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Traditional home-made dry sausages produced in Sardinia: a study of the microflora

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INTRODUCTION

Dry fermented meat products: history and principles

Raw meat is a nutrient matrix rich in protein with high water activity (a_w = 0.96-0.99) and favourable pH (5.6-6.0) for microbial growth. It allows a rapid spoilage process when microorganisms contamination occurs (Hammes *et al.*, 1990; Nychas and Arkoudelos, 1990; Garriga and Aymerich, 2007). Although the inner parts of animal tissues are sterile in healthy animals, meat contamination occurs during slaughtering and cutting (Petäjä-Kanninen and Puolanne, 2007). Gastrointestinal tract, feet, hides or skin of slaughtered animals are primary sources of contamination (Garriga and Aymerich, 2007).

Raw perishable materials need transformation processes to obtain stable foodstuffs that could be stored and transported. The transformation of raw materials to foods with higher keeping quality was well known in many ancient cultures (Zeuthen, 2007). Zeuthen (2007) thinks even devising methods to preserve foods was an important prerequisites for the development of civilization.

In ancient times salt was used to preserve many foods as meat cuts and fish. This process was usually combined with drying and/or smoking. Salt addition and drying reduce the free available water for microorganisms, lowering the water activity (a_w). Smoking contributes to preservation process by covering the surface of the meat with bacteriostatic and mycostatic compounds (Honikel, 2007). Spices are often added for flavour development and have also an inhibitory activity on microbial activity contributing to the preservation process (Andres *et al.*, 2007).

Besides above cited preserving processes, fermentation is one of the oldest and most important methods used to preserve food for long periods of time. Fermentation offers a means of producing safe, nutritious foods with desirable sensory qualities and extended storage stability (Smith and Palumbo, 1981). This process can be traced back to Babylonian times and was also practised in Asia and Europe (Hammes *et al.*, 1990).

The main effect of fermentation is pH lowering as a consequence of the conversion of sugar into lactic acid by bacteria. The decrease of pH in fermented meat products besides improving preservation aids the drying process. This is due to the decrease of water-holding capacity of meat protein when pH approaches their isoelectric value (Andres *et al.*, 2007). Some microorganisms have a specific bacteriostatic activity contributing to the microbial stabilization of the final product (Hugas and Monfort, 1997; Hammes and Hertel, 1998; Hugas, 1998; Lücke, 2000; Aymerich *et al.*, 2000; Andres *et al*., 2007).

Fermented foods differ from the starting material in texture, flavour and keeping quality. Fermentation causes changes in the nutritional content of foods. Microorganisms impart desirable flavour, improve texture and enhance digestibility of foods (Smith and Palumbo, 1981).

Dry sausages are cured fermented meats. The term “cured” is applied to numerous meat products but its meaning varies according to country and product. In the Mediterranean region the term is used to describe products which have undergone complicated biochemical modifications such as proteolysis and lipolysis during a long ripening process. In the North of Europe the term “cured”

has a more general meaning and it is mainly applied to meat products treated with nitrites (Flores, 1997).

Fermented sausages are often named *salami*. Cantoni *et al*. (1987) think this word comes from Medieval Latin *salumen* (a conjunction of salted things) (Garriga and Aymerich, 2007). Pederson (1971, 1979) suggested that the name could originate from the ancient town Salamis, on the Cyprian east cost, which was destroyed by an earthquake about 450 B.C., as reported by Garriga and Aymerich (2007) and Zeuthen (2007). The words *sausage, saucisse,* and *salchicha* (English, French, and Spanish, respectively) derive from the Italian *salsiccia* (Garriga and Aymerich, 2007). First references to fermented sausages first appeared around 3000 B.C., and more information is from China and the Mediterranean area around 2000 years ago (Varnam and Sutherland, 1995 in Petäjä-Kanninen and Puolanne, 2007). Sausages were well known in the Roman Empire (Lücke, 1974; Leistner, 1986; Zeuthen, 2007). Zeuthen (2007) quoted that some authors (Breasted, 1938; Pederson, 1979) stated that the success of Caesar's legions in the conquest of Gaul can be attributed to their use of dry sausage for their meat supply. The Roman butchers cut beef and pork into small pieces, added salt and spices, packed the blend into skins and placed these products in special rooms to dry. Sausage making spread throughout the Roman Empire (Zeuthen, 2007). The Romans inherited the usage of eating such a kind of foods from the Greeks. In the pre-Christian Rome large amounts of fermented sausages (*termicina*, *circeli*, *botuli*) were eaten during flower festivals and *lupercalias* (Ordónez and de la Hoz, 2007). Adams (1986) reported a fermented pork sausage from Thailand called *Nham* as an example of other area of production (Garriga

and Aymerich, 2007). A Chinese type of sausage, *Lup Cheong*, made from goat and lamb meat with salt and flavoured with green onion, bean sauce, ginger and pepper, from the North and Southern Dynasty (589-420 B.C.) is described by Leistner (1986) (Zeuthen, 2007). In quite recent times the production of fermented sausages has been spread in America, South Africa, and Australia by immigrants from European countries (Garriga and Aymerich, 2007).

Nowadays Europe is the major producer and consumer of dry-fermented sausages (Talon *et al.*, 2004; Lebert *et al.*, 2007a; Garriga and Aymerich, 2007); Germany, Italy, Spain and France account for approximately 95% of EU production (Gevers, 2002). A wide variety of dry fermented products can be find on the European market due to different raw materials, formulations and manufacturing processes, which come from the habits and customs of the different countries and regions (Talon *et al.*, 2007a). Among this variety of products two types of dry fermented sausages have been characterised: the Northern European products and the Mediterranean ones.

The Northern type is characterised by a rapid acidification: fermentation takes place at 20-32°C for 2-5 days; pH reaches value below 5 (4.8-4.9) with a lactate concentration 20-21 mmol/100 g of dry matter. These products are made with pork and/or beef meat, 1-2 mm particle with nitrite added and are 90 mm Ø. They undergo smoking and a short ripening (2-3 weeks) process. In general they have sour odour and low levels of spice and fruity notes (Ockerman and Basu, 2007). Rapid acidification to final pH below 5 and smoking ensure safety and shelf life; fermentation and drying are clearly separated periods (Demeyer *et al.*, 2000).

On the contrary Mediterranean dry sausages are characterised by slowly and lower acidification (Samelis *et al.*, 1998; Demeyer *et al.*, 2000; Parente *et al.*, 2001a; Aymerich *et al.*, 2003; Blaiotta *et al.*, 2004a, Martín *et al.*, 2006). Fermentation occurs at 10-24 °C in 3-7 days, pH reaches higher values than in the Northern type (5.2-5.8), sometimes it is higher than 5.8, with a lactate concentration 17 mmol/100g of dry matter. They are made with pork meat, 2-6 mm particle and are 40-60 mm Ø (Ockerman and Basu, 2007). The duration of ripening varies from 4-12 weeks. The temperature at which drying and ripening occurs ranges mainly between 10 and 14 °C (Italian, French, Greek sausages) (Talon *et al.***,** 2007). The a_w value is often below 0.90, low a_w in low acidic sausages are needed for good shelf life and safety reasons. In general, Mediterranean sausages are not smoked, spiced and with a limited sour taste (Garriga and Aymerich, 2007). Many Mediterranean sausages are mould ripened.

Moulds have an important role on the flavour of the sausages due to the enzymatic deamination of aminoacid to ammonia and the corresponding alpha-ketoacid. The liberation of ammonia raises the pH of these products (Flores, 1997); lactate oxidation and proteolysis occurring in these products lead to a taste markedly different from smoked northern sausages (Lucke, 2000).

Industrial development has led to the use of starter cultures to standardize and control sausage manufacturing in both categories. However, home-made slightly fermented sausages are a group of traditional Mediterranean products with a great regional diversity (Talon *et al.*, 2007a).

Europe has a great number of traditional foods as a result of its long history, diversity of cultures and different climates. These foods, which constitute an

important part of local economy, present a high variety of flavours, colours, textures. In most cases, the technologies involved in their production rely on traditional manufacturing processes (Toldrá and Navarro, 2000) in which fermentation and ripening/drying do not always constitute two separate steps and they can be carried out in natural dryer depending on climatic conditions (Talon *et al.*, 2007a)

Traditional low acid dry cured fermented sausages are very appreciated by European consumer in the Mediterranean area. They are produced without the use of starter cultures. Fermentation and ripening occur through the growth of the natural microbiota present in the meat and in the environment. These products have often a superior quality compared to those produced in industrial scale with starters. The nature of the meat, the production technology, the specific composition and metabolic activity of the indigenous microbiota are critical factors in the characterisation of a typical product in terms of its microbiological quality and sensory properties (Amato *et al.*, 1999; Samelis *et al.*, 1998; Aymerich *et al.*, 2003). Traditional manufacturing processes and their link with a given geographical region of production confer cured fermented meats specific properties (Blaiotta *et al*., 2004a).

Mediterranean dry fermented sausages: technological microbiota

The wide variety of traditional cured fermented product is mainly due to biodiversity of microorganisms involved in their manufacturing (Aymerich *et al.*, 2004; Rantsiou and Cocolin, 2006).

Lactic acid bacteria (LAB) and coagulase-negative cocci (CNC) are the main bacterial groups involved in the fermentation and ripening of traditional Mediterranean sausages (Nychas and Arkoudelos, 1990; Aymerich *et al.*, 2003). The indigenous microbiota which characterises some Mediterranean dry sausages is also constituted by some species of halotolerant moulds and yeasts (Flores, 1997; Lucke, 2000).

Lactic acid bacteria

In traditional sausages, LAB constitute the major microbiota. Even though their initial levels varies, they reaches numbers close to the one of industrial products manufactured with starter cultures at the end of the ripening stage (Talon *et al.*, 2007a). LAB colonize meat during slaughtering and manufacture and, from 10^2-10^3 cfu/g after stuffing, they can reach 10^7-10^9 cfu/g during ripening (Cocolin *et al.*, 2001a; Comi *et al.*, 2005, Rantsiou and Cocolin, 2006). LAB usually increase the very first days of fermentation, but they can increase during the process (Talon *et al.*, 2007a).

LAB are a heterogeneous group of Gram-positive, non spore-forming, strictly fermentative bacteria. They generally lack catalase, although some of them could have pseudo-catalase activity. They convert hexoses mainly to lactic acid (homofermentatives) or to lactic acid, carbon dioxide, ethanol and/or acetic acid (heterofermentatives) (Stiles and Holzapfel, 1997, Gevers, 2002).

Although LAB isolated from traditional dry sausages belong to different genera such as *Lactobacillus, Enterococcus, Leuconostoc, Weissella, Pediococcoccus, Lactococcus* (Torriani *et al.*, 1990; Hugas *et al.*, 1993; Samelis *et*

al., 1994a; Santos *et al*. 1998; Cocolin *et al*., 2000; Coppola *et al*., 2000; Parente *et al*., 2001a; Aymerich *et al*., 2003; Papamanoli *et al.*, 2003; Comi *et al*., 2005; Drosinos *et al.*, 2005; Greco *et al*., 2005; Martin *et al.*, 2005; Rantsiou *et al*., 2005a; Rantsiou *et al*., 2005b; Aymerich *et al.*, 2006; Urso *et al*., 2006; Garriga and Aymerich, 2007; Lebert *et al.*, 2007a), species belonging to genus *Lactobacillus* are the most frequently isolated (Lebert *et al*., 2007a).

The genus *Lactobacillus* is a wide and heterogeneous taxonomic unit, it numbers more than 100 different species with a large variety of phenotypic, biochemical and physiological properties (Axelsson, 2004), but few of them have been isolated from cured fermented meat products (Cocconcelli, 2007). The species most commonly found in different kinds of fermented sausages are *Lactobacillus sakei*, *Lactobacillus curvatus* and *Lactobacillus plantarum* (Cocconcelli, 2007; Garriga and Aymerich, 2007, Lebert *et al*., 2007a). These species belong to the subgroup of facultative heterofermentative lactobacilli (Stiles and Holzapfel, 1997). Other lactobacilli found in naturally fermented sausages include *Lactobacillus alimentarius, Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus casei, Lactobacillus coryniformis, Lactobacillus maltaromicus, Lactobacillus paracasei, Lactobacillus paraplantarum, Lactobacillus pentosus, Lactobacillus rhamnosus, Lactobacillus sanfranciscensis* (Aymerich *et al*., 2006; Comi *et al.*, 2005; Coppola *et al*., 2000; Drosinos *et al*., 2005; Papamanoli *et al*., 2003; Rantsiou *et al.*, 2005a; Urso *et al*., 2006).

LAB affect the quality of fermented sausages in several ways. Their most important action is the production of lactic acid from carbohydrate fermentation. This process has a key role for flavour, colour, and texture development (Hammes

et al., 1990; Cocconcelli, 2007). Lactic acid lowers the pH of fermented sausages, thereby enhancing the products keeping qualities (Smith and Palumbo, 1983). D-lactate which only originates from bacterial metabolism has been positively correlated to sour taste (Montel *et al.*, 1993). The decrease in pH also favours the coagulation of the muscle protein and the dehydration of the product by reducing the water retention capacity of the mixture, therefore LAB contribute to sliceability, firmness and cohesiveness of the product (Smith and Palumbo, 1983; Hugas and Monfort, 1997; Talon *et al.*, 2002; Talon *et al.*, 2008).

LAB also produce acetic acid that contributes to dry sausage aroma (Bedargué *et al.*, 1993). It contributes to inhibit food-born bacteria, but only low concentrations of acetic acid are acceptable from a sensory point of view (Lucke, 2000). However, it is more effective than lactic acid at the same concentration and pH (Lucke, 2000). In addition, LAB produce small amounts of low molecular weight compounds such as ethanol, diacetyl, acetoin, 1,3- and 2,3 butanediol, and acetaldehyde that contribute to the dry sausage aroma (Bedargué *et al.*, 1993).

Lactic acid bacteria produce bacteriocins (antibacterial peptides or proteins) with inhibitory effect against other Gram-positive (Hugas and Monfort, 1997; Hugas, 1998). Bacteriocins exert their inhibitory action by formation of pores in the cytoplasmatic membrane of sensitive cells (Lucke, 2000). Some bacteriocins inhibit *Listeria monocytogenes* (Hugas, 1998; Ennahar S. *et al.* 1999; Lucke, 2000; Leroy *et al.*, 2006a), but only few are effective against *Bacillus*, *Clostridium* and *Staphylococcus* (Lucke, 2000); bacteriocins are not effective against gram-negative pathogens (Lucke, 2000).

LAB peptidases contribute with muscle amino peptidases to the release of free amino acids important for taste formation (Bedarguè *et al.*, 1993; Demeyer *et al.*, 1995, 2000; Fadda *et al.*, 1999).

Lb. plantarum, *Lb. sakei* and *Lb. curvatus* in presence of oxygen hydrogen peroxide may be formed by LAB (Hammes and Hertel, 1998; Talon *et al.*, 2004) and it is often suggested that these LAB could be involved in colour or flavour faults however there are no data clearly indicating that LAB are responsible for these defects (Talon *et al.*, 2004). Some LAB could have antioxidant properties. In the last decade it was shown that strains of *Lb. sakei* and *Lb. plantarum* possess heme-dependant catalase, *Lb. plantarum* strains also have the so-called non-heme or manganese catalase (Hammes and Hertel, 1998; Cocconcelli, 2007). The genome analysis of *Lb. sakei* (Chaillou *et al.*, 2005) revealed that this species harbours Mn-dependent SOD (Cocconcelli, 2007). LAB can exhibit nitrate reductase, heme-dependent and heme-independent nitrite reductase activity that are involved in the formation of nitrosomyoglobin which is important for product reddening (Hammes *et al.*, 1990; Wolf *et al.*, 1990).

The release of intracellular enzymes after cell-lysis also contributes to the development of flavour in the sausage (Zambonelli *et al.*, 2002).

Some LAB with decarboxilase activity produce biogenic amine such as tyramine (Montel *et al.*, 1998; Demeyer *et al.*, 2000).

Coagulase negative cocci

CNC constitute the second microbiota at the end of ripening. From an initial count of 10^3-10^4, they can reach 10^5-10^8 cfu/g at the end of ripening. Their population is generally lower than the LAB one (Talon *et al*, 2007a). *Staphylococcus spp.* is the CNC genus most frequently isolated from traditional fermented sausages, although *Kokuria varians*, *Kokuria kristiniae*, *Micrococcus luteus* have been described (Garriga and Aymerich, 2007).

The *Staphylococcus* genus is ubiquitously distributed in nature, with some species inhabiting specific ecological niches. Staphylococci major habitats are skin, skin glands, and mucous membranes of mammals and birds (Cocconcelli, 2007; Irlinger, 2008). This genus comprises 38 species, 10 of which contain subspecies (21 subspecies) (DSMZ, 2008; Garrity *et el.*, 2007). Staphylococci are Gram-positive spherical bacteria that occur in microscopic clusters. They are aerobic or facultatively anaerobic, nutritionally undemanding and catalase-positive (Irlinger, 2008). They are able to survive environmental stress, such as high salt and low temperature, encountered during fermented meat ripening (Cocconcelli, 2007).

Staphylococcus xylosus is the species most frequently isolated from dry fermented sausages (Rantsiou and Cocolin, 2006) and it represents from 17% to 100% of the isolates according to the type of sausage (Talon *et al*., 2007a). It is the dominant species in Spanish and Italian sausages (Miralles *et al*., 1996; García-Varona *et al*. 2000, Aymerich *et al.*, 2003; Martin *et al.*, 2006; Coppola *et al.*, 1997; Cocolin *et al.*, 2001b; Mauriello *et al*., 2004; Blaiotta *et al*., 2004a;

Iacumin *et al.*, 2006a; Daga *et al.*, 2007). *Staphylococcus saprophyticus* is frequently isolated from Greek sausages and it has been reported to be the dominant species in these products (Samelis *et al.*, 1998; Papamanoli *et al.*, 2002; Drosinos *et al.*, 2005). Some authors reported that *S. equorum* was the dominant species in French small unit manufacturing traditional fermented sausages (Corbière Morot-Bizot *et al.*, 2006; Leroy *et al.*, 2006b); this species was also isolated from Italian products (Mauriello *et al.*, 2004; Iacumin *et al.*, 2006a; Daga *et al.*, 2007).

Other species reported in literature are represented by *Staphylococcus carnosus, Staphylococcus haemolitycus, Staphylococcus warneri, Staphylococcus cohnii, Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus capitis, Staphylococcus intermedius, Staphylococcus chromogenes, Staphylococcus pulvereri/vitulus, Staphylococcus succinus, Staphylococcus lentus, Staphylococcus pasteuri* (Blaiotta *et al.*, 2004a; Samelis *et al.*, 1998; Papamanoli *et al.*, 2002; Coppola *et al.*, 2000; Mauriello *et al.*, 2004; Martin *et al.*, 2006; Iacumin *et al.*, 2006a; Daga *et al.*, 2007).

Coagulase-negative staphylococci play a major role in the development of sensory characteristics of Mediterranean fermented sausages (Blaiotta *et al.*, 2004a). Different *Staphylococcus* species lead to different profile of aroma compounds (Bedargué *et al.*, 1993; Montel *et al.*, 1996).

The typical dry sausages aroma of Mediterranean products, correlated with high concentration of 3-methyl butanale, methyl ketones and ethyl esters, is due to *Staphylococcus* species metabolism (Stahnke, 1994; Stahnke *et al.*, 1995; Montel *et al.*, 1996; Søndergaard and Stahnke, 2002).

Staphylococci contribute to the sensory quality of sausages *via* the catabolism of carbohydrates and aminaoacids, the formation of esters and their interaction with fatty acids (Montel *et al.*, 1998). Although it has been demonstrated the importance of meat endogenous lipases and proteinases for lipolysis and proteolysis (Demeyer, 1992; Garcia *et al.*, 1992; Molly *et al.*, 1996; Hierro *et al.*, 1997), microbial lypolytic and proteolytic activities produce compounds that are important for the aroma and the flavour of the products (Samelis *et al.*, 1993; Miralles *et al.*, 1996; Hugas and Monfort, 1997; Talon and Montel, 1997; Olesen *et al.*, 2004; Casaburi *et al.*, 2008).

Lipolysis is the first step in the aroma formation process and is followed by oxidative degradation of fatty acids into alkanes, alkenes, alcohols, aldehydes, ketones and acids that enhance the development of the flavour (Hugas and Monfort, 1997; Montel *et al.*, 1998). Although the content of aldehydes, alcohols, ketones and acids in sausages is very low (of the order ppm), their low sensory threshold make them very important for flavour: they represent 60% of the aromatic fraction in sausages without spices (Bedargué, 1993). The presence of 2 methyl ketones (2-pentanone, 2-exanone, 2-heptanone) responsible of dry sausage aroma have been associated with *S. carnosus* and *S. xylosus* (Bedargué *et al.*, 1993; Stahnke, 1995; Montel *et al.*, 1996). Aldehydes such as hexanale and nonanale associated to rancid aroma can be produced by *S. warneri* and *S. saprophyticus* (Montel *et al.*, 1998).

Staphylococcus spp. are able to synthesize or hydrolyze esters (Talon and Montel, 1997; Montel *et al.*, 1998) and this capacity influences the content of esters in dry sausage (Edwards *et al.*, 1999). Ethyl-esters contribute to the fruity

notes of sausages aroma (Stahnke, 1994; Montel *et al.*, 1996) due to their low sensory threshold (Talon *et al.*, 1998). The esterase activity of Staphylococci is probably due to intracellular or cell-enveloped associated enzymes (Talon *et al.*, 1997; Casaburi *et al.*, 2006). It seems to be diversity of esterases in relation to activity levels, substrate specificity and sub-cellular localization among species and among strains of the same species (Casaburi *et al.*, 2006). *S. warneri* and *S. saprophyticus* have been shown to have more esterase activity than *S. xylosus* (Montel *et al.*, 1998)

Bacterial peptidases contribute to the initial breakdown of myofibrillar proteins, particularly myosin and actin, releasing free amino acids (Hughes *at al.*, 2002). The catabolism of branched-chain amino acids L-leucine, valine and leucine leads to formation of volatile compounds such as 2-methyl propanale and 2-3 methylbutanale (Waade and Stahnke, 1997) involved in sausage aroma. Their formation depends on the *Staphylococcus* species (Søndergaard and Stahnke, 2002).

Diacetyl and acetoin may be produced by *S. saprophyticus* and *S. warneri* from pyruvate catabolism (Bedarguè *et al.*, 1993).

It has been reported that staphylococci belonging to different species such as *S. xylosus*, *S. carnosus* and *S. simulans* posses anti oxidant enzymatic activities (Barriere *et al.*, 2001a; Casaburi *et al.*, 2005), thus they limit lipid oxidation through superoxide and peroxide decomposition (Talon *et al.*, 2000; Barriere *et al.*, 2001b). The inhibition of some unsaturated free fatty acid, such as linoleic acid, by *Staphylococcus* spp. has been reported by Talon *et. al.* (2004). The removal of peroxide produced in meat by various oxidative mechanisms

eliminates color defects resulting from peroxidative reactions (Smith and Palumbo, 1981).

Nitrate reductase activity of staphylococci is important for the development of red colour of the products (Cocconcelli, 2007) and to limit oxidation (Montel *et al.*, 1998). It has been detected in *S. xylosus*, *S. carnosus*, *S. epidermidis*, *S. equorum*, *S. lentus* and *S. simulans* (Talon *et al.*, 1999; Mauriello *et al.*, 2004; Cocconcelli, 2007).

Yeasts and moulds

Yeasts are habitual component of fermented sausages microbiota. Yeasts can grow at pH, water activity and temperature habitual in fermented sausages (Hammes and Knauf, 1994). Many species can grow at pH 4-6 and are able to maintain a neutral intracellular pH (Selgas and Garcia, 2007). Most species have a strictly aerobic metabolism although yeasts are able to grow in anaerobic media (Selgas and Garcia, 2007). Generally, their development is restricted to near the product surface, however they have been isolated in the innermost parts of products (Grazia *et al.*, 1986; Selgas *et al.*, 2003). The evolution of yeast population during ripening is influenced by different variables. Sausage diameter is an important factor influencing the counts of these microorganisms: higher counts have been found in smaller sausages due to the higher availability of oxygen in these sausages (Selgas and Garcia, 2007). Lower counts of yeasts have been observed in smoked sausages than in non-smoked kinds (Encinas *et al.*, 2000) because they are sensitive to the smoking process (Leistner, 1995).

The yeasts isolated from Mediteranean fermented sausages belong to genera *Candida, Cyteromyces, Cryptococcus, Debaryomyces, Galactomyces, Geotricum, Hansenula, Kluyveromyces, Pichia, Rhodotorula, Saccharomyces, Sporobolomyces, Torulaspora, Torulopsis, Trichosporon, Yarrowia* (Comi and Cantoni, 1983; Boissonet *et al.*, 1994; Samelis *et al.*, 1994b; Metaxopoulos *et al.*, 1996; Coppola *et al.*, 2000; Encinas *et al.*, 2000; Selgas *et al.*, 2003; Selgas and Garcia, 2007). The species most frequently isolated is *Debaryomyces hansenii* (Selgas and Garcia, 2007).

The main role of yeasts in fermented sausages seems to be related to the increase of ammonia and reduction of lactic acid content together with proteolytic activity (Garriga and Aymerich, 2007).

Yeasts possess lipolytic and proteolytic activity (Cook P.E., 1995; Jessen, 1995; Huerta *et al.*, 1988; Encinas *et al.*, 2000; Selgas *et al.*, 2003). They contribute to establishment of red pigment and inhibit the development of rancidity thanks to their consumption of oxygen and catalase production (Lücke and Heckelmann, 1987; Flores et *al.*, 2004). They are able to generate volatile compounds from branched-chain amino acids (Durà *et al.*, 2004) and metabolize organic acids mainly lactic and acetic acid (Selgas and Garcia, 2007).

Many dry sausages of Mediterranean area exhibit mould growth on the product surface (Flores, 1997). The moulds have an important role in developing of product flavour (Flores, 1997; Hammes and Hertel; 1998).

Penicillium, Aspergillus, Mucor and *Cladosporium* are the genera of moulds most frequently found (Garriga and Aymerich, 2007). Natural contamination results in a prevalence of xerotolerant strains belonging to the genus *Penicillium*

over those belonging to the genera *Aspergillus* or *Eurotium* (Spotti and Berni, 2007). Most *Penicillium* species grows well in environments with RH ranging from 85 to 92% and temperatures from 10-20 °C (Spotti and Berni, 2007), environmental conditions which are common during sausage ripening.

Moulds contribute to flavour development through lactate oxidation, proteolysis, degradation of amino acids, lipolysis, β-oxidation (Leistner, 1984; Grazia *et al.*, 1986; Lücke, 1997; Sunesen and Stahnke, 2003). The enzymatic oxidative de-amination of aminoacids leads to the release of ammonia and the corresponding alpha-ketoacids (Flores, 1997).

Moulds have an antioxidant effect by reducing the oxygen tension on the surface of sausage, metabolizing peroxide through catalase activity and protection against light (Bacus, 1986; Lucke and Hechelmann, 1987; Bruna *et al.*, 2001; Herranz *et al.*, 2004). The antioxidant action prevents rancidity and improves colour of the product (Sunesen and Stahnke, 2003; Herranz *et al.*, 2004; Spotti and Berni, 2007). The mycelium reduces water loss and leads to a slower and more homogenous dehydration of the product (Grazia *et al.*, 1986; Lücke, 1997), makes easier product skin peeling (Grazia *et al.*, 1986), and prevents growth of undesirable microorganisms (Smith and Palumbo, 1983). Moreover, the characteristic appearance of the mycelium is appreciated by many European consumers (Herranz *et al.*, 2004).

Molecular tools for Mediterranean dry fermented sausages technological microbiota study.

In the past microorganisms involved in the transformation of food were identified by using phenotypic tests. These tests take advantages of biochemical, physiological and biological phenomena. Most of them are based on the ability of a species to grow under given environmental conditions or the need of certain nutrients (Van Belkum, 1994). The identification by biochemical tests is based on the capability of a microorganism to produce certain metabolites on a given substrate. These methods often lead to an incorrect identification (Meugnier *et al.*, 1996; Forsnam *et al.*, 1997; Coppola *et al.*, 2000; Nigatu, 2000; Iacumin *et al.*, 2006a). The positive result for a certain character is done by colour change of culture medium and the interpretation of the result by the technician is often subjective (Rantsiou and Cocolin, 2006). Harbouring some character is often strain specific and it does not permit correct species identification of the isolate (Couret *et al.*, 2003). For example some commercial kit for coagulase negative staphylococci identification are designed for clinical isolates and lead to misidentification of food isolates (Blaiotta *et al.*, 2003a; Hoppe-Seyler *et al.*, 2004). Morover identification by biochemical tests is time consuming.

