with related expression data in the samples of cheese core and crust analysed in the second experiment. Abbreviations: CO, core; CR, crust; A, control ripening; B, ripened at high temperature.

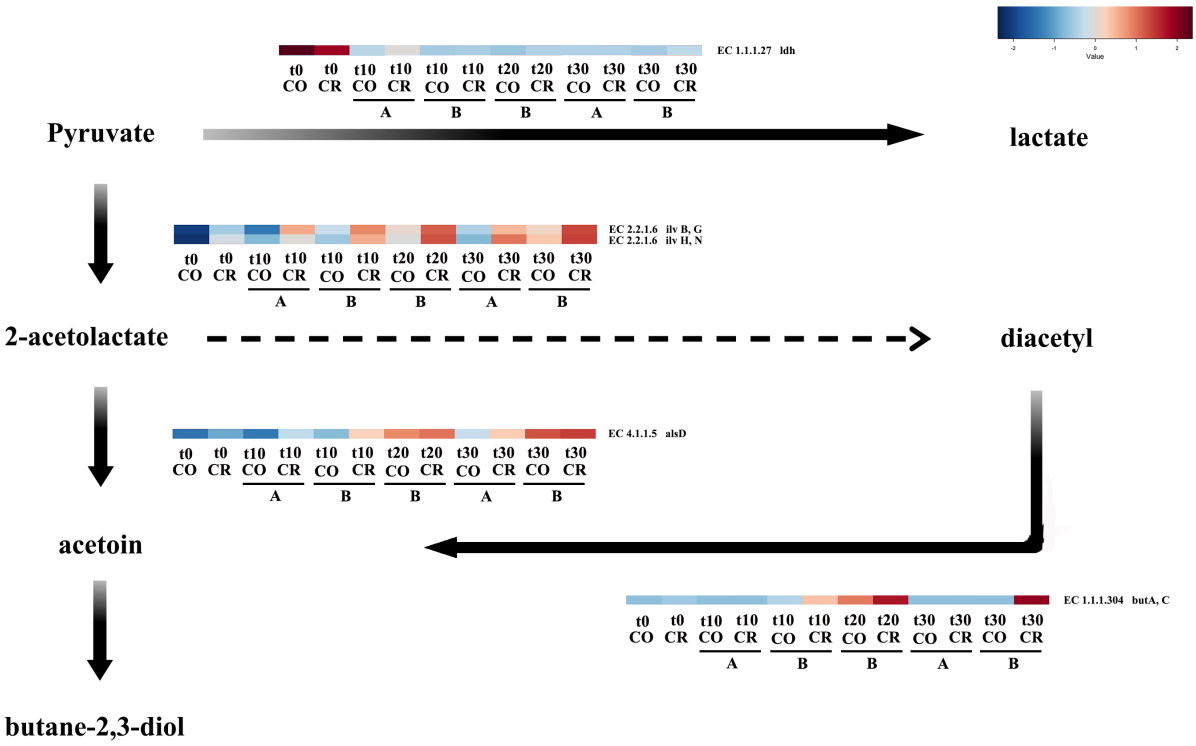

Figure 2.6.7 Pyruvate catabolism pathways leading to acetoin and lactate production, with related expression data in the samples of cheese core and crust analysed in the second experiment. Abbreviations: CO, core; CR, crust; A, control ripening; B, ripening at high temperature. Dashed line indicates chemical reaction.

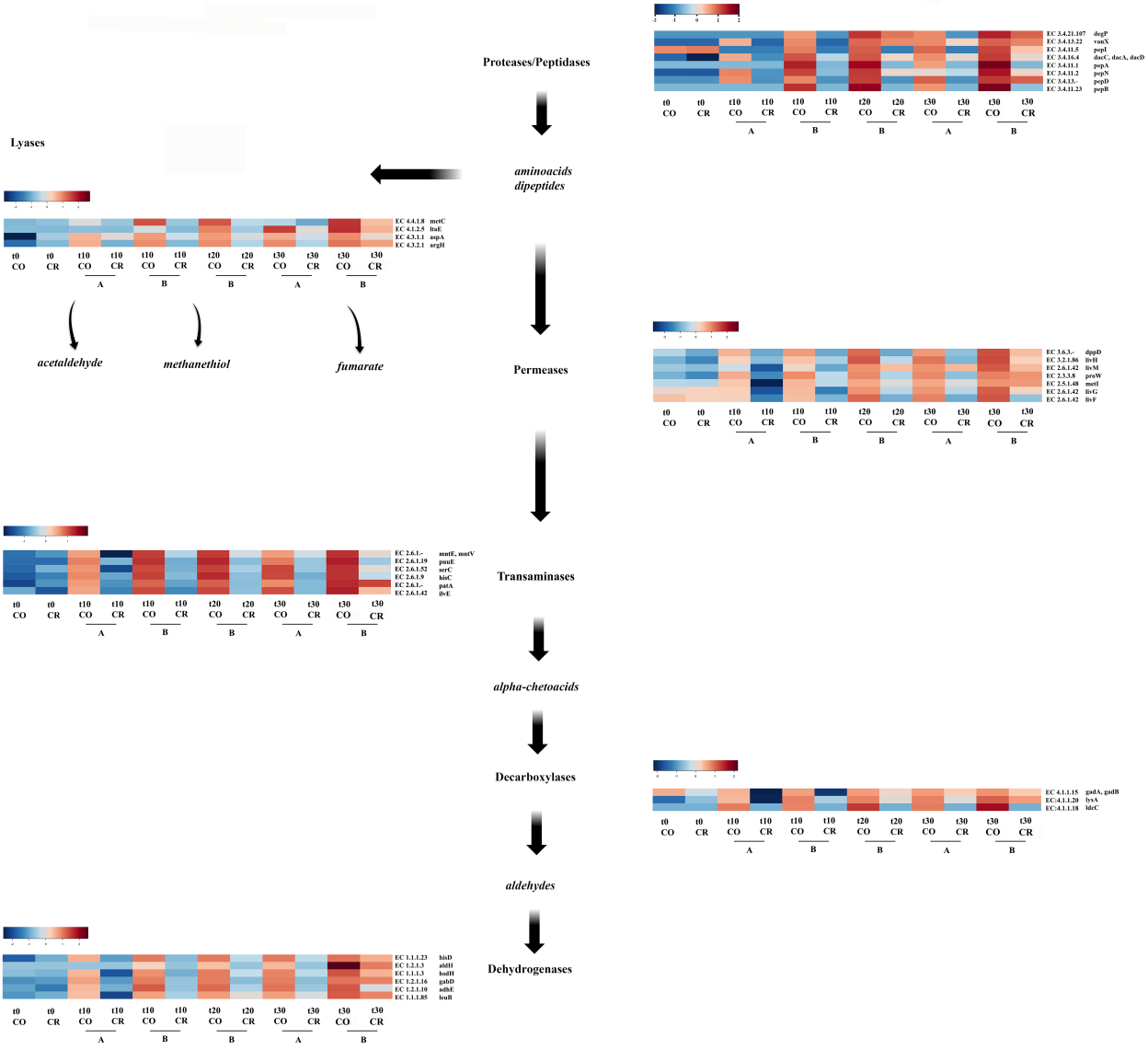

Figure 2.6.8 Genes related to proteolysis and aminoacids catabolism pathways leading to flavour compounds, with related expression data in the samples of cheese core and crust analysed in the second experiment. Abbreviations: CO, core; CR, crust; A, control ripening; B, ripening at high temperature.

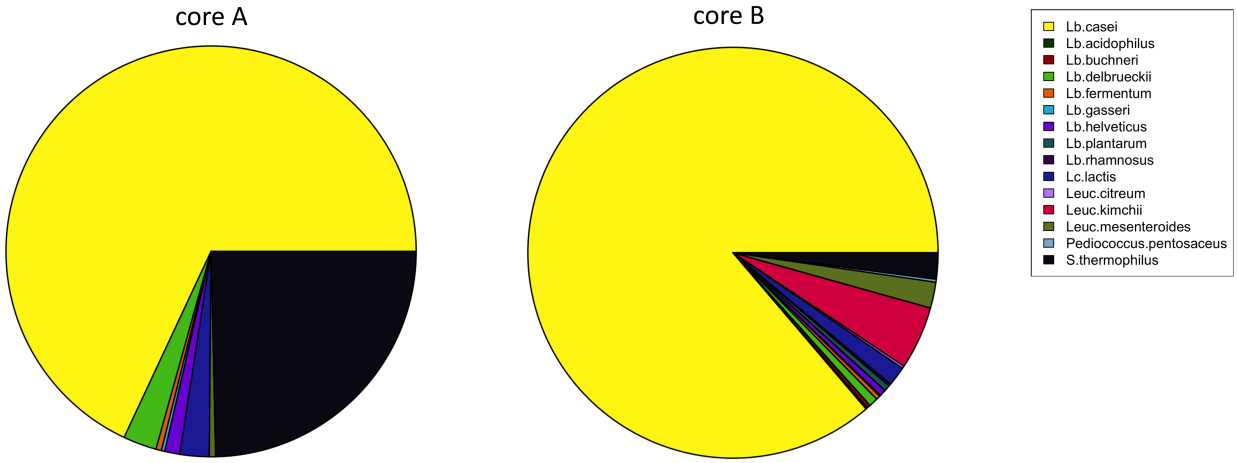

Figure 2.6.9 Taxonomic assignment of the genes belonging to aminoacid metabolisms in the samples of cheese core, regardless the time of ripening. Only species belonging to Firmicutes are reported. A, control ripening; B, ripening at high temperature.

Table 2.6.1 Sample codes and description.

Sample ID	Description	Experiment	Ripening conditions	16S pyrosequencing	RNA-seq
RM.1	Raw Milk	1	NA	yes	yes
TM.1	Thermized Milk	1	NA	yes	yes
NWC.1	Naural Whey Culture	1	NA	yes	yes
Cu.1	Curd before strechnig	1	NA	yes	yes
After.mol.1	Cheese after molding	1	NA	yes	yes
t0.co.1	Cheese after brining and drying, before entering the ripening cell - core	1	NA	yes	yes
t0.cr.1	Cheese after brining and drying, before entering the ripening cell - crust	1	NA	yes	yes
t10.co.1	Ripened cheese at 10 days - core	1	Standard	yes	yes
t10.cr.1	Ripened cheese at 10 days - crust	1	Standard	yes	yes
t20.co.1	Ripened cheese at 20 days - core	1	Standard	yes	yes
t20.cr.1	Ripened cheese at 20 days - crust	1	Standard	yes	yes
t30.co.1	Ripened cheese at 30 days - core	1	Standard	yes	yes
t30.cr.1	Ripened cheese at 30 days - crust	1	Standard	yes	yes
t60.co.1	Ripened cheese at 60 days - core	1	Standard	yes	yes
t60.cr.1	Ripened cheese at 10 days - crust	1	Standard	yes	yes
RM.2	Raw Milk	2	NA	yes	yes
TM.2	Thermized Milk	2	NA	yes	yes
NWC.2	Naural Whey Culture	2	NA	yes	yes
Cu.2	Curd before strechnig	2	NA	yes	yes
After.mol.2	Cheese after molding	2	NA	yes	yes
t0.co.2	Cheese after brining and drying, before entering the ripening cell - core	2	NA	yes	yes
t0.cr.2	Cheese after brining and drying, before entering the ripening cell - crust	2	NA	yes	yes
t10.coA.2	Ripened cheese at 10 days - core	2	Standard	yes	yes
t10.coB.2	Ripened cheese at 10 days - core	2	Higher temperature	yes	yes
t10.coC.2	Ripened cheese at 10 days - core	2	Lower UR	yes	no
t10.crA.2	Ripened cheese at 10 days - crust	2	Standard	yes	yes

t10.crB.2	Ripened cheese at 10 days - crust	2	Higher temperature	yes	yes
t10.crC.2	Ripened cheese at 10 days - crust	2	Lower UR	yes	no
t20.coA.2	Ripened cheese at 20 days - core	2	Standard	yes	no
t20.coB.2	Ripened cheese at 20 days - core	2	Higher temperature	yes	yes
t20.coC.2	Ripened cheese at 20 days - core	2	Lower UR	yes	no
t20.crA.2	Ripened cheese at 20 days - crust	2	Standard	yes	no
t20.crB.2	Ripened cheese at 20 days - crust	2	Higher temperature	yes	yes
t20.crC.2	Ripened cheese at 20 days - crust	2	Lower UR	yes	no
t30.coA.2	Ripened cheese at 30 days - core	2	Standard	yes	yes
t30.coB.2	Ripened cheese at 30 days - core	2	Higher temperature	yes	yes
t30.coC.2	Ripened cheese at 30 days - core	2	Lower UR	yes	no
t30.crA.2	Ripened cheese at 30 days - crust	2	Standard	yes	yes
t30.crB.2	Ripened cheese at 60 days - crust	2	Higher temperature	yes	yes
t30.crC.2	Ripened cheese at 10 days - crust	2	Lower UR	yes	no

NA: not applicable
A, standard: ripening at 16°C and 75% UR
B, higher temperature: ripening at 20°C and 75% UR
C, lower UR: ripening at 16°C and 65% UR
1, first experiment
2, second experiment

Table 2.6.2 Water activity values measured in samples of cheese core and crust in the two experiments carried out in this study.

Sample	a_w	Experiment
t0.cr.1	0.968	1
t10.cr.1	0.962	1
t20.cr.1	0.938	1
t30.cr.1	0.903	1
t0.co.1	0.972	1
t10.co.1	0.963	1
t20co.1	0.960	1
t30.co.1	0.956	1
t0.cr.2	0.969	2
t10.crA.2	0.950	2
t20.cr.A.2	0.937	2
t30.crA.2	0.907	2
t10.crB.2	0.940	2
t20.crB.2	0.927	2
t30.crB.2	0.890	2
t10.crC.2	0.933	2
t20.crC.2	0.905	2
t30.crC.2	0.872	2
t0.co.2	0.970	2
t10.coA.2	0.961	2
t20.co.A.2	0.961	2
t30.coA.2	0.951	2
t10.coB.2	0.959	2
t20.coB.2	0.958	2
t30.coB.2	0.951	2
t10.coC.2	0.953	2
t20.coC.2	0.950	2
t30.coC.2	0.950	2

Table 2.6.3 Bacterial genomes downloaded to build the reference database used in this study.

Genomes	NCBI genome ID
downloaded from the NCBI RefSeq database	
Enterobacter aerogenes KCTC 2190	NC_015663.1
Enterobacter cloacae EcWSU1	NC_016514.1
Enterobacter cloacae EcWSU1, plasmid pEcWSU1_A	NC_016515.1
Enterobacter cloacae SCF1	NC_014618.1
Enterobacter cloacae subsp. *cloacae* ATCC 13047	NC_014121.1
Enterobacter cloacae subsp. *cloacae* ATCC 13047, plasmid pECL_A	NC_014107.1
Enterobacter cloacae subsp. *cloacae* ATCC 13047, plasmid pECL_B	NC_014108.1
Enterococcus faecalis V583	NC_004668.1
Enterococcus faecalis V583, plasmid pTEF1	NC_004669.1
Enterococcus faecalis V583, plasmid pTEF2	NC_004671.1
Enterococcus faecalis V583, plasmid pTEF3	NC_004670.1
Erwinia amylovorans CFBP1430	NC_013961.1
Erwinia amylovorans CFBP1430, plasmid pEA29	NC_013957.1
Erwinia billingiae Eb661	NC_014306.1
Erwinia billingiae Eb661, plasmid pEB102	NC_014304.1
Erwinia billingiae Eb661, plasmid pEB170	NC_014305.1
Erwinia pyrifoliae Ep1/96	NC_012214.1
Erwinia pyrifoliae Ep1/96, plasmid pEP03	NC_013264.1
Erwinia pyrifoliae Ep1/96, plasmid pEP05	NC_013265.1
Erwinia pyrifoliae Ep1/96, plasmid pEP2.6	NC_013954.1
Erwinia pyrifoliae Ep1/96, plasmid pEP36	NC_013263.1
Erwinia tasmaniensis Et1/99	NC_010694.1
Erwinia tasmaniensis Et1/99, plasmid pET09	NC_010695.1
Erwinia tasmaniensis Et1/99, plasmid pET35	NC_010696.1
Erwinia tasmaniensis Et1/99, plasmid pET45	NC_010699.1
Erwinia tasmaniensis Et1/99, plasmid pET46	NC_010693.1
Erwinia tasmaniensis Et1/99, plasmid pET49	NC_010697.1
Escherichia coli 536	NC_008253.1
Escherichia coli ATCC 8739	NC_010468.1
Escherichia coli CF073	NC_004431.1
Escherichia coli K12 substr. DH10B	NC_010473.1
Escherichia coli K-12 substr. W3110	NC_007779.1
Escherichia coli O157:H7	NC_002655.2
Lactobacillus acidophilus 30SC	NC_015214.1
Lactobacillus acidophilus 30SC, plasmid pRKC30SC1	NC_015213.1
Lactobacillus acidophilus 30SC, plasmid pRKC30SC2	NC_015218.1
Lactobacillus acidophilus La-14	NC_021181.2
Lactobacillus acidophilus NCFM	NC_006814.3
Lactobacillus brevis ATCC367	NC_008497.1
Lactobacillus brevis ATCC367, plasmid 1	NC_008498.1
Lactobacillus brevis ATCC367, plasmid 2	NC_008499.1
Lactobacillus brevis KB290	NC_020819.1
Lactobacillus brevis KB290, plasmid pKB290-1	NC_020820.1
Lactobacillus brevis KB290, plasmid pKB290-2	NC_020821.1
Lactobacillus brevis KB290, plasmid pKB290-3	NC_020826.1
Lactobacillus brevis KB290, plasmid pKB290-4	NC_020822.1
Lactobacillus brevis KB290, plasmid pKB290-5	NC_020823.1
Lactobacillus brevis KB290, plasmid pKB290-6	NC_020827.1
Lactobacillus brevis KB290, plasmid pKB290-7	NC_020824.1
Lactobacillus brevis KB290, plasmid pKB290-8	NC_020828.1
Lactobacillus brevis KB290, plasmid pKB290-9	NC_020825.1
Lactobacillus buchneri CD034	NC_018610.1
Lactobacillus buchneri CD034, plasmid pCD034-1	NC_016035.1
Lactobacillus buchneri CD034, plasmid pCD034-2	NC_016034.1
Lactobacillus buchneri CD034, plasmid pCD034-3	NC_018611.1
Lactobacillus buchneri NRRL B-30929	NC_015428.1
Lactobacillus buchneri NRRL B-30929, plasmid pLBUC01	NC_015420.1
Lactobacillus buchneri NRRL B-30929, plasmid pLBUC02	NC_015429.1
Lactobacillus buchneri NRRL B-30929, plasmid pLBUC03	NC_015421.1
Lactobacillus casei ATCC334	NC_008526.1
Lactobacillus casei ATCC334, plasmid 1	NC_008502.1
Lactobacillus casei BD-II	NC_017474.1
Lactobacillus casei BD-II, plasmid pBD-II	NC_017476.1

