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YEAST DIVERSITY IN ARTISANAL CHEESES: BIOTECHNOLOGICAL APPLICATIONS

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La Dra. Paloma Mª Manzanares Mir, Investigadora Científica del Consejo Superior de Investigaciones Científicas (CSIC) y la Dra. Carmela Belloch Trinidad, Científica Titular del CSIC, en el Departamento de Biotecnología de Alimentos del Instituto de Agroquímica y Tecnología de Alimentos (IATA):

CERTIFICAN: Que Dña. Beatriz Padilla López, Licenciada en Ciencia y Tecnología de los Alimentos por la Universidad Politécnica de Valencia, ha realizado bajo su dirección el trabajo titulado: “Yeast diversity in artisanal cheeses: biotechnological applications”, que presenta para optar al grado de Doctor.

Y para que así conste a los efectos oportunos, firman el presente certificado en Valencia, a 7 de enero de 2014.

Dra. Paloma Mª Manzanares Mir Dra. Carmela Belloch Trinidad

El trabajo que aquí se expone se ha llevado a cabo en el Departamento de Biotecnología de los Alimentos del IATA (CSIC) y ha sido posible gracias a la concesión de una beca JAEPre del CSIC en el año 2009. Esta tesis doctoral se ha realizado en el marco de los proyectos Consolider Ingenio Fun-C-Food CSD2007-00063 e INIA RM2007-00010. A su vez, he de agradecer la concesión de una beca que me permitió la estancia en el Instituto de Investigación en Ciencias de la Alimentación (CIAL CSIC-UAM) otorgada por el CSIC.

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A los que buscan
aunque no encuentren.

A los que avanzan
aunque se pierdan.

A los que viven
aunque se mueran.

Mario Benedetti.

Defiende tu derecho a pensar,
porque incluso pensar de manera errónea
es mejor que no pensar.

Atribuido a Hypatia de Alejandría.

RESUM

L'impacte dels llevats en la producció, qualitat i seguretat d'aliments i begudes està íntimament relacionat amb la seva ecologia així com amb les seves activitats biològiques. Durant els últims anys, i com a conseqüència de la relació establerta entre dieta i salut, els llevats estan adquirint una posició rellevant com a nous probiòtics o amb la finalitat de produir determinats compostos bioactius. En els productes lactis, els llevats juguen un paper destacat en la proteòlisi, lipòlisi i fermentació de la lactosa durant la maduració dels formatges, contribuint així al desenvolupament de propietats organolèptiques d'interès, principalment l'aroma. Aquesta tesi doctoral aborda l'estudi de la diversitat de la població de llevats en formatges artesanals produïts amb llet crua d'ovella i cabra al Parc Natural de la Serra d'Espadà (Castelló). S'han emprat diferents tècniques moleculars amb l'objectiu de caracteritzar genèticament els aïllats de llevats i per tal d'estudiar la successió d'espècies durant el procés de maduració dels formatges. També s'ha avaluat la variabilitat intraespecífica de les dues espècies majoritàries identificades: *Debaryomyces hansenii* i *Kluyveromyces lactis.* A més, s'ha estudiat el potencial de les β-galactosidases de *Kluyveromyces marxianus* i *K. lactis* per produir oligosacàrids prebiòtics a partir de lactosa i de lactulosa. Per últim, s'ha explorat la capacitat dels aïllats de *Kluyveromyces* i de *Debaryomyces* per tal de generar compostos aromàtics d'interès en els formatges estudiats.