In the last two decades several typing methods based on microbial genotype or DNA sequence have been developed to overcome the shortcomings of phenotypically based typing methods (Olive and Bean, 1999).

Nowadys these methods based on microorganisms genome differences have practically replaced the phenotypic ones for the identification of microbial

isolates. They are reproducible and rapid (Charteris *et al.*, 1997; Olive and Bean, 1999; Rantsiou and Cocolin, 2006), and in some cases enable the establishment of large databases of characterized organisms (Olive and Bean, 1999).

Molecular methods such as ribosomal RNA probes (Nissen and Dainty, 1995; Hertel *et al.*, 1991), ribotyping (Zhong *et al.*, 1998), restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene (Sanz *et al.*, 1998; Lee *et al.*, 2004), randomly amplified polymorphic DNA (RAPD)-PCR analysis (Berthier and Ehrlich, 1999; Andrighetto *et al.*, 2001), species-specific PCR (Berthier and Ehrlich, 1998; Yost and Nattress, 2000; Rossi *et al.*, 2001; Blaiotta *et al.*, 2003a; Morot-Bizot *et al.*, 2003; Aymerich *et al.*, 2003; Greco *et al.*, 2005; Iacumin *et al.*, 2006a; Martin *et al.*, 2006; Aymerich *et al.*, 2006; Daga *et al.*, 2007), amplified ribosomal DNA restriction analysis (ARDRA) (Aquilanti *et al.*, 2007; Bonomo *et al.*, 2008) 16S rRNA gene sequencing (Daga *et al.*, 2007); multiplex PCR (Corbiere Morot-Bizot *et al.*, 2004; Martin *et al.*, 2005), PCR amplification of repetitive bacterial DNA elements (rep-PCR) (Gevers *et al.*, 2001), temperature gradient gel electrophoresis (TGGE) (Cocolin *et al.*, 2000) and denaturing gradient gel electrophoresis (DGGE) (Cocolin *et al.*, 2001b; Ercolini *et al.*, 2001; Blaiotta *et al.*, 2003b;Comi *et al.*, 2005; Urso *et al.*, 2006; Iacumin *et al.*, 2006a; Silvestri *et al.*, 2007) have been applied for the characterization of LAB and CNC microbiota of sausages.

Plasmid profiling (Martin *et al.*, 2005; Aymerich *et al.*, 2006; Martin *et al.*, 2006); Pulsed Field Gel Electrophoresis (PFGE) (Corbière Morot-Bizot, 2006) (RAPD)-PCR analysis (Rebecchi *et al.*, 1998; Berthier and Ehrlich, 1999; Martin *et al.*, 2005; Rantsiou *et al.*, 2005a; Comi *et al.*, 2005; Martin *et al.*, 2006;

Aymerich *et al.*, 2006; Rantsiou *et al.*, 2006; Iacumin *et al.*, 2006b; Urso *et al.*, 2006; Bonomo *et al.*, 2008), Sau-PCR analysis (Iacumin *et al.*, 2006b) and rep-PCR (Gevers *et al.*, 2003; Iacumin *et al.*, 2006b) have been used to characterize at strain level LAB and CNC isolated from sausages.

In this study 16s RNA gene sequencing and species-specific PCR have been used to characterize isolates at species level. Rep-PCR and RAPD-PCR analysis have been applied to type isolates within species at strain level.

All these molecular methods are based on polymerase chain reaction (PCR) developed by Mullis (1990).

PCR technique allows *in vitro* amplification of a specific DNA region. It is a very rapid, specific and sensible technique.

DNA is synthesized *in vitro* by using a thermostable DNA polymerase (*Taq* polymerase). *Taq* polymerase needs short single strand DNA molecules (primers) to originate a new DNA fragment by using a target DNA. Primers have to be complementary to DNA sequences flanking DNA region to amplify. PCR consists in the repeat of thermal cycles consisting of three steps each one:

Denaturation at 95 °C to allow the separation of the two DNA strands.

Annealing at variable temperature depending on primers sequence, during which primers anneal to target sequence.

Extension at 72°C during which *Taq* polymerase starts extension reaction of DNA strand.

The three steps are repeated variable times, usually 35-40 times. In this way a large number of copy of a certain DNA fragment can be obtained in few hours.

The amplification product is then subjected to gel electrophoresis and visualized under UV light after ethidium bromide or SYBR-green staining.

16S rRNA gene sequence analysis

Sequence analysis of the 16S rRNA gene represents a highly accurate and versatile method for bacterial identification.

The use of (rDNA)-based molecular identification can achieve identification of bacteria because of rDNA universal distribution among bacteria and the presence of species-specific variable sequences. It has been showed an excellent overall performance of this technique, since it was able to resolve almost 90% of identifications when applied to a large collection of phenotypically unidentifiable bacterial isolates (Drancourt *et al.,* 2000). Different regions can be recognized in 16S rDNA sequence: universal conserved regions with the same sequence in all bacteria; semi-conserved region and hyper-variable regions that have the same sequence in bacteria belonging to the same species (Woese, 1987). 16S rDNA is amplified by using universal primers designed on conserved regions, then the PCR product is subjected to sequencing reaction. The obtained rDNA sequence can be compared with those recorded in on-line databases by BLAST (Basic Local Alignment Search Tool) search program (Altschul *et al.*, 1997). A 16S rDNA sequence similarity higher than 97% with those recorded in databases for a certain species usually allows to identify the bacterial isolate by assigning it to the species.

Species-specific PCR

Species-specific PCR based assays have the same reliability of the other molecular techniques and it is more simple and rapid. It can be performed in less 5-6 h and a large number of isolates can be processed simultaneously (Blaiotta *et al.*, 2005)

Species-specific PCR is based on the use of primers annealing to DNA sequence specific of a bacterial species. If the primers found the target sequence, it will be synthesized a PCR product with a number of bp equal to that of the DNA region between the two primers used for the polymerase reaction. The presence of the expected size band visualized on a agarose gel after electrophoresis means that the tested microorganism belongs to the species which primers are specific for. Species-specific primers have been designed by utilizing DNA sequence differences among species of different gene or regions between genes. 16S rRNA gene, 16S-23S rRNA gene spacer region, *rec*A, *sod*A, *tuf, ddl* genes have been used to design species-specific primers (Berthier and Ehrlich,1998; Ke *et al.*, 1999; Knijff *et al.*, 2001; Ward and Timmins, 1999; Torriani *et al.*, 2001; Aymerich *et al.*, 2003; Ventura *et al.*, 2003; Blaiotta *et al.*, 2005; Blaiotta *et al.*, 2004b; Jackson *et al.*, 2004).

PCR fingerprinting

The basis of PCR fingerprinting is the amplification of polymorphic DNA through specific selection of primer annealing sites. Either constant primer sites bridge a single variable sequence domain or primers detect consensus sequences with variable distribution in the DNA. Differences in the distance between primer-

binding sites or existence of these sites lead to synthesis of amplified DNA fragments which differ in length. These differences can be detected by simple procedures such as gel electrophoresis (Van Belkum, 1994).

RAPD-PCR assays.

The random amplified polymorphic DNA (RAPD) assay was first described by Williams *et al.* (1990) and Welsh and McClelland (1990).

RAPD assay is based on the use of short random sequence primers, 9 to 10 bases in length, which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures such that they can be used to initiate amplification of regions of the bacterial genome. If two RAPD primers anneal within a few kilobases of each other in the proper orientation, a PCR product with a molecular length corresponding to the distance between the two primers results. In most cases the sequences of the RAPD primers which generate the best DNA pattern for differentiation must be determined empirically (Olive and Bean, 1999).

The number and location of these random primer sites vary for different strains of a bacterial species. The separation of the amplification products by agarose gel electrophoresis leads to a pattern of bands which, in theory, is characteristic of the particular bacterial strain (Olive and Bean, 1999).

It has been found that RAPD assay is more discriminating than RFLP analysis of either the 16S rRNA genes or the 16S-23S rRNA spacer region but less discriminating than Rep-PCR (Olive and Bean, 1999). Although it has been shown this method is less effective than other molecular methods, sometimes it

allows discrimination of strains that are indistinguishable by other techniques (Couret *et al.*, 2003).

RAPD analysis is a rapid and cheap typing method but it sometimes shows a poor reproducibility because the primers are not directed against any particular genetic locus and many of the priming events are the result of imperfect hybridization between the primer and the target site (Meunieur and Grimont, 1993; Olive and Bean, 1999; Couret *et al.*, 2003). Therefore to enhance the reproducibility of results a careful condition optimization is required (Couret *et al.*, 2003).

Rep-PCR analysis.

Prokaryotic genomes contain dispersed repetitive sequences separating longer single-copy DNA sequences (Lupski and Weinstock, 1992). Various classes of repeated sequences are present in diverse prokaryotic genomes. (REP, ERIC, BOX (Versalocic *et al.*, 1994; Olive and Bean, 1999). These sequences can be used as oligonucleotide primer binding sites for polymerase chain reaction mediated genomic fingerprinting (rep-PCR). Several of these interspersed repetitive elements are conserved in diverse genera of bacteria enabling the use of single primer sets for DNA fingerprinting of many different microorganisms (Versalovic *et al.*, 1994).

In rep-PCR (repetitive sequence element PCR) the primers bind to repetitive sequences in the prokaryotic genome, and if those primer binding sites are in the proper orientation and within a distance that can be spanned by *Taq* polymerase extension, an amplification product is obtained. Size fractionation of the

amplification products by agarose gel electrophoresis reveals a specific pattern or genomic fingerprint. These fingerprints appear to be species or even strain specific. Multiple colonies isolated from the same culture, as well as repeated isolates over time from the same strain reveal a specific pattern, demonstrating that the fingerprint is stable and specific to a given bacterial strain (Lupski and Weinstock, 1992; Versalovic *et al.*, 1994)

DNA fingerprinting patterns must be compared objectively. Between eight and fifteen bands per lane are desirable for rigorous comparative analyses. Electrophoresis conditions must be standardized (percent agarose, electrophoresis buffer, gel volume, separation times). DNA fingerprinting pattern may be compared by visual inspection of small sample sets in single gels, however quantitative (e.g. statistical) methods may be necessary for pattern comparisons if large sample sets are used and relative degree of similarity must be evaluated (Versalovic *et al.*, 1994).

Rep-PCR is a widely used method of DNA typing. The technique is easy to perform and can be applied to large or small numbers of isolates. Rep-PCR shows broader species applicability and better discriminatory power than either plasmid profiling or genomic fingerprinting. It has considerably better discriminatory power than restriction analysis of the 16S rRNA gene or the 16S-23S spacer region. Several studies have shown Rep-PCR to have good correlation with PFGE results but, in general, with slightly less discriminatory power (Olive and Bean, 1999).

Traditional homemade dry sausages: reasons for a study about them

Typical products manufactured with traditional raw materials using ingrained production methods have a preferential position in European consumers preferences (Conter *et al.*, 2008; Christieans *et al.*, 2006). Traditional products are often produced in small-scale facilities located in rural areas: such enterprises are frequently integrated into other sectors of local economy for instance tourism operators (Conter *et al.*, 2008).

Typical products are the result of the adaptation of technical skills to local resources, then they are strongly linked to the area of production (Scintu and Piredda, 2007; Conter *et al.*, 2008) and they are perceived as having less impact on the environment. Consumers consider traditional foods safer and healthier than the industrial ones especially since the occurrence of different alimentary crisis (BSE, dioxin, OGM) (Christieans *et al.*, 2006; Scintu and Piredda, 2007).

In traditional dry sausages produced without the addition of starter the processes of fermentation and ripening are due to the natural microbiota colonising raw materials and manufacturing environment. This is a complex ecosystem varying among different facilities.

In the last decade numerous studies concerning technological, chemical, microbiological and sensory characterization of traditional fermented meat products have been performed. In particular, autochthonous microbiota of Mediterranean dry fermented sausages produced without the addition of any starter have been received a great attention by scholars, as the numerous scientific published papers shows (Samelis *et al.*, 1994a, b; Coppola *et al.*, 1995a;

Metaxopoulos *et al.*, 1996; Coppola *et al.*, 1997; Coppola *et al.*, 1998; Samelis *et al.*, 1998; Santos *et al.*, 1998; Coppola *et al.*, 2000; Encinas *et al.*, 2000; García-Varona *et al.*, 2000; Cocolin *et al.*, 2001a, b; Parente *et al.*, 2001a, b; Papamanoli *et al.*, 2002; Aymerich *et al.*, 2003; Papamanoli *et al.*, 2003; Mauriello *et al.*, 2004; Blaiotta *et al.*, 2004a; Comi *et al.*, 2005; Drosinos *et al.*, 2005; Greco *et al.*, 2005; Martín *et al.*, 2005; Rantsiou *et al.*, 2005a, b; Aymerich *et al.*, 2006; Iacumin *et al.*, 2006; Martín *et al.*, 2006; Urso *et al.*, 2006; Daga *et al.*, 2007; Lebert *et al.*, 2007b; Bonomo *et al.*, 2008)

The study of natural microbiota of traditional fermented sausages is important to preserve their sensory characteristics. These characteristics are linked to the autochthonous microbiota that have been selected in the particular production environment.

Few species seem to be able to colonize such a kind of product and dominate the microbiota at the end of ripening. Neverthless, some authors pointed out a great biodiversity at strain level of microorganisms within species (Rantsiou *et al.*, 2005; Urso *et al.* 2006; Martin *et al.* 2006; Garriga e Aymerich, 2007).

The study of biodiversity at strain level of microorganisms important from a technological point of view represents one of future development in fermented meat microbiology for explaining the wide diversity of products (Cocolin e Rantsiou, 2006). In fact, sausages produced in different country but even in same country are characterized by different sensory profile that cannot be explained only by differences in used ingredients (Cocolin e Rantsiou, 2006). Furthermore, intraspecific biodiversity constitutes a source to select autochthonous strains that could be used as starters in traditional manufactures (Lebert *et al.*, 2006).

In Italy there is a wide variety of typical meat products, most of them are known only at local and regional level (Comi *et al*., 2005). Many typical fermented sausages are manufactured with traditional technologies, without selected starters added. The microbiota is closely related to the formulation, and the fermentation and ripening practices. Home-made products have a greater quality than those produced by using industrial starters, this phenomenon is due to the specific composition of the house microflora (Lebert *et al*., 2007; Bonomo *et al*., 2008).

Home production and consumption of traditional dry fermented sausages (*salsiccia*) is widespread in the island. Meat is processed during the coldest period of the year from November to March-April.

Pork meat, pork fat, salt, spices (black pepper, garlic, fennel seeds), and wine are used as ingredients; sometimes potassium nitrate is added. Some of the above-mentioned components may be absent. The proportion of ingredients varies depending on the production region and the family tradition.

Coarse minced meat is mixed with fat and the other ingredients manually. The mixture is left to stand, for a variable period, usually a few hours or overnight, after which it is stuffed in natural casings of pork or calf. Then the sausages are hung and trickled for 2-3 days at not controlled environmental conditions. After this stage, they may be smoked using wood of Mediterranean flora (e.g. oak, lentisk, myrtle, cistus). The smoking usually takes place for 2-3 hours a day for 3 days at not controlled temperature conditions.

The ripening lasts about 30 days and takes place in a cellar or 'farmhouse kitchen' where conditions of temperature and relative humidity are not controlled. The natural fermentation occurs through the growth of the microbiota present in the meat and in the environment, without the addition of any starter culture.

Despite the extent of pig farming and a thousand-year-old tradition of pork processing in Sardinia, in depth studies had never highlighted the quality and tradition of the island's cured meat products (Greco *et al*., 2005; Daga *et al.*, 2007)

To study the natural microbiota of traditional meat products produced in Sardinia and its biodiversity is an important step for product sensory characteristics preservation and valorisation.

Nowadays there is an increased interest in preserving biodiversity of microorganisms naturally involved in food transformation (Leroy *et al.*, 2006b; Scintu and Piredda, 2007).

The importance in biodiversity preserving have been defined precisely by the European Union with Council regulation (EC) No 870/2004 in which it is reaffirmed that "biological and genetic diversity in agriculture is essential to the sustainable development of agricultural production" and that "the work undertaken on the conservation, characterisation, collection and utilisation of genetic resources in agriculture can help maintain biological diversity, improve the quality of agricultural products, contribute to increase diversification in rural areas and reduce inputs and agricultural production costs".

The same regulation states the need to promote the *ex situ* and *in situ* conservation of microbial genetic resources in agriculture (including *in situ*/on-farm conservation and development).

The valorisation of naturally fermented products is a means of promoting *in situ* conservation of genetic resources in agriculture and the constitution of collection of microbial strains from traditional local products contribute to *ex situ* conservation of such resources that are or could prove useful for agriculture and rural development.

AIM OF THE STUDY AND MAIN OBJECTIVES

The aim of this work is to carry out an investigation on the microflora of home-made Sardinian sausage to valorise the product and to constitute a collection of strain isolated from this traditional product

The present study was focused on the next main objectives:

- ✓ To characterize microbial groups involved in fermentation and ripening process and their development.
- ✓ To characterize species of LAB and CNC and their distribution
- ✓ To evaluate biodiversity among isolates at strain level
- ✓ To constitute a collection of autochthonous strain isolated from naturally fermented sausages
- ✓ To set up a database containing the information about isolated strains

MATERIALS AND METHODS

Sausage manufacture

Five batches of sausages were produced according to traditional methods without the use of starter cultures by five different artisanal producers (hereinafter called producer AA, AB, AC, AD, AE respectively) located in different areas of Sardinia.

Table 1. Manufacturing conditions

	AA	AB	AC	AD	AE
Meat/fat ratio	95/5	70/30	65/35	70/30	
NaCl	21 g/Kg	26 g/Kg	31 g/Kg	33 g/Kg	20 g/Kg
Garlic	0.24 g/Kg	2 g/Kg	1 g/Kg		1g/Kg
KNO_3		0.5g/Kg			
Black pepper	1 g/Kg ground	1 g/Kg ground	3.8 g/Kg ground	2 g/Kg ground	2 g/Kg
Nutmeg				0.16 g/kg	
Fennel seeds			2.5 g/Kg		
White wine				5 ml/kg	
Vinegare	12 ml/Kg				22 ml/Kg
Preservative					0.5 g/kg
Mincing		electric 22 mm Ø	electric 18 mm Ø	electric 22 mm Ø	electric 22 mm Ø
Casing	Beef	Beef	Beef	Beef	Beef
Lenght-Ø	50 cm 4 cm	50/60 cm 4 cm	50 cm 4 cm	50 cm 3/3.5 cm	50 m
Smoking		2-3h/day Wood of Mediterranean flora	2 days After dripping/ 3 days		
Average T °C	8.72	9.7	9°	13.36	13.22
Average UR %	73.26	68	76	76.55	55.77

Sampling

Three samples of sausages at 0 days, 28 days and the end of ripening were analysed. The end of ripening was different for the five producers: 40 days for AA, AB and AC, 28 days for AD, 35 for AE.

pH and a_w measuraments

The pH was determined potentiometrically by inserting the pHmeter electrode (Crison Instruments, S.A., Barcelona, Spain) into the products g of sample with 90 ml of distilled water. A digital pH meter was used.

Water activity was determined at 25 °C using Novasina AW Sprint (Axair Ltd., Pfäffikon, Switzerland). Two independent measurements were made on each sample.

Microbiological analysis

Microbial counts

The sampling of the sausages was performed starting with the aseptic removal of the casings.

An aliquot of 20 g of each sample was taken aseptically and homogenised with 180 ml of sterile diluent containing peptone (1 g/l), NaCl (8.5 g/l) and Tween 80 (1 ml/l) in a Stomacher blender (Seward Medical, London, UK).

Ten-fold dilutions of the homogenates were made in sterile peptone water (1 g/l). Aliquots of dilutions were then plated in duplicate on: Plate Count Agar (PCA, Oxoid, Unipath Ltd. Basingstoke,UK) for mesophilic aerobic total count;

MRS agar for LAB incubated anaerobically (Gas Generating Kit, Oxoid, Unipath Ltd., Basingstoke, UK); Mannitol Salt Agar (MSA, Oxoid, Unipath Ltd., Basingstoke, UK) for *Micrococcaceae*; Slanetz and Bartley agar (SBA, Oxoid, Unipath Ltd., Basingstoke, UK), for enterococci; Violet Red Bile Glucose agar (VRBG, Oxoid, Unipath Ltd., Basingstoke, UK) for *Enterobacteriaceae*; Malt Extract Agar (MEA, Oxoid, Unipath Ltd., Basingstoke, UK) for yeasts and moulds.

The media used are described below.

MRS (De Man, Rogosa, Sharpe)

MRS medium is recommended for use in the enumeration (agar), isolation and cultivation of LAB

	g/L
Peptone	10.0
Meat extract	8.0
Yeast extract	4.0
D(+)-Glucose	20.0
Potassium phosphate	2.0
Ammonium citrate	2.0
Sodium acetate	5.0
Magnesium sulphate	0.2
Manganese sulphate	0.05
Tween 80	1 ml
Agar	10.0
pH 6.2 ± 0.2	

Brain Heart Infusion Broth (BHI)

BHI broth is a general-purpose liquid medium used for the cultivation of a wide variety of microorganisms, including bacteria, yeasts and moulds. It is a nutritious, buffered culture medium that contains infusions of brain and heart tissue and peptones to supply protein and other nutrients necessary to support the growth of fastidious and non fastidious microorganisms

	g/L
Calf brain infusion	12.5
Beef heart infusion	5.0
Peptones mix	10.0
D(+)-Glucose	2.0
Sodium Chloride	5.0
Disodium Phosphate	2.5
pH 7.4 ± 0.2	

Mannitol Salt Agar (MSA)

This is a selective medium used for the enumeration and isolation of CNS.

The selectivity of this medium is due to the high concentration of sodium chloride (7.5%) since CNS are able to tolerate high salt concentrations.

	g/L
Peptone	10.0
Beef Extract	1.0
D-Mannitol	10.0
Sodium Chloride	75.0

Phenol Red	0.025
Agar	15.0
pH 7.5 ± 0.2 .	

Malt Extract Agar (MEA)

Malt extract agar is used for isolating, cultivating and enumerating yeasts and moulds.

The acid pH of the medium allows the growth of yeasts and moulds and restricts bacterial growth.

	g/L
Malt extract	30.0
Mycological peptone	5.0
Agar	15.0
Supplemented with Lactic Acid 10% (2 ml/100 ml)	
pH 3.5 ± 0.2	

Plate Count Agar (PCA)

PCA medium is used for total count of aerobic bacteria and heterotrophic facultative anaerobes.

	g/L
Tryptone	5.0
Yeast extract	2.5
Dextrose	1.0
Agar	9.0
pH 7.0 ± 0.2	

Slanetz Bartley Agar (SBA)

Selective medium used for isolation and enumeration of enterococci in food. Sodium Azide inhibits the growth of Gram-negative bacteria and staphylococci. Enterococci reduce Triphenyltetrazolium Chloride (TTC) to formazan and grow forming red-pink colonies.

	g/L
Tryptose	20.0
Yeast Extract	5.0
Dextrose	2.0
Disodium Phosphate	4.0
Sodium Azide	0.4
Triphenyltetrazolium Chloride	0.1
Bacteriological Agar	10.0
pH 7.2 ± 0.2	

Violed Red Bile Glucose Agar (VRBGA)

VRBGA is a selective and differential medium used for the enumeration and isolation of *Enterobacteria* in foods. Bile salts and crystal violet inhibit Gram-positive bacteria growth. The acidification of the medium due to glucose fermentation by *Enterobacteria* brings about colour change of the indicator towards red-purple and precipitation of bile salts.

	g/L
Yeast Extract	3.0
Peptone	7.0
Bile Salts No. 3	1.5
Glucose	10.0
Sodium Chloride	5.0
Neutral Red	0.03
Crystal Violet	0.002
Agar	12.0
pH 7.4 ± 0.2	

The incubation conditions of different culture media are reported in Table 2.

Table 2. **Media and incubation conditions used for bacterial counts.**

Mediun	Microbial group	Incubation conditions
PCA	Mesophilic aerobic total count	30 °C - 72 h in aerobiosis
MRS	Lactic acid bacteria	30 °C - 72 h in anaerobiosis
MSA	*Micrococcaceae*	30 °C - 72 h in aerobiosis
SBA	Enterococci	45 °C - 48 h in aerobiosis
MEA	Moulds and Yeasts	25 °C -120 h in aerobiosis
VRBGA	*Enterobatteriaceae*	37 °C - 20 h in aerobiosis

Results were expressed as number of colony forming units/gram (CFU g^{-1}).

Isolation of presumptive LAB and CNS

About 15 colonies at each sampling time for each batch (5 for each of the three samples analysed) were isolated from MRS agar plates and MSA. The

colonies were randomly picked up from plates with the lowest countable number and cultivated in MRS or BHI broth depending on agar medium origin. The isolates were stored at –80 °C in MRS broth (Oxoid Unipath Ltd. Basingstoke, UK) (MRS isolates) or BHI broth (Oxoid, Unipath Ltd. Basingstoke,UK) (MSA isolates) containing 50% of sterile glycerol, before being purified.

Isolates coming from samples at 0, 28 days and the end of ripening (35-40 days) were purified to pure culture by streaking three times on MRS or MSA agar.

After checking for morphology, Gram stain, and catalase reaction, the pure isolates were submitted to molecular identification.

Molecular identification of isolates at species level.

Most isolates were identified by species-specific PCR. The isolates which were not identified by species-specific PCR were submitted to 16S rDNA amplification and sequencing.

The PCR-species-specific primers (Berthier and Ehrlich, 1998; Aymerich *et al*., 2003) are indicated in Table 3. The synthetic oligonucleotide primers P0 and P6, *Escherichia coli* position 27f and 1495r respectively (Grifoni *et al*., 1995), were used to amplify the 16S rDNA (Table 3)

Table 3 **Primers used in this study for species identification and 16S rRNA gene**

Microorganism or target gene	**Primer**	**Sequence**	**Product size (bp)**	**Reference**
Lactobacillus curvatus	16S	GCT GGA TCA CCT CCT TTC	222	Berthier & Erlich (1998)
	Lc	TTG GTA CTA TTT AAT TCT TAG		
Lactobacillus sakei	16S	GCT GGA TCA CCT CCT TTC	226	Berthier & Erlich (1998)
	Ls	ATG AAA CTA TTA AAT TGG TAC		
Staphylococcus xylosus	Sxyl FA	AAG TCG GTT GAA AAC CCA AA	417/317/217	Aymerich *et al*. (2003)
	Sxyl FB	AAA ATC GGC TGA AAA CCT AAA		
	Sxyl RA	CAT TGA CAT ATT GCA TTC AG		
	Sxyl RB	CAT TGA CAT ATT GTA TTC AG		
16S rDNA	P0	GAG AGT TTG ATC CTG GCT CAG	1468	Grifoni *et al*. (1995)
	P6	CTA CGG CTA CCT TGT TAC GA		

Microbial DNA template preparation protocol.

Colonies from a pure culture of each isolate were picked up from agar plates, suspended in 5 µl of sterile distilled water and treated in the microwave oven. The cells suspension was treated for 15 min at 700W (in order to avoid evaporation and dryness problems a beaker containing 200 ml of distilled water was put into the microwave oven). The MW lysed cells suspension was used to perform PCR (Mannu *et al.*, 2000).

Species-specific PCRs protocols.

PCRs were performed in a total volume of 25 µl in a Eppendorf Mastercycler® (Eppendorf AG, Hamburg, Germany).