Lactobacillus casei BL23	NC_010999.1
Lactobacillus casei LC2W	NC_017473.1
Lactobacillus casei LC2W, plasmid pLC2W	NC_017475.1
Lactobacillus casei LOCK919	NC_021721.1
Lactobacillus casei LOCK919, plasmid pLOCK919	NC_021722.1
Lactobacillus casei str. Zhang	NC_014334.1
Lactobacillus casei str. Zhang, plasmid plca36	NC_011352.1
Lactobacillus casei W56	NC_018641.1
Lactobacillus casei W56, plasmid pW56	NC_020057.1
Lactobacillus crispatus ST1	NC_014106.1
Lactobacillus delbrueckii subsp. *bulgaricus* 2038	NC_017469.1
Lactobacillus delbrueckii subsp. *bulgaricus* ATCC 11842	NC_008054.1
Lactobacillus delbrueckii subsp. *bulgaricus* ATCC BAA-365	NC_008529.1
Lactobacillus delbrueckii subsp. *bulgaricus* ND02	NC_014727.1
Lactobacillus delbrueckii subsp. *bulgaricus* ND02, plasmid unnamed	NC_014728.1
Lactobacillus fermentum CECT 5716	NC_017465.1
Lactobacillus fermentum F-6	NC_021235.1
Lactobacillus fermentum IFO 3956	NC_010610.1
Lactobacillus gasseri ATCC 33323	NC_008530.1
Lactobacillus helveticus CNRZ32	NC_021744.1
Lactobacillus helveticus DPC 4571	NC_010080.1
Lactobacillus helveticus H10	NC_017467.1
Lactobacillus helveticus H10, plasmid pH10	NC_017468.1
Lactobacillus helveticus R0052	NC_018528.1
Lactobacillus johnsonii FI9785	NC_012552.1
Lactobacillus johnsonii DPC 6026	NC_017477.1
Lactobacillus johnsonii FI9785	NC_013504.1
Lactobacillus johnsonii FI9786, plasmid p9785L	NC_013505.1
Lactobacillus johnsonii N6.2	NC_022909.1
Lactobacillus johnsonii NCC533	NC_005362.1
Lactobacillus kefiranofaciens ZW3	NC_015602.1
Lactobacillus kefiranofaciens ZW3, plasmid pWW1	NC_015598.1
Lactobacillus kefiranofaciens ZW3, plasmid pWW2	NC_015603.1
Lactobacillus paracasei subsp. *paracasei* 8700:2	NC_022112.1
Lactobacillus paracasei subsp. *paracasei* 8700:2, plasmid 1	NC_022114.1
Lactobacillus paracasei subsp. *paracasei* 8700:2, plasmid 2	NC_022123.1
Lactobacillus plantarum 16	NC_021514.1
Lactobacillus plantarum 16, plasmid Lp16A	NC_021515.1
Lactobacillus plantarum 16, plasmid Lp16B	NC_021525.1
Lactobacillus plantarum 16, plasmid Lp16C	NC_021516.1
Lactobacillus plantarum 16, plasmid Lp16D	NC_021526.1
Lactobacillus plantarum 16, plasmid Lp16E	NC_021517.1
Lactobacillus plantarum 16, plasmid Lp16F	NC_021518.1
Lactobacillus plantarum 16, plasmid Lp16G	NC_021527.1
Lactobacillus plantarum 16, plasmid Lp16H	NC_021519.1
Lactobacillus plantarum 16, plasmid Lp16I	NC_021528.1
Lactobacillus plantarum 16, plasmid Lp16L	NC_021520.1
Lactobacillus plantarum JDM1	NC_012984.1
Lactobacillus plantarum subsp. *plantarum* P-8	NC_021224.1
Lactobacillus plantarum subsp. *plantarum* P-8, plasmid LBPp1	NC_021233.1
Lactobacillus plantarum subsp. *plantarum* P-8, plasmid LBPp2	NC_021225.1
Lactobacillus plantarum subsp. *plantarum* P-8, plasmid LBPp3	NC_021226.1
Lactobacillus plantarum subsp. *plantarum* P-8, plasmid LBPp4	NC_021234.1
Lactobacillus plantarum subsp. *plantarum* P-8, plasmid LBPp5	NC_021227.1
Lactobacillus plantarum subsp. *plantarum* P-8, plasmid LBPp6	NC_021228.1
Lactobacillus plantarum subsp. *plantarum* ST-III	NC_014554.1
Lactobacillus plantarum subsp. *plantarum* ST-III, plasmid pST-III	NC_014558.2
Lactobacillus plantarum WCFS1	NC_004567.2
Lactobacillus plantarum WCFS1, plasmid pWCFS101	NC_006375.1
Lactobacillus plantarum WCFS1, plasmid pWCFS102	NC_006376.1
Lactobacillus plantarum WCFS1, plasmid pWCFS103	NC_006377.1
Lactobacillus plantarum ZJ316	NC_020229.1
Lactobacillus plantarum ZJ316, plasmid pLP-ZJ101	NC_021903.1
Lactobacillus plantarum ZJ316, plasmid pLP-ZJ102	NC_021904.1
Lactobacillus plantarum ZJ316, plasmid pLP-ZJ103	NC_021912.1
Lactobacillus reuteri DSM20016	NC_009513.1
Lactobacillus reuteri I5007	NC_021494.1
Lactobacillus reuteri I5007, plasmid pLRI01	NC_021503.1

Lactobacillus reuteri I5007, plasmid pLRI02	NC_021496.1
Lactobacillus reuteri I5007, plasmid pLRI03	NC_021495.1
Lactobacillus reuteri I5007, plasmid pLRI04	NC_021504.1
Lactobacillus reuteri I5007, plasmid pLRI05	NC_021497.1
Lactobacillus reuteri I5007, plasmid pLRI06	NC_021498.1
Lactobacillus reuteri JCM 1112	NC_010609.1
Lactobacillus reuteri SD2112	NC_015697.1
Lactobacillus reuteri SD2112, plasmid pLR580	NC_015699.1
Lactobacillus reuteri SD2112, plasmid pLR581	NC_015700.1
Lactobacillus reuteri SD2112, plasmid pLR584	NC_015701.1
Lactobacillus reuteri SD2112, plasmid pLR585	NC_015698.1
Lactobacillus reuteri TD1	NC_021872.1
Lactobacillus rhamnosus ATCC 8530	NC_017491.1
Lactobacillus rhamnosus GG ATCC 53103	NC_017482.1
Lactobacillus rhamnosus Lc 705	NC_013199.1
Lactobacillus rhamnosus Lc 705, plasmid pLC1	NC_013200.1
Lactobacillus rhamnosus LOCK900	NC_021723.1
Lactobacillus rhamnosus LOCK908	NC_021725.1
Lactobacillus ruminis ATCC 27782	NC_015975.1
Lactobacillus sakei subsp. *sakei* 23K	NC_007576.1
Lactobacillus salivarius CECT 5713	NC_017481.1
Lactobacillus salivarius CECT 5713, plasmid pHN1	NC_017479.1
Lactobacillus salivarius CECT 5713, plasmid pHN2	NC_017480.1
Lactobacillus salivarius CECT 5713, plasmid pHN3	NC_017499.1
Lactobacillus salivarius UCC118	NC_007929.1
Lactobacillus salivarius UCC118, plasmid pMP118	NC_007930.1
Lactobacillus salivarius UCC118, plasmid pSF118-20	NC_006529.1
Lactobacillus salivarius UCC118, plasmid pSF118-44	NC_006530.1
Lactobacillus sanfranciscensis TMW 11.304	NC_015978.1
Lactobacillus sanfranciscensis TMW 11.304, plasmid pLS1	NC_015979.1
Lactobacillus sanfranciscensis TMW 11.304, plasmid pLS2	NC_015980.1
Lactococcus garviae ATCC 49156	NC_015930.1
Lactococcus garviae Lg2	NC_017490.1
Lactococcus lactis subsp. *cremoris* A76	NC_017492.1
Lactococcus lactis subsp. *cremoris* A76, plasmid pQA504	NC_017497.1
Lactococcus lactis subsp. *cremoris* A76, plasmid pQA518	NC_017495.1
Lactococcus lactis subsp. *cremoris* A76, plasmid pQA549	NC_017493.1
Lactococcus lactis subsp. *cremoris* A76, plasmid pQA554	NC_017496.1
Lactococcus lactis subsp. *cremoris* KW2	NC_022369.1
Lactococcus lactis subsp. *cremoris* MG1363	NC_009004.1
Lactococcus lactis subsp. *cremoris* NZ9000	NC_017949.1
Lactococcus lactis subsp. *cremoris* plasmid 1	NC_008503.1
Lactococcus lactis subsp. *cremoris* plasmid 2	NC_008504.1
Lactococcus lactis subsp. *cremoris* plasmid 3	NC_008505.1
Lactococcus lactis subsp. *cremoris* plasmid 4	NC_008506.1
Lactococcus lactis subsp. *cremoris* plasmid 5	NC_008507.1
Lactococcus lactis subsp. *cremoris* SK11	NC_008527.1
Lactococcus lactis subsp. *cremoris* UC509.9	NC_019435.1
Lactococcus lactis subsp. *cremoris* UC509.9, plasmid pCIS1	NC_019438.1
Lactococcus lactis subsp. *cremoris* UC509.9, plasmid pCIS2	NC_019434.1
Lactococcus lactis subsp. *cremoris* UC509.9, plasmid pCIS3	NC_019433.1
Lactococcus lactis subsp. cremoris UC509.9, plasmid pCIS4	NC_019437.1
Lactococcus lactis subsp. cremoris UC509.9, plasmid pCIS5	NC_019432.1
Lactococcus lactis subsp. *cremoris* UC509.9, plasmid pCIS6	NC_019436.1
Lactococcus lactis subsp. *cremoris* UC509.9, plasmid pCIS7	NC_019431.1
Lactococcus lactis subsp. *cremoris* UC509.9, plasmid pCIS8	NC_019430.1
Lactococcus lactis subsp. *lactis* CV56	NC_017486.1
Lactococcus lactis subsp. *lactis* CV56, plasmid pCV56A	NC_017483.1
Lactococcus lactis subsp. *lactis* CV56, plasmid pCV56B	NC_017487.1
Lactococcus lactis subsp. *lactis* CV56, plasmid pCV56C	NC_017484.1
Lactococcus lactis subsp. *lactis* CV56, plasmid pCV56D	NC_017485.1
Lactococcus lactis subsp. *lactis* CV56, plasmid pCV56E	NC_017488.1
Lactococcus lactis subsp. *lactis* Il1403	NC_002662.1
Lactococcus lactis subsp. *lactis* IO-1	NC_020450.1
Lactococcus lactis subsp. *lactis* KF147	NC_013656.1
Lactococcus lactis subsp. *lactis* KF148, plasmid pKF147A	NC_013657.1
Lactococcus lactis subsp. *lactis* KLDS 4.0325	NC_022593.1
Leuconostoc carnosum JB16	NC_018673.1

Leuconostoc carnosum JB16, plasmid pKLC1	NC_018674.1
Leuconostoc carnosum JB16, plasmid pKLC2	NC_018698.1
Leuconostoc carnosum JB16, plasmid pKLC3	NC_018675.1
Leuconostoc carnosum JB16, plasmid pKLC4	NC_018699.1
Leuconostoc citreum KM20	NC_010471.1
Leuconostoc citreum KM20, plasmid pLCK1	NC_010470.1
Leuconostoc citreum KM20, plasmid pLCK2	NC_010466.1
Leuconostoc citreum KM20, plasmid pLCK3	NC_010467.1
Leuconostoc citreum KM20, plasmid pLCK4	NC_010469.1
Leuconostoc gasicomitatum LMG 18811	NC_014319.1
Leuconostoc gelidum JB7	NC_018631.1
Leuconostoc kimkii IMSNU 11154	NC_014136.1
Leuconostoc kimkii IMSNU 11154, plasmid LkipL4701	NC_014131.1
Leuconostoc kimkii IMSNU 11154, plasmid LkipL4704	NC_014132.1
Leuconostoc kimkii IMSNU 11154, plasmid LkipL4719	NC_014133.1
Leuconostoc kimkii IMSNU 11154, plasmid LkipL4726	NC_014134.1
Leuconostoc kimkii IMSNU 11154, plasmid LkipL48	NC_014135.1
Leuconostoc mesenteroides subsp. *mesenteroides* ATCC8293	NC_008531.1
Leuconostoc mesenteroides subsp. *mesenteroides* ATCC8293, plasmid pLEUM 1	NC_008496.1
Leuconostoc mesenteroides subsp. *mesenteroides* J18	NC_016805.1
Leuconostoc mesenteroides subsp. *mesenteroides* J18, plasmid pKLE01	NC_016827.1
Leuconostoc mesenteroides subsp. *mesenteroides* J18, plasmid pKLE02	NC_016820.1
Leuconostoc mesenteroides subsp. *mesenteroides* J18, plasmid pKLE03	NC_016821.1
Leuconostoc mesenteroides subsp. *mesenteroides* J18, plasmid pKLE04	NC_016828.1
Leuconostoc mesenteroides subsp. *mesenteroides* J18, plasmid pKLE05	NC_016806.1
Leuconostoc sp. C2	NC_015734.1
Pantoea ananatis LMG 20103	NC_013956.2
Pantoea sp. At-9b	NC_014837.1
Pantoea sp. At-9b, plasmid pPAT9B01	NC_014838.1
Pantoea sp. At-9b, plasmid pPAT9B02	NC_014839.1
Pantoea sp. At-9b, plasmid pPAT9B03	NC_014840.1
Pantoea sp. At-9b, plasmid pPAT9B04	NC_014841.1
Pantoea sp. At-9b, plasmid pPAT9B05	NC_014842.1
Pediococcus claussenii ATCC BAA-344	NC_016605.1
Pediococcus claussenii ATCC BAA-344, plasmid pPECL-1	NC_016635.1
Pediococcus claussenii ATCC BAA-344, plasmid pPECL-2	NC_016606.1
Pediococcus claussenii ATCC BAA-344, plasmid pPECL-3	NC_016636.1
Pediococcus claussenii ATCC BAA-344, plasmid pPECL-4	NC_016607.1
Pediococcus claussenii ATCC BAA-344, plasmid pPECL-5	NC_016608.1
Pediococcus claussenii ATCC BAA-344, plasmid pPECL-6	NC_017017.1
Pediococcus claussenii ATCC BAA-344, plasmid pPECL-7	NC_017018.1
Pediococcus claussenii ATCC BAA-344, plasmid pPECL-8	NC_017019.1
Pediococcus pentosaceus ATCC 25745	NC_008525.1
Pediococcus pentosaceus SL4	NC_022780.1
Propionibacterium acnes KPA17120	NC_006085.1
Propionibacterium freudenreichii subsp. *shermanii* CIRM-BIA1	NC_014215.1
Pseudomonas putida GB-1	NC_010322.1
Pseudomonas putida KT2440	NC_002947.3
Psychrobacter arcticus 273-4	NC_007204.1
Psychrobacter cryohalolentis K5	NC_007969.1
Psychrobacter cryohalolentis K5, plasmid 1	NC_007968.1
Psychrobacter sp. PRwf-1	NC_009524.1
Psychrobacter sp. PRwf-1, plasmid pRWF101	NC_009516.1
Psychrobacter sp. PRwf-1, plasmid pRWF102	NC_009517.1
Rahnella aquatilis ATCC 33071	NC_016818.1
Rahnella aquatilis ATCC 33071, plasmid	NC_016819.1
Rahnella aquatilis ATCC 33071, plasmid	NC_016835.1
Rahnella aquatilis ATCC 33071, plasmid	NC_017092.1
Ralstonia solanacearum Po82	NC_017574.1
Ralstonia solanacearum Po82, megaplasmid	NC_017575.1
Serratia proteamaculans 568	NC_009832.1
Serratia proteamaculans 568, plasmid pSPRO01	NC_009829.1
Shewanella denitrificans OS217	NC_007954.1
Shewanella frigidimarina NCIMB 400	NC_008345.1
Shewanella oneidensis MR-1	NC_004347.2
Shewanella oneidensis MR-1, plasmid	NC_004349.1
Shewanella piezotolerans WP3	NC_011566.1
Shewanella putrefaciens CN-32	NC_009438.1

Shewanella sp. ANA-3	NC_008577.1
Shewanella sp. ANA-3, plasmid 1	NC_008573.1
Shigella flexneri 2°	NC_004337.2
Shigella flexneri 2a, plasmid pCP301	NC_004851.1
Staphylococcus haemolyticus JCSC1435	NC_007168.1
Staphylococcus haemolyticus JCSC1435, plasmid pSHaeA	NC_007169.1
Staphylococcus haemolyticus JCSC1435, plasmid pSHaeB	NC_007170.1
Staphylococcus haemolyticus JCSC1435, plasmid pSHaeC	NC_007171.1
Staphylococcus pseudointermedius ED99	NC_017568.1
Sthaphylococcus aureus subsp. *aureus* NCTC 8325	NC_007795.1
Sthaphylococcus carnosus subsp. *carnosus* TM300	NC_012121.1
Streptococcus agalactiae NEM316	NC_004368.1
Streptococcus dysgalactiae subsp. *equisimilis* ATCC 12394	NC_017567.1
Streptococcus macedonicus ACA-DC 198	NC_016749.1
Streptococcus macedonicus ACA-DC 198, plasmid pSMA198	NC_016750.1
Streptococcus mitis B6	NC_013853.1
Streptococcus oralis Uo5	NC_015291.1
Streptococcus pneumoniae R6	NC_003098.1
Streptococcus pneumoniae TIGR4	NC_003028.3
Streptococcus pyogenes SF370	NC_002737.1
Streptococcus salivarius 57.I	NC_017594.1
Streptococcus suis 05ZYH33	NC_009442.1
Streptococcus thermophilus CNRZ1066	NC_006449.1
Streptococcus thermophilus JIM 8232	NC_017581.1
Streptococcus thermophilus LMD-9	NC_008532.1
Streptococcus thermophilus LMD-9, plasmid 1	NC_008500.1
Streptococcus thermophilus LMD-9, plasmid 2	NC_008501.1
Streptococcus thermophilus LMG18311	NC_006448.1
Streptococcus thermophilus MN-ZLW-002	NC_017927.1
Streptococcus thermophilus ND03	NC_017563.1
Streptococcus uberis 0140J	NC_012004.1
downloaded from http://patricbrc.org/portal/	NA
Enterococcus casseliflavus EC20	NA
Enterococcus gallinarum EG2	NA
Lactobacillus reuteri 100-23	NA
Pediococcus acidilactici DSM20284	NA

Table 2.6.4 Differentially expressed genes between core samples ripened at high temperature or in standard condition, according to DESeq analysis. Negative fold changes indicate genes over-expressed in condition B compared to A, regardless the time of ripening.

	log_2FoldChange	**Adjusted P value**
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];aapQ,bztB;generalL-aminoacidtransportsystempermeaseprotein	-5.79445058	0.021004259
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];ABC,FEV,S;ironcomplextransportsystemsubstrate-bindingprotein	-5.825832424	0.000379778
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];ABC,GLN1,A;putativeglutaminetransportsystemATP-bindingprotein[EC:3,6,3,-]	0.827662713	0.033036164
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];ABC,VB12,S1,btuF;vitaminB12transportsystemsubstrate-bindingprotein	-6.041019046	0.02302363
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];afuC,fbpC;iron(III)transportsystemATP-bindingprotein[EC:3,6,3,30]	-5.042539965	0.020748409
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];araF;L-arabinosetransportsystemsubstrate-bindingprotein	-4.699202506	0.043755579
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];artM;arginineтransportsystempermeaseprotein	-5.162185444	0.04356273
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];bioY;biotintransportsystemsubstrate-specificcomponent	-1.557141374	0.017244007
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];dppB;dipeptidetransportsystempermeaseprotein	-6.360916658	3.08E-05
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];dppD;dipeptidetransportsystemATP-bindingprotein	-5.020346936	0.014271914
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];gspL;generalsecretionpathwayproteinL	-5.162975173	0.044283932
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];livM;branched-chainaminoacidtransportsystempermeaseprotein	-5.761707722	0.000730056
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];malE;maltose/maltodextrintransportsystemsubstrate-bindingprotein	-4.394973416	0.018152474
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];malF;maltose/maltodextrintransportsystempermeaseprotein	-4.078495417	0.042930947
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];malG;maltose/maltodextrintransportsystempermeaseprotein	-3.617921368	0.037642814
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];malK,mtlK,thuK;multiplesugartransportsystemATP-bindingprotein[EC:3,6,3,-]	-4.686140514	0.019589753
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];mglA;methyl-galactosidetransportsystemATP-bindingprotein[EC:3,6,3,17]	-5.520371977	0.040303703
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];mglB;methyl-galactosidetransportsystemsubstrate-bindingprotein	-5.802875091	0.029813011
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];msbA;ATP-bindingcassette,subfamilyB,bacterialMsbA[EC:3,6,3,-]	-6.223001057	0.005758234

Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];msmX,msmK,malK,sugC,ggtA,msiK;multiplesugartransportsystemATP-bindingprotein	-5.08078914	0.000571588
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];nodI;lipooligosaccharidetransportsystemATP-bindingprotein	-5.508787084	0.002031792
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];nodJ;lipooligosaccharidetransportsystempermeaseprotein	-4.923850342	0.008424869
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];opuBD;osmoprotectanttransportsystempermeaseprotein	-3.826399874	0.012048496
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];opuC;osmoprotectanttransportsystemsubstrate-bindingprotein	-3.905138751	0.010739859
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];phnC;phosphonatetransportsystemATP-bindingprotein[EC:3,6,3,28]	-4.855635021	0.000308086
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];phnD;phosphonatetransportsystemsubstrate-bindingprotein	-5.147908585	3.13E-05
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];phnE;phosphonatetransportsystempermeaseprotein	-4.682026418	1.46E-05
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];proV;glycinebetaine/prolinetransportsystemATP-bindingprotein[EC:3,6,3,32]	-4.342908887	7.99E-06
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];proW;glycinebetaine/prolinetransportsystempermeaseprotein	-4.533502532	2.35E-05
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];proX;glycinebetaine/prolinetransportsystemsubstrate-bindingprotein	-4.50135696	2.04E-05
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];pstB;phosphatetransportsystemATP-bindingprotein[EC:3,6,3,27]	0.767093067	0.02975298
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];PTS-Aga-EIIA,agaF;PTSsystem,N-acetylgalactosamine-specificIIAcomponent[EC:2,7,1,69]	-4.846802103	3.93E-05
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];PTS-Bgl-EIIB,bglF;PTSsystem,beta-glucosides-specificIIBcomponent[EC:2,7,1,69]	-4.046032474	9.44E-05
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];PTS-Cel-EIIA,celC;PTSsystem,cellobiose-specificIIAcomponent[EC:2,7,1,69]	-5.152633964	7.50E-07
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];PTS-Cel-EIIB,celA;PTSsystem,cellobiose-specificIIBcomponent[EC:2,7,1,69]	-4.698040454	5.05E-06
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];PTS-Cel-EIIC,celB;PTSsystem,cellobiose-specificIICcomponent	-2.463007798	0.00032332
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];PTS-EI,PTSI,ptsI;phosphotransferasesystem,enzymeI,PtsI[EC:2,7,3,9]	-6.120948801	3.02E-05
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];PTS-Fru-EIIA,fruB;PTSsystem,fructose-specificIIAcomponent[EC:2,7,1,69]	-5.587499802	0.00039653
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];PTS-Fru-EIIB,fruA;PTSsystem,fructose-specificIIBcomponent[EC:2,7,1,69]	-5.588803896	0.00039653

Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];PTS-Fru-EIIC,fruA;PTSsystem,fructose-specificIICcomponent	-5.588803891	0.00039653
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];PTS-Gat-EIIC,gatC;PTSsystem,galactitol-specificIICcomponent	-3.263125977	0.018152474
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];PTS-Man-EIID,manZ;PTSsystem,mannose-specificIIDcomponent	1.688426934	0.029533322
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];PTS-Ula-EIIA,ulaC,sgaA;PTSsystem,ascorbate-specificIIAcomponent[EC:2,7,1,69]	-5.441430331	1.37E-05
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];PTS-Ula-EIIB,ulaB,sgaB;PTSsystem,ascorbate-specificIIBcomponent[EC:2,7,1,69]	-4.012856339	0.000231184
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];PTS-Ula-EIIC,ulaA,sgaT;PTSsystem,ascorbate-specificIICcomponent	-5.228916806	5.96E-06
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];rbsA;ribosetransportsystemATP-bindingprotein[EC:3,6,3,17]	-4.812403961	9.48E-05
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];rbsB;ribosetransportsystemsubstrate-bindingprotein	-5.604951479	2.42E-07
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];rbsC;ribosetransportsystempermeaseprotein	-5.017029039	1.30E-05
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];rbsD;D-ribosepyranase[EC:5,-,-,-]	-4.680373806	0.000234784
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];secE;preproteintranslocasesubunitSecE	1.14660634	0.012808524
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];secF;preproteintranslocasesubunitSecF	-4.676083612	0.032725985
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];tagG;teichoicacidtransportsystempermeaseprotein	-5.653678072	3.55E-07
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];tagH;teichoicacidtransportsystemATP-bindingprotein[EC:3,6,3,40]	-4.624431029	1.55E-05
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];ugpA;sn-glycerol3-phosphatetransportsystempermeaseprotein	-5.240890087	2.53E-06
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];ugpB;sn-glycerol3-phosphatetransportsystemsubstrate-bindingprotein	-4.806639566	3.71E-07
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];ugpC;sn-glycerol3-phosphatetransportsystemATP-bindingprotein[EC:3,6,3,20]	-5.28324773	5.02E-05
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];ugpE;sn-glycerol3-phosphatetransportsystempermeaseprotein	-4.922403666	1.17E-05
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];virD4,lvhD4;typeIVsecretionsystemproteinVirD4	-5.371159934	0.007319264
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];xylG;D-xylosetransportsystemATP-bindingprotein[EC:3,6,3,17]	-5.134973505	0.02302363
Environmentalinformationprocessing;Membranetransport;Ctransporters[PATH:ko02010];yojI;putativeATP-bindingcassettetransporter	-5.794042408	0.022225319

Environmentalinformationprocessing;Signaltrasduction;HIF-1signalingpathway[PATH:ko04066];ENO,eno;enolase[EC:4,2,1,11]	-5.950546634	6.12E-05
Environmentalinformationprocessing;Signaltrasduction;HIF-1signalingpathway[PATH:ko04066];GAPDH,gapA;glyceraldehyde3-phosphatedehydrogenase[EC:1,2,1,12]	-5.458656315	0.000134338
Environmentalinformationprocessing;Signaltrasduction;PI3K-Aktsignalingpathway[PATH:ko04151];htpG,HSP90A;molecularchaperoneHtpG	-5.070633139	0.037642814
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];agrA;two-componentsystem,AgrAfamily,responseregulatorAgrA	-3.436225515	0.000243456
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];agrC;two-componentsystem,AgrAfamily,sensorhistidinekinaseAgrC[EC:2,7,13,-]	-3.668420447	2.98E-05
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];atoE;short-chainfattyacidstransporter	-5.163257353	0.043141763
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];cheR;chemotaxisproteinmethyltransferaseCheR[EC:2,1,1,80]	-6.032709365	0.016092491
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];ciaH;two-componentsystem,OmpRfamily,sensorhistidinekinaseCiaH[EC:2,7,13,3]	-5.148019687	7.83E-05
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];ciaR;two-componentsystem,OmpRfamily,responseregulatorCiaR	-3.678644298	3.62E-06
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];citC;[citrate(pro-3S)-lyase]ligase[EC:6,2,1,22]	-6.306858006	2.32E-08
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];citD;citratelyasesubunitgamma(acylcarrierprotein)	-5.161523661	7.19E-05
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];citE;citratelyasesubunitbeta/citryl-CoAlyase[EC:4,1,3,34]	-5.705675918	2.75E-07
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];citF;citratelyasesubunitalpha/citrateCoA-transferase[EC:2,8,3,10]	-6.662554821	9.13E-08
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];citXG;holo-ACPsynthase/triphosphoribosyl-dephospho-CoAsynthase[EC:2,7,7,612,4,2,52]	-6.569294057	2.23E-08
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];cusA,silA;Cu(I)/Ag(I)effluxsystemmembraneproteinCusA/SilA	-5.136698074	0.024592108
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];cydA;cytochromedubiquinoloxidasesubunitI[EC:1,10,3,-]	-5.064875327	1.51E-05
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];cydB;cytochromedubiquinoloxidasesubunitII[EC:1,10,3,-]	-5.137486528	1.48E-06
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];degP,htrA;serineproteaseDo[EC:3,4,21,107]	-6.579035273	1.53E-05

Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];E2,3,1,9,atoB;acetyl-CoAC-acetyltransferase[EC:2,3,1,9]	-3.373422428	0.00346328
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];frdA;fumaratereductaseflavoproteinsubunit[EC:1,3,5,4]	-3.39479139	0.012913123
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];kdpA;K+-transportingATPaseATPaseAchain[EC:3,6,3,12]	-7.220993578	0.004572006
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];lrgA;holin-likeprotein	-4.837480365	0.000430229
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];lrgB;holin-likeproteinLrgB	-6.39764061	0.000312268
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];malR;two-componentsystem,CitBfamily,responseregulatorMalR	-5.823963576	0.001678448
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];mcp;methyl-acceptingchemotaxisprotein	-4.13974575	0.043106084
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];mdtC;RNDsuperfamily,multidrugtransportproteinMdtC	-12.23880014	0.004523313
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];narX;two-componentsystem,NarLfamily,nitrate/nitritesensorhistidinekinaseNarX[EC:2,7,13,3]	-11.80991106	0.016700344
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];phoB1,phoP;two-componentsystem,OmpRfamily,alkalinephosphatasesynthesisresponseregulatorPhoP	-6.000304319	2.31E-07
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];phoR;two-componentsystem,OmpRfamily,phosphateregulonsensorhistidinekinasePhoR[EC:2,7,13,3]	-5.97650818	2.36E-08
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];rstB;two-componentsystem,OmpRfamily,sensorhistidinekinaseRstB[EC:2,7,13,3]	-8.54976537	0.005903541
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];trg;methyl-acceptingchemotaxisproteinIII,riboseandgalactosesensorreceptor	-13.0990089	0.001572765
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];uhpC;MFStransporter,OPAfamily,sugarphosphatesensorproteinUhpC	-11.76854005	0.002995451
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];uhpT;MFStransporter,OPAfamily,hexosephosphatetransportproteinUhpT	-13.30306937	0.001164129
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];vraR;two-componentsystem,NarLfamily,vancomycinresistanceassociatedresponseregulatorVraR	-8.025730609	0.01063941
Environmentalinformationprocessing;Signaltrasduction;Two-componentsystem[PATH:ko02020];yufL,malK;two-componentsystem,CitBfamily,sensorhistidinekinaseMalK[EC:2,7,13,3]	-5.484163521	0.048249327
GeneticinformationandprocessingTranscription;Aminoacyl-tRNAbiosynthesis[PATH:ko00970];EARS,gltX;glutamyl-tRNAsynthetase[EC:6,1,1,17]	-8.769486459	0.039283267

GeneticinformationandprocessingTranscription;Aminoacyl-tRNAbiosynthesis[PATH:ko00970];gltX;nondiscriminatingglutamyl-tRNAsynthetase[EC:6,1,1,24]	-1.073337721	0.047440494
GeneticinformationandprocessingTranscription;Aminoacyl-tRNAbiosynthesis[PATH:ko00970];glyQ;glycyl-tRNAsynthetasealphachain[EC:6,1,1,14]	0.973594969	0.011586317
GeneticinformationandprocessingTranscription;Aminoacyl-tRNAbiosynthesis[PATH:ko00970];glyS;glycyl-tRNAsynthetasebetachain[EC:6,1,1,14]	-6.116192416	8.78E-05
GeneticinformationandprocessingTranscription;Aminoacyl-tRNAbiosynthesis[PATH:ko00970];MTFMT,fmt;methionyl-tRNAformyltransferase[EC:2,1,2,9]	0.89200961	0.002995451
GeneticinformationandprocessingTranscription;Aminoacyl-tRNAbiosynthesis[PATH:ko00970];QARS,glnS;glutaminyl-tRNAsynthetase[EC:6,1,1,18]	-11.75388851	0.001912743
GeneticinformationandprocessingTranscription;Aminoacyl-tRNAbiosynthesis[PATH:ko00970];RARS,argS;arginyl-tRNAsynthetase[EC:6,1,1,19]	0.727250117	0.004349097
GeneticinformationandprocessingTranscription;Aminoacyl-tRNAbiosynthesis[PATH:ko00970];WARS,trpS;tryptophanyl-tRNAsynthetase[EC:6,1,1,2]	-1.687480796	0.00540414
GeneticinformationandprocessingTranscription;Aminoacyl-tRNAbiosynthesis[PATH:ko00970];YARS,tyrS;tyrosyl-tRNAsynthetase[EC:6,1,1,1]	-3.021644096	0.005746486
GeneticInformationProcessing;Folding,sortinganddegradation;RNAdegradation[PATH:ko03018];dnaK;molecularchaperoneDnaK	-8.078495426	2.34E-08
GeneticInformationProcessing;Folding,sortinganddegradation;RNAdegradation[PATH:ko03018];ENO,eno;enolase[EC:4,2,1,11]	-5.951624563	6.12E-05
GeneticInformationProcessing;Folding,sortinganddegradation;RNAdegradation[PATH:ko03018];groEL,HSPD1;chaperoninGroEL	-9.178187903	1.46E-09
GeneticInformationProcessing;Folding,sortinganddegradation;RNAdegradation[PATH:ko03018];ppk;polyphosphatekinase[EC:2,7,4,1]	-5.057087872	2.54E-05
GeneticInformationProcessing;Folding,sortinganddegradation;RNAdegradation[PATH:ko03018];recQ;ATP-dependentDNAhelicaseRecQ[EC:3,6,4,12]	-6.427476407	1.42E-08
GeneticInformationProcessing;Folding,sortinganddegradation;RNAdegradation[PATH:ko03018];rhlB;ATP-dependentRNAhelicaseRhlB[EC:3,6,4,13]	-12.23457804	0.00346328
GeneticInformationProcessing;Folding,sortinganddegradation;RNAdegradation[PATH:ko03018];rho;Transcription;terminationfactorRho	-11.8085478	0.015753168
GeneticInformationProcessing;Folding,sortinganddegradation;RNAdegradation[PATH:ko03018];rnj;ribonucleaseJ[EC:3,1,-,-]	0.836349493	0.011983039
GeneticInformationProcessing;Folding,sortinganddegradation;Sulfurrelaysystem[PATH:ko04122];tusE,dsrC;tRNA2-thiouridinesynthesizingproteinE[EC:2,8,1,-]	-12.24908257	0.007708466
GeneticInformationProcessing;ReplicationandRepair;Baseexcisionrepair[PATH:ko03410];UNG,UDG;uracil-DNAglycosylase[EC:3,2,2,27]	-2.156528146	0.002100338
GeneticInformationProcessing;ReplicationandRepair;DNAreplication[PATH:ko03030];DPO3D1,holA;DNApolymeraseIIIsubunitdelta[EC:2,7,7,7]	-2.057134442	0.001769558
GeneticInformationProcessing;ReplicationandRepair;DNAreplication[PATH:ko03030];DPO3E,dnaQ;DNApolymeraseIIIsubunitepsilon[EC:2,7,7,7]	-2.281934658	0.014968485
GeneticInformationProcessing;ReplicationandRepair;DNAreplication[PATH:ko03030];E3,1,26,4A,RNASEH1,rnhA;ribonucleaseHI[EC:3,1,26,4]	-6.887346204	2.76E-07

GeneticInformationProcessing;ReplicationandRepair;Homologousrecombination[PATH:ko03440];DPO3D1,holA;DNApolymeraseIIIsubunitdelta[EC:2,7,7,7]	-2.057134442	0.001769558
GeneticInformationProcessing;ReplicationandRepair;Homologousrecombination[PATH:ko03440];DPO3E,dnaQ;DNApolymeraseIIIsubunitepsilon[EC:2,7,7,7]	-2.281934658	0.014968485
GeneticInformationProcessing;ReplicationandRepair;Homologousrecombination[PATH:ko03440];recA;recombinationproteinRecA	0.836567563	0.0271357
GeneticInformationProcessing;ReplicationandRepair;Homologousrecombination[PATH:ko03440];recB;exodeoxyribonucleaseVbetasubunit[EC:3,1,11,5]	-11.77951562	0.004389403
GeneticInformationProcessing;ReplicationandRepair;Homologousrecombination[PATH:ko03440];recC;exodeoxyribonucleaseVgammasubunit[EC:3,1,11,5]	-13.29246399	0.000592897
GeneticInformationProcessing;ReplicationandRepair;Homologousrecombination[PATH:ko03440];recF;DNAReplicationandRepair;proteinRecF	-1.245840594	0.034583942
GeneticInformationProcessing;ReplicationandRepair;Homologousrecombination[PATH:ko03440];recO;DNArepairproteinRecO(recombinationproteinO)	-3.194746372	0.000124049
GeneticInformationProcessing;ReplicationandRepair;Homologousrecombination[PATH:ko03440];recR;recombinationproteinRecR	-2.826461128	0.000868497
GeneticInformationProcessing;ReplicationandRepair;Homologousrecombination[PATH:ko03440];ruvB;hollidayjunctionDNAhelicaseRuvB[EC:3,6,4,12]	1.152675189	0.004963488
GeneticInformationProcessing;ReplicationandRepair;Mismatchrepair[PATH:ko03430];dam;DNAadeninemethylase[EC:2,1,1,72]	-5.547933664	0.001180211
GeneticInformationProcessing;ReplicationandRepair;Mismatchrepair[PATH:ko03430];DPO3D1,holA;DNApolymeraseIIIsubunitdelta[EC:2,7,7,7]	-2.057134442	0.001769558
GeneticInformationProcessing;ReplicationandRepair;Mismatchrepair[PATH:ko03430];DPO3E,dnaQ;DNApolymeraseIIIsubunitepsilon[EC:2,7,7,7]	-2.281934658	0.014968485
GeneticInformationProcessing;ReplicationandRepair;Mismatchrepair[PATH:ko03430];uvrD,pcrA;DNAhelicaseII/ATP-dependentDNAhelicasePcrA[EC:3,6,4,12]	-2.393805795	0.023107112
GeneticInformationProcessing;ReplicationandRepair;Nucleotideexcisionrepair[PATH:ko03420];uvrD,pcrA;DNAhelicaseII/ATP-dependentDNAhelicasePcrA[EC:3,6,4,12]	-2.393805795	0.023107112
GeneticInformationProcessing;Transcription;Ribosome[PATH:ko03010];RP-L20,MRPL20,rplT;largesubunitribosomalproteinL20	-2.496163215	0.007765704
GeneticInformationProcessing;Transcription;Ribosome[PATH:ko03010];RP-L24,MRPL24,rplX;largesubunitribosomalproteinL24	1.468966868	0.005713358
GeneticInformationProcessing;Transcription;Ribosome[PATH:ko03010];RP-L27,MRPL27,rpmA;largesubunitribosomalproteinL27	1.156896883	0.040207839
GeneticInformationProcessing;Transcription;Ribosome[PATH:ko03010];RP-L28,MRPL28,rpmB;largesubunitribosomalproteinL28	-2.253513018	0.012913123
GeneticInformationProcessing;Transcription;Ribosome[PATH:ko03010];RP-L34,MRPL34,rpmH;largesubunitribosomalproteinL34	-4.33611587	0.00057019
GeneticInformationProcessing;Transcription;Ribosome[PATH:ko03010];RP-L35,MRPL35,rpmI;largesubunitribosomalproteinL35	-1.924366964	0.043979192
GeneticInformationProcessing;Transcription;Ribosome[PATH:ko03010];RP-L36,MRPL36,rpmJ;largesubunitribosomalproteinL36	-2.800870899	0.00874987
GeneticInformationProcessing;Transcription;Ribosome[PATH:ko03010];RP-S10,MRPS10,rpsJ;smallsubunitribosomalproteinS10	-2.954335511	6.24E-05

GeneticInformationProcessing;Transcription;Ribosome[PATH:ko03010];RP-S13,rpsM;smallsubunitribosomalproteinS13	-1.519798236	0.033036164
GeneticInformationProcessing;Transcription;Ribosome[PATH:ko03010];RP-S18,MRPS18,rpsR;smallsubunitribosomalproteinS18	-2.63168196	0.003201241
GeneticInformationProcessing;Transcription;Ribosome[PATH:ko03010];RP-S20,rpsT;smallsubunitribosomalproteinS20	1.226072852	0.020069631
GeneticInformationProcessing;Transcription;Ribosome[PATH:ko03010];RP-S3,rpsC;smallsubunitribosomalproteinS3	1.272642324	0.007708466
GeneticInformationProcessing;Transcription;Ribosome[PATH:ko03010];RP-S4,rpsD;smallsubunitribosomalproteinS4	-1.822719232	0.016700344
GeneticInformationProcessing;Transcription;Ribosome[PATH:ko03010];RP-S6,MRPS6,rpsF;smallsubunitribosomalproteinS6	-2.771933346	0.00346328
GeneticInformationProcessing;Transcription;RNApolymerase[PATH:ko03020];rpoZ;DNA-directedRNApolymerasesubunitomega[EC:2,7,7,6]	1.140001556	0.003577006
Metabolism;Aminoacidmetabolism;Alanine,aspartateandglutamatemetabolism[PATH:ko00250];ald;alaninedehydrogenase[EC:1,4,1,1]	-5.521648089	0.009264107
Metabolism;Aminoacidmetabolism;Alanine,aspartateandglutamatemetabolism[PATH:ko00250];argG,ASS1;argininosuccinatesynthase[EC:6,3,4,5]	-2.488905339	0.001164129
Metabolism;Aminoacidmetabolism;Alanine,aspartateandglutamatemetabolism[PATH:ko00250];argH,ASL;argininosuccinatelyase[EC:4,3,2,1]	-3.714692095	1.59E-05
Metabolism;Aminoacidmetabolism;Alanine,aspartateandglutamatemetabolism[PATH:ko00250];aspA;aspartateammonia-lyase[EC:4,3,1,1]	-6.313900145	2.95E-06
Metabolism;Aminoacidmetabolism;Alanine,aspartateandglutamatemetabolism[PATH:ko00250];carA,CPA1;carbamoyl-phosphatesynthasesmallsubunit[EC:6,3,5,5]	-6.395428566	6.52E-05
Metabolism;Aminoacidmetabolism;Alanine,aspartateandglutamatemetabolism[PATH:ko00250];E5,1,1,13;aspartateracemase[EC:5,1,1,13]	-4.942524921	0.012913123
Metabolism;Aminoacidmetabolism;Alanine,aspartateandglutamatemetabolism[PATH:ko00250];gabD;succinate-semialdehydedehydrogenase/glutarate-semialdehydedehydrogenase[EC:1,2,1,161,2,1,791,2,1,20]	-4.549986885	1.37E-05
Metabolism;Aminoacidmetabolism;Alanine,aspartateandglutamatemetabolism[PATH:ko00250];gltB;glutamatesynthase(NADPH/NADH)largechain[EC:1,4,1,131,4,1,14]	-7.841446025	0.000132326
Metabolism;Aminoacidmetabolism;Alanine,aspartateandglutamatemetabolism[PATH:ko00250];gltD;glutamatesynthase(NADPH/NADH)smallchain[EC:1,4,1,131,4,1,14]	-12.19765424	0.00100271
Metabolism;Aminoacidmetabolism;Alanine,aspartateandglutamatemetabolism[PATH:ko00250];purB,ADSL;adenylosuccinatelyase[EC:4,3,2,2]	1.408979944	0.000660017
Metabolism;Aminoacidmetabolism;Alanine,aspartateandglutamatemetabolism[PATH:ko00250];putA;prolinedehydrogenase/delta1-pyrroline-5-carboxylatedehydrogenase[EC:1,5,5,21,2,1,88]	-13.47998144	0.000831604
Metabolism;Aminoacidmetabolism;Alanine,aspartateandglutamatemetabolism[PATH:ko00250];puuE;4-aminobutyrateaminotransferase[EC:2,6,1,19]	-5.839008597	1.17E-05
Metabolism;Aminoacidmetabolism;Alanine,aspartateandglutamatemetabolism[PATH:ko00250];pyrB,PYR2;aspartatecarbamoyltransferasecatalyticsubunit[EC:2,1,3,2]	0.766457577	0.047424644
Metabolism;Aminoacidmetabolism;Arginineandprolinemetabolism[PATH:ko00330];arcC;carbamatekinase[EC:2,7,2,2]	-11.15287326	0.003472935

Metabolism;Aminoacidmetabolism;Arginineandprolinemetabolism[PATH:ko00330];argC;N-acetyl-gamma-glutamyl-phosphatereductase[EC:1,2,1,38]	-3.656685122	0.004196998
Metabolism;Aminoacidmetabolism;Arginineandprolinemetabolism[PATH:ko00330];argD;acetylornithine/N-succinyldiaminopimelateaminotransferase[EC:2,6,1,112,6,1,17]	-11.79756423	0.009303631
Metabolism;Aminoacidmetabolism;Arginineandprolinemetabolism[PATH:ko00330];argG,ASS1;argininosuccinatesynthase[EC:6,3,4,5]	-2.488905339	0.001164129
Metabolism;Aminoacidmetabolism;Arginineandprolinemetabolism[PATH:ko00330];argH,ASL;argininosuccinatelyase[EC:4,3,2,1]	-3.714692095	1.59E-05
Metabolism;Aminoacidmetabolism;Arginineandprolinemetabolism[PATH:ko00330];astC;succinylornithineaminotransferase[EC:2,6,1,81]	-12.25415839	0.010072111
Metabolism;Aminoacidmetabolism;Arginineandprolinemetabolism[PATH:ko00330];codA;cytosinedeaminase[EC:3,5,4,1]	-3.331975975	0.02302363
Metabolism;Aminoacidmetabolism;Arginineandprolinemetabolism[PATH:ko00330];E1,5,1,2,proC;pyrroline-5-carboxylatereductase[EC:1,5,1,2]	-1.183816916	0.024547842
Metabolism;Aminoacidmetabolism;Arginineandprolinemetabolism[PATH:ko00330];E2,3,1,57,speG;diamineN-acetyltransferase[EC:2,3,1,57]	-6.028212858	2.42E-07
Metabolism;Aminoacidmetabolism;Arginineandprolinemetabolism[PATH:ko00330];E3,5,1,4,amiE;amidase[EC:3,5,1,4]	-9.159795129	0.012495682
Metabolism;Aminoacidmetabolism;Arginineandprolinemetabolism[PATH:ko00330];E3,5,3,6,arcA;argininedeiminase[EC:3,5,3,6]	-7.950039654	0.000119513
Metabolism;Aminoacidmetabolism;Arginineandprolinemetabolism[PATH:ko00330];E4,1,1,17,ODC1,speC,speF;ornithinedecarboxylase[EC:4,1,1,17]	-9.436250667	0.002995451
Metabolism;Aminoacidmetabolism;Arginineandprolinemetabolism[PATH:ko00330];OTC,argF,argI;ornithinecarbamoyltransferase[EC:2,1,3,3]	-2.288340744	0.002121995
Metabolism;Aminoacidmetabolism;Arginineandprolinemetabolism[PATH:ko00330];prr;aminobutyraldehydedehydrogenase[EC:1,2,1,19]	-11.81146043	0.018385998
Metabolism;Aminoacidmetabolism;Arginineandprolinemetabolism[PATH:ko00330];putA;prolinedehydrogenase/delta1-pyrroline-5-carboxylatedehydrogenase[EC:1,5,5,21,2,1,88]	-13.47998144	0.000831604
Metabolism;Aminoacidmetabolism;Cysteineandmethioninemetabolism[PATH:ko00270];E1,1,1,3;homoserinedehydrogenase[EC:1,1,1,3]	-1.457338016	0.02429635
Metabolism;Aminoacidmetabolism;Cysteineandmethioninemetabolism[PATH:ko00270];LDH,ldh;L-lactatedehydrogenase[EC:1,1,1,27]	1.073131866	0.038702186
Metabolism;Aminoacidmetabolism;Cysteineandmethioninemetabolism[PATH:ko00270];lysC;aspartatekinase[EC:2,7,2,4]	-2.470426967	0.000868497
Metabolism;Aminoacidmetabolism;Cysteineandmethioninemetabolism[PATH:ko00270];metC;cystathioninebeta-lyase[EC:4,4,1,8]	-5.996518004	0.000275868
Metabolism;Aminoacidmetabolism;Cysteineandmethioninemetabolism[PATH:ko00270];mmuM;homocysteineS-methyltransferase[EC:2,1,1,10]	-2.741641883	0.000767427
Metabolism;Aminoacidmetabolism;Cysteineandmethioninemetabolism[PATH:ko00270];thrA;bifunctionalaspartokinase/homoserinedehydrogenase1[EC:2,7,2,41,1,1,3]	-13.32943573	0.014525754
Metabolism;Aminoacidmetabolism;Glycine,serineandthreoninemetabolism[PATH:ko00260];DLD,lpd,pdhD;dihydrolipoamidedehydrogenase[EC:1,8,1,4]	-6.375720181	2.61E-05

Metabolism;Aminoacidmetabolism;Glycine,serineandthreoninemetabolism[PATH:ko00260];E1,1,1,3;homoserinedehydrogenase[EC:1,1,1,3]	-1.457338016	0.02429635
Metabolism;Aminoacidmetabolism;Glycine,serineandthreoninemetabolism[PATH:ko00260];gcvH,GCSH;glycinecleavagesystemHprotein	-7.572877698	0.003467562
Metabolism;Aminoacidmetabolism;Glycine,serineandthreoninemetabolism[PATH:ko00260];gcvT,AMT;aminomethyltransferase[EC:2,1,2,10]	-12.21984444	0.002190529
Metabolism;Aminoacidmetabolism;Glycine,serineandthreoninemetabolism[PATH:ko00260];GLDC,gcvP;glycinedehydrogenase[EC:1,4,4,2]	-8.292996831	0.024593612
Metabolism;Aminoacidmetabolism;Glycine,serineandthreoninemetabolism[PATH:ko00260];glyA,SHMT;glycinehydroxymethyltransferase[EC:2,1,2,1]	1.57364268	6.61E-07
Metabolism;Aminoacidmetabolism;Glycine,serineandthreoninemetabolism[PATH:ko00260];lysC;aspartatekinase[EC:2,7,2,4]	-2.470426967	0.000868497
Metabolism;Aminoacidmetabolism;Glycine,serineandthreoninemetabolism[PATH:ko00260];serA,PHGDH;D-3-phosphoglyceratedehydrogenase[EC:1,1,1,95]	-3.36727392	5.99E-05
Metabolism;Aminoacidmetabolism;Glycine,serineandthreoninemetabolism[PATH:ko00260];serC,PSAT1;phosphoserineaminotransferase[EC:2,6,1,52]	-2.871256379	0.000521046
Metabolism;Aminoacidmetabolism;Glycine,serineandthreoninemetabolism[PATH:ko00260];tdh;threonine3-dehydrogenase[EC:1,1,1,103]	-10.01944238	0.005129919
Metabolism;Aminoacidmetabolism;Glycine,serineandthreoninemetabolism[PATH:ko00260];thrA;bifunctionalaspartokinase/homoserinedehydrogenase1[EC:2,7,2,41,1,1,3]	-13.32943573	0.014525754
Metabolism;Aminoacidmetabolism;Glycine,serineandthreoninemetabolism[PATH:ko00260];thrB1;homoserinekinase[EC:2,7,1,39]	-1.925287818	0.00346328
Metabolism;Aminoacidmetabolism;Histidinemetabolism[PATH:ko00340];E3,1,3,15B;histidinol-phosphatase(PHPfamily)[EC:3,1,3,15]	-7.431575909	1.14E-08
Metabolism;Aminoacidmetabolism;Histidinemetabolism[PATH:ko00340];hisA;phosphoribosylformimino-5-aminoimidazolecarboxamideribotideisomerase[EC:5,3,1,16]	-1.487524168	0.046606821
Metabolism;Aminoacidmetabolism;Histidinemetabolism[PATH:ko00340];hisB;imidazoleglycerol-phosphatedehydratase[EC:4,2,1,19]	-2.975566053	0.000500234
Metabolism;Aminoacidmetabolism;Histidinemetabolism[PATH:ko00340];hisC;histidinol-phosphateaminotransferase[EC:2,6,1,9]	-4.325886491	1.99E-06
Metabolism;Aminoacidmetabolism;Histidinemetabolism[PATH:ko00340];hisD;histidinoldehydrogenase[EC:1,1,1,23]	-2.518577829	0.001771241
Metabolism;Aminoacidmetabolism;Histidinemetabolism[PATH:ko00340];hisE;phosphoribosyl-ATPpyrophosphohydrolase[EC:3,6,1,31]	-2.744707468	0.001947491
Metabolism;Aminoacidmetabolism;Histidinemetabolism[PATH:ko00340];hisF;cyclase[EC:4,1,3,-]	-2.060923175	0.010567335
Metabolism;Aminoacidmetabolism;Histidinemetabolism[PATH:ko00340];hisH;glutamineamidotransferase[EC:2,4,2,-]	-2.214467993	0.004442549
Metabolism;Aminoacidmetabolism;Histidinemetabolism[PATH:ko00340];hisI;phosphoribosyl-AMPcyclohydrolase[EC:3,5,4,19]	-2.184568523	0.008514636
Metabolism;Aminoacidmetabolism;Lysinebiosynthesis[PATH:ko00300];argD;acetylornithine/N-succinyldiaminopimelateaminotransferase[EC:2,6,1,112,6,1,17]	-11.79756423	0.009303631
Metabolism;Aminoacidmetabolism;Lysinebiosynthesis[PATH:ko00300];dapA;4-hydroxy-tetrahydrodipicolinatesynthase[EC:4,3,3,7]	-1.322063751	0.038521461

Metabolism;Aminoacidmetabolism;Lysinebiosynthesis[PATH:ko00300];dapB;4-hydroxy-tetrahydrodipicolinatereductase[EC:1,17,1,8]	1.3854821	2.54E-05
Metabolism;Aminoacidmetabolism;Lysinebiosynthesis[PATH:ko00300];dapC;N-succinyldiaminopimelateaminotransferase[EC:2,6,1,17]	-11.141225	0.001653568
Metabolism;Aminoacidmetabolism;Lysinebiosynthesis[PATH:ko00300];E1,1,1,3;homoserinedehydrogenase[EC:1,1,1,3]	-1.457338016	0.02429635
Metabolism;Aminoacidmetabolism;Lysinebiosynthesis[PATH:ko00300];lysC;aspartatekinase[EC:2,7,2,4]	-2.470426967	0.000868497
Metabolism;Aminoacidmetabolism;Lysinebiosynthesis[PATH:ko00300];murE;UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelateligase[EC:6,3,2,13]	-1.319193837	0.035849171
Metabolism;Aminoacidmetabolism;Lysinebiosynthesis[PATH:ko00300];patA;aminotransferase[EC:2,6,1,-]	-3.048929303	3.84E-05
Metabolism;Aminoacidmetabolism;Lysinebiosynthesis[PATH:ko00300];thrA;bifunctionalaspartokinase/homoserinedehydrogenase1[EC:2,7,2,41,1,1,3]	-13.32943573	0.014525754
Metabolism;Aminoacidmetabolism;Lysinedegradation[PATH:ko00310];E2,3,1,9,atoB;acetyl-CoAC-acetyltransferase[EC:2,3,1,9]	-3.373422428	0.00346328
Metabolism;Aminoacidmetabolism;Lysinedegradation[PATH:ko00310];E4,1,1,18,ldcC,cadA;lysinedecarboxylase[EC:4,1,1,18]	-11.79107023	0.007257142
Metabolism;Aminoacidmetabolism;Lysinedegradation[PATH:ko00310];fadJ;3-hydroxyacyl-CoAdehydrogenase/enoyl-CoAhydratase/3-hydroxybutyryl-CoAepimerase[EC:1,1,1,354,2,1,175,1,2,3]	-12.25582329	0.010739859
Metabolism;Aminoacidmetabolism;Lysinedegradation[PATH:ko00310];gabD;succinate-semialdehydedehydrogenase/glutarate-semialdehydedehydrogenase[EC:1,2,1,161,2,1,791,2,1,20]	-4.549986885	1.37E-05
Metabolism;Aminoacidmetabolism;Phenylalanine,tyrosineandtryptophanbiosynthesis[PATH:ko00400];aroA;3-phosphoshikimate1-carboxyvinyltransferase[EC:2,5,1,19]	1.24780215	0.005802781
Metabolism;Aminoacidmetabolism;Phenylalanine,tyrosineandtryptophanbiosynthesis[PATH:ko00400];AROA2,aroA;3-deoxy-7-phosphoheptulonatesynthase[EC:2,5,1,54]	-5.965682707	3.03E-05
Metabolism;Aminoacidmetabolism;Phenylalanine,tyrosineandtryptophanbiosynthesis[PATH:ko00400];aroB;3-dehydroquinatesynthase[EC:4,2,3,4]	0.925983145	0.048249327
Metabolism;Aminoacidmetabolism;Phenylalanine,tyrosineandtryptophanbiosynthesis[PATH:ko00400];E2,7,1,71,aroK,aroL;shikimatekinase[EC:2,7,1,71]	1.050467488	0.021690776
Metabolism;Aminoacidmetabolism;Phenylalanine,tyrosineandtryptophanbiosynthesis[PATH:ko00400];hisC;histidinol-phosphateaminotransferase[EC:2,6,1,9]	-4.325886491	1.99E-06
Metabolism;Aminoacidmetabolism;Phenylalanine,tyrosineandtryptophanbiosynthesis[PATH:ko00400];trpC;indole-3-glycerolphosphatesynthase[EC:4,1,1,48]	-4.542391636	0.000331713
Metabolism;Aminoacidmetabolism;Phenylalanine,tyrosineandtryptophanbiosynthesis[PATH:ko00400];trpF;phosphoribosylanthranilateisomerase[EC:5,3,1,24]	0.705447491	0.034342378
Metabolism;Aminoacidmetabolism;Phenylalaninemetabolism[PATH:ko00360];E3,5,1,4,amiE;amidase[EC:3,5,1,4]	-9.159795129	0.012495682
Metabolism;Aminoacidmetabolism;Phenylalaninemetabolism[PATH:ko00360];hcaD;3-phenylpropionate/trans-cinnamatedioxygenaseferredoxinreductasesubunit[EC:1,18,1,3]	-11.80808924	0.015307575
Metabolism;Aminoacidmetabolism;Phenylalaninemetabolism[PATH:ko00360];hisC;histidinol-phosphateaminotransferase[EC:2,6,1,9]	-4.325886491	1.99E-06
Metabolism;Aminoacidmetabolism;Phenylalaninemetabolism[PATH:ko00360];katG;catalase-peroxidase[EC:1,11,1,21]	-11.79536398	0.007765704

Metabolism;Aminoacidmetabolism;Phenylalaninemetabolism[PATH:ko00360];paaJ;acetyl-CoAacetyltransferase[EC:2,3,1,-]	-11.80808924	0.015307575
Metabolism;Aminoacidmetabolism;Tryptophanmetabolism[PATH:ko00380];E2,3,1,9,atoB;acetyl-CoAC-acetyltransferase[EC:2,3,1,9]	-3.373422428	0.00346328
Metabolism;Aminoacidmetabolism;Tryptophanmetabolism[PATH:ko00380];E3,5,1,4,amiE;amidase[EC:3,5,1,4]	-9.159795129	0.012495682
Metabolism;Aminoacidmetabolism;Tryptophanmetabolism[PATH:ko00380];fadJ;3-hydroxyacyl-CoAdehydrogenase/enoyl-CoAhydratase/3-hydroxybutyryl-CoAepimerase[EC:1,1,1,354,2,1,175,1,2,3]	-12.25582329	0.010739859
Metabolism;Aminoacidmetabolism;Tryptophanmetabolism[PATH:ko00380];katG;catalase-peroxidase[EC:1,11,1,21]	-11.79536398	0.007765704
Metabolism;Aminoacidmetabolism;Tyrosinemetabolism[PATH:ko00350];adhE;acetaldehydededehydrogenase/alcoholdehydrogenase[EC:1,2,1,101,1,1,1]	-2.237328876	0.038521461
Metabolism;Aminoacidmetabolism;Tyrosinemetabolism[PATH:ko00350];adhP;alcoholdehydrogenase,propanol-preferring[EC:1,1,1,1]	-3.523029673	2.61E-05
Metabolism;Aminoacidmetabolism;Tyrosinemetabolism[PATH:ko00350];gabD;succinate-semialdehydededehydrogenase/glutarate-semialdehydededehydrogenase[EC:1,2,1,161,2,1,791,2,1,20]	-4.549986885	1.37E-05
Metabolism;Aminoacidmetabolism;Tyrosinemetabolism[PATH:ko00350];hisC;histidinol-phosphateaminotransferase[EC:2,6,1,9]	-4.325886491	1.99E-06
Metabolism;Aminoacidmetabolism;Valine,leucineandisoleucinebiosynthesis[PATH:ko00290];E2,6,1,42,ilvE;branched-chainaminoacidaminotransferase[EC:2,6,1,42]	-2.752257097	0.000346456
Metabolism;Aminoacidmetabolism;Valine,leucineandisoleucinebiosynthesis[PATH:ko00290];leuB;3-isopropylmalatedehydrogenase[EC:1,1,1,85]	-1.446471659	0.024792564
Metabolism;Aminoacidmetabolism;Valine,leucineandisoleucinebiosynthesis[PATH:ko00290];leuD;3-isopropylmalate/(R)-2-methylmalatedehydratasesmallsubunit[EC:4,2,1,334,2,1,35]	-2.749564252	0.000118783
Metabolism;Aminoacidmetabolism;Valine,leucineandisoleucinedegradation[PATH:ko00280];DLD,lpd,pdhD;dihydrolipoamidedehydrogenase[EC:1,8,1,4]	-6.375717808	2.61E-05
Metabolism;Aminoacidmetabolism;Valine,leucineandisoleucinedegradation[PATH:ko00280];E2,3,1,9,atoB;acetyl-CoAC-acetyltransferase[EC:2,3,1,9]	-3.373422428	0.00346328
Metabolism;Aminoacidmetabolism;Valine,leucineandisoleucinedegradation[PATH:ko00280];E2,6,1,42,ilvE;branched-chainaminoacidaminotransferase[EC:2,6,1,42]	-8.117069891	1.09E-07
Metabolism;Aminoacidmetabolism;Valine,leucineandisoleucinedegradation[PATH:ko00280];E6,4,1,4B;3-methylcrotonyl-CoAcarboxylasebetasubunit[EC:6,4,1,4]	-11.79536398	0.007765704
Metabolism;Aminoacidmetabolism;Valine,leucineandisoleucinedegradation[PATH:ko00280];fadJ;3-hydroxyacyl-CoAdehydrogenase/enoyl-CoAhydratase/3-hydroxybutyryl-CoAepimerase[EC:1,1,1,354,2,1,175,1,2,3]	-12.25582329	0.010739859
Metabolism;Biosynthesisofothersecondarymetabolites;Carbapenembiosynthesis[PATH:ko00332];proA;glutamate-5-semialdehydededehydrogenase[EC:1,2,1,41]	-6.929452986	8.78E-05
Metabolism;Biosynthesisofothersecondarymetabolites;Flavoneandflavonolbiosynthesis[PATH:ko00944];uidA,GUSB;beta-glucuronidase[EC:3,2,1,31]	-11.78042809	0.003902356
Metabolism;Biosynthesisofothersecondarymetabolites;Novobiocinbiosynthesis[PATH:ko00401];hisC;histidinol-phosphateaminotransferase[EC:2,6,1,9]	-4.325886491	1.99E-06