PCR mixture for *Lb. curvatus* species-specific PCR

DNA template	5	µl	
Reagent	**Amount**		**Final concentration**
Buffer 10X	2,5	µl	1X
$MgCl_2$ 50 mM	1.25	µl	2.5 mM
dNTPs 2 mM	2.5	µl	0.2 mM
P16 7.5 µM	1	µl	0.3 µM
Lc 7.5 µM	1	µl	0.3 µM
Taq	0.65	U	

PCR mixture for *Lb. sakei* species-specific PCR

DNA template	5	µl	
Reagent	**Amount**		**Final concentration**
Buffer 10X	2,5	µl	1X
$MgCl_2$ 50 mM	1.25	µl	2.5 mM
dNTPs 2 mM	2.5	µl	0.2 mM
P16 7.5 µM	1	µl	0.3 µM
Ls7.5 µM	1	µl	0.3 µM
Taq	0.65	U	

PCR mixture for *Staph. xylosus* species-specific PCR

DNA template	5	µl	
Reagent	**Amount**		**Final concentration**
Buffer 10X	2,5	µl	1X
$MgCl_2$ 50 mM	1.5	µl	3 mM
dNTPs 2 mM	2.5	µl	0.2 mM
Sxyl FA 10 µM	1.25	µl	0.5 µM
Sxyl FB 10 µM	1.25	µl	0.5 µM
Sxyl RA 10 µM	1.25	µl	0.5 µM
Sxyl RB 10 µM	1.25	µl	0.5 µM
Taq	1	U	

PCR conditions for *Lb. curvatus*

A	starting denaturation	94 °C	5 min	
B	denaturation	94 °C	1 min	
C	annealing	50 °C	1 min	Repeat cycles B-D for 25 times
D	extension	72 °C	1 min	
E	last extension	72 °C	7 min	

PCR conditions for *Lb. sakei*

A	starting denaturation	94 °C	5 min	
B	denaturation	94 °C	1 min	
C	annealing	45 °C	1 min	Repeat cycles B-D for 25 times
D	extension	72 °C	1 min	
E	last extension	72 °C	7 min	

PCR conditions for *Staph. xylosus*

A	starting denaturation	94 °C	5 min	
B	denaturation	94 °C	1 min	
C	annealing	58°C	1 min	Repeat cycles B-D for 35 times
D	extension	72 °C	1 min	
E	last extension	72 °C	5 min	

16S rDNA PCR protocol

PCR mixture for 16S rDNA amplification

DNA template	5	µl	
Reagent	**Amount**		**Final concentration**
Buffer 10X	2,5	µl	1X
$MgCl_2$ 50 mM	1.25	µl	2.5 mM
dNTPs 2 mM	2.5	µl	0.2 mM
P0 150 ng/µl (23.15 µM)	1	µl	0.93 µM
P6 150 ng/µl (24.7 µM)	1	µl	0.99 µM
Taq	1.25	U	

PCR conditions for 16S rDNA amplification

A	starting denaturation	94 °C	3 min	
B	denaturation	94 °C	30 sec	
C	annealing	58°C	30 sec	Repeat cycles B-D for 30 times
D	extension	72 °C	2 min	
E	last extension	72 °C	7 min	

The species-specific PCR products were resolved by electrophoresis on 2% (w/v) agarose gel in Tris-acetate buffer at 100V and then stained in ethidium bromide solution (0.5 µg ml^{-1}); the ladder 1kb plus by Invitrogen was used.

16S rDNA amplification product was purified with Montage® PCR Centrifugal Filter Devices (Microcone, Millipore, Billerica, USA). This purification system allows sample clean-up of salts, primers and unincorporated dNTPs with no solvents or chemicals required. After quantification PCR products were sent to a commercial facility service for sequencing (.BMR Genomics, Padova, Italy). The primer P0 was used for sequencing.

The partial 16S rDNA sequences obtained were edited with Chromas version 1.43 (Griffith University, Brisbane, Qld., Australia) and aligned with those of

GenBank with the BLAST (Altschul *et al.*, 1997) search program (http://www.ncbi.nml.nih.gov/). The identities of the isolates were determined on the highest score basis.

Strain typing

After species identification isolates were characterised at strain level. LAB and CNS were typed by $(GTG)_5$ REP-PCR (Versalovic *et al.*, 1994; Gevers *et al.*, 2001) and RAPD-PCR with primer M13R2 (Martin *et al.*, 2006) respectively. Among CNS, isolates belonging to *Staphylococcus xylosus* species were also typed by $(GTG)_5$ rep-PCR. Primers used for strain typing are reported in table 4.

Table 4. **Primers used in this study for typing at strain level**

Microbial group	Primer	Sequence	Reference
LAB/CNS	$(GTG)_5$	GTG GTG GTG GTG GTG	Gevers *et al.*, 2001
CNS	M13R2	GGA AAC AGC TAT GAC CAT GA	Martin *et al.*, 2006

For strain typing of isolates by repetitive-element PCR **(rep-PCR**) technique, using $(GTG)_5$ primer, DNA was prepared by FTA® Technology (Whatman International Ltd., Maidstone, UK). This technology uses cellulose cards impregnated with a patented chemical formula that lyses cell membranes and denatures proteins on contact. FTA works by lysis of cells releasing the nucleic acid within the matrix of the card, where the nucleic acid will be entrapped among the cellulose fibres.

Protocol for DNA preparation by FTA technology

1. Spot 5 µl of an overnight culture on FTA Card.
2. Dry by air at least 1 hour.
3. Take a sample disc from the dried spot with a micro-punch 2mm diameter.
4. Place disc in a PCR amplification tube.
5. Add 180 µl of FTA Purification Reagent® to PCR tube.
6. Incubate for 5 min at room temperature giving tube moderate manual mixing to disrupt the debris and aid in washing.
7. Remove and discard all used FTA Purification Reagent with a pipette.
8. Repeat steps 5-7 for a total of 2 washes with FTA Purification Reagent.
9. Add 180 µl of TE^{-1} Buffer (10mM Tris-HCl, 0.1 mM EDTA, pH 8).
10. Incubate for 5 min. at room temperature.
11. Remove and discard all used TE^{-1} Buffer with a pipette.
12. Repeat steps 9-11 for a total of 2 washes with TE^{-1} Buffer.
13. Ensure that all the liquid has been removed before performing the analysis.
14. Use the washed and eventually dried disc directly to perform PCR.

For strain typing of isolates by RAPD with primer M13R2 genomic DNA was extracted from overnight BHI cultures using DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to protocol reported below (Martin, 2005).

Protocol for DNA preparation

1. Centrifuge 1.5 ml of an overnight bacterial culture in 1.5 ml tube for 10 min at 8000 rpm (Centrifuge 5402, Eppendorf AG, Hamburg, Germany).
2. Resuspend bacterial pellet in 180 µl enzymatic lysis buffer (Tris 20 mM, EDTA 2 mM, Triton® X-100 1.2 %, pH 8) and add 30 µl lysozime solution (50 mg/ml in Tris-HCl 25 mM pH 8.0), 10 µl mutanolysine (2U/ µl in Tris HCl 20 mM pH 8.2), 1 µl lysostaphine solution (10 mg/ ml in sodium acetate (pH 4.5).
3. Incubate for 1 hour at 37 °C.
4. Add 25 µl di proteinase K (20 mg/ ml) and 200 µl buffer AL (Qiagen). Mix by vortexing.
5. Incubate for 30 min at 56 °C.
6. Add 4 µl di RNase (100 mg/ ml in sodium acetate 0.01 M pH 5.2). Incubate for 2 min at room temperature.
7. Add 200 µl ethanol (96-100%) and mix thoroughly by vortexing.
8. Pipet the mixture into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at 8000 rpm for 1 min. Discard collection tube with liquid.
9. Place the mini column in a new 2 ml collection tube (Qiagen). Add 500 µl buffer AW1 (Qiagen). Centrifuge at 8000 rpm for 1 min. Discard collection tube with liquid.

10. Place the mini column in a new 2 ml collection tube (Qiagen). Add 500 µl buffer AW2 (Qiagen). Centrifuge at 14000 rpm for 3 min. Discard collection tube with liquid.
11. Place the mini column in a clean 1.5 ml tube. Pipet 200 µl buffer AE (Qiagen) directly onto the DNeasy membrane. Incubate at room temperature for 1 min.
12. Centrifuge at 8000 rpm for 1 min and collect the eluate.
13. Repeat elution steps (11 and 12).
14. Add 40 µl NaCl 5M and 980 µl cold ethanol. Mix by inversion.
15. Keep at -80°C for at least 30 min.
16. Centrifuge at 14000 rpm for 5 min.
17. Discard the supernatant and wash pellet in 1 ml ice-cold 70% ethanol.
18. Centrifuge at 14000 rpm for 5 min.
19. Discard the supernatant and let to dry.
20. Add distilled sterile water to resuspend DNA. Let at room temperature to allow DNA resuspension.
21. Conserve at -20°C.

$(GTG)_5$ Rep-PCR

A washed FTA® disc was used directly to perform PCR. PCR reaction was performed in a total volume of 25 µl (24 µl MegaMix ™ Labogen, 1 µl 50 µM $(GTG)_5$ primer).

PCR conditions for rep-$(GTG)_5$ amplification

A	starting denaturation	95 °C	7 min	
B	denaturation	90°C	30 sec	Repeat cycles B-D for 30 times
C	annealing	40 °C	1 min	
D	extension	65 °C	8 min	
E	last extension	65 °C	16 min	

Rep-PCR products were resolved by electrophoresis on 1.8 % (w/v) agarose gel in Tris-acetate buffer at 90V (Volthour 222) and then stained in ethidium bromide solution (0.5 µg ml^{-1}); the ladder 1kb plus by Invitrogen was used.

RAPD-PCR

A 100 ng DNA template was used for RAPD-PCR with primer M13R2. PCR was performed in a total volume of 25 µl

DNA template	100	ng	
Reagent	**Amount**		**Final concentration**
Buffer 10X	2,5	µl	1X
$MgCl_2$ 50 mM	0.75	µl	1.5 mM
dNTPs 2 mM	2.5	µl	0.2 mM
M13R2 20 µM	1	µl	0.8 µM
Taq	1	U	

PCR conditions for RAPD amplification with M13R2

A	starting denaturation	94°C	5 min	
B	denaturation	94°C	1 min	Repeat cycles B-D for 35times
C	annealing	38 °C	1 min	
D	extension	72 °C	1 min	
E	last extension	72°C	5 min	

RAPD-PCR products were electrophoresed in 1.5% (w/v) gel agarose for 100 min at 80 mA and then stained in ethidium bromide solution (0.5 μg ml^{-1}); the ladder 1kb by Invitrogen was used.

Cluster analysis

Digital photos of gels were taken with Kodak DC 120 Camera (Kodak Digital Science 1D LE 3.0 Software).

The resulting fingerprints were analysed by the BioNumerics® V 4.5 software package (Applied Maths, Ghent, Belgium). The similarity among profiles was calculated using the Pearson correlation coefficient for $(GTG)_5$ profiles and Dice coefficient for RAPD profiles. Dendrograms were obtained by Unweighted Pair group Method using Arithmetic averages (UPGMA) (Vauterin and Vauterin, 1992).

RESULTS AND DISCUSSION

Physicochemical analysis

The pH during ripening of the sausages from the five producers (AA, AB, AC, AD and AE) is shown in table 5.

Table 5
Changes in pH during ripening

Time of ripening (days)	Batches				
	AA	AB	AC	AD	AE
0	5.52	5.85	5.95	5.46	5.47
7	5.47	5.88	5.85	5.37	5.50
14	5.86	5.82	5.62	5.28	5.34
21	5.41	5.89	6.10	5.40	5.34
28	5.51	5.82	6.20	5.60	5.38
35	-	-	-	-	5.44
40	5.57	5.93	6.08	-	-

The pH at the start of the ripening was different in the five batches. The pH of batch AA, AB, AD, AE did not widely vary during ripening. Batch AC had a higher variation in pH values: it varied from 5.95 at the start of ripening to the minimum value of 5.62 on the 14th day of ripening and then reached a maximum of 6.2 on the 28th day. All batches reached the minimum pH value on the 14th day of ripening except batch AA that showed the maximum pH value (5.86) at that time of ripening. Batch AB and AC showed higher pH values during ripening than other producer. The pH of all batches never reached values below 5.3.

The relatively high pH values detected in the products of the five batches indicate a weak acidification as found in other naturally fermented traditional Mediterranean dry sausages, like Spanish *chorizo*, and *fuet* (González-Fandos *et*

al., 1999; Aymerich *et al*., 2003), French *Saucisson sec du Massif central* (Lebert *et al*., 2006b), Portuguese *Salpicão de Vinhais* and *Chouriça de Vinhais* (Ferreira *et al*., 2007) and Italian sausages (*salsiccia sotto sugna* salami, *soppressata* salami, *salsiccia* salami; *Ciauscolo* salami) (Coppola *et al*., 1995b; Forastiero *et al*., 1999; Amato *et al*., 1999; Parente *et al*., 2001b; Blaiotta *et al*., 2004a; Rantsiou *et al*., 2005; Aquilanti *et al*., 2007). The high pH values may be due either to the fact that no fermentable sugars were added to the meat or to a limited acidifying activity of the indigenous microbiota. A high pH may positively affect the activity of lipolytic enzymes produced by some *Staphylococcus* spp. (Montel *et al*., 1998) with the consequent release of free fatty acids that may affect the aroma and the taste of the product.

The a_w during ripening is shown in table 6.

Table 6
Changes in a_w during ripening

Time of ripening (days)	**Batches**				
	AA	**AB**	**AC**	**AD**	**AE**
0	0.96	0.96	0.96	0.97	0.96
7	0.95	0.95	0.95	0.95	0.95
14	0.95	0.93	0.95	0.90	0.95
21	0.94	0.90	0.93	0.86	0.93
28	0.93	0.88	0.91	0.81	0.93
35	-	-	-	-	0.89
40	0.88	0.83	0.82	-	-

At the start of ripening a_w was almost the same (0.96/0.97) in all batches. It decreased during ripening at different rate in the different batches. For example the value of about 0.88 was detected on the 28^{th} , 35^{th}, 40^{th} day of ripening in batch AB, AE, AA respectively. A fast decrease in a_w was observed in batch AD: a_w reached the value 0.81 on the 28^{th} day of ripening. This is the lowest value

detected among the products at the end of ripening of all batches. The slowest decreasing rate was observed in producers AA and AE until the 28 day of ripening, the fall in a_w was more rapid from 28 day to the end of ripening. The values detected during ripening are similar to those found in other typical naturally fermented dry sausages of Mediterranean regions (Aymerich *et al.*, 2003; Blaiotta *et al.*, 2004; Lebert *et al.*, 2006; Aquilanti *et al.*, 2007; Gounadaki *et al.*, 2008). The products at the end of ripening showed different values of a_w among producers ranging from 0.81 to 0.89. Low a_w values are fundamental for keeping low acid products such as Mediterranean traditional dry sausages (Talon, 2006).

Microbiological analysis

Microbial counts

Figure from 1 to 6 shows the evolution of the viable counts in the different media of the different producers' sausages throughout ripening.

Aerobic mesophilic count in PCA (Fig. 1) at the start of ripening was very different in the five producers ($1.96x10^4$ / $1x10^7$ CFU g^{-1}) probably due to the different environmental and meat level of contamination.

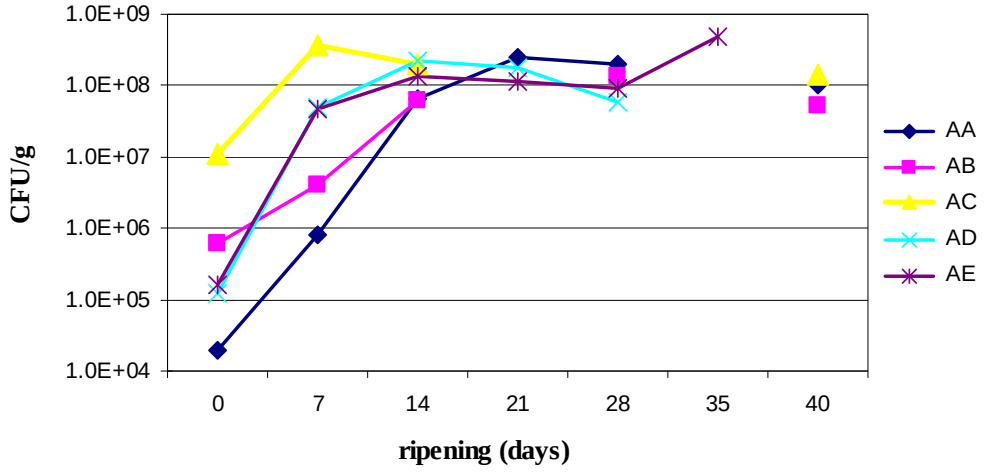


Fig. 1. Evolution of PCA counts during ripening.

The high variability of environmental microbial levels of small scale-processing units due to different cleaning and manufacturing practices has been recently reported (Talon *et al.*, 2007b). A rapid increase was observed till 14 day of ripening for all producers except producer AC that reached the maximum at 7 days. Aerobic mesophilic count had a superimposable trend in producers AD and AE till the 21 day of ripening. The maximum count was detected at different time of ripening in the different producers. The number of aerobic mesophilic bacteria at the end of ripening was close in the five producers in a order of $10^7/10^8$ CFU g^{-1}, the highest count ($5x10^8$ CFU g^{-1}) was detected in producer AE. Although aerobic mesophilic count was very high at the beginning of ripening in AC (10^7 CFU g^{-1}), it did not increase a lot during ripening (1.2 log). The counts detected are comparable with those found in other Italian home-made dry sausages like *soppressata* and *salsiccia* salami produced in the South of Italy (Forastiero *et al.*, 1999).

Initial presumptive lactic acid bacteria levels (Fig. 2) were different in the five producers ranging from 3x10 CFU g^{-1} (AB) to 1.68x10^4 CFU g^{-1} (AA).

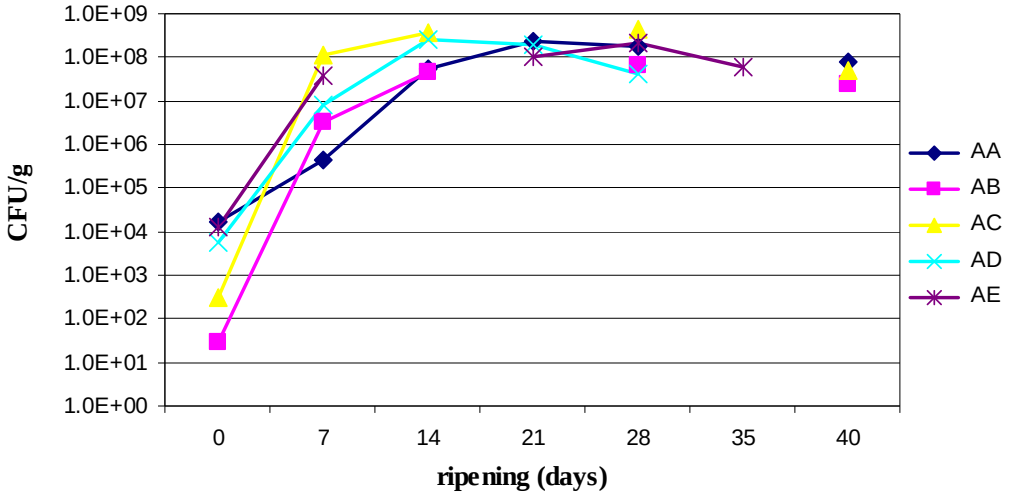


Fig. 2. Evolution of MRS counts during ripening

These counts were similar to those reported by other author for traditional dry sausages (Parente *et al.*, 2001a, b; Papamanoli *et al.*, 2003; Drosinos *et al.*, 2005; Greco *et al.*, 2005; Rantsiou and Cocolin, 2006; Urso *et al.*, 2006). Their number rapidly increased in the first seven days of ripening in all batches except AA where their increase was more slowly probably due to the low environmental temperatures occurred (T below 6 °C). In the second week of ripening presumptive LAB counts had about 1 log increase in all batches except AA in which there was an increase of 2 log. At the end of ripening presumptive LAB reached high counts in the order of $10^7/10^8$ CFU g^{-1} as found in other Mediterranean dry sausages (Cocolin *et al.*, 2001a; Aymerich *et al.*, 2003; Papamanoli *et al.*, 2003; Drosinos *et al.*, 2005; Comi *et al.*, 2005; Urso *et al.*, 2006; Silvestri *et al.*, 2007). As reported in other home-made Mediterranean dry sausages (Coppola *et al.*, 1995b; Forastiero *et al.*, 1999; Amato *et al.*, 1999;

Parente *et al.*, 2001b), the lack of added fermentable carbohydrates to the meat did not prevent the growth of LAB.

The evolution of presumptive CNC counted in MSA is showed in Fig. 3.

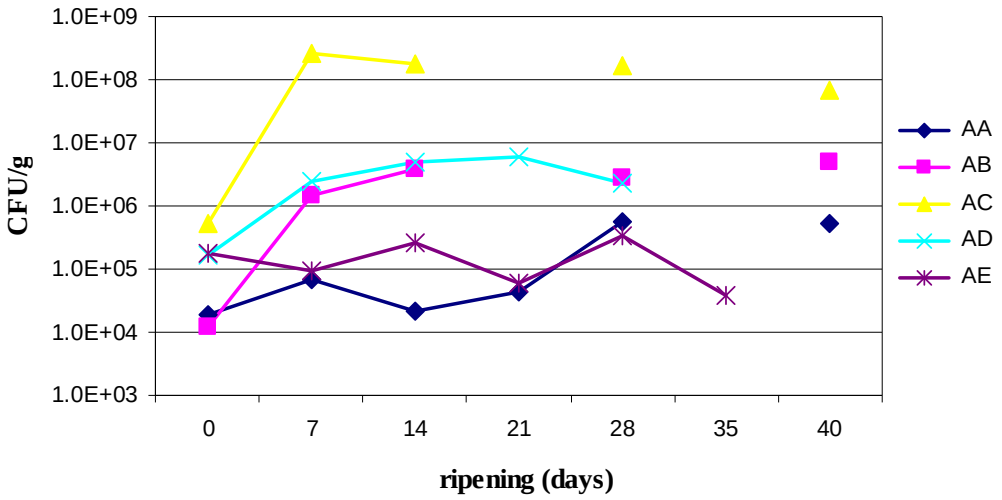


Fig. 3. Evolution of MSA counts during ripening.

Unlike presumptive LAB and total mesophilic bacteria, there was not a wide variation of CNC initial levels among producers. The initial number of CNC was similar in all the producers varying from 10^4 to $5x10^5$ CFU g^{-1} and close to that found in other dry fermented sausages (Coppola *et al.*, 1997; Rebecchi *et al.*, 1998; Samelis *et al.*, 1998; Cocolin *et al.*, 2001b; Parente *et al.*, 2001b; Blaiotta *et al.*, 2004a; Mauriello *et al.*, 2004; Drosinos *et al.*, 2005, 2007; Morot-Bizot *et al.*, 2006; Iacumin *et al.*, 2006; Gounadaki *et al.*, 2008).

The trend of this microbial group was different in the five producers. In AA they had a fluctuating trend and did not vary a lot till the 21 day of ripening. Then they increased and reached $5.78x10^5$ CFU g^{-1} at the end of ripening (40 days).

A fluctuating trend was also observed in AE, but their number at the end of ripening (35 days) was lower ($3.95x10^4$ CFU g^{-1}) than at the start.

In the other three producers an increase of MSA counts was observed at 7 days of ripening. The highest increase (3 log) was observed in AC after 7 days with a count of $3.95x10^4$ CFU g^{-1}, their number was stable till 28 days, then it decreased to $7x10^7$ CFU g^{-1} at the end of ripening (40 days). In AB and AD their number remained lower and reached $5x10^6$ and $2.4x10^6$ CFU g^{-1} respectively at the end of ripening (40, 28 days).

At the end of ripening the number of presumptive CNC was close to that found in other traditional dry products (Coppola *et al.*, 1995b; Coppola *et al.*, 1997; Samelis *et al.*, 1998; Coppola *et al.*, 2000; Parente *et al.*, 2001b; Aymerich *et al.*, 2003; Blaiotta *et al.*, 2004a; Mauriello *et al.*, 2004; Comi *et al.*, 2005; Rantsiou *et al.*, 2005b; Iacumin *et al.*, 2006; Aquilanti *et al.*, 2007; Drosinos *et al.*, 2007; Martin *et al.*, 2007; Silvestri *et al.*, 2007).

The initial number of moulds and yeasts was in the order of 10^2 CFU g^{-1} except AD ($5.17\ 10^4$ CFU g^{-1}). Their counts increased during ripening in all producers from 1 log (AD) to 4 log (AA) and ranged from 10^4 to 10^6 (AA, AC) CFU g^{-1}. Similar values were detected in several traditional Mediterranean dry sausages (Amato *et al.*, 1999; Coppola *et al.*, 2000; Martuscelli *et al.*, 2000; Aquilanti *et al.*, 2007; Silvestri *et al.*, 2007; Gounadaki *et al.*, 2000)

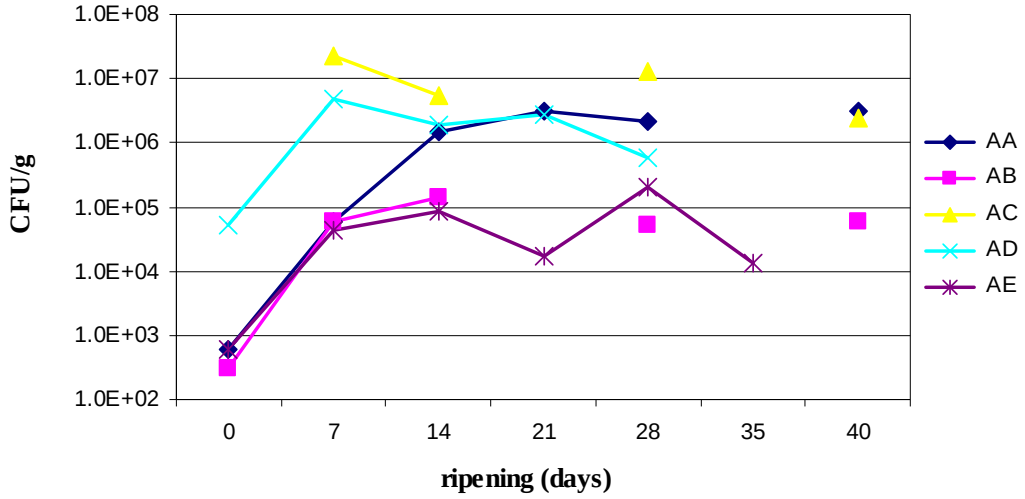


Fig. 4. Evolution of MEA counts during ripening.

Figure 5 represents the evolution of Enterococci during ripening.

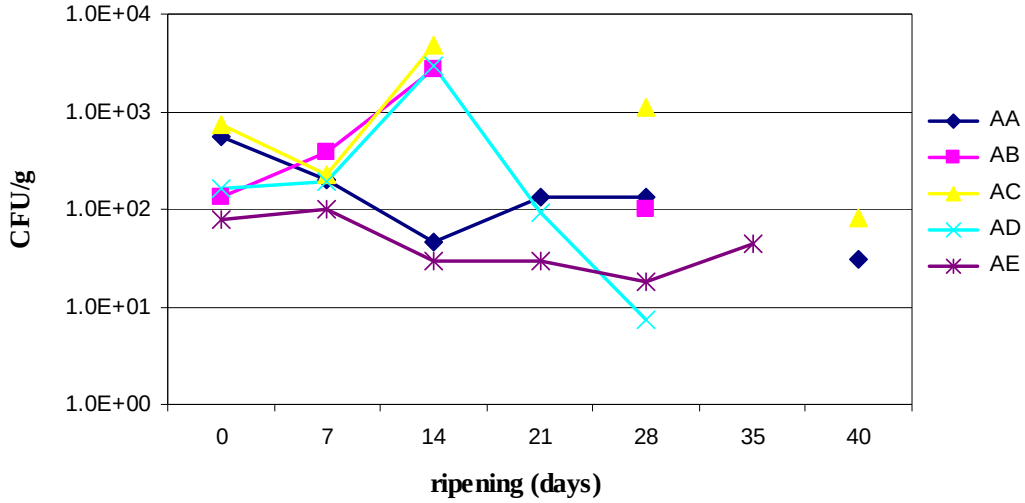


Fig. 5. Evolution of SBA counts during ripening.

Enterococci are present in the gastrointestinal tract of animals and can contaminate meat at the time of slaughter (Franz *et al.*, 1999). Enterococci can be used as indicators of faecal contamination (Franz *et al.*, 1999). They multiply during ripening (Giraffa, 2002) and may compete well in fermented sausages (Samelis *et al.*, 1998) since they are very resistant to extremes in temperatures, pH

and salinity (Giraffa, 2002). They usually contaminate raw meat in the range of 10^2-10^4 CFU g^{-1} (Giraffa, 2002) and are always present in fermented sausages (Hugas *et al.*, 2003). Some authors consider enterococci important for ripening and for flavour development of certain traditional sausages (Franz et *al.*, 2003). Though they could be opportunistic pathogens and not considered "generally recognised as safe" (GRAS) (Giraffa *et al.*, 1997), studies indicate that enterococci isolated from food have a lower pathogenicity potential than clinical strains (Hugas *et al.*, 2003; Mannu *et al.*, 2003). Their ability to produce bacteriocins could have an antimicrobial effect against pathogens and microorganisms responsible for meat spoilage (Hugas *et al.*, 2003). Enterococci could be involved in the production of biogenic amines, especially tyramine (Lebert *et al.*, 2007b).

At the start of ripening the level of enterococci was in the order of 10^2 CFU g^{-1} for all the producers except AE (<100). They increased of 1 log at 14 days of ripening in producers AB, AC, AD. Their counts were <100 CFU g^{-1} at the end of ripening for all producers. The counts detected were similar to those found in some lots of Spanish *chorizo* and *fuet* (Aymerich *et al.*, 2003). They were slightly lower than those detected in different Italian dry sausages (Cocolin *et al.*, 2001a; Luongo *et al.*, 2001) and much lower than those detected in French, Portoguese and Greek traditional sausages (Lebert *et al.*, 2006a; Lebert *et al.*, 2007b; Samelis *et al.*, 1998; Drosinos *et al.*, 2005; Ferreira *et al.*, 2007; Gounadaki *et al.*, 2008) and in Italian *soppressata* and *salsiccia* (Forastiero *et al.*, 1999).

The evolution of *Enterobacteriaceae* enumerated in VRBGA is shown in figure 6.

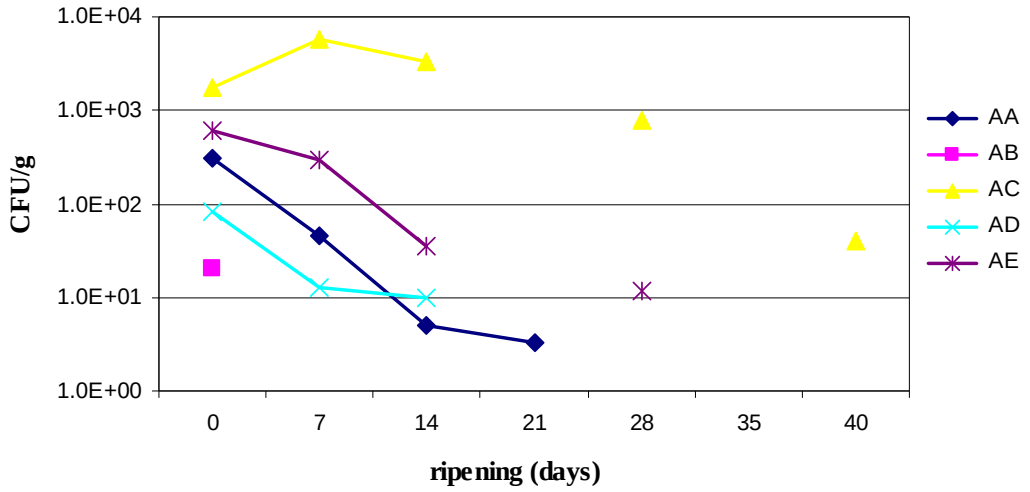


Fig. 6. Evolution of VRBGA counts during ripening.

Enterobacteria are natural inhabitants of the gastrointestinal tract of animals. They are used as hygiene indicators (FSAI, 2001), although the EU regulation on microbiological criteria for foodstuffs, Reg. EC N. 2073/2005 (European Commission, 2005), does not provide for this microbial group as regards dry sausages. They can produce histamine and diamines (Bover-Cid *et al.*, 2003; Lebert *et al.*, 2007b).

At the start of ripening their number varied among producers from 20 to $8.17x10^3$ CFU g^{-1} and was similar to those detected in other traditional dry sausages (Samelis *et al.*, 1998; Drosinos *et al.*, 2005; Rantsiou *et al.*, 2005b; Aquilanti *et al.*, 2007; Comi *et al.*, 2005; Parente et al., 2001b; Forastiero *et al.*, 1999).

They progressively decreased during ripening. In the product at time of consumption they were absent or in low number (<100 CFU g^{-1}) as found in other traditional products without starter cultures added (Samelis *et al.*, 1998; Aymerich

et al., 2003; Drosinos *et al.*, 2005; Aquilanti *et al.*, 2007; Silvestri *et al.*, 2007), indicating a good hygienic quality of the product.

Molecular characterization of LAB and CNC microbiota

Species identification

A total of 245 colonies from MRS and 250 colonies from MSA were isolated. Thirty-eight isolates from MSA appeared as mobile bacilli at microscope observation and were not purified. The information about isolates were recorded in a database.

As regards isolates from MRS, 74.3% of them were identified as *Lb. sakei* (Fig. 8). The second main species isolated was *Lb. curvatus* (16.3%). The proportion of *Lb. sakei* and *Lb. curvatus* found in this study was similar to the that reported for Spanish sausages (Aymerich *et al.*, 2006).

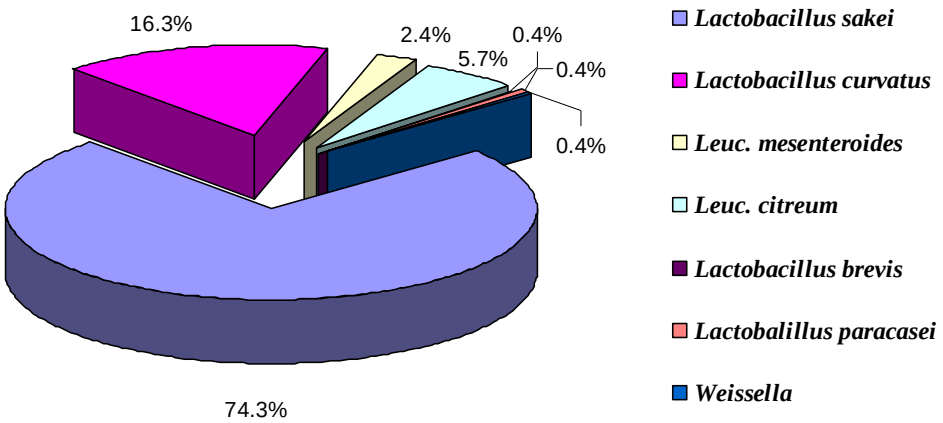


Fig.8. LAB distribution among species

The prevalence of *Lb. sakei* and *Lb. curvatus* in naturally-fermented sausages is well documented in literature (Hugas *et al.*,1993; Samelis *et al.*, 1994a,1998; Coppola *et al.*, 1998, 2000; Santos *et al.*, 1998; Parente *et al.*, 2001a; Papamanoli *et al.*, 2003; Rantsiou *et al.*, 2005a; Comi *et al.*, 2005; Aymerich *et al.*, 2006; Rantsiou *et al.*, 2006b; Urso *et al.*, 2006; Silvestri *et al.*, 2007). These two species are the most adapted species to the fermented sausages environment (Samelis *et al.*, 1994). They are psychrotrophic species and compete well when low temperature of ripening are used (Silvestri *et al.*, 2007).

The recent analysis of the 1.8 Mb genome sequence (Chaillou *et al.*, 2005) has revealed that this organism has evolved to adapt itself to the meat environment (Cocconcelli, 2007).

Psychrotrophic character and salt tolerance of *Lb. sakei* may be due to its ability to efficiently accumulate osmo- and cryoprotective solutes such as betaine and carnitina (Chaillou *et al.*, 2005). A combination of mechanisms including modification of carbohydrates metabolism and stimulation of oxidative stress may also increase its resilience to cold (Marceu *et al.*, 2004).

Lb. sakei was the most frequently isolated species in all the producers even though its proportion to *Lb. curvatus* varied among producers.

Other species isolated were *Leuconostoc citreum* (5.7 %), *Leuconostoc mesenteroides* (2.4%) , *Lb paracasei* (0.4%), *Lb. brevis* (0.4%) and *Weissella spp.* (0.4%). *Leuc. mesenteroides* was isolated from two producers (AC, AE), while *Leuc. citreum* was isolated only from the producers AE (Fig. 9). *Lb paracasei*, *Lb. brevis* and *Weissella spp.* were isolated occasionally in the producer AA and were represented only by one isolate (Fig. 9).

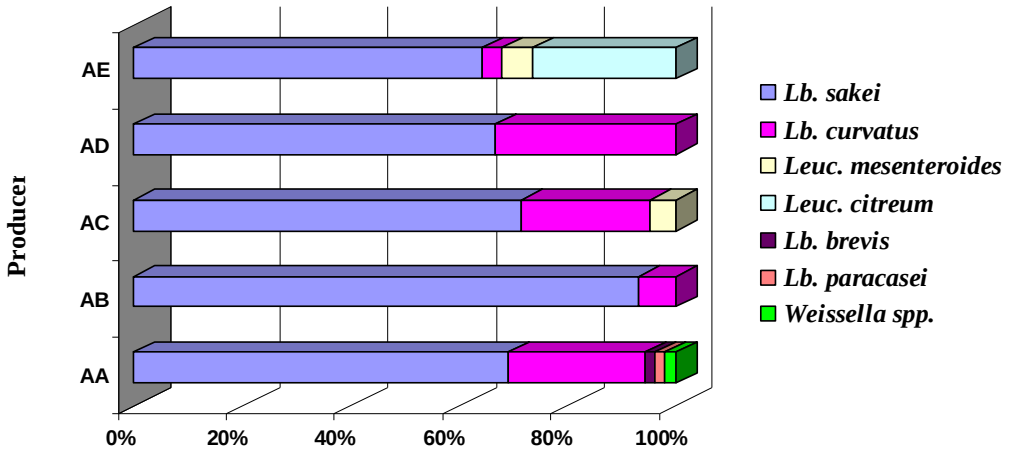


Fig. 9. Distribution of isolates among LAB species in the different producers

Lb. sakei and *Lb. curvatus* were isolated at every sampling point, but not in all producers (Fig.10)

Species	0	28	end
Lb. sakei	■✱	■▲♦●✱	■▲♦✱
Lb. curvatus	■♦	■▲♦●✱	■▲♦✱
Lb. paracasei			■
Lb. brevis			■
Weissella			■
Leuc. mesenteroides	♦✱		
Leuc. citreum	✱		

AA	AB	AC	AD	AE
■	▲	♦	●	✱

Fig. 10. Distribution of LAB species according to ripening period and producer

Leuconostoc spp. were isolated only at the start of ripening as reported by Papamanoli *et al.* (2003*). Lb. plantarum* detected by other authors (Greco *et al.*, 2005) in dry sausages produced in Sardinia was not found in this study, probably

due to the very low temperatures occurred during ripening as *Lb. plantarum* has low competitiveness at ripening temperatures below 18-25°C (Samelis *et al.*, 1994; Parente *et al.*, 2001a, b).

Among CNC, *Staphylococcus xylosus* was the main species isolated (81.5% of isolates) (Fig.11).

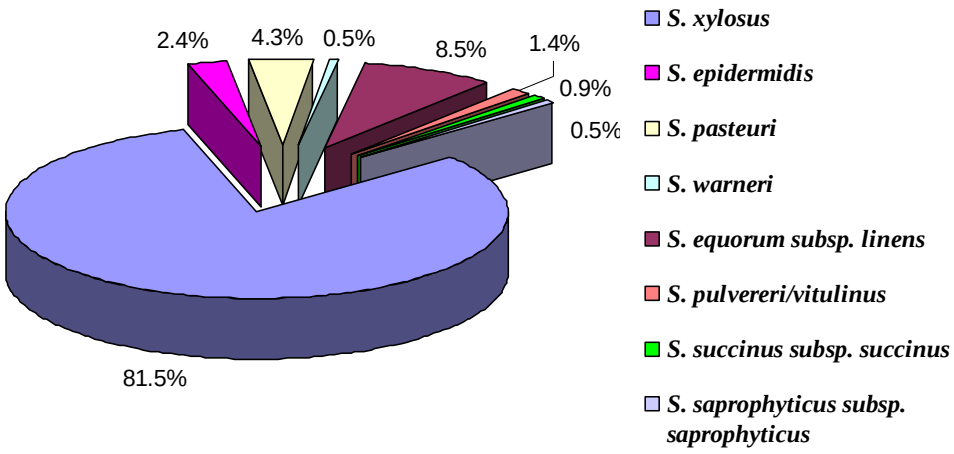


Fig. 11. CNC distribution among species

The percentage of *S. xylosus* detected in this study is similar to that found in Spanish dry sausages such as *fuet* and *chorizo* (Martin *et al.*, 2006).

S. xylosus was detected in all producers at percentage higher than 60% (Fig.12) and at all stages of ripening except AA in which it was not detected at the start of ripening (Fig. 13).

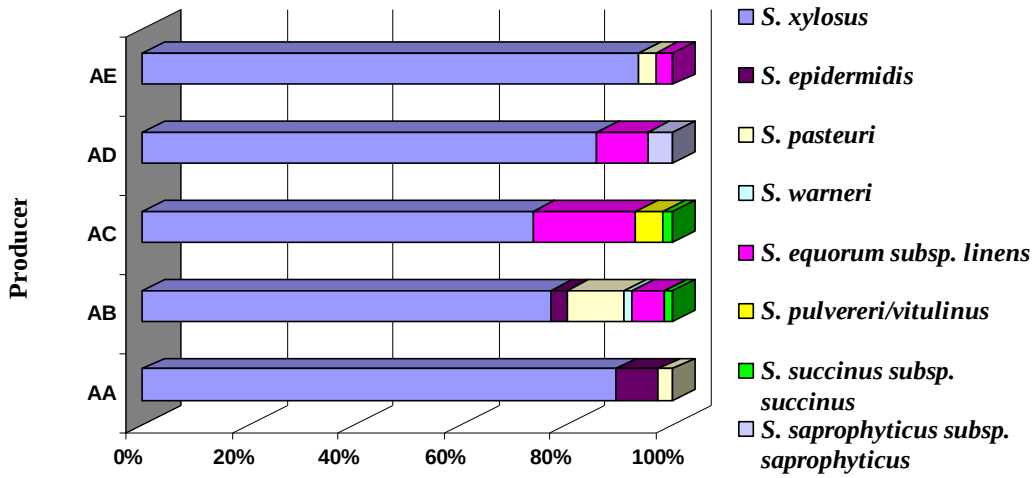


Fig. 12. Distribution of isolates among CNC species in the different producers

This species is often described as the dominant species among staphylococci isolated from naturally fermented sausages (Rebecchi *et al.*, 1998; Garcia-Varona *et al.*, 2000; Cocolin *et al.*, 2001b; Papamanoli *et al.*, 2002; Aymerich *et al.*, 2003; Blaiotta *et al.*, 2004a; Mauriello *et al.*, 2004; Iacumin *et al.*, 2006; Martin *et al.*, 2007; García Fontán *et al.*, 2007).

The predominance of *S. xylosus* in traditional slightly fermented sausages indicates high adaptation properties of this species in this kind of product, it is one of the most resistant to unfavourable environmental conditions (Mc Meekin *et al.*, 1987; García Fontán *et al.*, 2007). Strains of *S. xylosus* are included in commercial starter cultures in order to achieve aromatic products because they contribute to develop “rounded aroma” and a less acid taste in sausages (Papamanoli *et al.*, 2002; Sorensen, 1997).

	Ripening period		
Species	**0**	**28**	**end**
S. xylosus	▲♦●✱	■▲♦●✱	■▲♦✱
S. epidermidis	■▲		
S. pasteuri	■▲✱		
S. warneri	▲		
S. equorum subsp. *linens*	♦●	▲♦●	▲♦✱
S. pulvereri/vitulinus	♦	♦	♦
S. succinus subsp. *succinus*		▲	♦
S. saprophyticus subsp. *saprophyticus*		●	

AA ■ **AB** ▲ **AC** ♦ **AD** ● **AE** ✱

Fig. 13. Distribution of CNC species according to ripening period and producer

The second main species found was *S. equorum* (8.5%). It was present at all stages of ripening, but not in all the producers (Fig.12). It was detected in almost all producers, excepted AA. The presence of *S. equorum* in traditional dry sausages is believed to be underestimated in the past due to the use of phenotypical methods of identification (Mauriello *et al.*, 2004). *S. equorum* was isolated also in other Italian traditional dry sausages (Mauriello *et al.*, 2004; Blaiotta *et al.*, 2004a; Iacumin *et al.*, 2006). *S. equorum* was found to be the dominant species of French traditional dry sausages produced in a small manufacturing unit (Corbiere Morot-Bizot *et al.*, 2006).

S. saprophyticus was isolated only from producer AD (Fig. 12) at the end of ripening (28 days) (Fig.13). This species was shown to be the dominant one in Greek dry sausages (Samelis *et al.*, 1998; Papamanoli *et al.*, 2002; Drosinos *et al.*,

2005, 2007) and it was highly represented in other fermented sausages of Southern Italy (Mauriello *et al.*, 2004). *S. pulvereri* was isolated only in producer AC at all stages of ripening, while *S. warneri* was isolated only in producer AB (Fig. 12, 13). *S. pulvereri* was found also in other Southern Italy traditional sausages (*soppressata*) (Mauriello *et al.*, 2004). *S. warneri* was sporadically isolated in Italian traditional fermented sausages (Blaiotta *et al.*, 2004a; Mauriello *et al.*, 2004) and was the second species found in Spanish sausages (Martin *et al.*, 2006). The species *S. epidermidis, S. pasteuri* and *S. warneri* were detected only at the start of ripening (Fig. 13). *S. succinus* was detected in two producers. *S. succinus* was the second species isolated in traditional French sausages (Corbiere Morot-Bizot *et al.*, 2006). The highest number of species was found in producer AB (6 species).

Strain typing

The dendrogram derived from numerical analysis of rep-$(GTG)_5$ profiles of LAB isolates is shown in figure 14. A clear separation of isolates belonging to different species in different clusters was obtained.

A similarity index of 90 % between three banding patterns of the same strain was obtained. Then, a cut off value of 90% similarity was used to discriminate different rep-biotype. Isolates with a similarity <90% were considered different biotypes. Biodiversity was expressed as percentage of n° biotypes/ n° isolates.

Biodiversity within producer evaluated by considering all LAB biotype and all species found is shown in figure 15. A high heterogeneity among strains was

found; a biodiversity higher than 50% was detected within all producers. The highest biodiversity among LAB isolates was observed within producer AA.

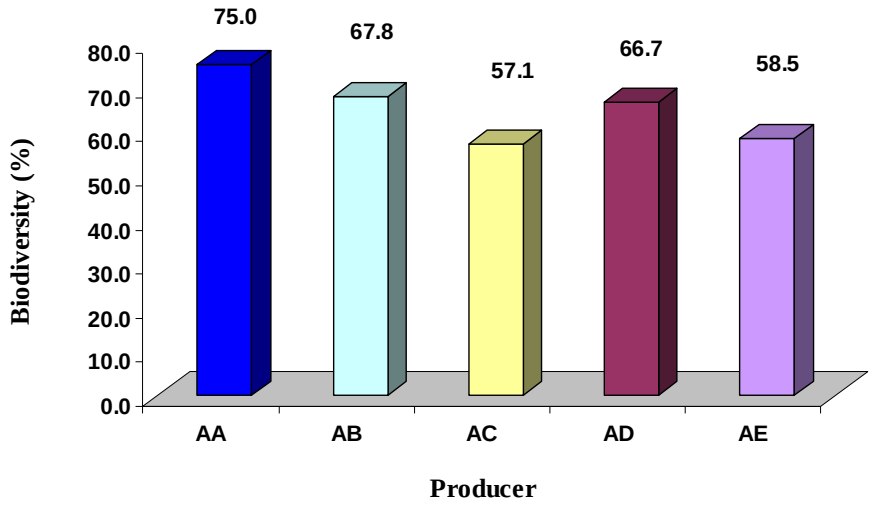


Fig. 15. Total LAB biodiversity within producer

Among *Lb. curvatus* isolates 33 biotypes were found, only one of them was detected in different producers (Fig. 14a). Among 104 biotypes detected for *Lb. sakei*, seven patterns were shared by different producers (Fig. 14b). *Leuconostoc citreum* (14 isolates from only one producer) and *Leuconostoc mesenteroides* (6 isolates from two producers) were represented by 7 biotypes and 4 biotypes respectively (Fig. 14c, d). Figure 15 shows the biodiversity found within species in the different producers. A higher biodiversity within *Lb. curvatus* species than *Lb. sakei* was detected. Most of *Lb. curvatus* profiles were represented by only one isolate (Fig. 14a), while 35 profiles were represented by more than one isolate among *Lb. sakei* isolates (Fig. 14b).

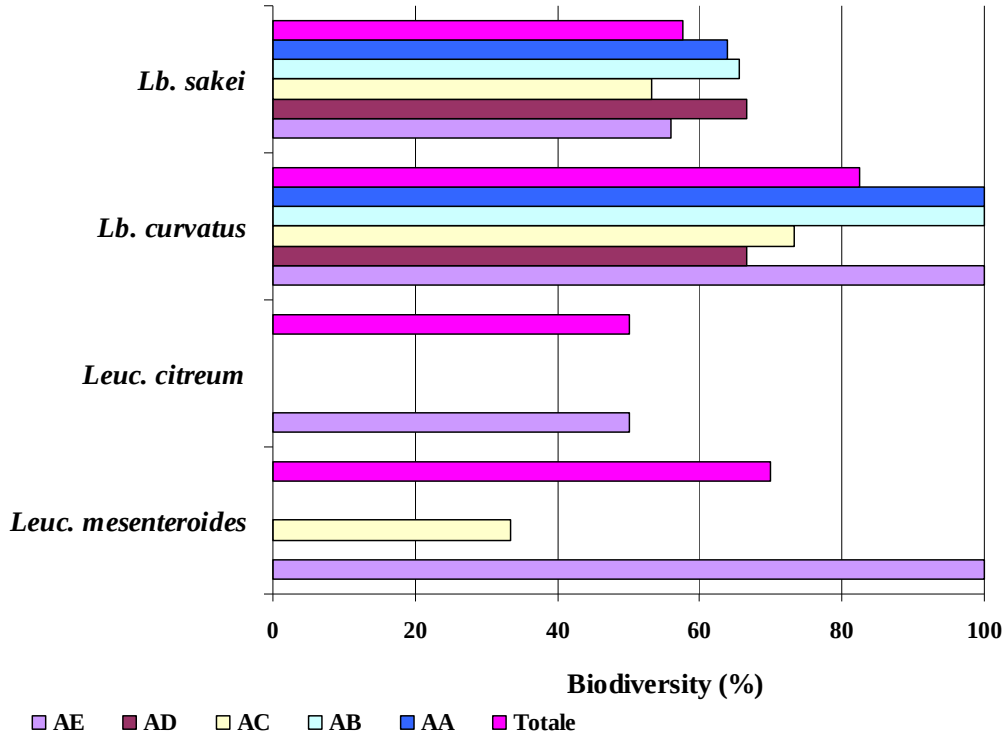


Fig. 16. LAB biodiversity (%) within species in the different producers

The dendrogram derived from numerical analysis RAPD-PCR profiles with primer M13R2 is shown in figure 17. This technique did not give good results in our experimental conditions. It was not possible to discriminate isolates belonging to different specie by using the cut off value 80% (similarity index between three banding patterns of the same strain) and many isolates had profiles with low band number. Therefore it was decided to type isolates belonging to *S. xylosus*, the most frequently isolated species among staphylococci, by using Rep-$(GTG)_5$ PCR. The results obtained by numerical analysis of rep-profiles are shown in figure 18. 81 different biotypes were discriminated by using a cut off value of 90% similarity. Twelve biotypes were common to different producers. About 65% of biotypes found constituted a unique profile (represented by only one isolate). The calculated biodiversity within this species was 47.9%. A high variability of

biodiversity within *S. xylosus* among different producers was detected, it ranged from 37.5% (AA) to 95% (AD) (Fig. 19). A biodiversity lower than 50 % was detected in producer AA and AB. As shown in figures 18a, 18b there was three dominant biotypes among isolates of producer AA and one dominant profile among AB isolates, indicating the presence of well adapted biotypes that prevailed. The highest biodiversity was found in producer AD: 16 biotype /17 were unique profile (Fig. 18d). Figure 18 points out that in producer AA and AB the relatively low biodiversity found among *S. xylosus* isolates was offset by the high biodiversity among LAB isolates.

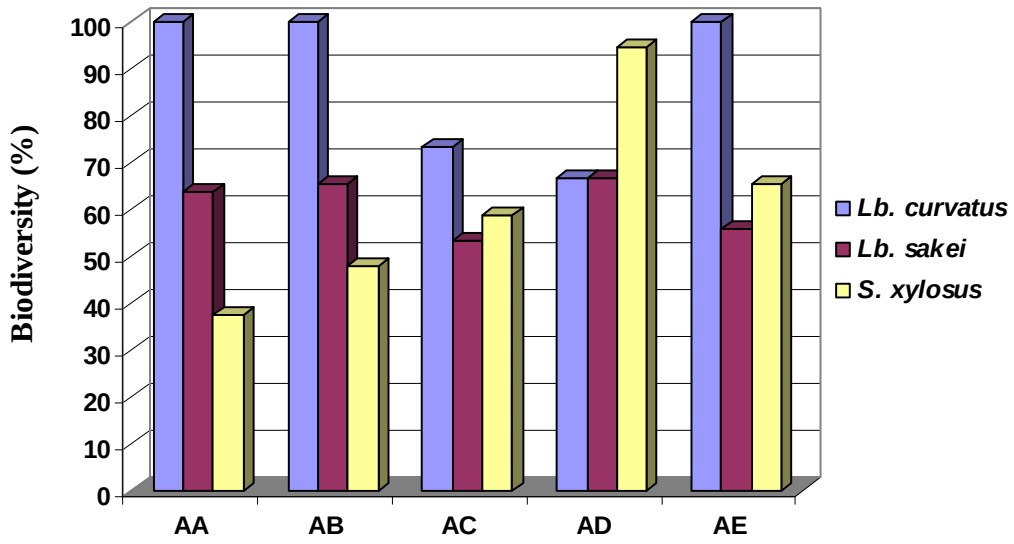


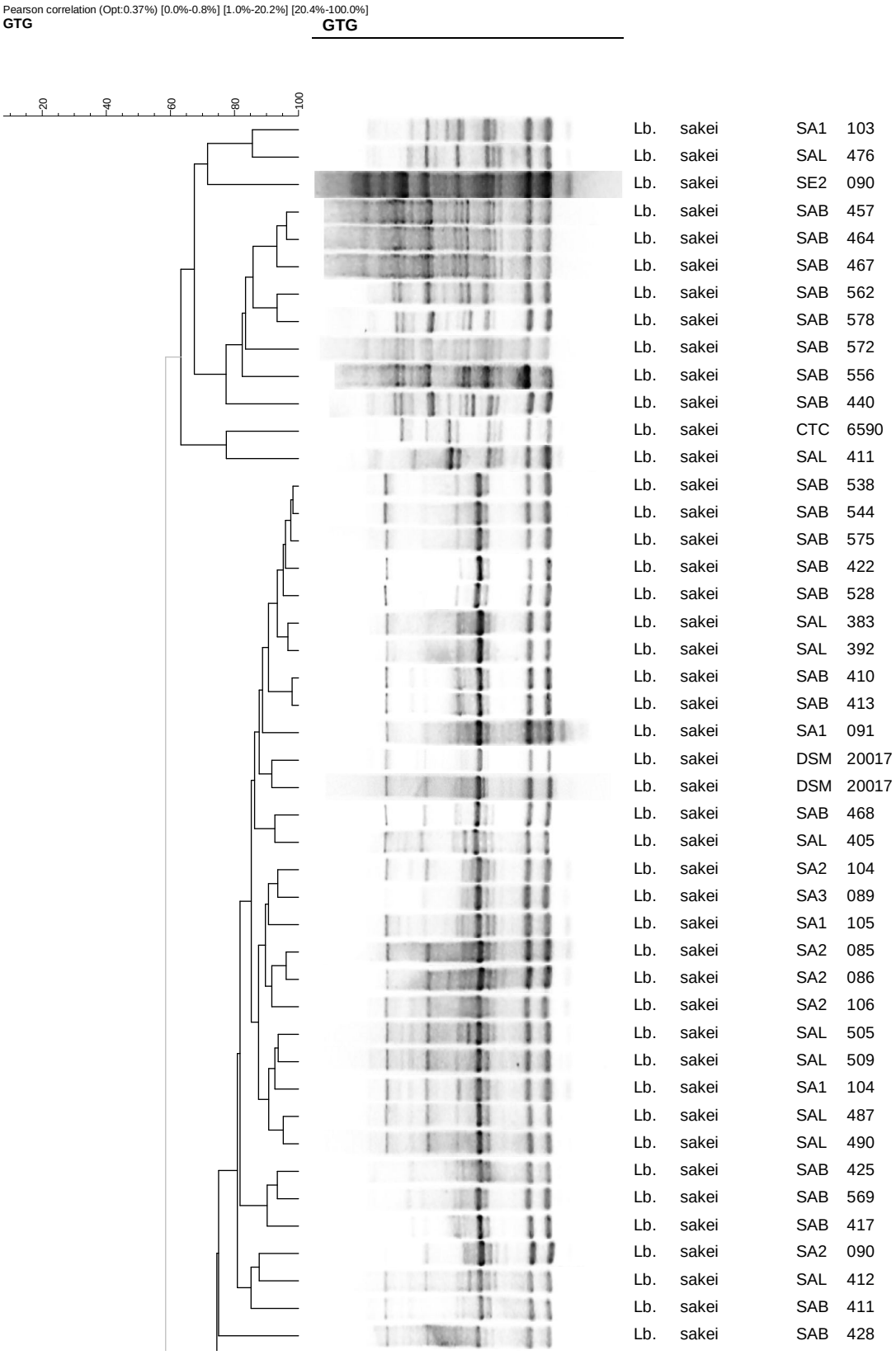
Fig. 19 Biodiversity within the dominant species in the different producers (rep-PCR analysis).