Metabolism;Biosynthesisofothersecondarymetabolites;Phenylpropanoidbiosynthesis[PATH:ko00940];bglX;beta-glucosidase[EC:3,2,1,21]	-11.77203009	0.003118189
Metabolism;Biosynthesisofothersecondarymetabolites;Phenylpropanoidbiosynthesis[PATH:ko00940];katG;catalase-peroxidase[EC:1,11,1,21]	-11.79536398	0.007765704
Metabolism;Biosynthesisofothersecondarymetabolites;Streptomycinbiosynthesis[PATH:ko00521];E4,2,1,46,rfbB,rffG;dTDP-glucose4,6-dehydratase[EC:4,2,1,46]	1.072919012	0.035526824
Metabolism;Biosynthesisofothersecondarymetabolites;Tropane,piperidineandpyridinealkaloidbiosynthesis[PATH:ko00960];E4,1,1,18,ldcC,cadA;lysinedecarboxylase[EC:4,1,1,18]	-11.79107023	0.007257142
Metabolism;Biosynthesisofothersecondarymetabolites;Tropane,piperidineandpyridinealkaloidbiosynthesis[PATH:ko00960];hisC;histidinol-phosphateaminotransferase[EC:2,6,1,9]	-4.325886491	1.99E-06
Metabolism;Carbohydratemetabolism;Aminosugarandnucleotidesugarmetabolism[PATH:ko00520];arnA,pmrI;UDP-4-amino-4-deoxy-L-arabinoseformyltransferase/UDP-glucuronicaciddehydrogenase(UDP-4-keto-hexauronicaciddecarboxylating)[EC:2,1,2,131,1,1,305]	-9.206051617	0.024593612
Metabolism;Carbohydratemetabolism;Aminosugarandnucleotidesugarmetabolism[PATH:ko00520];arnC,pmrF;undecaprenyl-phosphate4-deoxy-4-formamido-L-arabinosetransferase[EC:2,4,2,53]	-12.59923004	0.00766499
Metabolism;Carbohydratemetabolism;Aminosugarandnucleotidesugarmetabolism[PATH:ko00520];glmU;bifunctionalUDP-N-acetylglucosaminepyrophosphorylase/Glucosamine-1-phosphateN-acetyltransferase[EC:2,7,7,232,3,1,157]	-1.289772237	0.042564457
Metabolism;Carbohydratemetabolism;Aminosugarandnucleotidesugarmetabolism[PATH:ko00520];PTS-Man-EIID,manZ;PTSsystem,mannose-specificIIDcomponent	1.689056976	0.029533322
Metabolism;Carbohydratemetabolism;Aminosugarandnucleotidesugarmetabolism[PATH:ko00520];UGDH,ugd;UDPglucose6-dehydrogenase[EC:1,1,1,22]	-10.06115434	0.000112561
Metabolism;Carbohydratemetabolism;Aminosugarandnucleotidesugarmetabolism[PATH:ko00520];UGP2,galU,galF;UTP--glucose-1-phosphateuridylyltransferase[EC:2,7,7,9]	0.713947129	0.032725985
Metabolism;Carbohydratemetabolism;Aminosugarandnucleotidesugarmetabolism[PATH:ko00520];wbpO;UDP-N-acetyl-D-galactosaminedehydrogenase[EC:1,1,1,-]	-12.24908257	0.007708466
Metabolism;Carbohydratemetabolism;Aminosugarandnucleotidesugarmetabolism[PATH:ko00520];wecB;UDP-N-acetylglucosamine2-epimerase(non-hydrolysing)[EC:5,1,3,14]	-5.899726023	0.006457012
Metabolism;Carbohydratemetabolism;Aminosugarandnucleotidesugarmetabolism[PATH:ko00520];xynB;xylan1,4-beta-xylosidase[EC:3,2,1,37]	-6.215045099	1.45E-06
Metabolism;Carbohydratemetabolism;Citratecycle(TCAcycle)[PATH:ko00020];aceE;pyruvatedehydrogenaseE1component[EC:1,2,4,1]	-11.79245895	0.00766499
Metabolism;Carbohydratemetabolism;Citratecycle(TCAcycle)[PATH:ko00020];DLD,lpd,pdhD;dihydrolipoamidedehydrogenase[EC:1,8,1,4]	-6.375720181	2.61E-05
Metabolism;Carbohydratemetabolism;Citratecycle(TCAcycle)[PATH:ko00020];E4,2,1,2B,fumC;fumaratehydratase,classII[EC:4,2,1,2]	-3.270061347	0.027939722
Metabolism;Carbohydratemetabolism;Citratecycle(TCAcycle)[PATH:ko00020];frdA;fumaratereductaseflavoproteinsubunit[EC:1,3,5,4]	-3.39479139	0.012913123
Metabolism;Carbohydratemetabolism;Citratecycle(TCAcycle)[PATH:ko00020];IDH1,IDH2,icd;isocitratedehydrogenase[EC:1,1,1,42]	-2.763414464	0.038521461
Metabolism;Carbohydratemetabolism;Citratecycle(TCAcycle)[PATH:ko00020];sdhB;succinatedehydrogenaseiron-sulfursubunit[EC:1,3,5,1]	-9.543035019	0.016387029

Metabolism;Carbohydratemetabolism;Fructoseandmannosemetabolism[PATH:ko00051];FBA,fbaA;fructose-bisphosphatealdolase,classII[EC:4,1,2,13]	-6.482770493	1.53E-05
Metabolism;Carbohydratemetabolism;Fructoseandmannosemetabolism[PATH:ko00051];FBP,fbp;fructose-1,6-bisphosphataseI[EC:3,1,3,11]	-11.79608401	0.008749896
Metabolism;Carbohydratemetabolism;Fructoseandmannosemetabolism[PATH:ko00051];fruK;1-phosphofructokinase[EC:2,7,1,56]	1.272668773	0.001504431
Metabolism;Carbohydratemetabolism;Fructoseandmannosemetabolism[PATH:ko00051];fucA;L-fuculose-phosphatealdolase[EC:4,1,2,17]	-12.24908257	0.007708466
Metabolism;Carbohydratemetabolism;Fructoseandmannosemetabolism[PATH:ko00051];fucI;L-fucoseisomerase[EC:5,3,1,25]	-11.79719596	0.007765704
Metabolism;Carbohydratemetabolism;Fructoseandmannosemetabolism[PATH:ko00051];PTS-Fru-EIIA,fruB;PTSsystem,fructose-specificIIAcomponent[EC:2,7,1,69]	-5.590205876	0.000401184
Metabolism;Carbohydratemetabolism;Fructoseandmannosemetabolism[PATH:ko00051];PTS-Fru-EIIB,fruA;PTSsystem,fructose-specificIIBcomponent[EC:2,7,1,69]	-5.59151029	0.000401184
Metabolism;Carbohydratemetabolism;Fructoseandmannosemetabolism[PATH:ko00051];PTS-Fru-EIIC,fruA;PTSsystem,fructose-specificIICcomponent	-5.591510284	0.000401184
Metabolism;Carbohydratemetabolism;Fructoseandmannosemetabolism[PATH:ko00051];PTS-Man-EIID,manZ;PTSsystem,mannose-specificIIDcomponent	1.689056976	0.029533322
Metabolism;Carbohydratemetabolism;Fructoseandmannosemetabolism[PATH:ko00051];PTS-Mtl-EIIB,mtlA;PTSsystem,mannitol-specificIIBcomponent[EC:2,7,1,69]	-11.12095281	0.001653568
Metabolism;Carbohydratemetabolism;Fructoseandmannosemetabolism[PATH:ko00051];PTS-Mtl-EIIC,mtlA;PTSsystem,mannitol-specificIICcomponent	-11.12095281	0.001653568
Metabolism;Carbohydratemetabolism;Fructoseandmannosemetabolism[PATH:ko00051];rhaD;rhamnulose-1-phosphatealdolase[EC:4,1,2,19]	-9.817164929	0.011904792
Metabolism;Carbohydratemetabolism;Fructoseandmannosemetabolism[PATH:ko00051];rhmA;2-dehydro-3-deoxy-L-rhamnonatealdolase[EC:4,1,2,53]	-11.80991106	0.016700344
Metabolism;Carbohydratemetabolism;Fructoseandmannosemetabolism[PATH:ko00051];xylA;xyloseisomerase[EC:5,3,1,5]	-6.406312281	9.13E-08
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];bgaB,lacA;beta-galactosidase[EC:3,2,1,23]	-10.58581923	2.42E-07
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];E3,2,1,10;oligo-1,6-glucosidase[EC:3,2,1,10]	-5.480195436	2.42E-07
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];E3,2,1,22B,galA,rafA;alpha-galactosidase[EC:3,2,1,22]	-5.507313066	2.05E-05
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];ebgA;evolvedbeta-galactosidasesubunitalpha[EC:3,2,1,23]	-7.749288161	0.015307292
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];glf;UDP-galactopyranosemutase[EC:5,4,99,9]	-1.683382157	0.00882928
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];lacC;tagatose6-phosphatekinase[EC:2,7,1,144]	-3.600257432	0.041405281
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];lacZ;beta-galactosidase[EC:3,2,1,23]	-5.671584301	3.48E-05
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];malZ;alpha-glucosidase[EC:3,2,1,20]	-9.006626157	2.85E-07

Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];melA;alpha-galactosidase[EC:3,2,1,22]	-12.25454019	0.010249226
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];PTS-Aga-EIIA,agaF;PTSsystem,N-acetylgalactosamine-specificIIAcomponent[EC:2,7,1,69]	-6.128638867	1.43E-06
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];PTS-Gat-EIIA,gatA;PTSsystem,galactitol-specificIIAcomponent[EC:2,7,1,69]	-3.296106652	0.048719099
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];PTS-Gat-EIIC,gatC;PTSsystem,galactitol-specificIICcomponent	-3.771901762	0.005986024
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];PTS-Ula-EIIA,ulaC,sgaA;PTSsystem,ascorbate-specificIIAcomponent[EC:2,7,1,69]	-9.175545735	2.62E-07
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];PTS-Ula-EIIB,ulaB,sgaB;PTSsystem,ascorbate-specificIIBcomponent[EC:2,7,1,69]	-4.517957613	0.000103131
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];PTS-Ula-EIIC,ulaA,sgaT;PTSsystem,ascorbate-specificIICcomponent	-5.798272313	1.36E-06
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];UGDH,ugd;UDPglucose6-dehydrogenase[EC:1,1,1,22]	-10.06115434	0.000112561
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];UGP2,galU,galF;UTP--glucose-1-phosphateuridylyltransferase[EC:2,7,7,9]	0.713947129	0.032725985
Metabolism;Carbohydratemetabolism;Galactosemetabolism[PATH:ko00052];ulaG;L-ascorbate6-phosphatelactonase[EC:3,1,1,-]	-6.489220836	4.17E-07
Metabolism;Carbohydratemetabolism;Glycolysis/Gluconeogenesis[PATH:ko00010];aceE;pyruvatedehydrogenaseE1component[EC:1,2,4,1]	-11.79245895	0.00766499
Metabolism;Carbohydratemetabolism;Glycolysis/Gluconeogenesis[PATH:ko00010];adhE;acetaldehydedehydrogenase/alcoholdehydrogenase[EC:1,2,1,101,1,1,1]	-2.237328876	0.038521461
Metabolism;Carbohydratemetabolism;Glycolysis/Gluconeogenesis[PATH:ko00010];adhP;alcoholdehydrogenase,propanol-preferring[EC:1,1,1,1]	-3.523029673	2.61E-05
Metabolism;Carbohydratemetabolism;Glycolysis/Gluconeogenesis[PATH:ko00010];DLD,lpd,pdhD;dihydrolipoamidedehydrogenase[EC:1,8,1,4]	-6.375720181	2.61E-05
Metabolism;Carbohydratemetabolism;Glycolysis/Gluconeogenesis[PATH:ko00010];E3,2,1,86B,bglA;6-phospho-beta-glucosidase[EC:3,2,1,86]	-1.560020113	0.012785221
Metabolism;Carbohydratemetabolism;Glycolysis/Gluconeogenesis[PATH:ko00010];ENO,eno;enolase[EC:4,2,1,11]	-5.951624563	6.12E-05
Metabolism;Carbohydratemetabolism;Glycolysis/Gluconeogenesis[PATH:ko00010];FBA,fbaA;fructose-bisphosphatealdolase,classII[EC:4,1,2,13]	-6.482770493	1.53E-05
Metabolism;Carbohydratemetabolism;Glycolysis/Gluconeogenesis[PATH:ko00010];FBP,fbp;fructose-1,6-bisphosphataseI[EC:3,1,3,11]	-11.79608401	0.008749896
Metabolism;Carbohydratemetabolism;Glycolysis/Gluconeogenesis[PATH:ko00010];GAPDH,gapA;glyceraldehyde3-phosphatedehydrogenase[EC:1,2,1,12]	-5.46010237	0.000134338
Metabolism;Carbohydratemetabolism;Glycolysis/Gluconeogenesis[PATH:ko00010];LDH,ldh;L-lactatedehydrogenase[EC:1,1,1,27]	1.073131866	0.038702186
Metabolism;Carbohydratemetabolism;Glycolysis/Gluconeogenesis[PATH:ko00010];PTS-Arb-EIIB,glvB;PTSsystem,arbutin-likeIIBcomponent[EC:2,7,1,69]	-6.651053486	0.011057468

Metabolism;Carbohydratemetabolism;Glycolysis/Gluconeogenesis[PATH:ko00010];PTS-Arb-EIIC,glvC;PTSsystem,arbutin-likeIICcomponent	-6.651053486	0.011057468
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];accB,bccP;acetyl-CoAcarboxylasebiotincarboxylcarrierprotein	0.846959361	0.049618655
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];adhE;acetaldehydedehydrogenase/alcoholdehydrogenase[EC:1,2,1,101,1,1,1]	-2.237328876	0.038521461
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];aldA;lactaldehydedehydrogenase/glycolaldehydedehydrogenase[EC:1,2,1,221,2,1,21]	-9.591392669	0.000124308
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];butA,budC;meso-butanedioldehydrogenase/(S,S)-butanedioldehydrogenase/diacetylreductase[EC:1,1,1,-1,1,1,761,1,1,304]	-3.717248269	2.80E-05
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];E2,3,1,54,pflD;formateC-acetyltransferase[EC:2,3,1,54]	-5.45284466	0.002584061
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];E2,3,1,9,atoB;acetyl-CoAC-acetyltransferase[EC:2,3,1,9]	-3.373422428	0.00346328
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];E4,1,1,5,alsD;acetolactatedecarboxylase[EC:4,1,1,5]	0.973137017	0.001422087
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];E5,3,1,22,gip;hydroxypyruvateisomerase[EC:5,3,1,22]	-8.01390043	0.024792564
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];fadJ;3-hydroxyacyl-CoAdehydrogenase/enoyl-CoAhydratase/3-hydroxybutyryl-CoAepimerase[EC:1,1,1,354,2,1,175,1,2,3]	-12.25582329	0.010739859
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];fdoG,fdfH;formatedehydrogenasemajorsubunit[EC:1,2,1,2]	-6.762554455	0.004407149
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];frdA;fumaratereductaseflavoproteinsubunit[EC:1,3,5,4]	-3.39479139	0.012913123
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];gabD;succinate-semialdehydedehydrogenase/glutarate-semialdehydedehydrogenase[EC:1,2,1,161,2,1,791,2,1,20]	-4.549986885	1.37E-05
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];gcvH,GCSH;glycinecleavagesystemHprotein	-7.572877698	0.003467562
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];glcD;glycolateoxidase[EC:1,1,3,15]	-8.769486459	0.039283267
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];gldA;glyceroldehydrogenase[EC:1,1,1,6]	-5.911646545	2.42E-07
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];glyA,SHMT;glycinehydroxymethyltransferase[EC:2,1,2,1]	1.57364268	6.61E-07
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];LDH,ldh;L-lactatedehydrogenase[EC:1,1,1,27]	1.073131866	0.038702186
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];leuB;3-isopropylmalatedehydrogenase[EC:1,1,1,85]	-1.446471659	0.024792564
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];leuD;3-isopropylmalate/(R)-2-methylmalatedehydratasesmallsubunit[EC:4,2,1,334,2,1,35]	-2.749564252	0.000118783

Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];puuE;4-aminobutyrateaminotransferase[EC:2,6,1,19]	-5.839008597	1.17E-05
Metabolism;Carbohydratemetabolism;Glyoxylateanddicarboxylatemetabolism[PATH:ko00630];sdhB;succinatedehydrogenaseiron-sulfursubunit[EC:1,3,5,1]	-9.543035019	0.016387029
Metabolism;Carbohydratemetabolism;Pentoseandglucuronateinterconversions[PATH:ko00040];araA;L-arabinoseisomerase[EC:5,3,1,4]	-5.847898782	4.20E-06
Metabolism;Carbohydratemetabolism;Pentoseandglucuronateinterconversions[PATH:ko00040];araD;L-ribulose-5-phosphate4-epimerase[EC:5,1,3,4]	-5.477300859	1.23E-07
Metabolism;Carbohydratemetabolism;Pentoseandglucuronateinterconversions[PATH:ko00040];kduD;2-deoxy-D-gluconate3-dehydrogenase[EC:1,1,1,125]	-5.405432446	1.51E-05
Metabolism;Carbohydratemetabolism;Pentoseandglucuronateinterconversions[PATH:ko00040];rhaD;rhamnulose-1-phosphatealdolase[EC:4,1,2,19]	-9.817164929	0.011904792
Metabolism;Carbohydratemetabolism;Pentoseandglucuronateinterconversions[PATH:ko00040];sgbH;3-dehydro-L-gulonate-6-phosphatedecarboxylase[EC:4,1,1,85]	-5.457570519	4.37E-05
Metabolism;Carbohydratemetabolism;Pentoseandglucuronateinterconversions[PATH:ko00040];sgbU;hexulose-6-phosphateisomerase[EC:5,-,-,-]	-4.509274122	4.02E-05
Metabolism;Carbohydratemetabolism;Pentoseandglucuronateinterconversions[PATH:ko00040];UGDH,ugd;UDPglucose6-dehydrogenase[EC:1,1,1,22]	-10.06115434	0.000112561
Metabolism;Carbohydratemetabolism;Pentoseandglucuronateinterconversions[PATH:ko00040];UGP2,galU,galF;UTP--glucose-1-phosphateuridylyltransferase[EC:2,7,7,9]	0.713947129	0.032725985
Metabolism;Carbohydratemetabolism;Pentoseandglucuronateinterconversions[PATH:ko00040];uidA,GUSB;beta-glucuronidase[EC:3,2,1,31]	-11.78042809	0.003902356
Metabolism;Carbohydratemetabolism;Pentoseandglucuronateinterconversions[PATH:ko00040];xylA;xyloseisomerase[EC:5,3,1,5]	-6.406312281	9.13E-08
Metabolism;Carbohydratemetabolism;Pentoseandglucuronateinterconversions[PATH:ko00040];xylB,XYLB;xylulokinase[EC:2,7,1,17]	-7.128084003	1.81E-09
Metabolism;Carbohydratemetabolism;Pentosephosphatepathway[PATH:ko00030];E2,2,1,2,talA,talB;transaldolase[EC:2,2,1,2]	-11.76644974	0.002995451
Metabolism;Carbohydratemetabolism;Pentosephosphatepathway[PATH:ko00030];E2,7,1,12,gntK,idnK;gluconokinase[EC:2,7,1,12]	-5.702007645	4.30E-05
Metabolism;Carbohydratemetabolism;Pentosephosphatepathway[PATH:ko00030];E4,1,2,9;phosphoketolase[EC:4,1,2,9]	-4.035204468	0.000344147
Metabolism;Carbohydratemetabolism;Pentosephosphatepathway[PATH:ko00030];FBA,fbaA;fructose-bisphosphatealdolase,classII[EC:4,1,2,13]	-6.482770493	1.53E-05
Metabolism;Carbohydratemetabolism;Pentosephosphatepathway[PATH:ko00030];FBP,fbp;fructose-1,6-bisphosphataseI[EC:3,1,3,11]	-11.79608401	0.008749896
Metabolism;Carbohydratemetabolism;Pentosephosphatepathway[PATH:ko00030];G6PD,zwf;glucose-6-phosphate1-dehydrogenase[EC:1,1,1,49]	-4.965135325	4.77E-07
Metabolism;Carbohydratemetabolism;Pentosephosphatepathway[PATH:ko00030];kdgK;2-dehydro-3-deoxygluconokinase[EC:2,7,1,45]	-6.233354308	7.70E-08
Metabolism;Carbohydratemetabolism;Pentosephosphatepathway[PATH:ko00030];PGD,gnd;6-phosphogluconatedehydrogenase[EC:1,1,1,44]	-4.864027831	2.76E-07
Metabolism;Carbohydratemetabolism;Pentosephosphatepathway[PATH:ko00030];pgl;6-phosphogluconolactonase[EC:3,1,1,31]	-6.034832737	6.61E-07