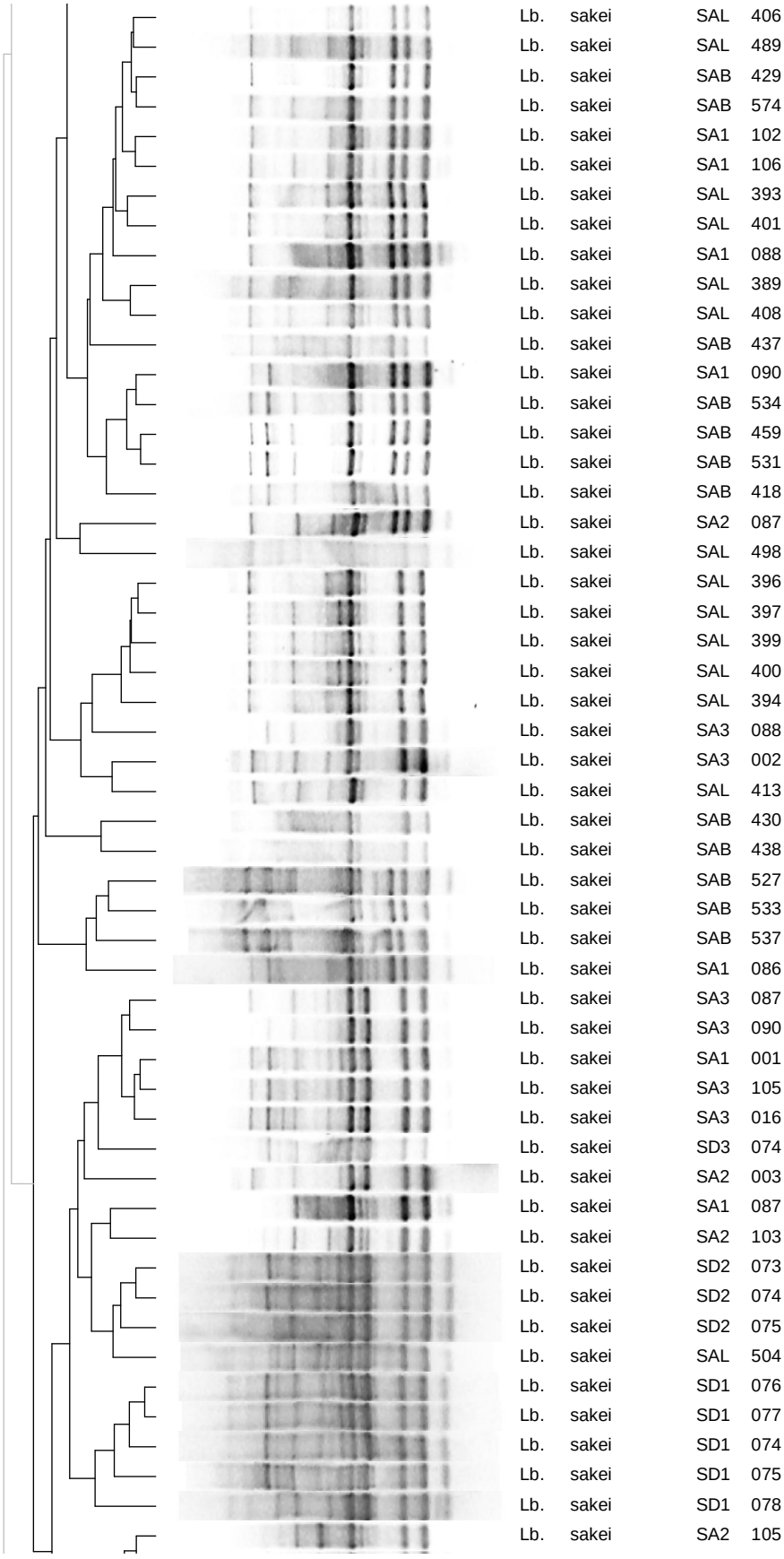
The biodiversity within the same dominant species was very different according to producer and all the dominant species detected in this study showed to possess a high genetic diversity (Fig.19). The low number of biotypes shared

(20/218) by different producer indicate that each product is a particular ecosystem with its own flora.

These results highlight the importance of traditional products valorisation as an important means to preserve biodiversity.

Fig.14. Dendrogram generated after cluster analysis of the digitized (GTG)5-PCR fingerprints of LAB isolates

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Lb. sakei SAB 429
Lb. sakei SAB 574
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Lb. sakei SA1 106
Lb. sakei SAL 393
Lb. sakei SAL 401
Lb. sakei SA1 088
Lb. sakei SAL 389
Lb. sakei SAL 408
Lb. sakei SAB 437
Lb. sakei SA1 090
Lb. sakei SAB 534
Lb. sakei SAB 459
Lb. sakei SAB 531
Lb. sakei SAB 418
Lb. sakei SA2 087
Lb. sakei SAL 498
Lb. sakei SAL 396
Lb. sakei SAL 397
Lb. sakei SAL 399
Lb. sakei SAL 400
Lb. sakei SAL 394
Lb. sakei SA3 088
Lb. sakei SA3 002
Lb. sakei SAL 413
Lb. sakei SAB 430
Lb. sakei SAB 438
Lb. sakei SAB 527
Lb. sakei SAB 533
Lb. sakei SAB 537
Lb. sakei SA1 086
Lb. sakei SA3 087
Lb. sakei SA3 090
Lb. sakei SA1 001
Lb. sakei SA3 105
Lb. sakei SA3 016
Lb. sakei SD3 074
Lb. sakei SA2 003
Lb. sakei SA1 087
Lb. sakei SA2 103
Lb. sakei SD2 073
Lb. sakei SD2 074
Lb. sakei SD2 075
Lb. sakei SAL 504
Lb. sakei SD1 076
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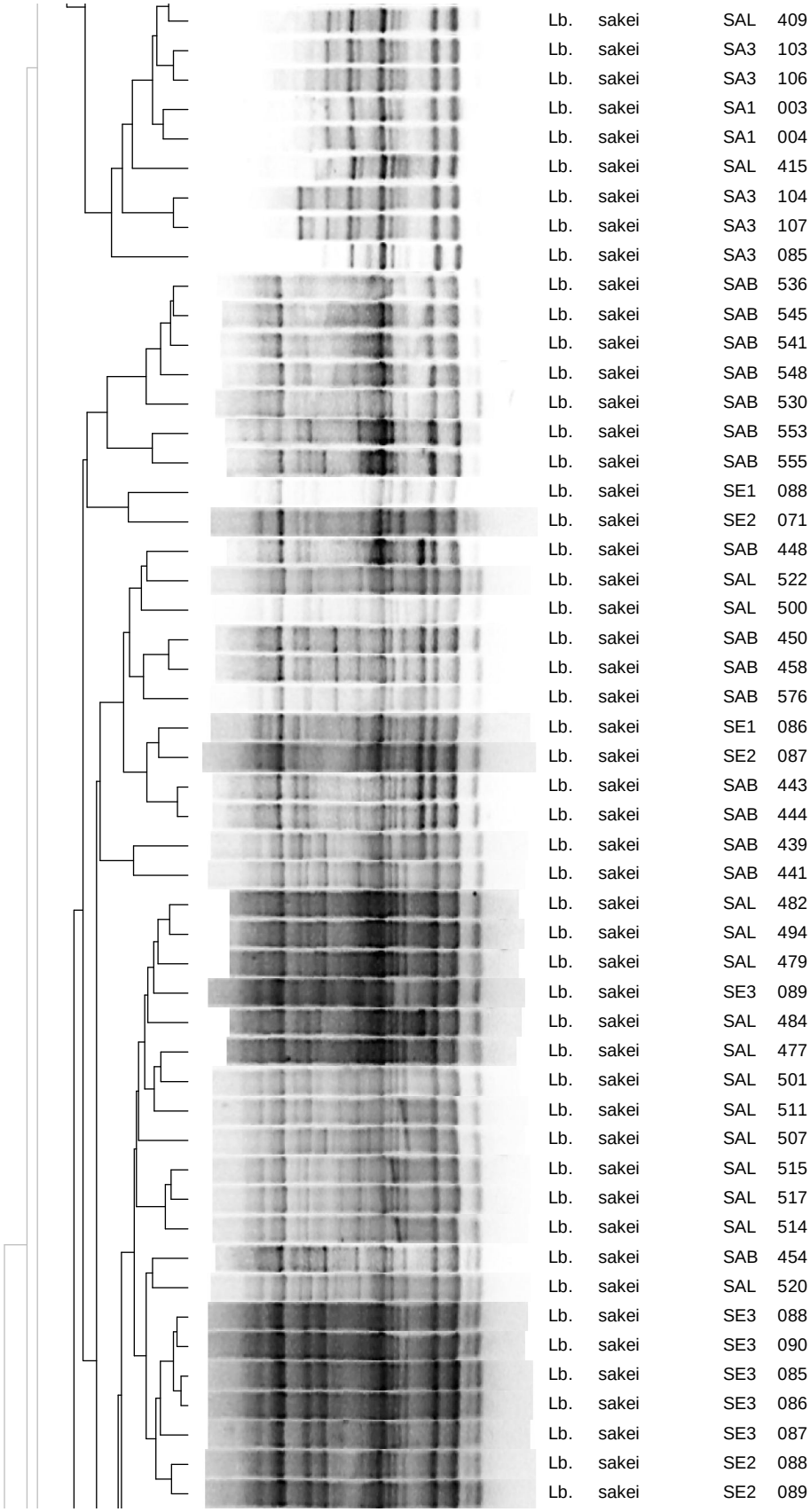
Lb. sakei SA2 105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Lb. sakei SA3 106
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Lb. sakei SAL 415
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Lb. sakei SA3 107
Lb. sakei SA3 085
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Lb. sakei SAB 541
Lb. sakei SAB 548
Lb. sakei SAB 530
Lb. sakei SAB 553
Lb. sakei SAB 555
Lb. sakei SE1 088
Lb. sakei SE2 071
Lb. sakei SAB 448
Lb. sakei SAL 522
Lb. sakei SAL 500
Lb. sakei SAB 450
Lb. sakei SAB 458
Lb. sakei SAB 576
Lb. sakei SE1 086
Lb. sakei SE2 087
Lb. sakei SAB 443
Lb. sakei SAB 444
Lb. sakei SAB 439
Lb. sakei SAB 441
Lb. sakei SAL 482
Lb. sakei SAL 494
Lb. sakei SAL 479
Lb. sakei SE3 089
Lb. sakei SAL 484
Lb. sakei SAL 477
Lb. sakei SAL 501
Lb. sakei SAL 511
Lb. sakei SAL 507
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Lb. sakei SAL 517
Lb. sakei SAL 514
Lb. sakei SAB 454
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Lb. sakei SE3 090
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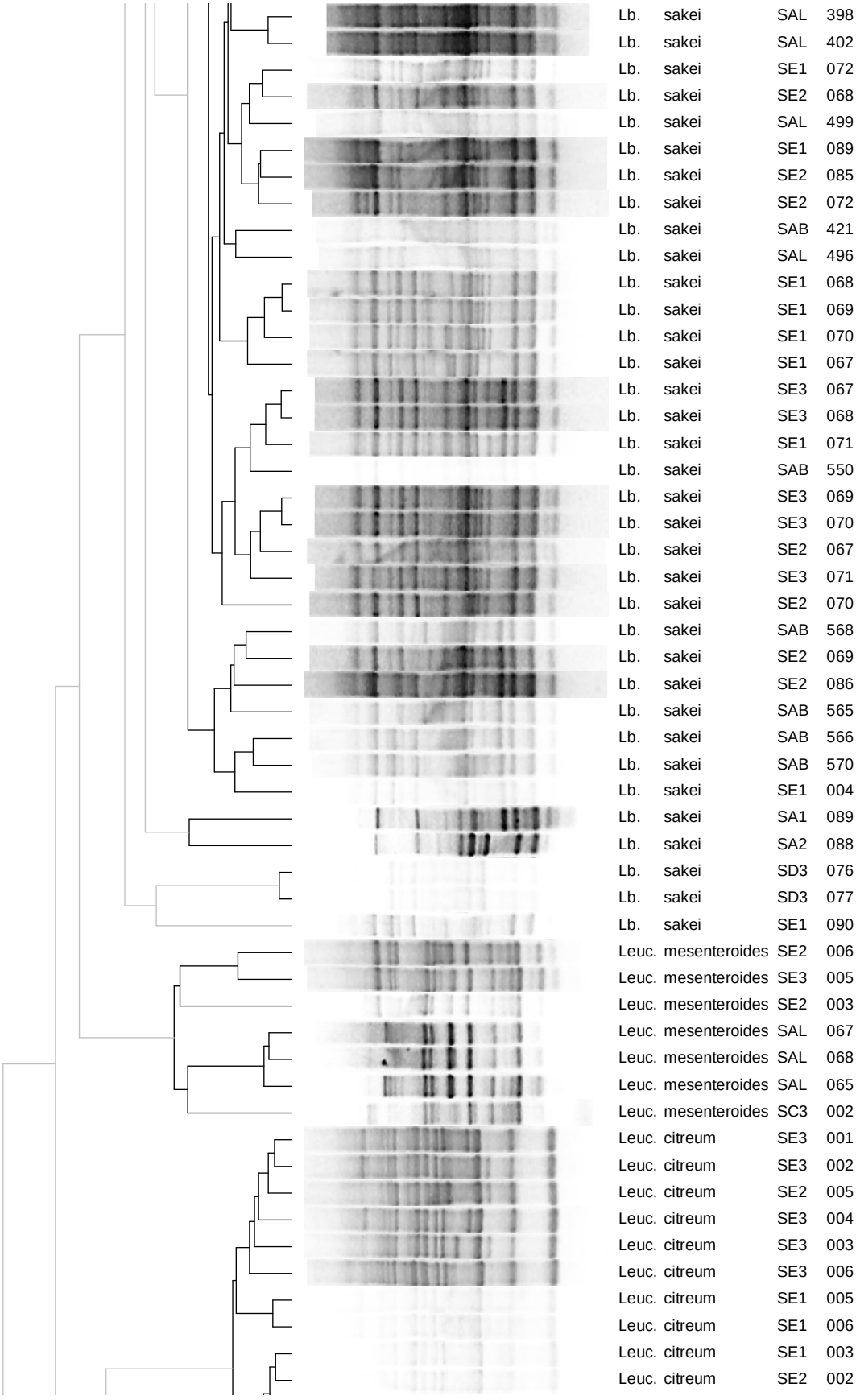
Lb. sakei SE2 089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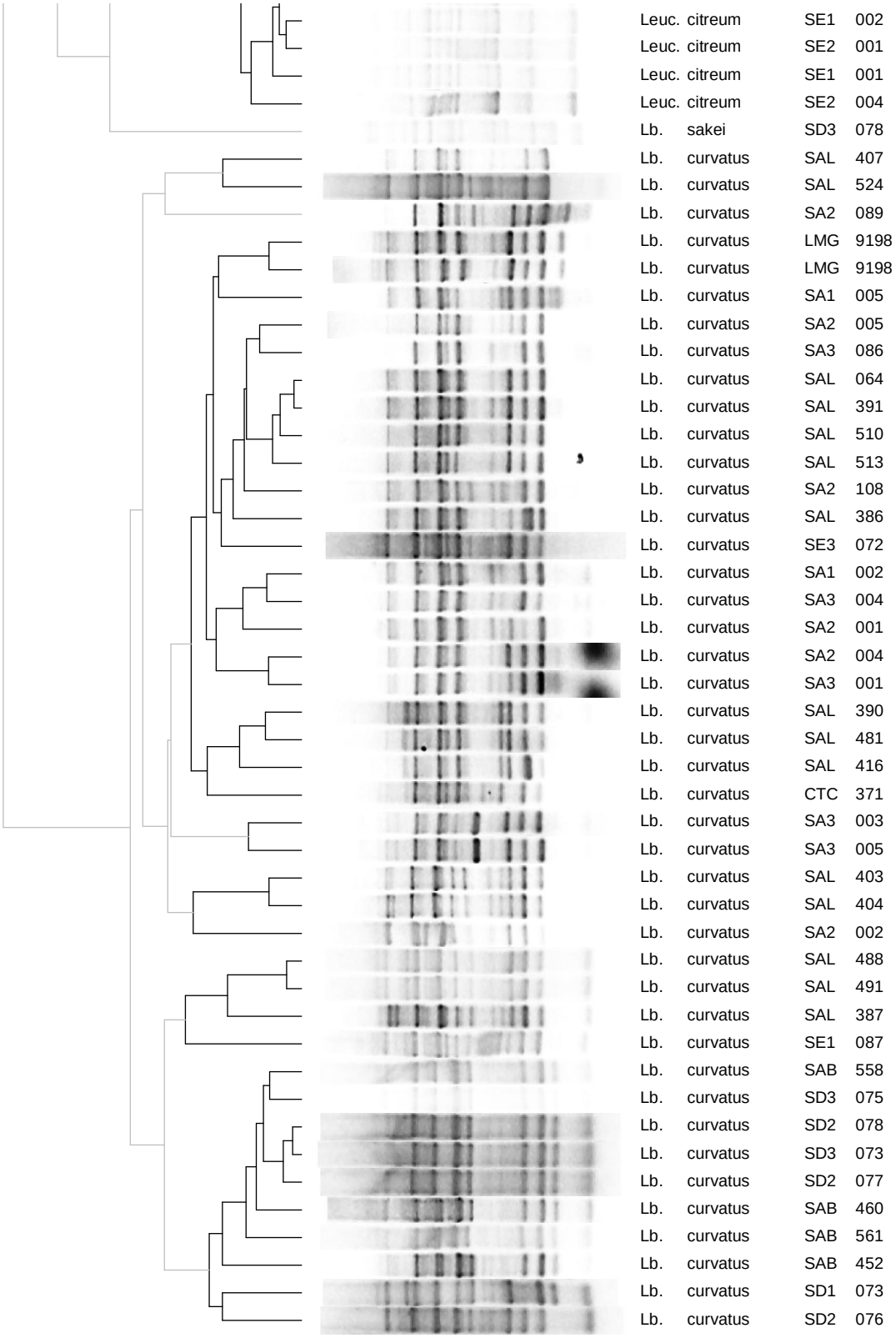
Lb. sakei SE2 085
Lb. sakei SE2 072
Lb. sakei SAB 421
Lb. sakei SAL 496
Lb. sakei SE1 068
Lb. sakei SE1 069
Lb. sakei SE1 070
Lb. sakei SE1 067
Lb. sakei SE3 067
Lb. sakei SE3 068
Lb. sakei SE1 071
Lb. sakei SAB 550
Lb. sakei SE3 069
Lb. sakei SE3 070
Lb. sakei SE2 067
Lb. sakei SE3 071
Lb. sakei SE2 070
Lb. sakei SAB 568
Lb. sakei SE2 069
Lb. sakei SE2 086
Lb. sakei SAB 565
Lb. sakei SAB 566
Lb. sakei SAB 570
Lb. sakei SE1 004
Lb. sakei SA1 089
Lb. sakei SA2 088
Lb. sakei SD3 076
Lb. sakei SD3 077
Lb. sakei SE1 090
Leuc. mesenteroides SE2 006
Leuc. mesenteroides SE3 005
Leuc. mesenteroides SE2 003
Leuc. mesenteroides SAL 067
Leuc. mesenteroides SAL 068
Leuc. mesenteroides SAL 065
Leuc. mesenteroides SC3 002
Leuc. citreum SE3 001
Leuc. citreum SE3 002
Leuc. citreum SE2 005
Leuc. citreum SE3 004
Leuc. citreum SE3 003
Leuc. citreum SE3 006
Leuc. citreum SE1 005
Leuc. citreum SE1 006
Leuc. citreum SE1 003
Leuc. citreum SE2 002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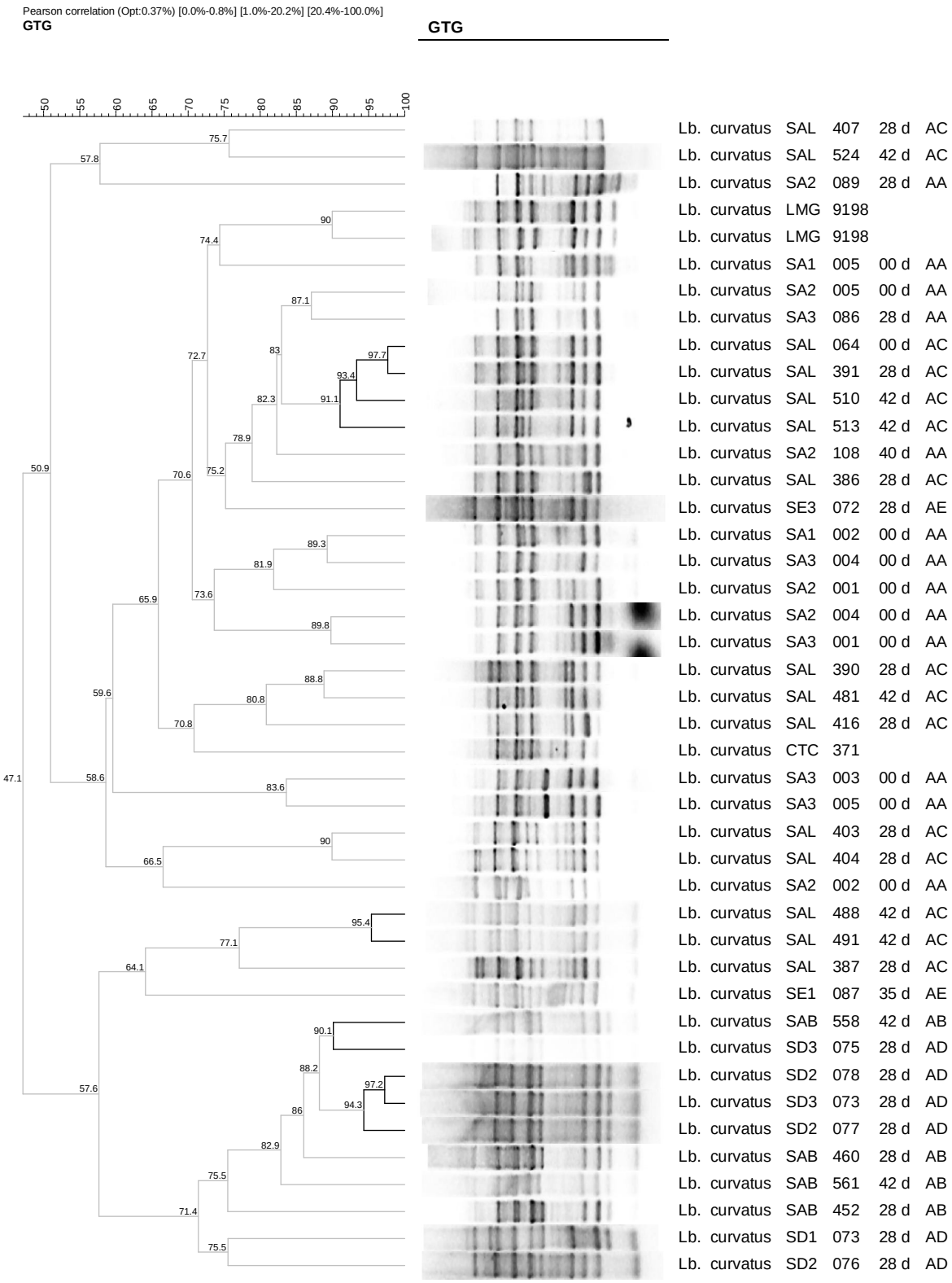
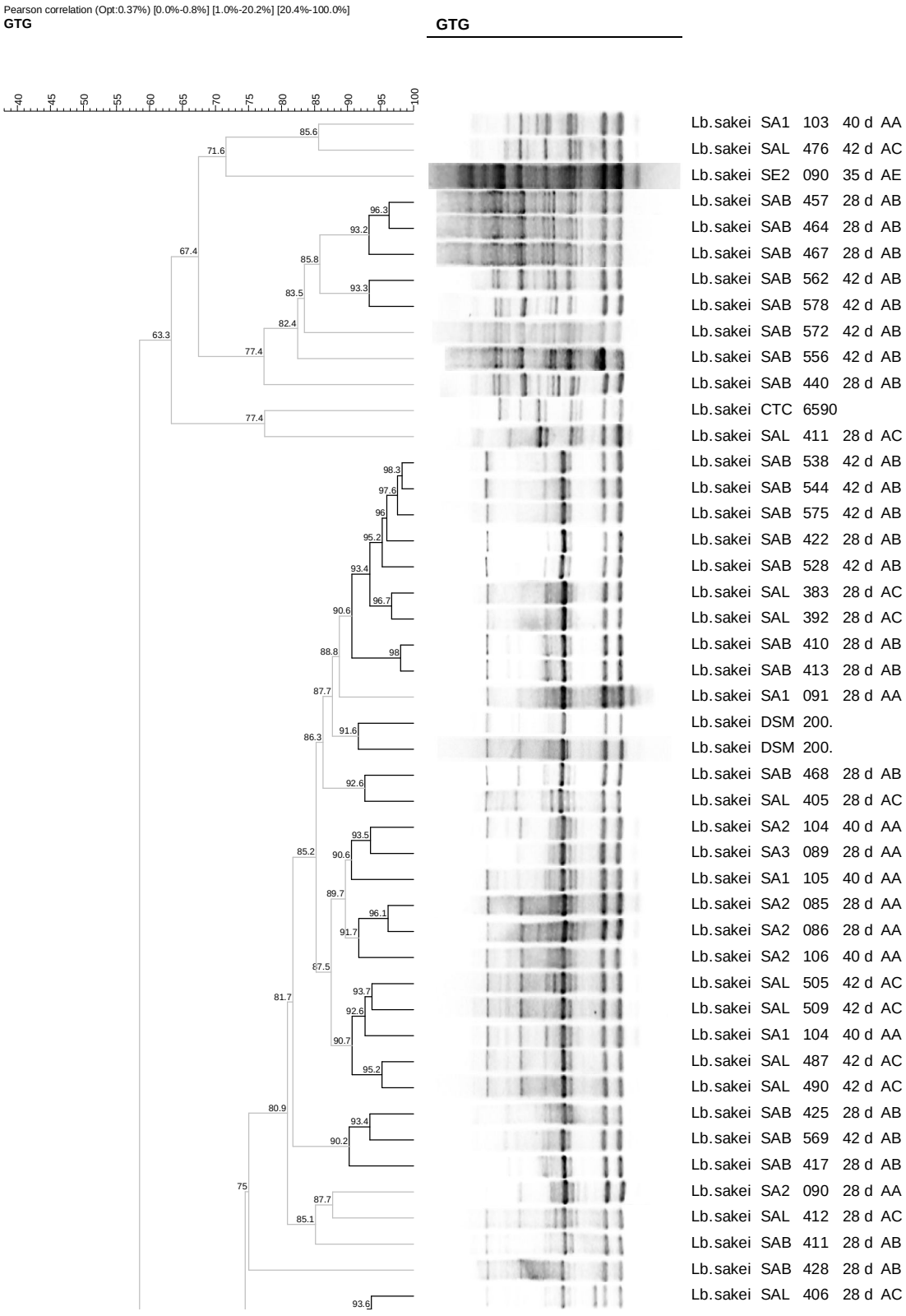
Leuc. citreum SE2 001
Leuc. citreum SE1 001
Leuc. citreum SE2 004
Lb. sakei SD3 078
Lb. curvatus SAL 407
Lb. curvatus SAL 524
Lb. curvatus SA2 089
Lb. curvatus LMG 9198
Lb. curvatus LMG 9198
Lb. curvatus SA1 005
Lb. curvatus SA2 005
Lb. curvatus SA3 086
Lb. curvatus SAL 064
Lb. curvatus SAL 391
Lb. curvatus SAL 510
Lb. curvatus SAL 513
Lb. curvatus SA2 108
Lb. curvatus SAL 386
Lb. curvatus SE3 072
Lb. curvatus SA1 002
Lb. curvatus SA3 004
Lb. curvatus SA2 001
Lb. curvatus SA2 004
Lb. curvatus SA3 001
Lb. curvatus SAL 390
Lb. curvatus SAL 481
Lb. curvatus SAL 416
Lb. curvatus CTC 371
Lb. curvatus SA3 003
Lb. curvatus SA3 005
Lb. curvatus SAL 403
Lb. curvatus SAL 404
Lb. curvatus SA2 002
Lb. curvatus SAL 488
Lb. curvatus SAL 491
Lb. curvatus SAL 387
Lb. curvatus SE1 087
Lb. curvatus SAB 558
Lb. curvatus SD3 075
Lb. curvatus SD2 078
Lb. curvatus SD3 073
Lb. curvatus SD2 077
Lb. curvatus SAB 460
Lb. curvatus SAB 561
Lb. curvatus SAB 452
Lb. curvatus SD1 073
Lb. curvatus SD2 076

Fig.14a. Dendrogram generated after cluster analysis of the digitized (GTG)5- PCR fingerprints of Lb. curvatus isolates




Pearson correlation (Opt:0.37%) [0.0%-0.8%] [1.0%-20.2%] [20.4%-100.0%]
GTG
GTG
40 45 50 55 60 65 70 75 80 85 90 95 100
Lb.sakei SA1 103 40 d AA
Lb.sakei SAL 476 42 d AC
Lb.sakei SE2 090 35 d AE
Lb.sakei SAB 457 28 d AB
Lb.sakei SAB 464 28 d AB
Lb.sakei SAB 467 28 d AB
Lb.sakei SAB 562 42 d AB
Lb.sakei SAB 578 42 d AB
Lb.sakei SAB 572 42 d AB
Lb.sakei SAB 556 42 d AB
Lb.sakei SAB 440 28 d AB
Lb.sakei CTC 6590
Lb.sakei SAL 411 28 d AC
Lb.sakei SAB 538 42 d AB
Lb.sakei SAB 544 42 d AB
Lb.sakei SAB 575 42 d AB
Lb.sakei SAB 422 28 d AB
Lb.sakei SAB 528 42 d AB
Lb.sakei SAL 383 28 d AC
Lb.sakei SAL 392 28 d AC
Lb.sakei SAB 410 28 d AB
Lb.sakei SAB 413 28 d AB
Lb.sakei SA1 091 28 d AA
Lb.sakei DSM 200.
Lb.sakei DSM 200.
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Lb.sakei SAL 405 28 d AC
Lb.sakei SA2 104 40 d AA
Lb.sakei SA3 089 28 d AA
Lb.sakei SA1 105 40 d AA
Lb.sakei SA2 085 28 d AA
Lb.sakei SA2 086 28 d AA
Lb.sakei SA2 106 40 d AA
Lb.sakei SAL 505 42 d AC
Lb.sakei SAL 509 42 d AC
Lb.sakei SA1 104 40 d AA
Lb.sakei SAL 487 42 d AC
Lb.sakei SAL 490 42 d AC
Lb.sakei SAB 425 28 d AB
Lb.sakei SAB 569 42 d AB
Lb.sakei SAB 417 28 d AB
Lb.sakei SA2 090 28 d AA
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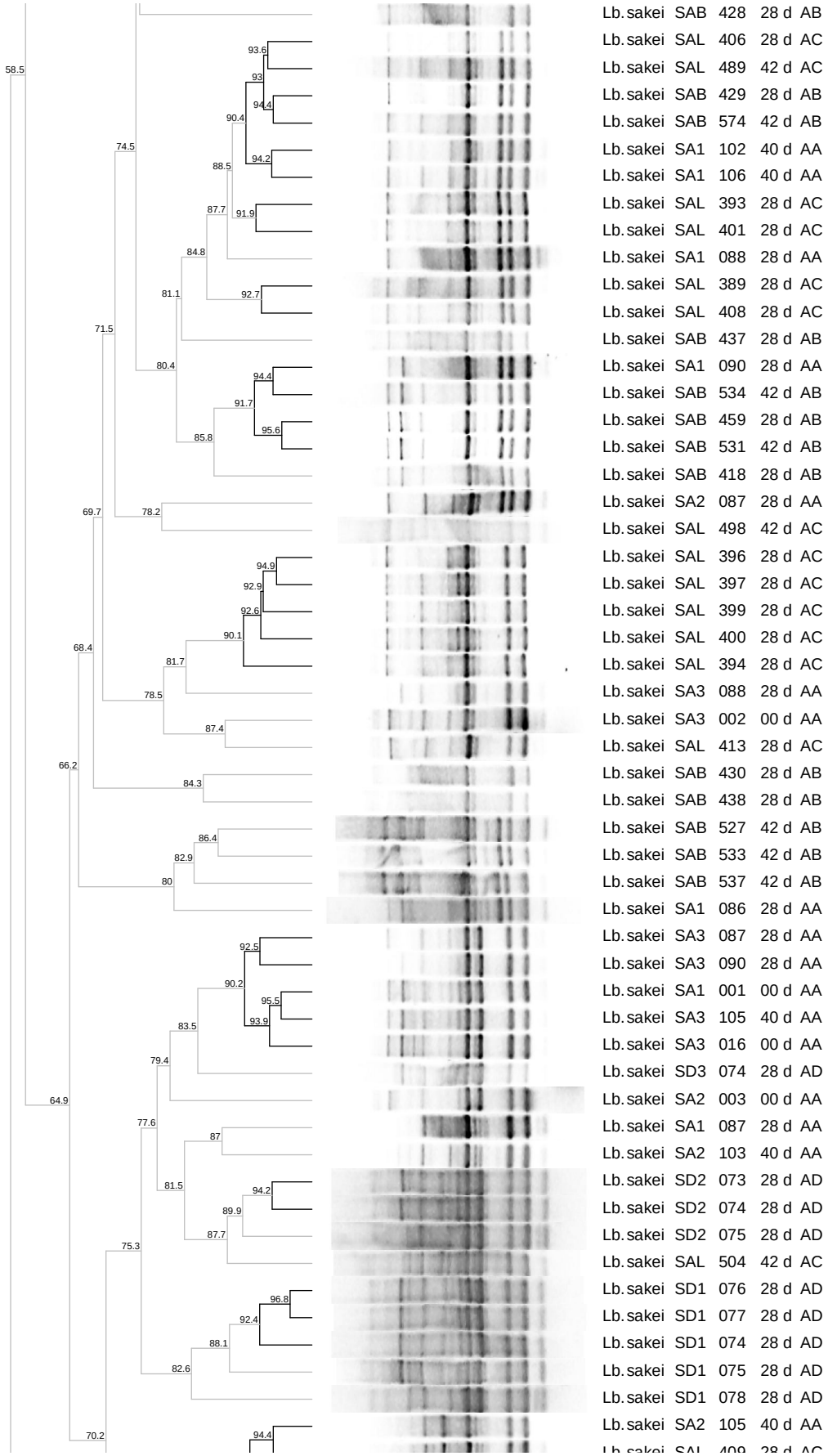
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Lb. sakei SA1 106 40 d AA
Lb. sakei SAL 393 28 d AC
Lb. sakei SAL 401 28 d AC
Lb. sakei SA1 088 28 d AA
Lb. sakei SAL 389 28 d AC
Lb. sakei SAL 408 28 d AC
Lb. sakei SAB 437 28 d AB
Lb. sakei SA1 090 28 d AA
Lb. sakei SAB 534 42 d AB
Lb. sakei SAB 459 28 d AB
Lb. sakei SAB 531 42 d AB
Lb. sakei SAB 418 28 d AB
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Lb. sakei SAL 498 42 d AC
Lb. sakei SAL 396 28 d AC
Lb. sakei SAL 397 28 d AC
Lb. sakei SAL 399 28 d AC
Lb. sakei SAL 400 28 d AC
Lb. sakei SAL 394 28 d AC
Lb. sakei SA3 088 28 d AA
Lb. sakei SA3 002 00 d AA
Lb. sakei SAL 413 28 d AC
Lb. sakei SAB 430 28 d AB
Lb. sakei SAB 438 28 d AB
Lb. sakei SAB 527 42 d AB
Lb. sakei SAB 533 42 d AB
Lb. sakei SAB 537 42 d AB
Lb. sakei SA1 086 28 d AA
Lb. sakei SA3 087 28 d AA
Lb. sakei SA3 090 28 d AA
Lb. sakei SA1 001 00 d AA
Lb. sakei SA3 105 40 d AA
Lb. sakei SA3 016 00 d AA
Lb. sakei SD3 074 28 d AD
Lb. sakei SA2 003 00 d AA
Lb. sakei SA1 087 28 d AA
Lb. sakei SA2 103 40 d AA
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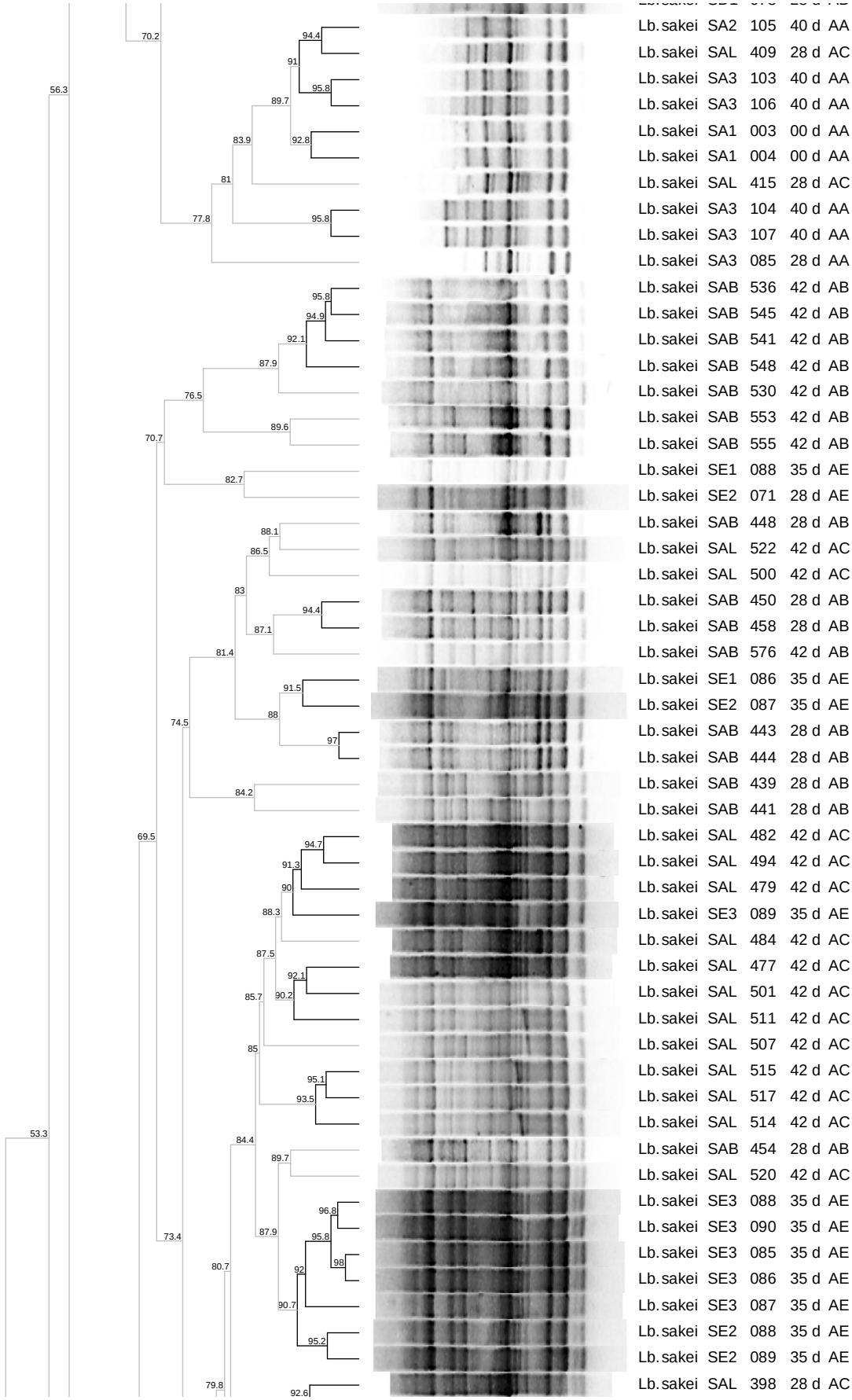
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Lb. sakei SA1 004 00 d AA
Lb. sakei SAL 415 28 d AC
Lb. sakei SA3 104 40 d AA
Lb. sakei SA3 107 40 d AA
Lb. sakei SA3 085 28 d AA
Lb. sakei SAB 536 42 d AB
Lb. sakei SAB 545 42 d AB
Lb. sakei SAB 541 42 d AB
Lb. sakei SAB 548 42 d AB
Lb. sakei SAB 530 42 d AB
Lb. sakei SAB 553 42 d AB
Lb. sakei SAB 555 42 d AB
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Lb. sakei SE2 071 28 d AE
Lb. sakei SAB 448 28 d AB
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Lb. sakei SAL 500 42 d AC
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Lb. sakei SAB 458 28 d AB
Lb. sakei SAB 576 42 d AB
Lb. sakei SE1 086 35 d AE
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Lb. sakei SAL 494 42 d AC
Lb. sakei SAL 479 42 d AC
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Lb. sakei SAL 484 42 d AC
Lb. sakei SAL 477 42 d AC
Lb. sakei SAL 501 42 d AC
Lb. sakei SAL 511 42 d AC
Lb. sakei SAL 507 42 d AC
Lb. sakei SAL 515 42 d AC
Lb. sakei SAL 517 42 d AC
Lb. sakei SAL 514 42 d AC
Lb. sakei SAB 454 28 d AB
Lb. sakei SAL 520 42 d AC
Lb. sakei SE3 088 35 d AE
Lb. sakei SE3 090 35 d AE
Lb. sakei SE3 085 35 d AE
Lb. sakei SE3 086 35 d AE
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Lb. sakei SAL 398 28 d AC

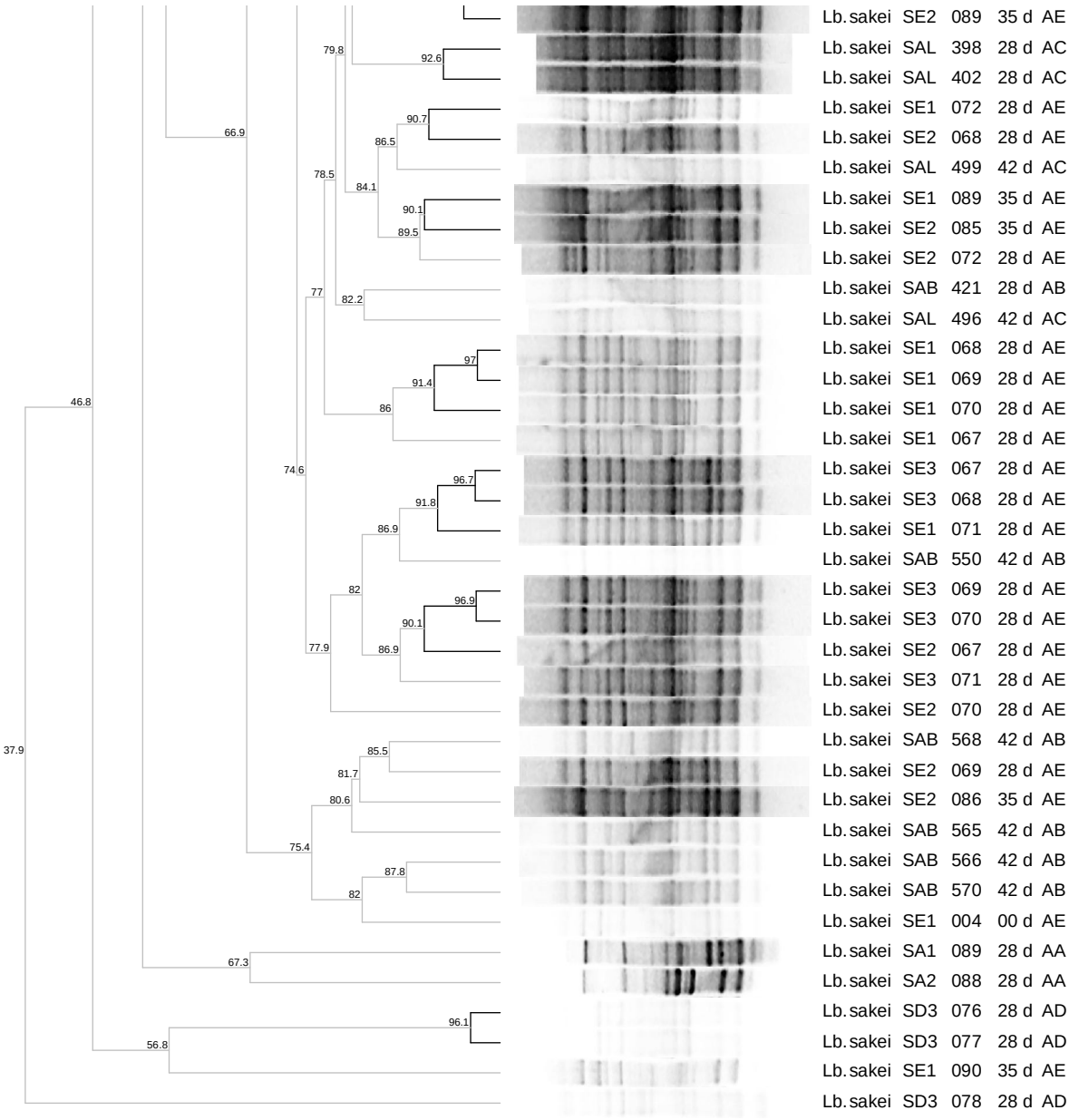


Fig. 14b. Dendrogram generated after cluster analysis of the digitized $(GTG)_5$-PCR profiles oth the Leuc. citreum isolates

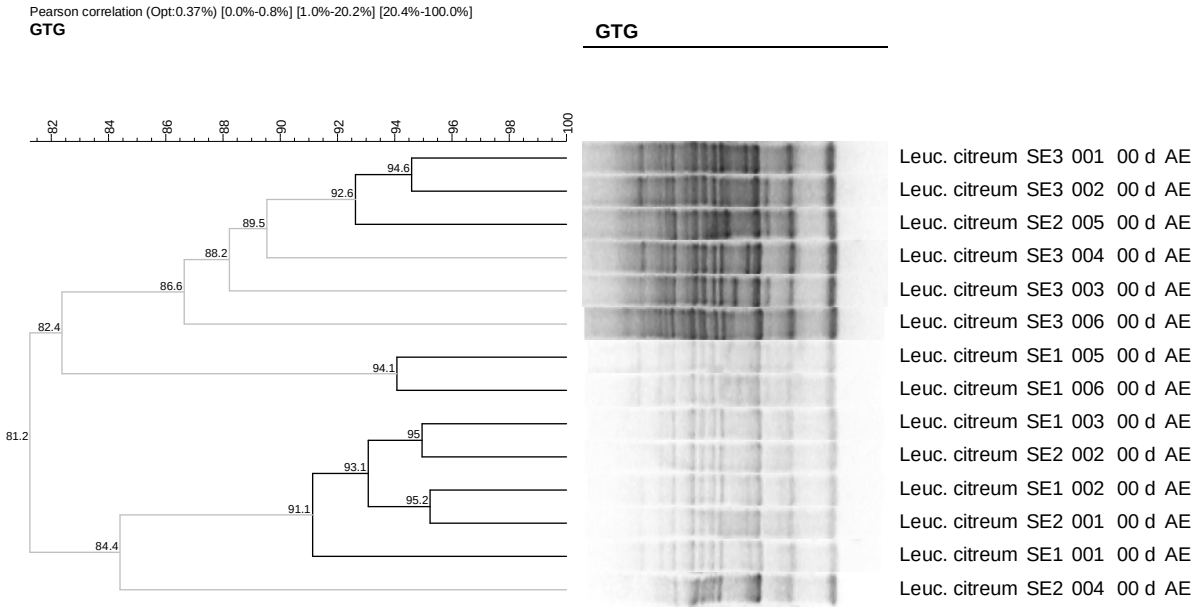


Fig. 14c. Dendrogram generated after cluster analysis of the digitized $(GTG)_5$-PCR profiles oth the Leuc. citreum isolates. (

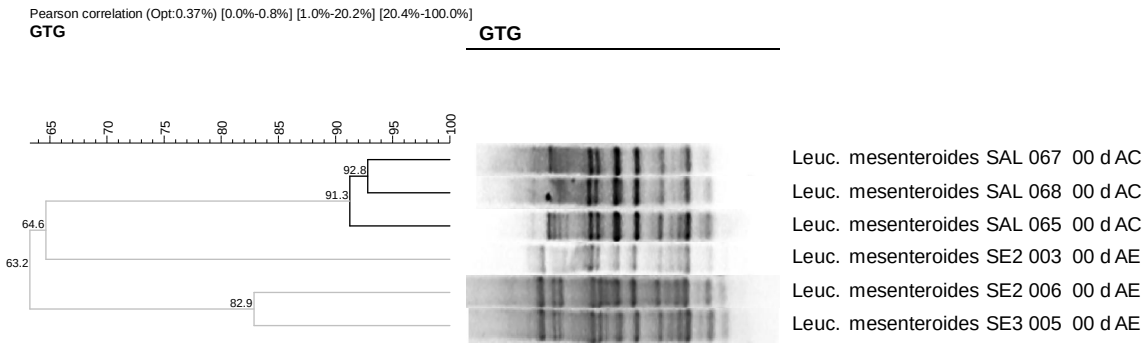
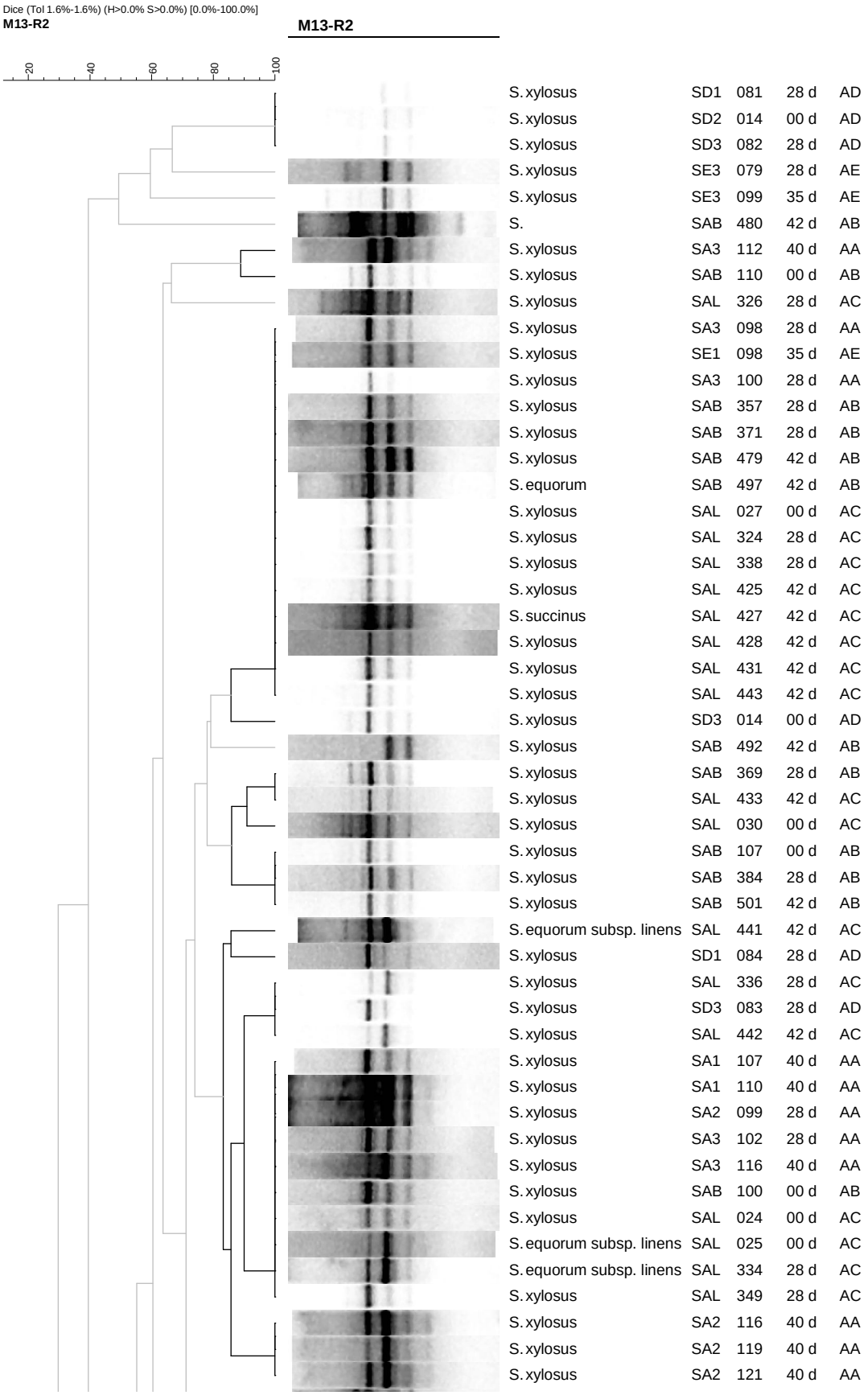
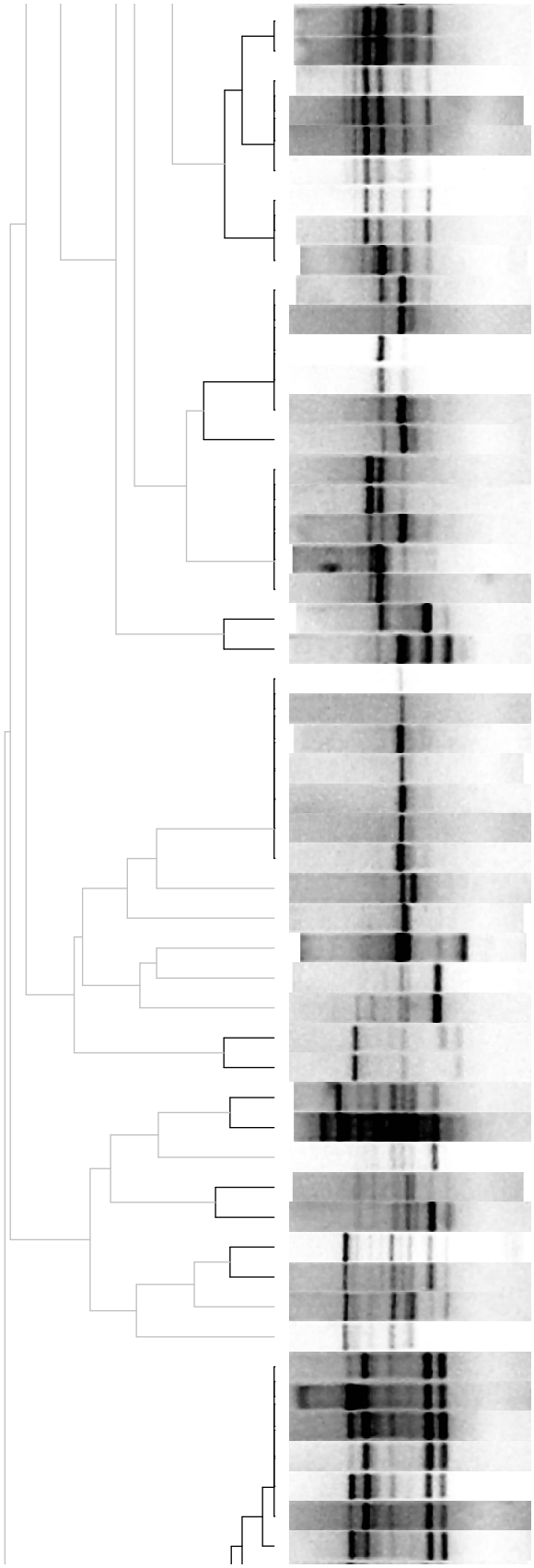
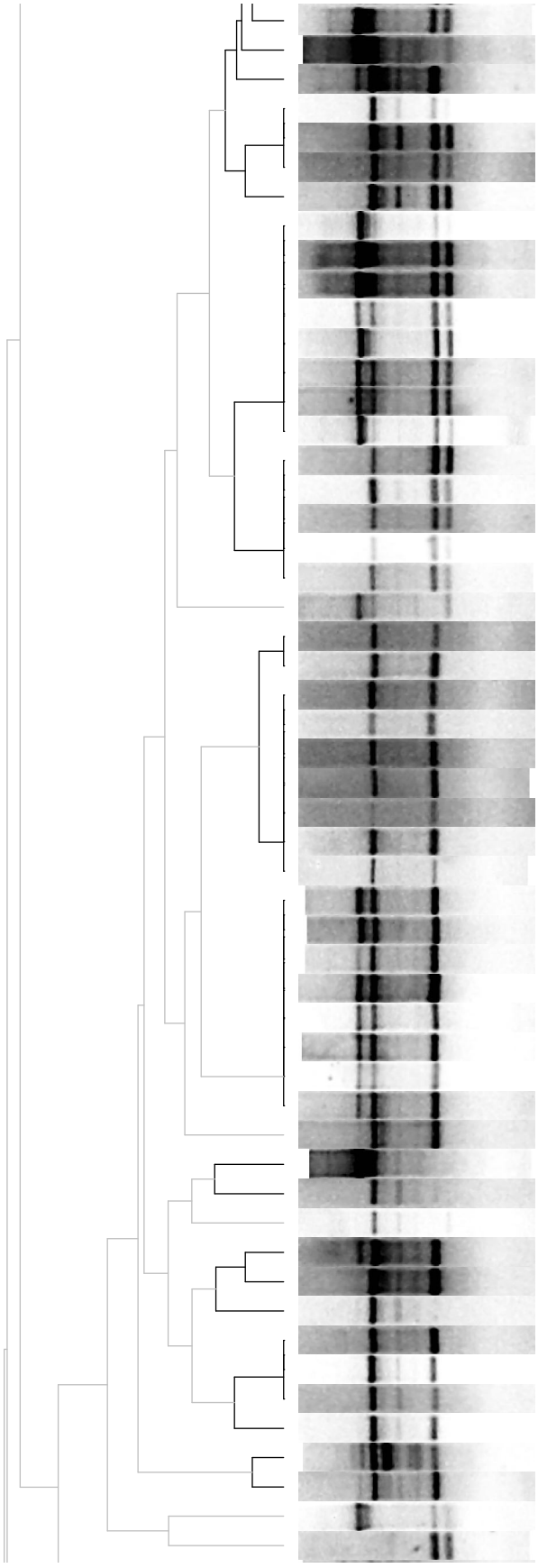


Fig. 14d. Dendrogram generated after cluster analysis of the digitized $(GTG)_5$-PCR profiles oth the Leuc. mesenteroides isolates.