Metabolism;Carbohydratemetabolism;Pentosephosphatepathway[PATH:ko00030];rbsK,RBKS;ribokinase[EC:2,7,1,15]	-5.105090944	7.53E-07
Metabolism;Carbohydratemetabolism;Pyruvatemetabolism[PATH:ko00620];accB,bccP;acetyl-CoAcarboxylasebiotincarboxylcarrierprotein	0.846959361	0.049618655
Metabolism;Carbohydratemetabolism;Pyruvatemetabolism[PATH:ko00620];aceE;pyruvatedehydrogenaseE1component[EC:1,2,4,1]	-11.79245895	0.00766499
Metabolism;Carbohydratemetabolism;Pyruvatemetabolism[PATH:ko00620];acyP;acylphosphatase[EC:3,6,1,7]	-2.916448414	0.002995451
Metabolism;Carbohydratemetabolism;Pyruvatemetabolism[PATH:ko00620];adhE;acetaldehydededehydrogenase/alcoholdehydrogenase[EC:1,2,1,101,1,1,1]	-2.237328876	0.038521461
Metabolism;Carbohydratemetabolism;Pyruvatemetabolism[PATH:ko00620];aldA;lactaldehydededehydrogenase/glycolaldehydededehydrogenase[EC:1,2,1,221,2,1,21]	-9.591392669	0.000124308
Metabolism;Carbohydratemetabolism;Pyruvatemetabolism[PATH:ko00620];DLD,lpd,pdhD;dihydrolipoamidedehydrogenase[EC:1,8,1,4]	-6.375720181	2.61E-05
Metabolism;Carbohydratemetabolism;Pyruvatemetabolism[PATH:ko00620];dld;D-lactatedehydrogenase[EC:1,1,1,28]	-5.496561247	3.05E-05
Metabolism;Carbohydratemetabolism;Pyruvatemetabolism[PATH:ko00620];E1,2,3,3,poxL;pyruvateoxidase[EC:1,2,3,3]	-3.748915635	5.00E-05
Metabolism;Carbohydratemetabolism;Pyruvatemetabolism[PATH:ko00620];E2,3,1,54,pflD;formateC-acetyltransferase[EC:2,3,1,54]	-5.45284466	0.002584061
Metabolism;Carbohydratemetabolism;Pyruvatemetabolism[PATH:ko00620];E2,3,1,9,atoB;acetyl-CoAC-acetyltransferase[EC:2,3,1,9]	-3.373422428	0.00346328
Metabolism;Carbohydratemetabolism;Pyruvatemetabolism[PATH:ko00620];E4,2,1,2B,fumC;fumaratehydratase,classII[EC:4,2,1,2]	-3.270061347	0.027939722
Metabolism;Carbohydratemetabolism;Pyruvatemetabolism[PATH:ko00620];frdA;fumaratereductaseflavoproteinsubunit[EC:1,3,5,4]	-3.39479139	0.012913123
Metabolism;Carbohydratemetabolism;Pyruvatemetabolism[PATH:ko00620];LDH,ldh;L-lactatedehydrogenase[EC:1,1,1,27]	1.073131866	0.038702186
Metabolism;Carbohydratemetabolism;Pyruvatemetabolism[PATH:ko00620];ldhA;D-lactatedehydrogenase[EC:1,1,1,28]	-4.97732117	3.71E-07
Metabolism;Carbohydratemetabolism;Pyruvatemetabolism[PATH:ko00620];ppdK;pyruvate,orthophosphatedikinase[EC:2,7,9,1]	-9.358232779	0.000641867
Metabolism;Carbohydratemetabolism;Pyruvatemetabolism[PATH:ko00620];pps,ppsA;pyruvate,waterdikinase[EC:2,7,9,2]	-3.962193451	0.029219419
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];bcsA;cellulosesynthase(UDP-forming)[EC:2,4,1,12]	-4.859827727	0.003902356
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];bglX;beta-glucosidase[EC:3,2,1,21]	-11.77203009	0.003118189
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];E2,4,1,1,glgP,PYG;starchphosphorylase[EC:2,4,1,1]	-6.048917334	0.001103521
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];E2,4,1,7;sucrosephosphorylase[EC:2,4,1,7]	-6.528183172	1.48E-07
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];E2,4,1,8,mapA;maltosephosphorylase[EC:2,4,1,8]	-5.503252856	6.90E-07

Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];E3,2,1,10;oligo-1,6-glucosidase[EC:3,2,1,10]	-5.480195436	2.42E-07
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];E3,2,1,122,glvA;maltose-6'-phosphateglucosidase[EC:3,2,1,122]	-7.062080919	0.002705541
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];E3,2,1,28,treA,treF;alpha,alpha-trehalase[EC:3,2,1,28]	-13.30306937	0.001164129
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];malQ;4-alpha-glucanotransferase[EC:2,4,1,25]	-6.694938881	0.003049744
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];malZ;alpha-glucosidase[EC:3,2,1,20]	-9.006626157	2.85E-07
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];otsA;trehalose6-phosphatesynthase[EC:2,4,1,15]	-12.2522972	0.009026929
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];pgmB;beta-phosphoglucomutase[EC:5,4,2,6]	-7.691695791	0.003902356
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];treC;trehalose-6-phosphatehydrolase[EC:3,2,1,93]	-3.017443165	0.038521461
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];treX,glgX;glycogenoperonprotein[EC:3,2,1,-]	-12.24041236	0.003851629
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];UGDH,ugd;UDPglucose6-dehydrogenase[EC:1,1,1,22]	-10.06115434	0.000112561
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];UGP2,galU,galF;UTP--glucose-1-phosphateuridylyltransferase[EC:2,7,7,9]	0.713947129	0.032725985
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];uidA,GUSB;beta-glucuronidase[EC:3,2,1,31]	-11.78042809	0.003902356
Metabolism;Carbohydratemetabolism;Starchandsucrosemetabolism[PATH:ko00500];xynB;xylan1,4-beta-xylosidase[EC:3,2,1,37]	-6.215045099	1.45E-06
Metabolism;Energymetabolism;Nitrogenmetabolism[PATH:ko00910];arcC;carbamatekinase[EC:2,7,2,2]	-11.15287326	0.003472935
Metabolism;Energymetabolism;Nitrogenmetabolism[PATH:ko00910];cynT,can;carbonicanhydrase[EC:4,2,1,1]	2.451903192	0.016049109
Metabolism;Energymetabolism;Nitrogenmetabolism[PATH:ko00910];gltB;glutamatesynthase(NADPH/NADH)largechain[EC:1,4,1,131,4,1,14]	-7.841446025	0.000132326
Metabolism;Energymetabolism;Nitrogenmetabolism[PATH:ko00910];gltD;glutamatesynthase(NADPH/NADH)smallchain[EC:1,4,1,131,4,1,14]	-12.19765424	0.00100271
Metabolism;Energymetabolism;Nitrogenmetabolism[PATH:ko00910];ncd2,npd;nitronatemonooxygenase[EC:1,13,12,16]	-10.33978025	0.042908627
Metabolism;Energymetabolism;Nitrogenmetabolism[PATH:ko00910];nirB;nitritereductase(NADH)largesubunit[EC:1,7,1,15]	-11.78042809	0.003902356
Metabolism;Energymetabolism;Nitrogenmetabolism[PATH:ko00910];NRT,narK,nrtP,nasA;MFStransporter,NNPfamily,nitrate/nitritetransporter	-12.87188337	0.003472935
Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];ATPF0A,atpB;F-typeH+-transportingATPasesubunita	-1.529677463	0.023044057
Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];ATPF0B,atpF;F-typeH+-transportingATPasesubunitb	1.185859681	0.043106084
Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];ATPF1B,atpD;F-typeH+-transportingATPasesubunitbeta[EC:3,6,3,14]	-7.079413701	1.13E-05

Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];ATPF1D,atpH;F-typeH+-transportingATPasesubunitdelta	1.004732623	0.022656255
Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];ATPF1G,atpG;F-typeH+-transportingATPasesubunitgamma	-5.858814818	0.000372819
Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];coxA;cytochromecoxidasesubunitI[EC:1,9,3,1]	-11.80991106	0.016700344
Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];cydA;cytochromedubiquinoloxidasesubunitI[EC:1,10,3,-]	-5.329192263	1.17E-05
Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];cydB;cytochromedubiquinoloxidasesubunitII[EC:1,10,3,-]	-6.06163902	1.23E-07
Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];cyoB;cytochromeoubiquinoloxidasesubunitI[EC:1,10,3,-]	-9.486563045	0.006880452
Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];frdA;fumaratereductaseflavoproteinsubunit[EC:1,3,5,4]	-3.39479139	0.012913123
Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];ndh;NADHdehydrogenase[EC:1,6,99,3]	-4.997215855	5.99E-05
Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];nuoA;NADH-quinoneoxidoreductasesubunitA[EC:1,6,5,3]	-8.619570321	0.026503529
Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];nuoG;NADH-quinoneoxidoreductasesubunitG[EC:1,6,5,3]	-9.154070597	0.011532103
Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];nuoI;NADH-quinoneoxidoreductasesubunitI[EC:1,6,5,3]	-8.619570321	0.026503529
Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];nuoM;NADH-quinoneoxidoreductasesubunitM[EC:1,6,5,3]	-11.79536398	0.007765704
Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];ppk;polyphosphatekinase[EC:2,7,4,1]	-5.057087872	2.54E-05
Metabolism;Energymetabolism;Oxidativephosphorylation[PATH:ko00190];sdhB;succinatedehydrogenaseiron-sulfursubunit[EC:1,3,5,1]	-9.543035019	0.016387029
Metabolism;Glycanbiosynthesisandmetabolism;Glycosaminoglycandegradation[PATH:ko00531];uidA,GUSB;beta-glucuronidase[EC:3,2,1,31]	-11.78042809	0.003902356
Metabolism;Glycanbiosynthesisandmetabolism;Glycosphingolipidbiosynthesis-globoseries[PATH:ko00603];E3,2,1,22B,galA,rafA;alpha-galactosidase[EC:3,2,1,22]	-5.507313066	2.05E-05
Metabolism;Glycanbiosynthesisandmetabolism;Glycosphingolipidbiosynthesis-globoseries[PATH:ko00603];melA;alpha-galactosidase[EC:3,2,1,22]	-12.25454019	0.010249226
Metabolism;Glycanbiosynthesisandmetabolism;Lipopolysaccharidebiosynthesis[PATH:ko00540];kdsA;2-dehydro-3-deoxyphosphooctonatealdolase(KDO8-Psynthase)[EC:2,5,1,55]	-5.708904552	3.55E-07
Metabolism;Glycanbiosynthesisandmetabolism;Lipopolysaccharidebiosynthesis[PATH:ko00540];lpxB;lipid-A-disaccharidesynthase[EC:2,4,1,182]	-12.87221147	0.003552338
Metabolism;Glycanbiosynthesisandmetabolism;Lipopolysaccharidebiosynthesis[PATH:ko00540];waaB,rfaB;UDP-D-galactose:(glucosyl)LPSalpha-1,6-D-galactosyltransferase[EC:2,4,1,-]	-12.84017433	0.000658913
Metabolism;Glycanbiosynthesisandmetabolism;Lipopolysaccharidebiosynthesis[PATH:ko00540];waaL,rfaL;O-antigenligase[EC:6,-,-,-]	-12.24990814	0.007909432

Metabolism;Glycanbiosynthesisandmetabolism;Lipopolysaccharidebiosynthesisproteins[BR:ko01005];mrdA;penicillin-bindingprotein2	-12.25582329	0.010739859
Metabolism;Glycanbiosynthesisandmetabolism;Lipopolysaccharidebiosynthesisproteins[BR:ko01005];murE;UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelateligase[EC:6,3,2,13]	-1.319193837	0.035849171
Metabolism;Glycanbiosynthesisandmetabolism;Lipopolysaccharidebiosynthesisproteins[BR:ko01005];pbpA;penicillin-bindingprotein1[EC:3,4,-,-]	-13.09558937	0.001238169
Metabolism;Glycanbiosynthesisandmetabolism;Lipopolysaccharidebiosynthesisproteins[BR:ko01005];pbpB;penicillin-bindingprotein2B	-5.410198846	3.05E-05
Metabolism;Glycanbiosynthesisandmetabolism;Otherglycandegradation[PATH:ko00511];ebgA;evolvedbeta-galactosidasesubunitalpha[EC:3,2,1,23]	-7.749288161	0.015307292
Metabolism;Glycanbiosynthesisandmetabolism;Otherglycandegradation[PATH:ko00511];lacZ;beta-galactosidase[EC:3,2,1,23]	-5.671584301	3.48E-05
Metabolism;Glycanbiosynthesisandmetabolism;Otherglycandegradation[PATH:ko00511];NEU1;sialidase-1[EC:3,2,1,18]	-8.737726906	0.025817995
Metabolism;Lipidmetabolism;Arachidonicacidmetabolism[PATH:ko00590];E1,11,1,9;glutathioneperoxidase[EC:1,11,1,9]	-5.514768616	3.08E-05
Metabolism;Lipidmetabolism;Biosynthesisofunsaturatedfattyacids[PATH:ko01040];fadJ;3-hydroxyacyl-CoAdehydrogenase/enoyl-CoAhydratase/3-hydroxybutyryl-CoAepimerase[EC:1,1,1,354,2,1,175,1,2,3]	-12.25582329	0.010739859
Metabolism;Lipidmetabolism;Fattyacidbiosynthesis[PATH:ko00061];accB,bccP;acetyl-CoAcarboxylasebiotincarboxylcarrierprotein	0.846959361	0.049618655
Metabolism;Lipidmetabolism;Fattyacidbiosynthesis[PATH:ko00061];fabI;enoyl-[acyl-carrierprotein]reductaseI[EC:1,3,1,91,3,1,10]	-4.200496577	0.026264115
Metabolism;Lipidmetabolism;Fattyaciddegradation[PATH:ko00071];adhE;acetaldehydedehydrogenase/alcoholdehydrogenase[EC:1,2,1,101,1,1,1]	-7.78137147	3.62E-06
Metabolism;Lipidmetabolism;Fattyaciddegradation[PATH:ko00071];adhP;alcoholdehydrogenase,propanol-preferring[EC:1,1,1,1]	-3.523029673	2.61E-05
Metabolism;Lipidmetabolism;Fattyaciddegradation[PATH:ko00071];E2,3,1,9,atoB;acetyl-CoAC-acetyltransferase[EC:2,3,1,9]	-3.373422428	0.00346328
Metabolism;Lipidmetabolism;Fattyaciddegradation[PATH:ko00071];fadJ;3-hydroxyacyl-CoAdehydrogenase/enoyl-CoAhydratase/3-hydroxybutyryl-CoAepimerase[EC:1,1,1,354,2,1,175,1,2,3]	-12.25582329	0.010739859
Metabolism;Lipidmetabolism;Fattyaciddegradation[PATH:ko00071];hcaD;3-phenylpropionate/trans-cinnamatedioxygenaseferredoxinreductasesubunit[EC:1,18,1,3]	-11.80808924	0.015307575
Metabolism;Lipidmetabolism;GlyceroLipidmetabolism;[PATH:ko00561];E3,2,1,22B,galA,rafA;alpha-galactosidase[EC:3,2,1,22]	-5.507313066	2.05E-05
Metabolism;Lipidmetabolism;GlyceroLipidmetabolism;[PATH:ko00561];gldA;glyceroldehydrogenase[EC:1,1,1,6]	-5.911646545	2.42E-07
Metabolism;Lipidmetabolism;GlyceroLipidmetabolism;[PATH:ko00561];glpK,GK;glycerolkinase[EC:2,7,1,30]	-4.25237447	0.02262111
Metabolism;Lipidmetabolism;GlyceroLipidmetabolism;[PATH:ko00561];melA;alpha-galactosidase[EC:3,2,1,22]	-12.25454019	0.010249226
Metabolism;Lipidmetabolism;GlyceroLipidmetabolism;[PATH:ko00561];plsC;1-acyl-sn-glycerol-3-phosphateacyltransferase[EC:2,3,1,51]	-4.18632634	1.53E-05

Metabolism;Lipidmetabolism;GlycerophosphoLipidmetabolism;[PATH:ko00564];E3,1,4,46,glpQ,ugpQ;glycerophosphoryldiesterphosphodiesterase[EC:3,1,4,46]	-5.491990218	2.31E-07
Metabolism;Lipidmetabolism;GlycerophosphoLipidmetabolism;[PATH:ko00564];glpA,glpD;glycerol-3-phosphatedehydrogenase[EC:1,1,5,3]	-11.17474535	0.015307575
Metabolism;Lipidmetabolism;GlycerophosphoLipidmetabolism;[PATH:ko00564];plsC;1-acyl-sn-glycerol-3-phosphateacyltransferase[EC:2,3,1,51]	-4.18632634	1.53E-05
Metabolism;Lipidmetabolism;GlycerophosphoLipidmetabolism;[PATH:ko00564];ybhO;putativecardiolipinsynthase[EC:2,7,8,-]	-11.80991106	0.016700344
Metabolism;Lipidmetabolism;SphingoLipidmetabolism;[PATH:ko00600];E3,1,6,1,aslA;arylsulfatase[EC:3,1,6,1]	-12.24908257	0.007708466
Metabolism;Lipidmetabolism;SphingoLipidmetabolism;[PATH:ko00600];E3,2,1,22B,galA,rafA;alpha-galactosidase[EC:3,2,1,22]	-5.507313066	2.05E-05
Metabolism;Lipidmetabolism;SphingoLipidmetabolism;[PATH:ko00600];lacZ;beta-galactosidase[EC:3,2,1,23]	-5.671584301	3.48E-05
Metabolism;Lipidmetabolism;SphingoLipidmetabolism;[PATH:ko00600];melA;alpha-galactosidase[EC:3,2,1,22]	-12.25454019	0.010249226
Metabolism;Lipidmetabolism;SphingoLipidmetabolism;[PATH:ko00600];NEU1;sialidase-1[EC:3,2,1,18]	-8.737726906	0.025817995
Metabolism;Lipidmetabolism;Steroidhormonebiosynthesis[PATH:ko00140];E3,1,6,1,aslA;arylsulfatase[EC:3,1,6,1]	-12.24908257	0.007708466
Metabolism;Metabolismofcofactorsandvitamins;Biotinmetabolism[PATH:ko00780];bioA;adenosylmethionine-8-amino-7-oxononanoateaminotransferase[EC:2,6,1,62]	-8.238907281	0.019121562
Metabolism;Metabolismofcofactorsandvitamins;Biotinmetabolism[PATH:ko00780];bioF;8-amino-7-oxononanoatesynthase[EC:2,3,1,47]	-11.17670975	0.00346328
Metabolism;Metabolismofcofactorsandvitamins;Biotinmetabolism[PATH:ko00780];birA;BirAfamilyTranscription;alregulator,biotinoperonrepressor/biotin-[acetyl-CoA-carboxylase]ligase[EC:6,3,4,15]	-1.906216563	0.003762574
Metabolism;Metabolismofcofactorsandvitamins;Biotinmetabolism[PATH:ko00780];fabI;enoyl-[acyl-carrierprotein]reductaseI[EC:1,3,1,91,3,1,10]	-4.200496577	0.026264115
Metabolism;Metabolismofcofactorsandvitamins;Lipoicacidmetabolism[PATH:ko00785];lplA;lipoate-proteinligaseA[EC:2,7,7,63]	0.662250911	0.042708858
Metabolism;Metabolismofcofactorsandvitamins;Nicotinateandnicotinamidemetabolism[PATH:ko00760];pncB,NAPRT1;nicotinatephosphoribosyltransferase[EC:6,3,4,21]	-2.194474502	0.012065563
Metabolism;Metabolismofcofactorsandvitamins;Nicotinateandnicotinamidemetabolism[PATH:ko00760];pntA;NAD(P)transhydrogenasesubunitalpha[EC:1,6,1,2]	-11.771402	0.003262402
Metabolism;Metabolismofcofactorsandvitamins;Nicotinateandnicotinamidemetabolism[PATH:ko00760];pntB;NAD(P)transhydrogenasesubunitbeta[EC:1,6,1,2]	-7.584308884	0.001497268
Metabolism;Metabolismofcofactorsandvitamins;Nicotinateandnicotinamidemetabolism[PATH:ko00760];sthA,udhA;NAD(P)transhydrogenase[EC:1,6,1,1]	-8.72149831	0.020955328
Metabolism;Metabolismofcofactorsandvitamins;Nicotinateandnicotinamidemetabolism[PATH:ko00760];ushA;5'-nucleotidase/UDP-sugardiphosphatase[EC:3,1,3,53,6,1,45]	-11.14418291	0.003902356
Metabolism;Metabolismofcofactorsandvitamins;Onecarbonpoolbyfolate[PATH:ko00670];E6,3,3,2;5-formyltetrahydrofolatecyclo-ligase[EC:6,3,3,2]	4.351269696	2.73E-06
Metabolism;Metabolismofcofactorsandvitamins;Onecarbonpoolbyfolate[PATH:ko00670];folD;methylenetetrahydrofolatedehydrogenase(NADP+)/methenyltetrahydrofolatecyclohydrolase[EC:1,5,1,53,5,4,9]	1.161964243	1.48E-06
Metabolism;Metabolismofcofactorsandvitamins;Onecarbonpoolbyfolate[PATH:ko00670];gcvT,AMT;aminomethyltransferase[EC:2,1,2,10]	-12.21984444	0.002190529