Dice (Tol 1.6%-1.6%) (H>0.0% S>0.0%) [0.0%-100.0%]
M13-R2
M13-R2
20
40
60
80
100
S. xylosus SD1 081 28 d AD
S. xylosus SD2 014 00 d AD
S. xylosus SD3 082 28 d AD
S. xylosus SE3 079 28 d AE
S. xylosus SE3 099 35 d AE
S. SAB 480 42 d AB
S. xylosus SA3 112 40 d AA
S. xylosus SAB 110 00 d AB
S. xylosus SAL 326 28 d AC
S. xylosus SA3 098 28 d AA
S. xylosus SE1 098 35 d AE
S. xylosus SA3 100 28 d AA
S. xylosus SAB 357 28 d AB
S. xylosus SAB 371 28 d AB
S. xylosus SAB 479 42 d AB
S. equorum SAB 497 42 d AB
S. xylosus SAL 027 00 d AC
S. xylosus SAL 324 28 d AC
S. xylosus SAL 338 28 d AC
S. xylosus SAL 425 42 d AC
S. succinus SAL 427 42 d AC
S. xylosus SAL 428 42 d AC
S. xylosus SAL 431 42 d AC
S. xylosus SAL 443 42 d AC
S. xylosus SD3 014 00 d AD
S. xylosus SAB 492 42 d AB
S. xylosus SAB 369 28 d AB
S. xylosus SAL 433 42 d AC
S. xylosus SAL 030 00 d AC
S. xylosus SAB 107 00 d AB
S. xylosus SAB 384 28 d AB
S. xylosus SAB 501 42 d AB
S. equorum subsp. linens SAL 441 42 d AC
S. xylosus SD1 084 28 d AD
S. xylosus SAL 336 28 d AC
S. xylosus SD3 083 28 d AD
S. xylosus SAL 442 42 d AC
S. xylosus SA1 107 40 d AA
S. xylosus SA1 110 40 d AA
S. xylosus SA2 099 28 d AA
S. xylosus SA3 102 28 d AA
S. xylosus SA3 116 40 d AA
S. xylosus SAB 100 00 d AB
S. xylosus SAL 024 00 d AC
S. equorum subsp. linens SAL 025 00 d AC
S. equorum subsp. linens SAL 334 28 d AC
S. xylosus SAL 349 28 d AC
S. xylosus SA2 116 40 d AA
S. xylosus SA2 119 40 d AA
S. xylosus SA2 121 40 d AA


S. xylosus SA3 114 40 d AA
S. xylosus SA3 113 40 d AA
S. xylosus SA1 098 28 d AA
S. xylosus SA1 109 40 d AA
S. xylosus SA2 120 40 d AA
S. equorum subsp. linens SAL 439 42 d AC
S. xylosus SA1 111 40 d AA
S. xylosus SA3 099 28 d AA
S. equorum SAB 484 42 d AB
S. xylosus SA2 098 28 d AA
S. xylosus SA2 117 40 d AA
S. xylosus SA3 117 40 d AA
S. xylosus SAB 380 28 d AB
S. xylosus SAL 434 42 d AC
S. xylosus SAB 493 42 d AB
S. xylosus DSM 20266
S. xylosus DSM 20266
S. xylosus SA1 100 28 d AA
S. xylosus SE1 102 35 d AE
S. xylosus SE2 017 00 d AE
S. xylosus SA1 108 40 d AA
S. xylosus SD2 086 28 d AD
S. xylosus SA1 097 28 d AA
S. xylosus SA2 100 28 d AA
S. xylosus SA2 118 40 d AA
S. xylosus SAL 347 28 d AC
S. xylosus SD2 015 00 d AD
S. xylosus SD2 084 28 d AD
S. xylosus SD3 015 00 d AD
S. xylosus SD3 018 00 d AD
S. xylosus SAL 422 42 d AC
S. epidermidis SAB 114 00 d AB
S. pulvereri/vitulus SAL 028 00 d AC
S. pulvereri SAL 436 42 d AC
S. xylosus SD2 016 00 d AD
S. xylosus SD3 016 00 d AD
S. pasteuri SA3 011 00 d AA
S. pasteuri SAB 109 00 d AB
S. xylosus SAL 026 00 d AC
S. pasteuri SAB 112 00 d AB
S. pasteuri SE3 016 00 d AE
S. pasteuri SAB 102 00 d AB
S. pasteuri SAB 105 00 d AB
S. pasteuri SAB 111 00 d AB
S. pasteuri SAB 101 00 d AB
S. xylosus SAB 366 28 d AB
S. xylosus SAB 367 28 d AB
S. xylosus SAB 482 42 d AB
S. xylosus SAB 489 42 d AB
S. xylosus SAB 495 42 d AB
S. xylosus SAL 332 28 d AC
S. xylosus SAB 477 42 d AB


S. xylosus SAB 359 28 d AB
S. equorum SD2 017 00 d AD
S. xylosus SAB 486 42 d AB
S. xylosus SAB 378 28 d AB
S. xylosus SAL 327 28 d AC
S. equorum subsp. linens SAL 429 42 d AC
S. xylosus SAB 383 28 d AB
S. equorum SAB 355 28 d AB
S. xylosus SAB 372 28 d AB
S. xylosus SAB 375 28 d AB
S. xylosus SAB 376 28 d AB
S. xylosus SAB 478 42 d AB
S. xylosus SAB 496 42 d AB
S. xylosus SAB 498 42 d AB
S. xylosus SAL 424 42 d AC
S. xylosus SA2 102 28 d AA
S. xylosus SAB 377 28 d AB
S. xylosus SAB 485 42 d AB
S. xylosus SAL 440 42 d AC
S. xylosus SE1 100 35 d AE
S. xylosus SA1 099 28 d AA
S. xylosus SAL 331 28 d AC
S. xylosus SAL 339 28 d AC
S. equorum subsp. linens SAL 325 28 d AC
S. xylosus SAL 330 28 d AC
S. xylosus SAL 335 28 d AC
S. xylosus SAL 348 28 d AC
S. equorum subsp. linens SAL 420 42 d AC
S. xylosus SAL 421 42 d AC
S. xylosus SAL 435 42 d AC
S. xylosus SA2 097 28 d AA
S. xylosus SA3 097 28 d AA
S. xylosus SA3 101 28 d AA
S. xylosus SAB 381 28 d AB
S. xylosus SAB 499 42 d AB
S. xylosus SAL 328 28 d AC
S. xylosus SAL 438 42 d AC
S. equorum subsp. linens SE3 100 35 d AE
S. equorum subsp. linens SAL 346 28 d AC
S. equorum SAB 488 42 d AB
S. xylosus SAL 445 42 d AC
S. xylosus SD3 084 28 d AD
S. xylosus SAB 373 28 d AB
S. xylosus SAB 487 42 d AB
S. xylosus SAB 491 42 d AB
S. xylosus SAB 365 28 d AB
S. xylosus SAL 419 42 d AC
S. xylosus SAL 437 42 d AC
S. xylosus SAB 379 28 d AB
S. xylosus SA1 112 40 d AA
S. xylosus SAB 494 42 d AB
S. xylosus SAB 356 28 d AB
S. xylosus SAL 333 28 d AC

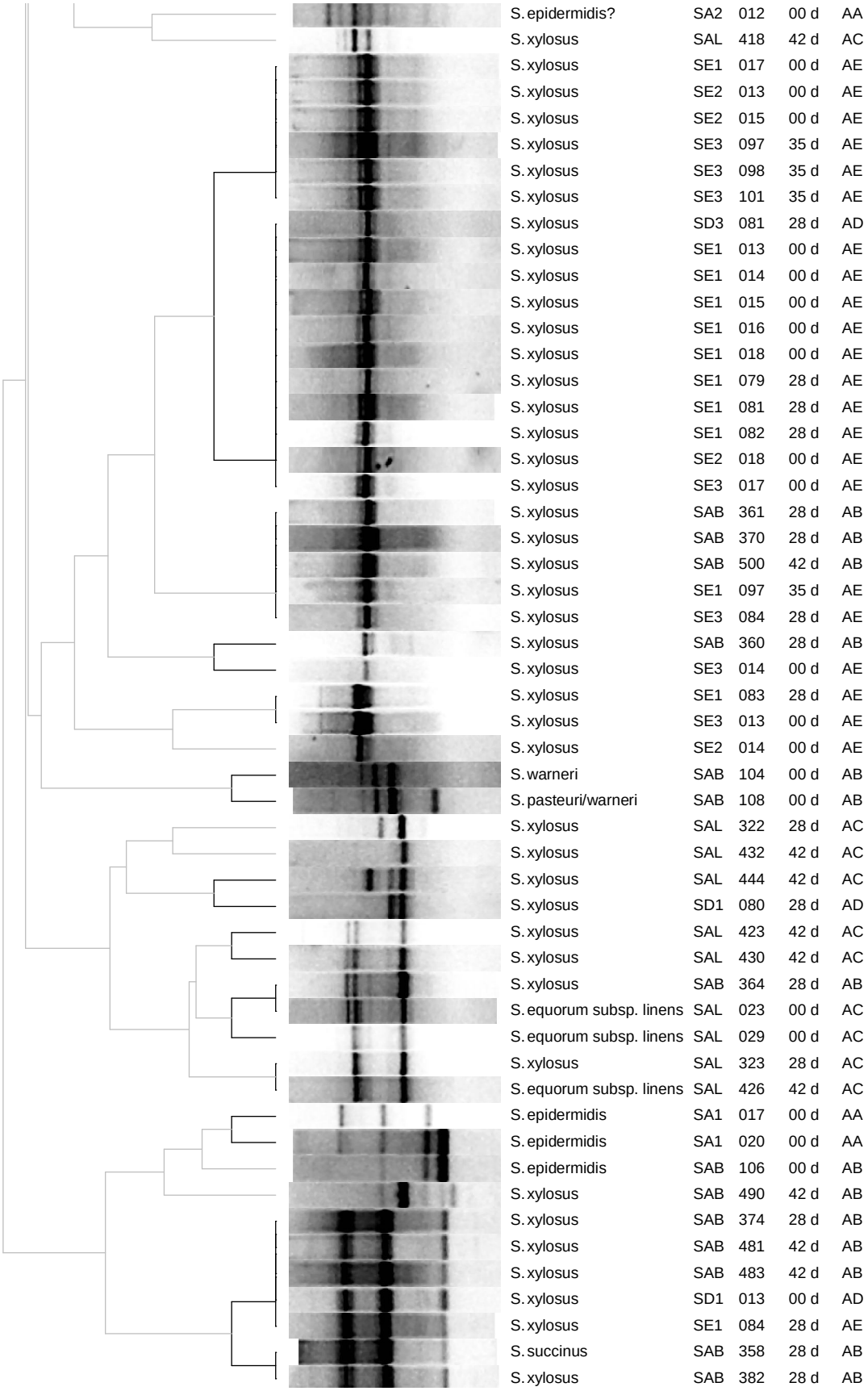
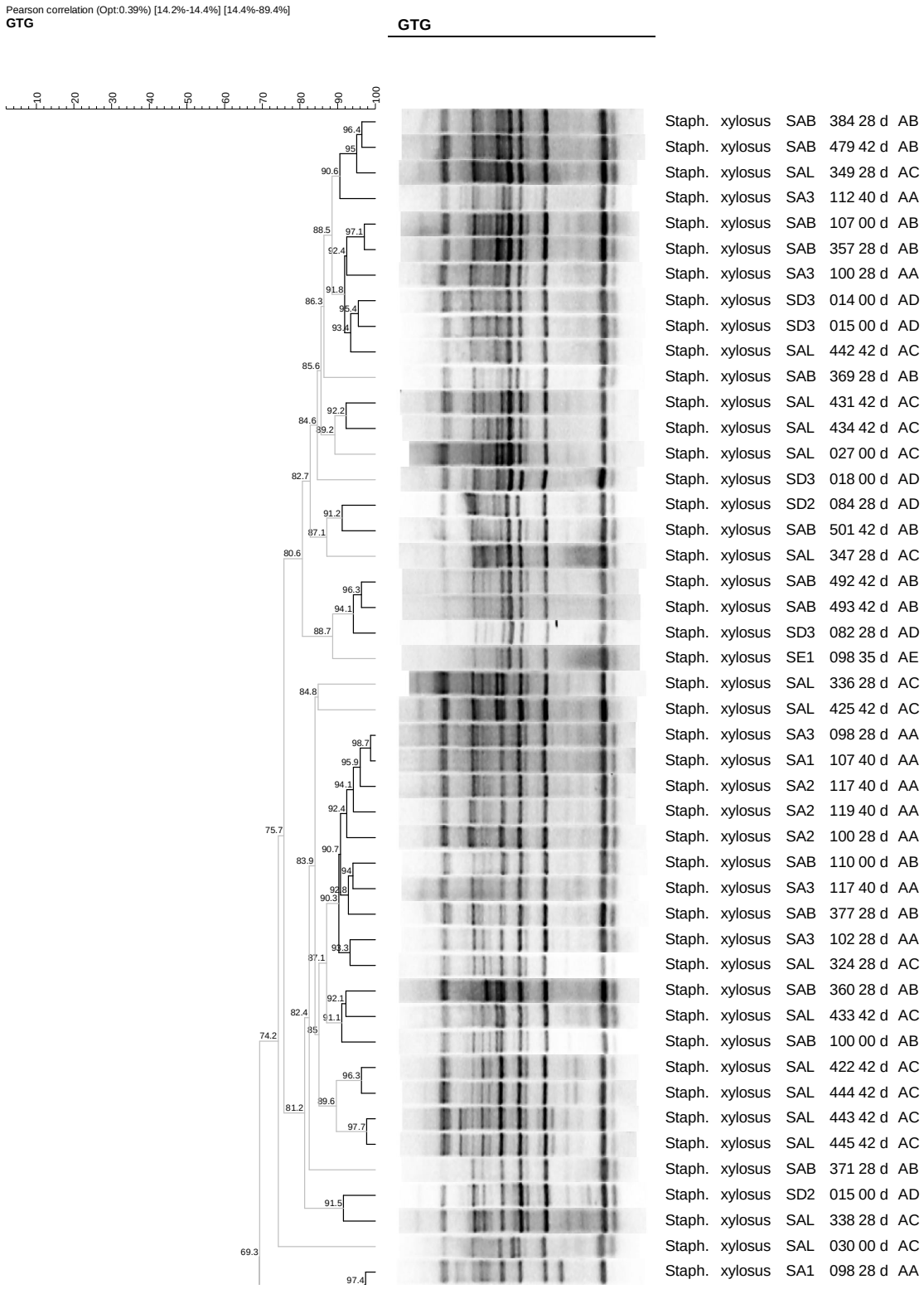
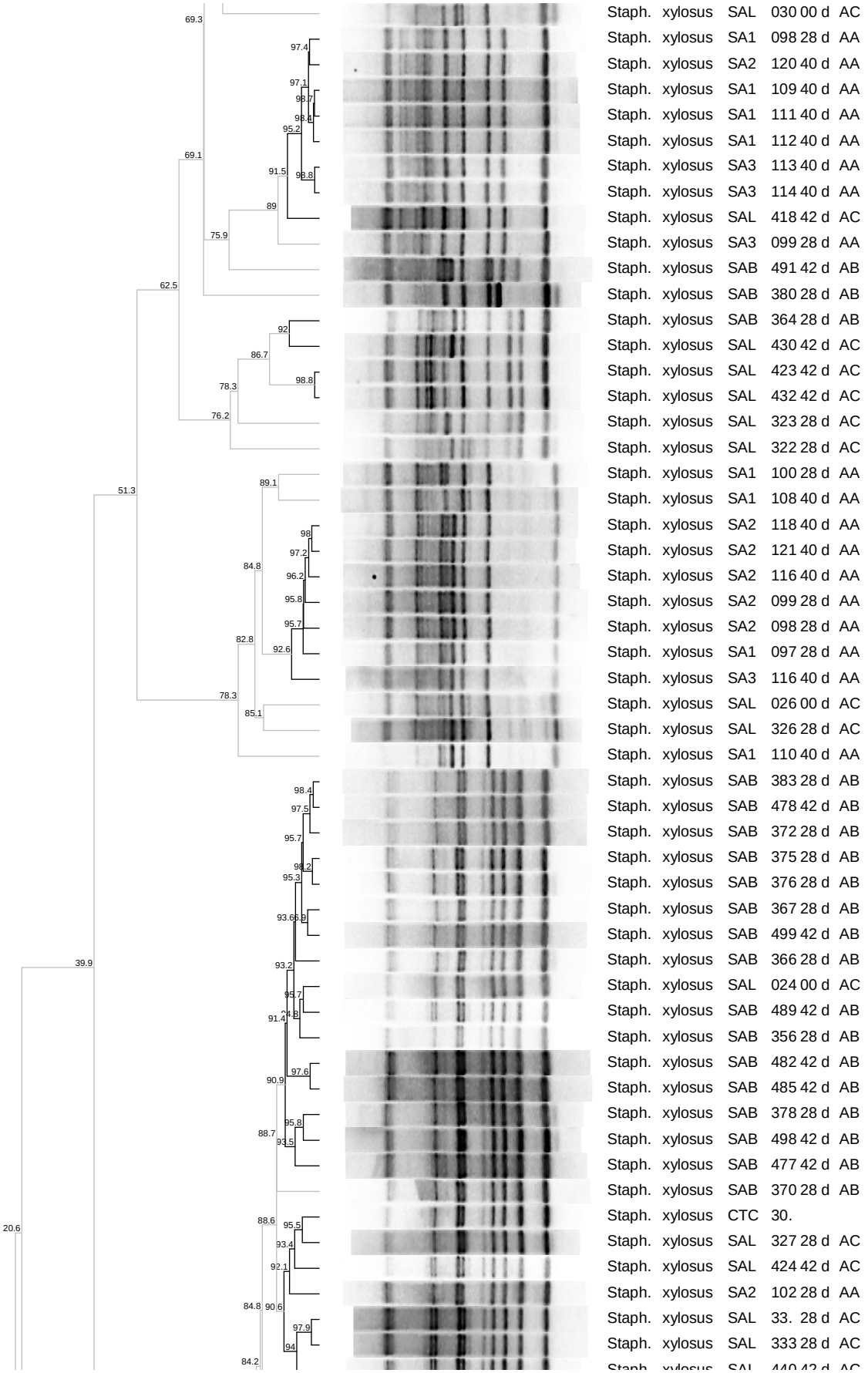
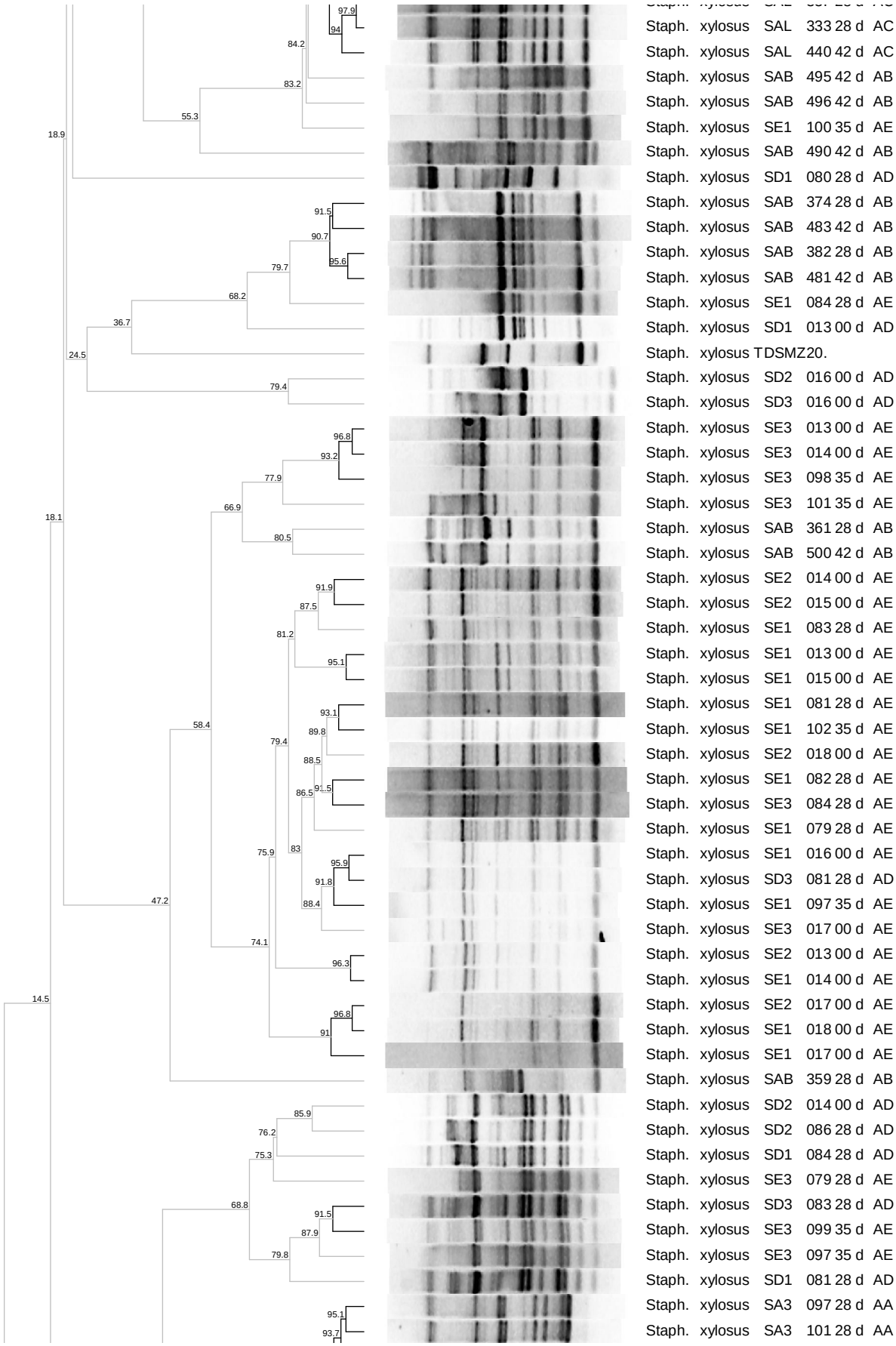


Fig. 17. Dendrogram generated after cluster analysis of RAPD-PCR profiles of the staphylococci species

Fig. 18. Dendrogram generated after ckuster analysis of GTG5-PCR profiles of the S.xylosus isolates.




Staph. xylosus SAL 030 00 d AC
Staph. xylosus SA1 098 28 d AA
Staph. xylosus SA2 120 40 d AA
Staph. xylosus SA1 109 40 d AA
Staph. xylosus SA1 111 40 d AA
Staph. xylosus SA1 112 40 d AA
Staph. xylosus SA3 113 40 d AA
Staph. xylosus SA3 114 40 d AA
Staph. xylosus SAL 418 42 d AC
Staph. xylosus SA3 099 28 d AA
Staph. xylosus SAB 491 42 d AB
Staph. xylosus SAB 380 28 d AB
Staph. xylosus SAB 364 28 d AB
Staph. xylosus SAL 430 42 d AC
Staph. xylosus SAL 423 42 d AC
Staph. xylosus SAL 432 42 d AC
Staph. xylosus SAL 323 28 d AC
Staph. xylosus SAL 322 28 d AC
Staph. xylosus SA1 100 28 d AA
Staph. xylosus SA1 108 40 d AA
Staph. xylosus SA2 118 40 d AA
Staph. xylosus SA2 121 40 d AA
Staph. xylosus SA2 116 40 d AA
Staph. xylosus SA2 099 28 d AA
Staph. xylosus SA2 098 28 d AA
Staph. xylosus SA1 097 28 d AA
Staph. xylosus SA3 116 40 d AA
Staph. xylosus SAL 026 00 d AC
Staph. xylosus SAL 326 28 d AC
Staph. xylosus SA1 110 40 d AA
Staph. xylosus SAB 383 28 d AB
Staph. xylosus SAB 478 42 d AB
Staph. xylosus SAB 372 28 d AB
Staph. xylosus SAB 375 28 d AB
Staph. xylosus SAB 376 28 d AB
Staph. xylosus SAB 367 28 d AB
Staph. xylosus SAB 499 42 d AB
Staph. xylosus SAB 366 28 d AB
Staph. xylosus SAL 024 00 d AC
Staph. xylosus SAB 489 42 d AB
Staph. xylosus SAB 356 28 d AB
Staph. xylosus SAB 482 42 d AB
Staph. xylosus SAB 485 42 d AB
Staph. xylosus SAB 378 28 d AB
Staph. xylosus SAB 498 42 d AB
Staph. xylosus SAB 477 42 d AB
Staph. xylosus SAB 370 28 d AB
Staph. xylosus CTC 30.
Staph. xylosus SAL 327 28 d AC
Staph. xylosus SAL 424 42 d AC
Staph. xylosus SA2 102 28 d AA
Staph. xylosus SAL 33. 28 d AC
Staph. xylosus SAL 333 28 d AC


Staph. xylosus SAL 333 28 d AC
Staph. xylosus SAL 440 42 d AC
Staph. xylosus SAB 495 42 d AB
Staph. xylosus SAB 496 42 d AB
Staph. xylosus SE1 100 35 d AE
Staph. xylosus SAB 490 42 d AB
Staph. xylosus SD1 080 28 d AD
Staph. xylosus SAB 374 28 d AB
Staph. xylosus SAB 483 42 d AB
Staph. xylosus SAB 382 28 d AB
Staph. xylosus SAB 481 42 d AB
Staph. xylosus SE1 084 28 d AE
Staph. xylosus SD1 013 00 d AD
Staph. xylosus TDSMZ20.
Staph. xylosus SD2 016 00 d AD
Staph. xylosus SD3 016 00 d AD
Staph. xylosus SE3 013 00 d AE
Staph. xylosus SE3 014 00 d AE
Staph. xylosus SE3 098 35 d AE
Staph. xylosus SE3 101 35 d AE
Staph. xylosus SAB 361 28 d AB
Staph. xylosus SAB 500 42 d AB
Staph. xylosus SE2 014 00 d AE
Staph. xylosus SE2 015 00 d AE
Staph. xylosus SE1 083 28 d AE
Staph. xylosus SE1 013 00 d AE
Staph. xylosus SE1 015 00 d AE
Staph. xylosus SE1 081 28 d AE
Staph. xylosus SE1 102 35 d AE
Staph. xylosus SE2 018 00 d AE
Staph. xylosus SE1 082 28 d AE
Staph. xylosus SE3 084 28 d AE
Staph. xylosus SE1 079 28 d AE
Staph. xylosus SE1 016 00 d AE
Staph. xylosus SD3 081 28 d AD
Staph. xylosus SE1 097 35 d AE
Staph. xylosus SE3 017 00 d AE
Staph. xylosus SE2 013 00 d AE
Staph. xylosus SE1 014 00 d AE
Staph. xylosus SE2 017 00 d AE
Staph. xylosus SE1 018 00 d AE
Staph. xylosus SE1 017 00 d AE
Staph. xylosus SAB 359 28 d AB
Staph. xylosus SD2 014 00 d AD
Staph. xylosus SD2 086 28 d AD
Staph. xylosus SD1 084 28 d AD
Staph. xylosus SE3 079 28 d AE
Staph. xylosus SD3 083 28 d AD
Staph. xylosus SE3 099 35 d AE
Staph. xylosus SE3 097 35 d AE
Staph. xylosus SD1 081 28 d AD
Staph. xylosus SA3 097 28 d AA
Staph. xylosus SA3 101 28 d AA

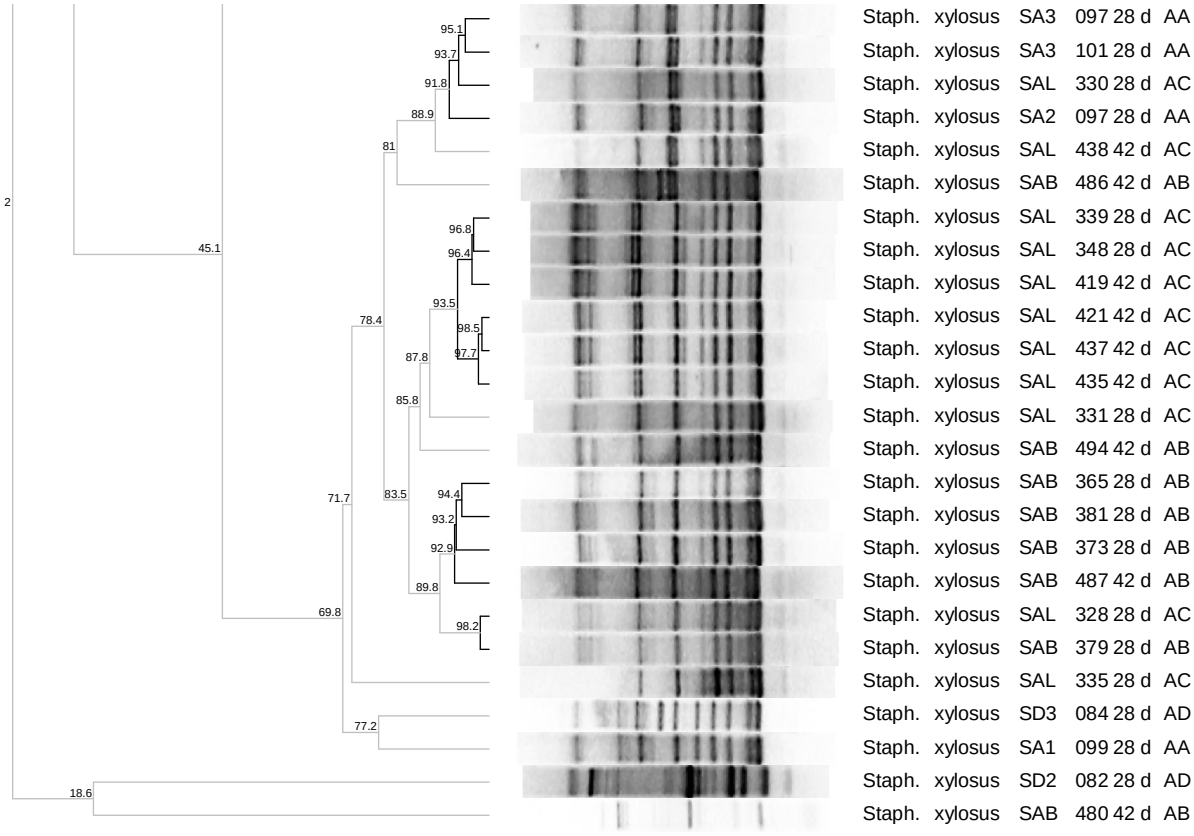
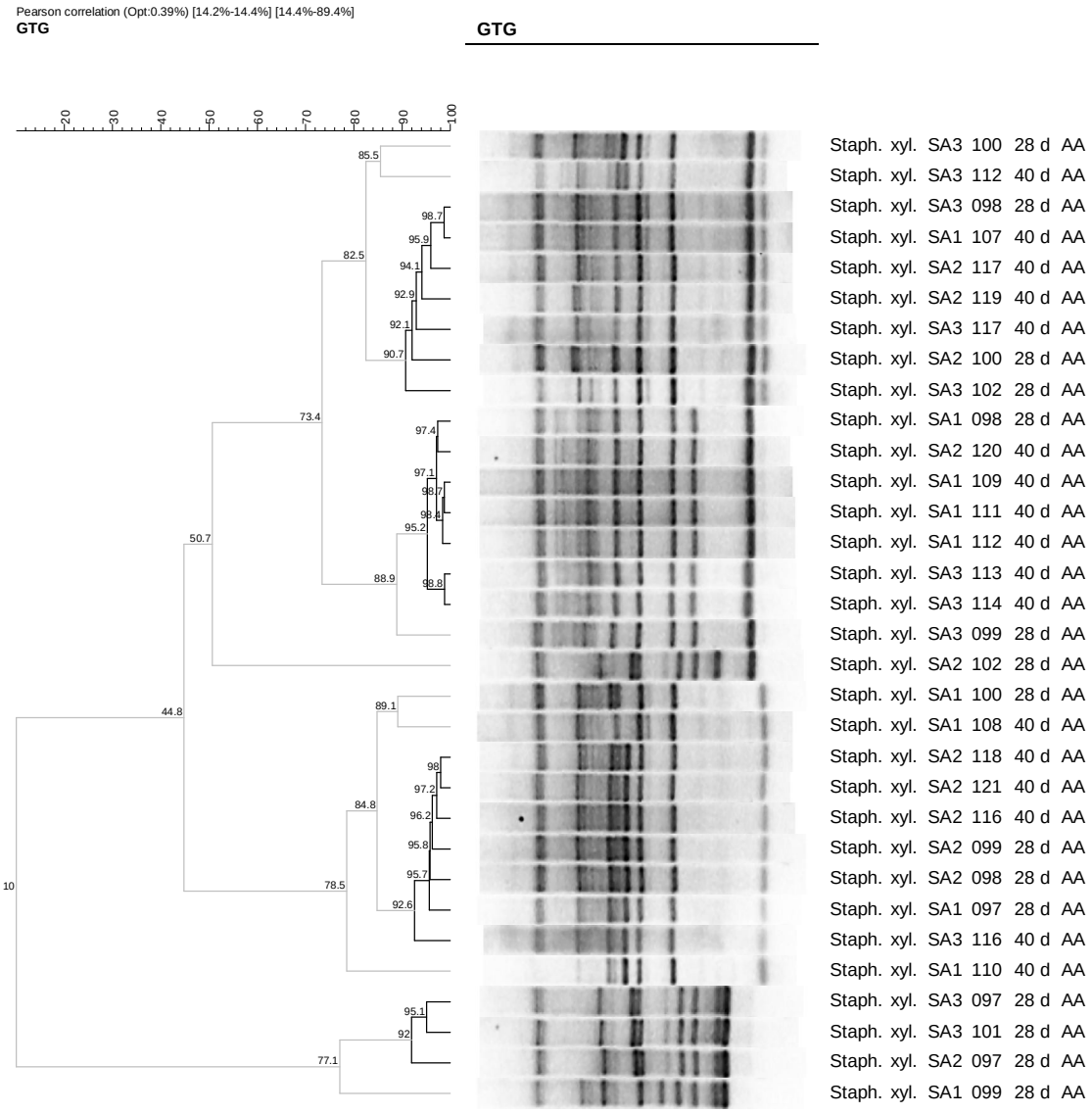
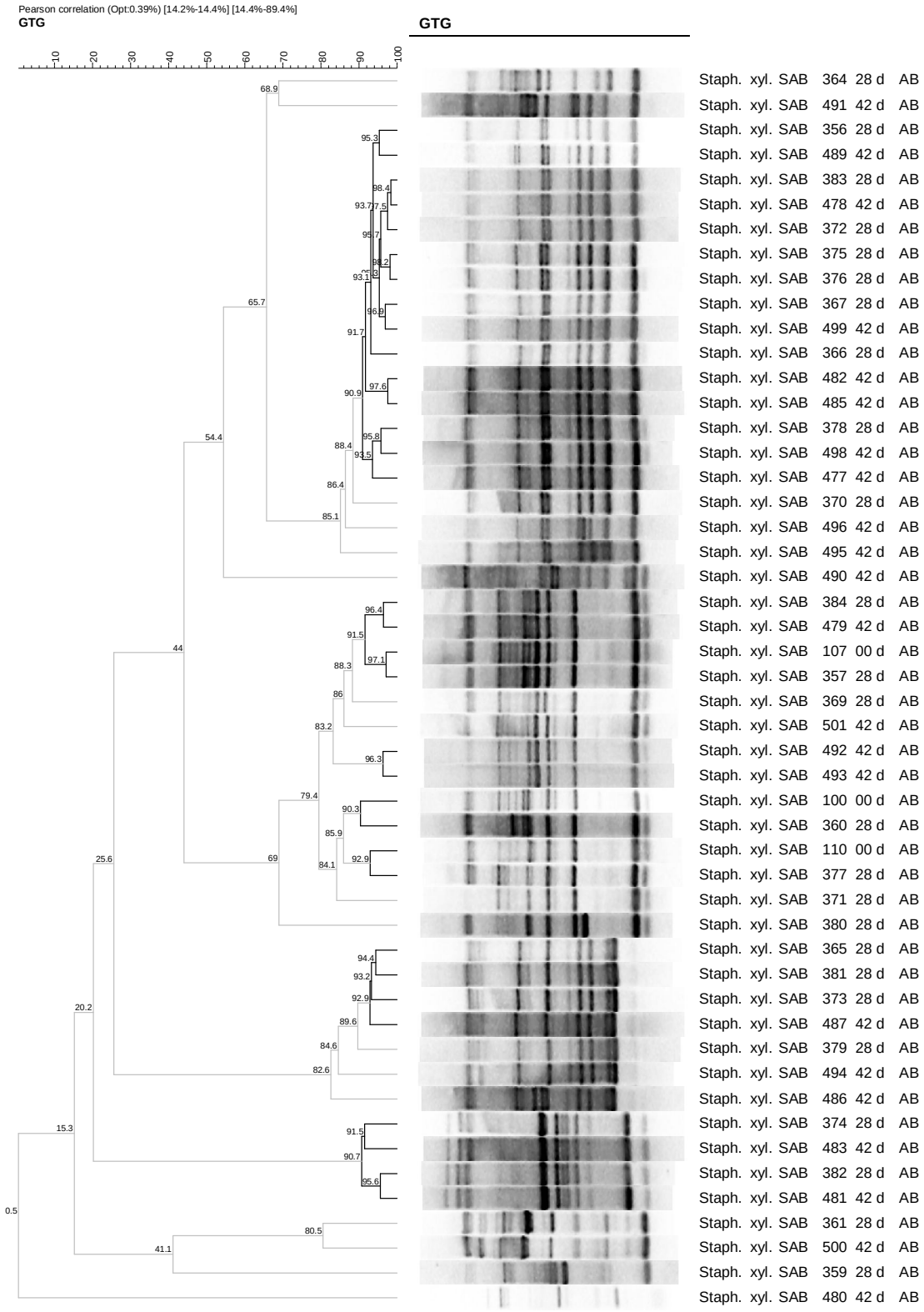
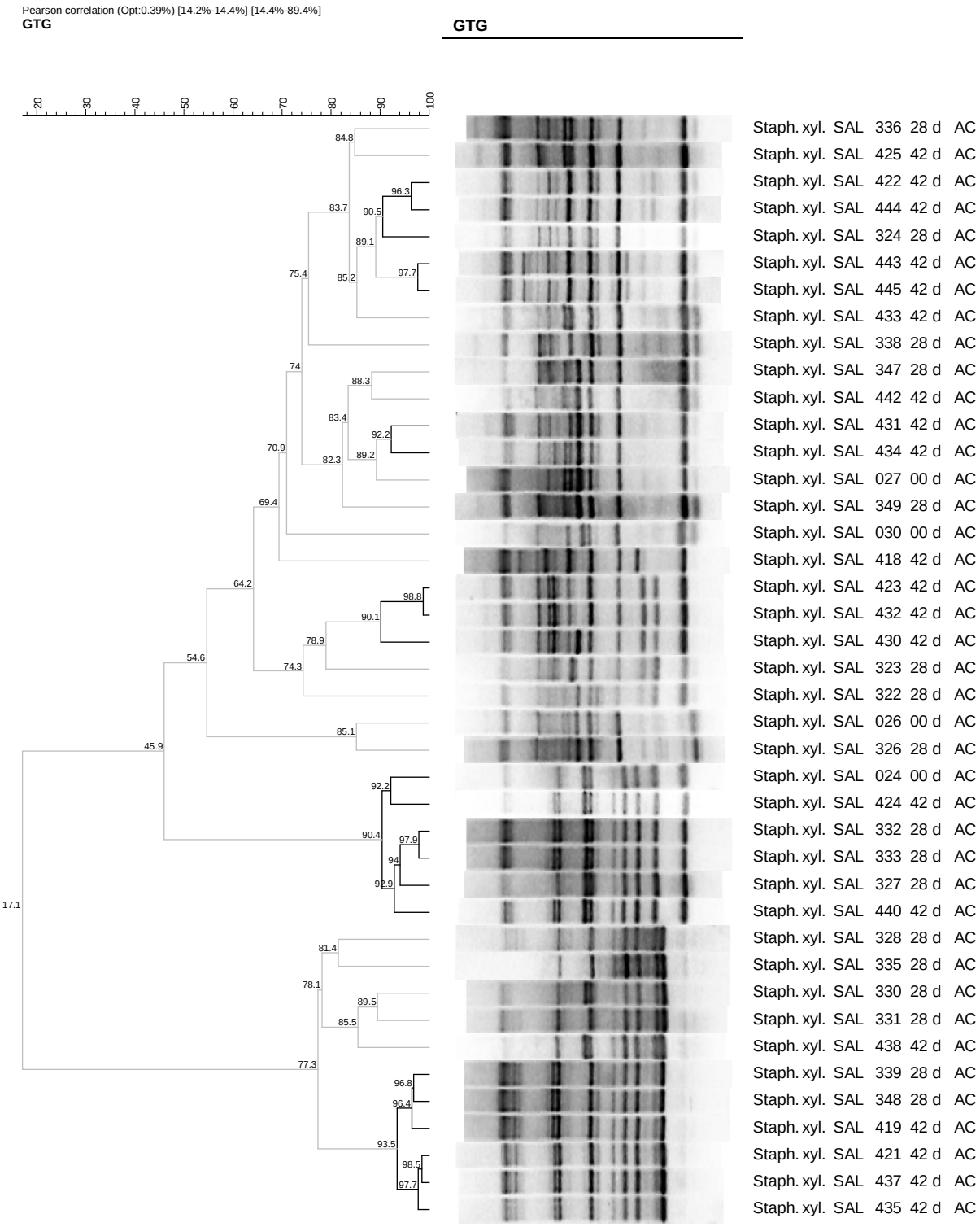

95.1
93.7
91.8
88.9
81
2
96.8
96.4
45.1
93.5
78.4
98.5
97.7
87.8
85.8
94.4
71.7
83.5
93.2
92.9
89.8
69.8
98.2
77.2
18.6
Staph. xylosus SA3 097 28 d AA
Staph. xylosus SA3 101 28 d AA
Staph. xylosus SAL 330 28 d AC
Staph. xylosus SA2 097 28 d AA
Staph. xylosus SAL 438 42 d AC
Staph. xylosus SAB 486 42 d AB
Staph. xylosus SAL 339 28 d AC
Staph. xylosus SAL 348 28 d AC
Staph. xylosus SAL 419 42 d AC
Staph. xylosus SAL 421 42 d AC
Staph. xylosus SAL 437 42 d AC
Staph. xylosus SAL 435 42 d AC
Staph. xylosus SAL 331 28 d AC
Staph. xylosus SAB 494 42 d AB
Staph. xylosus SAB 365 28 d AB
Staph. xylosus SAB 381 28 d AB
Staph. xylosus SAB 373 28 d AB
Staph. xylosus SAB 487 42 d AB
Staph. xylosus SAL 328 28 d AC
Staph. xylosus SAB 379 28 d AB
Staph. xylosus SAL 335 28 d AC
Staph. xylosus SD3 084 28 d AD
Staph. xylosus SA1 099 28 d AA
Staph. xylosus SD2 082 28 d AD
Staph. xylosus SAB 480 42 d AB

Fig. 18a, b, c, d, e. Dendrogram after cluster analysis of (GTG)5- PCR profiles S: xylosus. Producer AA, AB, AC, AD, AE




Pearson correlation (Opt:0.39%) [14.2%-14.4%] [14.4%-89.4%]
GTG
GTG
Staph. xyl. SAB 364 28 d AB
Staph. xyl. SAB 491 42 d AB
Staph. xyl. SAB 356 28 d AB
Staph. xyl. SAB 489 42 d AB
Staph. xyl. SAB 383 28 d AB
Staph. xyl. SAB 478 42 d AB
Staph. xyl. SAB 372 28 d AB
Staph. xyl. SAB 375 28 d AB
Staph. xyl. SAB 376 28 d AB
Staph. xyl. SAB 367 28 d AB
Staph. xyl. SAB 499 42 d AB
Staph. xyl. SAB 366 28 d AB
Staph. xyl. SAB 482 42 d AB
Staph. xyl. SAB 485 42 d AB
Staph. xyl. SAB 378 28 d AB
Staph. xyl. SAB 498 42 d AB
Staph. xyl. SAB 477 42 d AB
Staph. xyl. SAB 370 28 d AB
Staph. xyl. SAB 496 42 d AB
Staph. xyl. SAB 495 42 d AB
Staph. xyl. SAB 490 42 d AB
Staph. xyl. SAB 384 28 d AB
Staph. xyl. SAB 479 42 d AB
Staph. xyl. SAB 107 00 d AB
Staph. xyl. SAB 357 28 d AB
Staph. xyl. SAB 369 28 d AB
Staph. xyl. SAB 501 42 d AB
Staph. xyl. SAB 492 42 d AB
Staph. xyl. SAB 493 42 d AB
Staph. xyl. SAB 100 00 d AB
Staph. xyl. SAB 360 28 d AB
Staph. xyl. SAB 110 00 d AB
Staph. xyl. SAB 377 28 d AB
Staph. xyl. SAB 371 28 d AB
Staph. xyl. SAB 380 28 d AB
Staph. xyl. SAB 365 28 d AB
Staph. xyl. SAB 381 28 d AB
Staph. xyl. SAB 373 28 d AB
Staph. xyl. SAB 487 42 d AB
Staph. xyl. SAB 379 28 d AB
Staph. xyl. SAB 494 42 d AB
Staph. xyl. SAB 486 42 d AB
Staph. xyl. SAB 374 28 d AB
Staph. xyl. SAB 483 42 d AB
Staph. xyl. SAB 382 28 d AB
Staph. xyl. SAB 481 42 d AB
Staph. xyl. SAB 361 28 d AB
Staph. xyl. SAB 500 42 d AB
Staph. xyl. SAB 359 28 d AB
Staph. xyl. SAB 480 42 d AB


Pearson correlation (Opt:0.39%) [14.2%-14.4%] [14.4%-89.4%]
GTG
GTG
20
30
40
50
60
70
80
90
100
Staph. xyl. SAL 336 28 d AC
Staph. xyl. SAL 425 42 d AC
Staph. xyl. SAL 422 42 d AC
Staph. xyl. SAL 444 42 d AC
Staph. xyl. SAL 324 28 d AC
Staph. xyl. SAL 443 42 d AC
Staph. xyl. SAL 445 42 d AC
Staph. xyl. SAL 433 42 d AC
Staph. xyl. SAL 338 28 d AC
Staph. xyl. SAL 347 28 d AC
Staph. xyl. SAL 442 42 d AC
Staph. xyl. SAL 431 42 d AC
Staph. xyl. SAL 434 42 d AC
Staph. xyl. SAL 027 00 d AC
Staph. xyl. SAL 349 28 d AC
Staph. xyl. SAL 030 00 d AC
Staph. xyl. SAL 418 42 d AC
Staph. xyl. SAL 423 42 d AC
Staph. xyl. SAL 432 42 d AC
Staph. xyl. SAL 430 42 d AC
Staph. xyl. SAL 323 28 d AC
Staph. xyl. SAL 322 28 d AC
Staph. xyl. SAL 026 00 d AC
Staph. xyl. SAL 326 28 d AC
Staph. xyl. SAL 024 00 d AC
Staph. xyl. SAL 424 42 d AC
Staph. xyl. SAL 332 28 d AC
Staph. xyl. SAL 333 28 d AC
Staph. xyl. SAL 327 28 d AC
Staph. xyl. SAL 440 42 d AC
Staph. xyl. SAL 328 28 d AC
Staph. xyl. SAL 335 28 d AC
Staph. xyl. SAL 330 28 d AC
Staph. xyl. SAL 331 28 d AC
Staph. xyl. SAL 438 42 d AC
Staph. xyl. SAL 339 28 d AC
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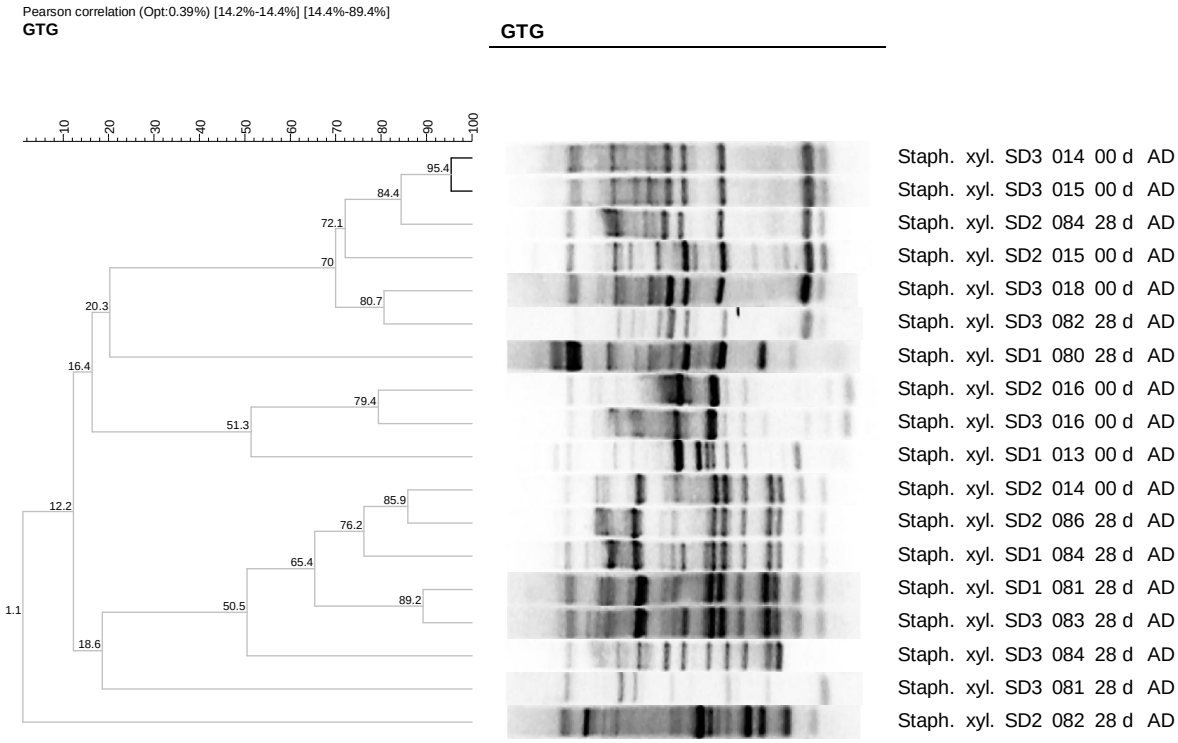
Staph. xyl. SAL 419 42 d AC
Staph. xyl. SAL 421 42 d AC
Staph. xyl. SAL 437 42 d AC
Staph. xyl. SAL 435 42 d AC


Pearson correlation (Opt:0.39%) [14.2%-14.4%] [14.4%-89.4%]
GTG
GTG
10 20 30 40 50 60 70 80 90 100
95.4
84.4
72.1
70
80.7
20.3
16.4
79.4
51.3
12.2
85.9
76.2
65.4
89.2
50.5
1.1
18.6
Staph. xyl. SD3 014 00 d AD
Staph. xyl. SD3 015 00 d AD
Staph. xyl. SD2 084 28 d AD
Staph. xyl. SD2 015 00 d AD
Staph. xyl. SD3 018 00 d AD
Staph. xyl. SD3 082 28 d AD
Staph. xyl. SD1 080 28 d AD
Staph. xyl. SD2 016 00 d AD
Staph. xyl. SD3 016 00 d AD
Staph. xyl. SD1 013 00 d AD
Staph. xyl. SD2 014 00 d AD
Staph. xyl. SD2 086 28 d AD
Staph. xyl. SD1 084 28 d AD
Staph. xyl. SD1 081 28 d AD
Staph. xyl. SD3 083 28 d AD
Staph. xyl. SD3 084 28 d AD
Staph. xyl. SD3 081 28 d AD
Staph. xyl. SD2 082 28 d AD

CONCLUSIONS

The results above reported indicates that the product object of this study is a typical Mediterranean type of dry, naturally-fermented sausage, characterised by low acidification rates and high final pH. The bacterial microflora is mainly represented by LAB and CNC that reach high counts from the 7th day of ripening.

The main species isolated were *Lb. sakei* and *Lb. curvatus* among LAB and *S. xylosus* among CNC.

A high biodiversity within species was highlighted by numerical analysis of rep-PCR profiles .Moreover most of biotypes found were exclusive of a producer.

Further studies are needed to verify if the genetic diversity found is correlated with differences in technological properties of the biotypes

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