Metabolism;Metabolismofcofactorsandvitamins;Onecarbonpoolbyfolate[PATH:ko00670];glyA,SHMT;glycinehydroxymethyltransferase[EC:2,1,2,1]	1.57364268	6.61E-07
Metabolism;Metabolismofcofactorsandvitamins;Onecarbonpoolbyfolate[PATH:ko00670];metF,MTHFR;methylenetetrahydrofolatereductase(NADPH)[EC:1,5,1,20]	-1.73540505	0.007781973
Metabolism;Metabolismofcofactorsandvitamins;Onecarbonpoolbyfolate[PATH:ko00670];MTFMT,fmt;methionyl-tRNAformyltransferase[EC:2,1,2,9]	0.89200961	0.002995451
Metabolism;Metabolismofcofactorsandvitamins;PantothenateandCoAbiosynthesis[PATH:ko00770];acpS;holo-[acyl-carrierprotein]synthase[EC:2,7,8,7]	1.797149853	3.02E-05
Metabolism;Metabolismofcofactorsandvitamins;PantothenateandCoAbiosynthesis[PATH:ko00770];coaA;typeIpantothenatekinase[EC:2,7,1,33]	-3.183807136	0.000132326
Metabolism;Metabolismofcofactorsandvitamins;PantothenateandCoAbiosynthesis[PATH:ko00770];coaBC,dfp;phosphopantothenoylcysteinedecarboxylase/phosphopantothenate--cysteineligase[EC:4,1,1,366,3,2,5]	-4.62304432	0.000161726
Metabolism;Metabolismofcofactorsandvitamins;PantothenateandCoAbiosynthesis[PATH:ko00770];E2,6,1,42,ilvE;branched-chainaminoacidaminotransferase[EC:2,6,1,42]	-2.752257097	0.000346456
Metabolism;Metabolismofcofactorsandvitamins;PantothenateandCoAbiosynthesis[PATH:ko00770];LYS5,acpT;4'-phosphopantetheinyltransferase[EC:2,7,8,-]	-8.454479247	0.000571588
Metabolism;Metabolismofcofactorsandvitamins;Retinolmetabolism[PATH:ko00830];adhP;alcoholdehydrogenase,propanol-preferring[EC:1,1,1,1]	-3.523029673	2.61E-05
Metabolism;Metabolismofcofactorsandvitamins;Retinolmetabolism[PATH:ko00830];cysG;uroporphyrin-IIIC-methyltransferase/precorrin-2dehydrogenase/sirohydrochlorinferrochelatase[EC:2,1,1,1071,3,1,764,99,1,4]	-12.25721895	0.011710324
Metabolism;Metabolismofcofactorsandvitamins;Retinolmetabolism[PATH:ko00830];EARS,gltX;glutamyl-tRNAsynthetase[EC:6,1,1,17]	-8.769486459	0.039283267
Metabolism;Metabolismofcofactorsandvitamins;Retinolmetabolism[PATH:ko00830];uidA,GUSB;beta-glucuronidase[EC:3,2,1,31]	-11.78042809	0.003902356
Metabolism;Metabolismofcofactorsandvitamins;Riboflavinmetabolism[PATH:ko00740];ribE,RIB5;riboflavinsynthase[EC:2,5,1,9]	-11.16547026	0.010541687
Metabolism;Metabolismofcofactorsandvitamins;Thiaminemetabolism[PATH:ko00730];dxs;1-deoxy-D-xylulose-5-phosphatesynthase[EC:2,2,1,7]	-6.229850367	0.019768035
Metabolism;Metabolismofcofactorsandvitamins;Thiaminemetabolism[PATH:ko00730];tenA;thiaminase(Transcription;alactivatorTenA)[EC:3,5,99,2]	-7.098204951	4.37E-08
Metabolism;Metabolismofcofactorsandvitamins;Thiaminemetabolism[PATH:ko00730];thiD;hydroxymethylpyrimidine/phosphomethylpyrimidinekinase[EC:2,7,1,492,7,4,7]	-5.462044542	3.40E-07
Metabolism;Metabolismofcofactorsandvitamins;Thiaminemetabolism[PATH:ko00730];thiE;thiamine-phosphatepyrophosphorylase[EC:2,5,1,3]	-4.842846205	0.000680763
Metabolism;Metabolismofcofactorsandvitamins;Thiaminemetabolism[PATH:ko00730];thiM;hydroxyethylthiazolekinase[EC:2,7,1,50]	-6.265058553	2.32E-07
Metabolism;Metabolismofcofactorsandvitamins;Ubiquinoneandotherterpenoid-quinonebiosynthesis[PATH:ko00130];menA;1,4-dihydroxy-2-naphthoateoctaprenyltransferase[EC:2,5,1,742,5,1,-]	-3.705492221	2.05E-05
Metabolism;Metabolismofcofactorsandvitamins;Ubiquinoneandotherterpenoid-quinonebiosynthesis[PATH:ko00130];menB;naphthoatesynthase[EC:4,1,3,36]	-5.662348449	2.85E-07

Metabolism;Metabolismofcofactorsandvitamins;Ubiquinoneandotherterpenoid-quinonebiosynthesis[PATH:ko00130];menC;O-succinylbenzoatesynthase[EC:4,2,1,113]	-6.150562841	5.96E-06
Metabolism;Metabolismofcofactorsandvitamins;Ubiquinoneandotherterpenoid-quinonebiosynthesis[PATH:ko00130];menD;2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylatesynthase[EC:2,2,1,9]	-5.67901844	2.76E-07
Metabolism;Metabolismofcofactorsandvitamins;Ubiquinoneandotherterpenoid-quinonebiosynthesis[PATH:ko00130];menE;O-succinylbenzoicacid--CoAligase[EC:6,2,1,26]	-5.130738797	2.75E-05
Metabolism;Metabolismofcofactorsandvitamins;Ubiquinoneandotherterpenoid-quinonebiosynthesis[PATH:ko00130];menF;menaquinone-specificisochorismatesynthase[EC:5,4,4,2]	-6.584201842	2.31E-07
Metabolism;Metabolismofcofactorsandvitamins;Ubiquinoneandotherterpenoid-quinonebiosynthesis[PATH:ko00130];menH;2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylatesynthase[EC:4,2,99,20]	-8.457238479	2.32E-08
Metabolism;Metabolismofcofactorsandvitamins;Ubiquinoneandotherterpenoid-quinonebiosynthesis[PATH:ko00130];ubiE;ubiquinone/menaquinonebiosynthesismethyltransferase[EC:2,1,1,1632,1,1,201]	-5.869040184	5.45E-06
Metabolism;Metabolismofcofactorsandvitamins;VitaminB6metabolism[PATH:ko00750];pdxK,pdxY;pyridoxinekinase[EC:2,7,1,35]	-1.729055447	0.00766499
Metabolism;Metabolismofcofactorsandvitamins;VitaminB6metabolism[PATH:ko00750];serC,PSAT1;phosphoserineaminotransferase[EC:2,6,1,52]	-2.871256379	0.000521046
Metabolism;Metabolismofotheraminoacids;beta-Alaninemetabolism[PATH:ko00410];fadJ;3-hydroxyacyl-CoAdehydrogenase/enoyl-CoAhydratase/3-hydroxybutyryl-CoAepimerase[EC:1,1,1,354,2,1,175,1,2,3]	-12.25582329	0.010739859
Metabolism;Metabolismofotheraminoacids;beta-Alaninemetabolism[PATH:ko00410];prr;aminobutyraldehydedehydrogenase[EC:1,2,1,19]	-11.81146043	0.018385998
Metabolism;Metabolismofotheraminoacids;beta-Alaninemetabolism[PATH:ko00410];puuE;4-aminobutyrateaminotransferase[EC:2,6,1,19]	-5.839008597	1.17E-05
Metabolism;Metabolismofotheraminoacids;Cyanoaminoacidmetabolism[PATH:ko00460];bglX;beta-glucosidase[EC:3,2,1,21]	-11.77203009	0.003118189
Metabolism;Metabolismofotheraminoacids;Cyanoaminoacidmetabolism[PATH:ko00460];glyA,SHMT;glycinehydroxymethyltransferase[EC:2,1,2,1]	1.57364268	6.61E-07
Metabolism;Metabolismofotheraminoacids;Glutathionemetabolism[PATH:ko00480];CARP,pepA;leucylaminopeptidase[EC:3,4,11,1]	-5.161530567	0.000625531
Metabolism;Metabolismofotheraminoacids;Glutathionemetabolism[PATH:ko00480];E1,11,1,9;glutathioneperoxidase[EC:1,11,1,9]	-5.514768616	3.08E-05
Metabolism;Metabolismofotheraminoacids;Glutathionemetabolism[PATH:ko00480];E4,1,1,17,ODC1,speC,speF;ornithinedecarboxylase[EC:4,1,1,17]	-9.436250667	0.002995451
Metabolism;Metabolismofotheraminoacids;Glutathionemetabolism[PATH:ko00480];G6PD,zwf;glucose-6-phosphate1-dehydrogenase[EC:1,1,1,49]	-4.965135325	4.77E-07
Metabolism;Metabolismofotheraminoacids;Glutathionemetabolism[PATH:ko00480];IDH1,IDH2,icd;isocitratedehydrogenase[EC:1,1,1,42]	-2.763414464	0.038521461
Metabolism;Metabolismofotheraminoacids;Glutathionemetabolism[PATH:ko00480];pepB;PepBaminopeptidase[EC:3,4,11,23]	-5.706615237	0.000287658

Metabolism;Metabolismofotheraminoacids;Glutathionemetabolism[PATH:ko00480];pepD;dipeptidaseD[EC:3,4,13,-]	-7.596152366	0.000603532
Metabolism;Metabolismofotheraminoacids;Glutathionemetabolism[PATH:ko00480];pepN;aminopeptidaseN[EC:3,4,11,2]	-7.651072919	1.47E-06
Metabolism;Metabolismofotheraminoacids;Glutathionemetabolism[PATH:ko00480];PGD,gnd;6-phosphogluconatedehydrogenase[EC:1,1,1,44]	-4.864027831	2.76E-07
Metabolism;Metabolismofotheraminoacids;Selenocompoundmetabolism[PATH:ko00450];metC;cystathioninebeta-lyase[EC:4,4,1,8]	1.143510255	0.007708466
Metabolism;Metabolismofotheraminoacids;Taurineandhypotaurinemetabolism[PATH:ko00430];ald;alaninedehydrogenase[EC:1,4,1,1]	-5.521648089	0.009264107
Metabolism;Metabolismofterpenoidsandpolyketides;Biosynthesisofsiderophoregroupnonribosomalpeptides[PATH:ko01053];menF;menaquinone-specificisochorismatesynthase[EC:5,4,4,2]	-6.584201842	2.31E-07
Metabolism;Metabolismofterpenoidsandpolyketides;Biosynthesisofvancomycingroupantibiotics[PATH:ko01055];E4,2,1,46,rfbB,rffG;dTDP-glucose4,6-dehydratase[EC:4,2,1,46]	1.072919012	0.035526824
Metabolism;Metabolismofterpenoidsandpolyketides;Carotenoidbiosynthesis[PATH:ko00906];crtB;phytoenesynthase[EC:2,5,1,32]	-6.365725146	6.94E-05
Metabolism;Metabolismofterpenoidsandpolyketides;Carotenoidbiosynthesis[PATH:ko00906];crtN;4,4'-diapophytoenedesaturase[EC:1,3,8,2]	-13.30782884	3.48E-05
Metabolism;Metabolismofterpenoidsandpolyketides;Geranioldegradation[PATH:ko00281];fadJ;3-hydroxyacyl-CoAdehydrogenase/enoyl-CoAhydratase/3-hydroxybutyryl-CoAepimerase[EC:1,1,1,354,2,1,175,1,2,3]	-12.25582329	0.010739859
Metabolism;Metabolismofterpenoidsandpolyketides;Limoneneandpinenedegradation[PATH:ko00903];fadJ;3-hydroxyacyl-CoAdehydrogenase/enoyl-CoAhydratase/3-hydroxybutyryl-CoAepimerase[EC:1,1,1,354,2,1,175,1,2,3]	-12.25582329	0.010739859
Metabolism;Metabolismofterpenoidsandpolyketides;Nonribosomalpeptidestructures[PATH:ko01054];E5,1,1,13;aspartateracemase[EC:5,1,1,13]	-4.942524921	0.012913123
Metabolism;Metabolismofterpenoidsandpolyketides;Polyketidesugarunitbiosynthesis[PATH:ko00523];E4,2,1,46,rfbB,rffG;dTDP-glucose4,6-dehydratase[EC:4,2,1,46]	1.072919012	0.035526824
Metabolism;Metabolismofterpenoidsandpolyketides;Terpenoidbackbonebiosynthesis[PATH:ko00900];dxs;1-deoxy-D-xylulose-5-phosphatesynthase[EC:2,2,1,7]	-6.229850367	0.019768035
Metabolism;Metabolismofterpenoidsandpolyketides;Terpenoidbackbonebiosynthesis[PATH:ko00900];E1,17,7,1,gcpE,ispG;(E)-4-hydroxy-3-methylbut-2-enyl-diphosphatesynthase[EC:1,17,7,1]	-11.16530237	0.001348297
Metabolism;Metabolismofterpenoidsandpolyketides;Terpenoidbackbonebiosynthesis[PATH:ko00900];E2,3,1,9,atoB;acetyl-CoAC-acetyltransferase[EC:2,3,1,9]	-3.373422428	0.00346328
Metabolism;Metabolismofterpenoidsandpolyketides;Terpenoidbackbonebiosynthesis[PATH:ko00900];hepST;heptaprenyldiphosphatesynthase[EC:2,5,1,30]	-5.468788894	7.53E-07
Metabolism;Metabolismofterpenoidsandpolyketides;Terpenoidbackbonebiosynthesis[PATH:ko00900];ispD;2-C-methyl-D-erythritol4-phosphatecytidylyltransferase[EC:2,7,7,60]	-11.77098915	0.00346328
Metabolism;Metabolismofterpenoidsandpolyketides;Terpenoidbackbonebiosynthesis[PATH:ko00900];MVD,mvaD;diphosphomevalonatedecarboxylase[EC:4,1,1,33]	0.613401277	0.039704551
Metabolism;Metabolismofterpenoidsandpolyketides;Tetracyclinebiosynthesis[PATH:ko00253];accB,bccP;acetyl-CoAcarboxylasebiotincarboxylcarrierprotein	0.846959361	0.049618655

Metabolism;Metabolismofterpenoidsandpolyketides;Zeatin biosynthesis[PATH:ko00908];miaA,TRIT1;tRNAdimethylallyltransferase[EC:2,5,1,75]	-1.383946873	0.03049584
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];ade;adeninedeaminase[EC:3,5,4,2]	-5.402962856	2.50E-05
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];APRT,apt;adeninephosphoribosyltransferase[EC:2,4,2,7]	-1.931540789	0.010739859
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];arcC;carbamatekinase[EC:2,7,2,2]	-11.15287326	0.003472935
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];dgt;dGTPase[EC:3,1,5,1]	-12.184505	0.000642141
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];DPO3D1,holA;DNApolymeraseIIIsubunitdelta[EC:2,7,7,7]	-2.057134442	0.001769558
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];DPO3E,dnaQ;DNApolymeraseIIIsubunitepsilon[EC:2,7,7,7]	-2.281934658	0.014968485
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];E1,17,4,1A,nrdA,nrdE;ribonucleoside-diphosphatereductasealphachain[EC:1,17,4,1]	-1.864005504	0.016073075
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];E1,17,4,1B,nrdB,nrdF;ribonucleoside-diphosphatereductasebetachain[EC:1,17,4,1]	-1.671837133	0.016557857
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];E1,7,1,7,guaC;GMPreductase[EC:1,7,1,7]	-7.20639124	0.000767427
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];nudF;ADP-ribosepyrophosphatase[EC:3,6,1,13]	-1.93227125	0.009171493
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];ppx-gppA;exopolyphosphatase/guanosine-5'-triphosphate,3'-diphosphatepyrophosphatase[EC:3,6,1,113,6,1,40]	-5.507859918	7.70E-08
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];purB,ADSL;adenylosuccinatelyase[EC:4,3,2,2]	1.408979944	0.000660017
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];purD;phosphoribosylamine--glycineligase[EC:6,3,4,13]	0.959229612	4.69E-05
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];purE;5-(carboxyamino)imidazoleribonucleotidemutase[EC:5,4,99,18]	0.900720113	0.001873837
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];purK;5-(carboxyamino)imidazoleribonucleotidesynthase[EC:6,3,4,18]	0.747616177	0.00158021
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];purL,PFAS;phosphoribosylformylglycinamidinesynthase[EC:6,3,5,3]	-5.800440217	0.000280125
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];rpoZ;DNA-directedRNApolymerasesubunitomega[EC:2,7,7,6]	1.140001556	0.003577006
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];ushA;5'-nucleotidase/UDP-sugardiphosphatase[EC:3,1,3,53,6,1,45]	-11.14418291	0.003902356
Metabolism;Nucleotidemetabolism;Purinemetabolism[PATH:ko00230];ygeT,xdhB;xanthinedehydrogenaseFAD-bindingsubunit[EC:1,17,1,4]	-9.543035019	0.016387029
Metabolism;Nucleotidemetabolism;Pyrimidinemetabolism[PATH:ko00240];codA;cytosinedeaminase[EC:3,5,4,1]	-3.331975975	0.02302363
Metabolism;Nucleotidemetabolism;Pyrimidinemetabolism[PATH:ko00240];DPO3D1,holA;DNApolymeraseIIIsubunitdelta[EC:2,7,7,7]	-2.057134442	0.001769558
Metabolism;Nucleotidemetabolism;Pyrimidinemetabolism[PATH:ko00240];DPO3E,dnaQ;DNApolymeraseIIIsubunitepsilon[EC:2,7,7,7]	-2.281934658	0.014968485

Metabolism;Nucleotidemetabolism;Pyrimidinemetabolism [PATH:ko00240];E1,17,4,1A,nrdA,nrdE;ribonucleoside-diphosphatereductasealphachain[EC:1,17,4,1]	-1.864005504	0.016073075
Metabolism;Nucleotidemetabolism;Pyrimidinemetabolism [PATH:ko00240];E1,17,4,1B,nrdB,nrdF;ribonucleoside-diphosphatereductasebetachain[EC:1,17,4,1]	-1.671837133	0.016557857
Metabolism;Nucleotidemetabolism;Pyrimidinemetabolism [PATH:ko00240];E2,4,2,6;nucleosidedeoxyribosyltransferase[EC:2,4,2,6]	-5.649851152	2.54E-05
Metabolism;Nucleotidemetabolism;Pyrimidinemetabolism [PATH:ko00240];pyrB,PYR2;aspartatecarbamoyltransferasecatalyticsubunit[EC:2,1,3,2]	0.766457577	0.047424644
Metabolism;Nucleotidemetabolism;Pyrimidinemetabolism [PATH:ko00240];pyrE;orotatephosphoribosyltransferase[EC:2,4,2,10]	1.328705156	0.002813251
Metabolism;Nucleotidemetabolism;Pyrimidinemetabolism [PATH:ko00240];pyrR;pyrimidineoperonattenuationprotein/uracilphosphoribosyltransferase[EC:2,4,2,9]	-2.227924036	0.004861897
Metabolism;Nucleotidemetabolism;Pyrimidinemetabolism [PATH:ko00240];rpoZ;DNA-directedRNApolymerasesubunitomega[EC:2,7,7,6]	1.140001556	0.003577006
Metabolism;Nucleotidemetabolism;Pyrimidinemetabolism [PATH:ko00240];tdk,TK;thymidinekinase[EC:2,7,1,21]	-1.497942543	0.011295792
Metabolism;Nucleotidemetabolism;Pyrimidinemetabolism [PATH:ko00240];tmk,DTYMK;dTMPkinase[EC:2,7,4,9]	-1.232727497	0.03859021
Metabolism;Nucleotidemetabolism;Pyrimidinemetabolism [PATH:ko00240];udk,UCK;uridinekinase[EC:2,7,1,48]	-2.215277024	0.002602884
Metabolism;Nucleotidemetabolism;Pyrimidinemetabolism [PATH:ko00240];udp,UPP;uridinephosphorylase[EC:2,4,2,3]	-8.044482887	0.002872349
Metabolism;Nucleotidemetabolism;Pyrimidinemetabolism [PATH:ko00240];upp,UPRT;uracilphosphoribosyltransferase[EC:2,4,2,9]	1.001057449	0.014968485
Metabolism;Nucleotidemetabolism;Pyrimidinemetabolism [PATH:ko00240];ushA;5'-nucleotidase/UDP-sugardiphosphatase[EC:3,1,3,53,6,1,45]	-11.14418291	0.003902356
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];argC;N-acetyl-gamma-glutamyl-phosphatereductase[EC:1,2,1,38]	-3.656685122	0.004196998
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];argD;acetylornithine/N-succinyldiaminopimelateaminotransferase[EC:2,6,1,112,6,1,17]	-11.79756423	0.009303631
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];argG,ASS1;argininosuccinatesynthase[EC:6,3,4,5]	-2.488905339	0.001164129
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];argH,ASL;argininosuccinatelyase[EC:4,3,2,1]	-3.714692095	1.59E-05
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];aroA;3-phosphoshikimate1-carboxyvinyltransferase[EC:2,5,1,19]	1.24780215	0.005802781
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];AROA2,aroA;3-deoxy-7-phosphoheptulonatesynthase[EC:2,5,1,54]	-5.965682707	3.03E-05
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];aroB;3-dehydroquinatesynthase[EC:4,2,3,4]	0.925983145	0.048249327
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];dapA;4-hydroxy-tetrahydrodipicolinatesynthase[EC:4,3,3,7]	-1.322063751	0.038521461
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];dapB;4-hydroxy-tetrahydrodipicolinatereductase[EC:1,17,1,8]	1.3854821	2.54E-05
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];dapC;N-succinyldiaminopimelateaminotransferase[EC:2,6,1,17]	-11.141225	0.001653568
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];E1,1,1,3;homoserinedehydrogenase[EC:1,1,1,3]	-1.457338016	0.02429635

Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];E1,5,1,2,proC;pyrroline-5-carboxylatereductase[EC:1,5,1,2]	-1.183816916	0.024547842
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];E2,2,1,2,talA,talB;transaldolase[EC:2,2,1,2]	-11.76644974	0.002995451
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];E2,6,1,42,ilvE;branched-chainaminoacidaminotransferase[EC:2,6,1,42]	-2.752257097	0.000346456
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];E2,7,1,71,aroK,aroL;shikimatekinase[EC:2,7,1,71]	1.050467488	0.021690776
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];E3,1,3,15B;histidinol-phosphatase(PHPfamily)[EC:3,1,3,15]	-7.431575909	1.14E-08
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];ENO,eno;enolase[EC:4,2,1,11]	-5.951624563	6.12E-05
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];FBA,fbaA;fructose-bisphosphatealdolase,classII[EC:4,1,2,13]	-6.482770493	1.53E-05
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];GAPDH,gapA;glyceraldehyde3-phosphatedehydrogenase[EC:1,2,1,12]	-5.46010237	0.000134338
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];gltB;glutamatesynthase(NADPH/NADH)largechain[EC:1,4,1,131,4,1,14]	-7.841446025	0.000132326
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];gltD;glutamatesynthase(NADPH/NADH)smallchain[EC:1,4,1,131,4,1,14]	-12.19765424	0.00100271
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];glyA,SHMT;glycinehydroxymethyltransferase[EC:2,1,2,1]	1.57364268	6.61E-07
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];hisA;phosphoribosylformimino-5-aminoimidazolecarboxamideribotideisomerase[EC:5,3,1,16]	-1.487524168	0.046606821
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];hisB;imidazoleglycerol-phosphatedehydratase[EC:4,2,1,19]	-2.975566053	0.000500234
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];hisC;histidinol-phosphateaminotransferase[EC:2,6,1,9]	-4.325886491	1.99E-06
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];hisD;histidinoldehydrogenase[EC:1,1,1,23]	-2.518577829	0.001771241
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];hisE;phosphoribosyl-ATPpyrophosphohydrolase[EC:3,6,1,31]	-2.744707468	0.001947491
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];hisF;cyclase[EC:4,1,3,-]	-2.060923175	0.010567335
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];hisH;glutamineamidotransferase[EC:2,4,2,-]	-2.214467993	0.004442549
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];hisI;phosphoribosyl-AMPcyclohydrolase[EC:3,5,4,19]	-2.184568523	0.008514636
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];IDH1,IDH2,icd;isocitratedehydrogenase[EC:1,1,1,42]	-2.763414464	0.038521461
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];leuB;3-isopropylmalatedehydrogenase[EC:1,1,1,85]	-1.446471659	0.024792564
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];leuD;3-isopropylmalate/(R)-2-methylmalatedehydratasesmallsubunit[EC:4,2,1,334,2,1,35]	-2.749564252	0.000118783
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];lysC;aspartatekinase[EC:2,7,2,4]	-2.470426967	0.000868497
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Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];OTC,argF,argI;ornithinecarbamoyltransferase[EC:2,1,3,3]	-2.288340744	0.002121995
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Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];serA,PHGDH;D-3-phosphoglyceratedehydrogenase[EC:1,1,1,95]	-3.36727392	5.99E-05
Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];serC,PSAT1;phosphoserineaminotransferase[EC:2,6,1,52]	-2.871256379	0.000521046
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Metabolism;OverviewBiosynthesisofaminoacids[PATH:ko01230];trpF;phosphoribosylanthranilateisomerase[EC:5,3,1,24]	0.705447491	0.034342378
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];accB,bccP;acetyl-CoAcarboxylasebiotincarboxylcarrierprotein	0.846959361	0.049618655
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];aceE;pyruvatedehydrogenaseE1component[EC:1,2,4,1]	-11.79245895	0.00766499
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];arcC;carbamatekinase[EC:2,7,2,2]	-11.15287326	0.003472935
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];DLD,lpd,pdhD;dihydrolipoamidedehydrogenase[EC:1,8,1,4]	-6.375720181	2.61E-05
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];E2,2,1,2,talA,talB;transaldolase[EC:2,2,1,2]	-11.76644974	0.002995451
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];E2,3,1,9,atoB;acetyl-CoAC-acetyltransferase[EC:2,3,1,9]	-3.373422428	0.00346328
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];E2,7,1,12,gntK,idnK;gluconokinase[EC:2,7,1,12]	-5.702007645	4.30E-05
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];E4,2,1,2B,fumC;fumaratehydratase,classII[EC:4,2,1,2]	-3.270061347	0.027939722
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];ENO,eno;enolase[EC:4,2,1,11]	-5.951624563	6.12E-05
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];fadJ;3-hydroxyacyl-CoAdehydrogenase/enoyl-CoAhydratase/3-hydroxybutyryl-CoAepimerase[EC:1,1,1,354,2,1,175,1,2,3]	-12.25582329	0.010739859
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];FBA,fbaA;fructose-bisphosphatealdolase,classII[EC:4,1,2,13]	-6.482770493	1.53E-05
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];FBP,fbp;fructose-1,6-bisphosphataseI[EC:3,1,3,11]	-11.79608401	0.008749896
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];fdoG,fdfH;formatedehydrogenasemajorsubunit[EC:1,2,1,2]	-6.762554455	0.004407149
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];folD;methylenetetrahydrofolatedehydrogenase(NADP+)/methenyltetrahydrofolatecyclohydrolase[EC:1,5,1,53,5,4,9]	1.161964243	1.48E-06
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];frdA;fumaratereductaseflavoproteinsubunit[EC:1,3,5,4]	-3.39479139	0.012913123
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];G6PD,zwf;glucose-6-phosphate1-dehydrogenase[EC:1,1,1,49]	-4.965135325	4.77E-07
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];GAPDH,gapA;glyceraldehyde3-phosphatedehydrogenase[EC:1,2,1,12]	-5.46010237	0.000134338

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Metabolism;OverviewCarbonmetabolism[PATH:ko01200];GLDC,gcvP;glycinedehydrogenase[EC:1,4,4,2]	-8.292996831	0.024593612
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];glyA,SHMT;glycinehydroxymethyltransferase[EC:2,1,2,1]	1.57364268	6.61E-07
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];IDH1,IDH2,icd;isocitratedehydrogenase[EC:1,1,1,42]	-2.763414464	0.038521461
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];kdgK;2-dehydro-3-deoxygluconokinase[EC:2,7,1,45]	-6.233354308	7.70E-08
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];metF,MTHFR;methylenetetrahydrofolatereductase(NADPH)[EC:1,5,1,20]	-1.73540505	0.007781973
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Metabolism;OverviewCarbonmetabolism[PATH:ko01200];pgl;6-phosphogluconolactonase[EC:3,1,1,31]	-6.034832737	6.61E-07
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];ppdK;pyruvate,orthophosphatedikinase[EC:2,7,9,1]	-9.358232779	0.000641867
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];pps,ppsA;pyruvate,waterdikinase[EC:2,7,9,2]	-3.962193451	0.029219419
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];sdhB;succinatedehydrogenaseiron-sulfursubunit[EC:1,3,5,1]	-9.543035019	0.016387029
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];serA,PHGDH;D-3-phosphoglyceratedehydrogenase[EC:1,1,1,95]	-3.36727392	5.99E-05
Metabolism;OverviewCarbonmetabolism[PATH:ko01200];serC,PSAT1;phosphoserineaminotransferase[EC:2,6,1,52]	-2.871256379	0.000521046
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Metabolism;OverviewDegradationofaromaticcompounds[PATH:ko01220];hcaD;3-phenylpropionate/trans-cinnamatedioxygenaseferredoxinreductasesubunit[EC:1,18,1,3]	-11.80808924	0.015307575
Metabolism;OverviewDegradationofaromaticcompounds[PATH:ko01220];pcaC;4-carboxymuconolactonedecarboxylase[EC:4,1,1,44]	-11.2802081	0.000379071
Metabolism;OverviewFattyacidmetabolism[PATH:ko01212];accB,bccP;acetyl-CoAcarboxylasebiotincarboxylcarrierprotein	0.846959361	0.049618655
Metabolism;OverviewFattyacidmetabolism[PATH:ko01212];E2,3,1,9,atoB;acetyl-CoAC-acetyltransferase[EC:2,3,1,9]	-3.373422428	0.00346328
Metabolism;OverviewFattyacidmetabolism[PATH:ko01212];fabI;enoyl-[acyl-carrierprotein]reductaseI[EC:1,3,1,91,3,1,10]	-4.200496577	0.026264115
Metabolism;OverviewFattyacidmetabolism[PATH:ko01212];fadJ;3-hydroxyacyl-CoAdehydrogenase/enoyl-CoAhydratase/3-hydroxybutyryl-CoAepimerase[EC:1,1,1,354,2,1,175,1,2,3]	-12.25582329	0.010739859
Metabolism;OverviewOxocarboxylicacidmetabolism[PATH:ko01210];argC;N-acetyl-gamma-glutamyl-phosphatereductase[EC:1,2,1,38]	-3.656685122	0.004196998
Metabolism;OverviewOxocarboxylicacidmetabolism[PATH:ko01210];argD;acetylornithine/N-succinyldiaminopimelateaminotransferase[EC:2,6,1,112,6,1,17]	-11.79756423	0.009303631
Metabolism;OverviewOxocarboxylicacidmetabolism[PATH:ko01210];E2,6,1,42,ilvE;branched-chainaminoacidaminotransferase[EC:2,6,1,42]	-2.752257097	0.000346456

Metabolism;OverviewOxocarboxylicacidmetabolism[PATH:ko01210];IDH1,IDH2,icd;isocitratedehydrogenase[EC:1,1,1,42]	-2.763414464	0.038521461
Metabolism;OverviewOxocarboxylicacidmetabolism[PATH:ko01210];leuB;3-isopropylmalatedehydrogenase[EC:1,1,1,85]	-1.446471659	0.024792564
Metabolism;OverviewOxocarboxylicacidmetabolism[PATH:ko01210];leuD;3-isopropylmalate/(R)-2-methylmalatedehydratasesmallsubunit[EC:4,2,1,334,2,1,35]	-2.749564252	0.000118783
Metabolism;OverviewOxocarboxylicacidmetabolism[PATH:ko01210];lysC;aspartatekinase[EC:2,7,2,4]	-2.470426967	0.000868497
Organismalsystem;Cellmobility;Bacterialchemotaxis[PATH:ko02030];cheR;chemotaxisproteinmethyltransferaseCheR[EC:2,1,1,80]	-13.11070865	0.003472935
Organismalsystem;Cellmobility;Bacterialchemotaxis[PATH:ko02030];cheV;two-componentsystem,chemotaxisfamily,responseregulatorCheV	-11.80327053	0.012048496
Organismalsystem;Cellmobility;Bacterialchemotaxis[PATH:ko02030];malE;maltose/maltodextrintransportsystemsubstrate-bindingprotein	-7.030153597	0.001616572
Organismalsystem;Cellmobility;Bacterialchemotaxis[PATH:ko02030];mcp;methyl-acceptingchemotaxisprotein	-4.13974575	0.043106084
Organismalsystem;Cellmobility;Bacterialchemotaxis[PATH:ko02030];rbsB;ribosetransportsystemsubstrate-bindingprotein	-6.526422376	1.42E-08
Organismalsystem;Cellmobility;Bacterialchemotaxis[PATH:ko02030];trg;methyl-acceptingchemotaxisproteinIII,riboseandgalactosesensorreceptor	-13.0990089	0.001572765

3 GENERAL CONCLUSIONS

Cheese is a biologically and biochemically dynamic food in which the microbiota structure is shaped by the environmental conditions and the interactions among microorganisms during manufacturing and ripening. Cheese microorganisms can be either deliberately added as starters or adventitious contaminants, that is non-starter organisms. In this PhD thesis different dairy ecosystems were investigated following cheese manufacturing and ripening by using a culture-independent HTS approach, in order to evaluate the specific role played by microorganisms in each cheese manufacturing and in each step of cheese production. Very different cheese productions were taken into account: fresh and medium-ripened pasta-filata cheeses (Mozzarella and Caciocavallo Silano) and long-ripened cheeses (Grana Padano, Parmigiano Reggiano). The microbiota of natural whey cultures (NWC) used in Mozzarella di bufala Campana, Parmigiano Reggiano and Grana Padano manufacturing was studied, in order to define which species are responsible of curd fermentation. All curds and NWCs were dominated by a naturally selected core microbiota, characterized by few thermophilic lactic acid bacteria (LAB), such as *Lactobacillus helveticus*, *Lb. delbrueckii*, *Lb. fermentum* and *Streptococcus thermophilus*, that drove the curd fermentation and were present in all the dairy productions considered, although varying in abundance. Moreover, the presence of sub-dominant species was identified, mainly environmental microbial contaminants, such as *Enterobacteriaceae*, *Propionibacterium* spp. and *Acinetobacter* spp. Mozzarella manufactures showed higher diversity in terms of sub-populations, due to the lower level of industrialization, as well as the lower selective pressure applied during the manufacturing.

Thermophilic LAB dominated also in Mozzarella di bufala PDO cheese and intermediates of production from different areas (Caserta and Salerno). Microbiota of raw milk, although very complex, did not develop during the fermentation. In fact, the species present at high abundance in raw milk, like *S. macedonicus* and *Lactococcus lactis*, were not found in the intermediates, as well as in the final product. The fermentation was driven by the microorganisms added through the NWC, specific for each production area. The spoilage dynamics of commercial high-moisture Mozzarella cheeses at the end of the shelf-life were also investigated. The microbiota composition allows to discriminate between the acidification method used (natural starters or direct addition of citric acid). In fact, cheeses produced by addition of defined starter cultures had the lowest diversity in terms of LAB composition, with *S. thermophilus* always dominating, as well as the lowest contamination of psychrotrophic bacteria. Nevertheless, the pattern of occurrence of spoilage microorganisms seemed to be more related to lot to lot variability rather than to plant or product-specific spoilage associations

Furthermore, the evolution of the metabolically active microbiota during manufacturing and ripening of Fontina PDO cheese was studied through a "RNA- based" approach. The milk samples coming from three lactation phases (oestrus, post-partum and early gestation) were extremely different: in the oestrus dominated almost completely *Lb. casei* group, while a high biodiversity was found in the milk from the other lactation phases, where *Propionibacterium acnes*, *Staphylococcus* spp., *Pseudomonas* spp., *Psychrobacter* spp. and several *Enterobacteriaceae* were part of the active microbiota. However, after the 24h curd fermentation, the microorganisms of the selected starter took over and outcompeted other microbes, regardless the composition of the milk. *Lb. delbrueckii*, *S. thermophilus* and *Lb. casei* group dominated in the curds and after 84 days of ripening.

The "RNA-based" approach was used also for the study of the microbiome involved in Caciocavallo Silano PDO cheese manufacturing and ripening. Moreover, experimental ripenings were carried out, in order to verify the possibility of accelerating the ripening through the manipulation of the technological parameters (temperature and relative humidity). Thermophilic LAB dominated curd fermentation and the cheeses after molding and brining, while non-starter LAB (*Lb. casei* group, *Lb. buchneri* group, *Lb. fermentum*) progressively increased during the ripening, reaching higher abundance in the cheese core compared to the crust. The metatranscriptome analysis revealed that genes involved in carbohydrates metabolism reached higher expression levels on the crust, while genes related to proteolysis and aminoacids catatabolic pathways were over-expressed in the core. The higher ripening temperature seemed to promote the expression of genes involved in protein degradation and aminoacid catabolism, as well as NSLAB growth, possibly accelerating the ripening.

Finally, the application of HTS for *S. thermophilus* strain monitoring was firstly highlighted, through sequencing of *lacS* gene amplicons. Twenty-eight *lacS* sequence types were identified, but this gene did not prove enough variable for this approach. In fact, one sequence type prevailed in all the curd and NWC samples analysed. Nevertheless, Mozzarella manufactures showed higher diversity compared to Grana Padano and Parmigiano Reggiano and *lacS* sequence types specific for Caserta or Salerno production area

were identified. Therefore, the HTS-based strain-monitoring has been shown to be a promising application, if genes highly variable within a species are selected.

Studies of the cheese microbiota can address several questions that are important for the improvement of dairy production and the monitoring of microbial species during manufacture and ripening can give important insights to understand the process dynamics and work out conditions that can assure a premium quality. Therefore, understanding microbial behavior during cheese manufacturing is a pivotal step in order to ensure safety and quality in dairy productions. Overall, thanks to the different HTS approaches it was possible to obtain a complete picture of the microbial *consortia* involved in each dairy production with high levels of speed, reliability and sensitivity.

4 ACKNOWLEDGEMENTS

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5 APPENDIX I – LIST OF PUBLICATIONS INCLUDED IN THE THESIS

Guidone, A., Matera, A., Ricciardi, A., Zotta, T., **De Filippis, F.**, Ercolini, D., Parente, E. (2015) The microbiota of high-moisture Mozzarella cheese produced with different acidification methods. Applied Environ. Microbiol., *in press*.

De Filippis, F., La Storia, A., Stellato, G., Gatti, M., Ercolini, D. (2014) A selected core microbiome drives the early stages of three popular Italian cheese manufactures. PLoS One 9(2):e89680.

Dolci, P., **De Filippis, F.**, La Storia, A., Ercolini, D., Cocolin, L. (2014) rRNA-based monitoring of the microbiota involved in Fontina PDO cheese production in relation to different stages of cow lactation. Int. J. Food Microbiol. 185:127-135.

Ercolini, D., **De Filippis, F.**, La Storia, A., Iacono, M. (2012) "Remake" by high-throughput sequencing of the microbiota involved in the production of water buffalo Mozzarella cheese. Appl. Environ. Microbiol. 78:8142-8145.

6 APPENDIX II – LIST OF PUBLICATIONS NOT INCLUDED IN THE THESIS

De Filippis, F., Pellegrini, N., Vannini, L., Jeffery, I.B., La Storia, A., Laghi, L., Serrazanetti, D.I., Di Cagno, R., Ferrocino, I., Lazzi, C., Turroni, S., Cocolin, L., Brigidi, P., Neviani, E., Gobbetti, M., O'Toole, P.W., Ercolini, D. (2015) Consumption of a Mediterranean diet can beneficially impact the gut microbiota and associated metabolome. Gut, *under review*.

Ercolini, D., Francavilla, R., Vannini, L., **De Filippis, F.**, Capriati, T., Di Cagno, R., Iacono, G., De Angelis, M., Gobbetti, M. (2015) Italian-style gluten-free diet alters the salivary microbiota and metabolome of Africans (Saharawi) celiac children. ISME J., *in press*.

Rizzello, C.G., Cavoski, I., Turk, J., Ercolini, D., Nionelli, L., Pontonio, E., De Angelis, M., **De Filippis, F.**, Gobbetti, M., Di Cagno, R. (2015) The organic cultivation of *Triticum turgidum* spp. *durum* reflects on the axis flour, sourdough fermentation and bread. Applied. Environ. Microbiol., doi: 10.1128/AEM.04161-14.

Garofalo, C., Osimani, A., Milanović, V., Aquilanti, L., **De Filippis, F.**, Stellato, G., Buzzini, P., Turchetti, B., Di Mauro, S., Ercolini, D., Clementi, F. (2015) Bacteria and yeast microbiota in milk kefir grains from different Italian regions. Food Microbiol. 49:123-133.

De Filippis, F., Vannini, L., La Storia, A., Laghi, L., Piombino, P., Stellato, G., Serrazanetti, D.I., Gozzi, G., Turroni, S., Ferrocino, I., Lazzi, C., Di Cagno, R., Gobbetti, M., Ercolini, D. (2014) The same microbiota and a potentially discriminant metabolome in the saliva of omnivore, ovo-lacto-vegetarian and vegan individuals. PLoS One 9(11):e112373.

Francavilla, R., Ercolini, D., Piccolo, M., Vannini, L., Siragusa, S., **De Filippis, F.**, De Pasquale, I., Di Cagno, R., Di Toma, M., Gozzi, G., Serrazanetti, D.I., De Angelis, M., Gobbetti, M. (2014) Salivary microbiota and metabolome associated with celiac disease. Appl. Environ. Microbiol. 80(11):3416-3425.

Casaburi, A., **De Filippis, F.**, Villani, F., Ercolini, D. (2014) Activities of strains of Brochothrix thermosphacta in vitro and in meat. Food Res. Intern. 62:366-374.

De Filippis, F., La Storia, A., Stellato, G., Gatti, M., Ercolini, D. (2014) A selected core microbiome drives the early stages of three popular Italian cheese manufactures. PLoS One 9(2): e89680.

Cruciata, M., Sannino, C., Ercolini, D., Scatassa, M.L., **De Filippis, F.**, Mancuso, I., La Storia, A., Moschetti, G., Settanni, L. (2014) Animal rennets as sources of dairy lactic acid bacteria. Appl. Environ. Microbiol. 80(7):2050-2061.

Ercolini, D., Pontonio, E., **De Filippis, F.**, Minervini, F., La Storia, A., Gobbetti, M., Di Cagno, R. (2013) Microbial ecology dynamics during rye and wheat sourdough preparation. Appl. Environ. Microbiol. 79 (24):7827-7836.

De Filippis, F., La Storia, A., Villani, F., Ercolini, D. (2013) Exploring the sources of bacterial spoilers in beefsteaks by culture-independent high-throughput sequencing. PLoS One 8 (7):e70222.

Cocolin, L., Alessandria, V., Botta, C., Gorra, R., **De Filippis, F.**, Ercolini, D., Rantsiou, K. (2013) NaOH-debittering induces changes in bacterial ecology during table olives fermentation. PLoS One 8 (7):e69074.

De Filippis, F., Pennacchia, C., Di Pasqua, R., Fiore, A., Fogliano, V., Villani, F., Ercolini, D. (2013) Decarboxylase gene expression and cadaverine and putrescine production by *Serratia proteamaculans in vitro* and in beef. Int. J. Food Microbiol. 165:332-338.