RESUMEN

El impacto de las levaduras en la producción, calidad y seguridad de alimentos y bebidas está íntimamente relacionado con su ecología y sus actividades biológicas. En los últimos años, y como consecuencia de la conexión entre dieta y salud, las levaduras están adquiriendo una posición relevante como nuevos probióticos o para la producción de determinados compuestos bioactivos. En los productos lácteos, las levaduras juegan un papel destacado en la proteolisis, lipolisis y fermentación de la lactosa durante la maduración de los quesos, contribuyendo al desarrollo de propiedades organolépticas de interés, principalmente del aroma. Esta tesis doctoral aborda el estudio de la diversidad de la población levaduriforme en quesos artesanales producidos a partir de leche cruda de oveja y de cabra en el Parque Natural de la Serra d'Espadà (Castellón). Para ello se han empleado diferentes técnicas moleculares con el objetivo de caracterizar genéticamente los aislados de levaduras y para estudiar la sucesión de especies durante el proceso de maduración de los quesos. También se ha evaluado la variabilidad intraespecífica de las dos especies mayoritarias identificadas: *Debaryomyces hansenii* y *Kluyveromyces lactis*. Además, se ha estudiado el potencial de las β-galactosidasas de *Kluyveromyces marxianus* y *K. lactis* para producir oligosacáridos prebióticos a partir de lactosa y de lactulosa. Por último se ha explorado la capacidad de los aislados de *Kluyveromyces* y de *Debaryomyces* para producir compuestos aromáticos de interés en los quesos estudiados.

ABSTRACT

The impact of yeasts on food production, quality and safety is closely linked with their ecology and biological activities. Recently, as a consequence of the relationship between diet and health, yeasts are becoming relevant as new probiotics or for the production of bioactive compounds. In dairy products, yeasts play a key role in proteolysis, lipolysis and lactose fermentation during cheese ripening, promoting the development of sensory properties, particularly aroma. This thesis focuses on the yeast diversity in artisanal cheeses produced in the Natural Park Serra d'Espadà (Castelló) from ewes' and goats' raw milk. Different molecular techniques have been employed in order to characterize yeast isolates. Moreover, the succession of species along the cheese ripening process was studied. The intraspecific variability of the most abundant identified species *Debaryomyces hansenii* and *Kluyveromyces lactis* was also assessed. Additionally, the potential of *Kluyveromyces marxianus* and *K. lactis* β-galactosidases to synthetize prebiotic oligosaccharides from lactose and lactulose was tested. Finally, *Kluyveromyces* and *Debaryomyces* isolates were investigated for the production of cheese aromatic compounds.

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ABBREVIATIONS

AU	Abundance units
CCE	Crude cell extract
CECT	Spanish Type Culture Collection
cfu	Colony-forming unit
CLM	Cheese-like medium
Dh	*Debaryomyces hansenii*
DNA	Deoxyribonucleic acid
DVB/CAR/PDMS	Divinylbenzene/carboxen/polydimethylsiloxane
FFA	Free fatty acids
GC-MS	Gas chromatography coupled with mass spectrometry
GOS	Galactooligosaccharides
GPY	Glucose, peptone, yeast extract
GRAS	Generally recognized as safe
HPAEC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
ITS	Internal transcribed spacers
Kl	*Kluyveromyces lactis*
Km	*Kluyveromyces marxianus*
LPY	Lactose, peptone and yeast extract
LRI	Linear retention indices
LSD	Least significant difference
mtDNA	mitochondrial DNA
nd	Not detected
NSLAB	Non-starter lactic acid bacteria
OD	Optical density
*o*NPG	*o*-nitrophenyl β-D-galactopyranoside
OsLu	Oligosaccharides derived from lactulose
PAB	Propionic acid bacteria
PCA	Principal component analysis
PCR	Polymerase chain reaction
POD	Protected designation of origin
RAPD	Randomly amplified polymorphic DNA
rDNA	ribosomal DNA

ABBREVIATIONS

RFLP	Restriction fragment length polymorphism
RI	Reliability of identification
RNA	Ribonucleic acid
SD	Standard deviation
SPME	Solid phase microextraction
TIC	Total ion chromatogram
UPGMA	Unweighted pair-group method using arithmetic averages

Introduction

Cheese can be defined as “a consolidated curd of milk solids in which milk fat is entrapped by coagulated caseins” (Adams and Moss, 2000). This description comprises a heterogeneous group of fermented milk-based food products, which are worldwide elaborated and present a broad range of flavors and forms. It is commonly believed that cheese emerged from a geographical area between the Tigris and Euphrates rivers, around 8000 years ago. Moreover, it came together with the “Agricultural Revolution” with the domestication of plants and animals, specifically goats and sheep (Fox and McSweeney, 2004).

Initially, the main objective of cheesemaking was to extend the shelf life and to conserve the nutritious components of milk (Beresford et al., 2001). Nowadays, cheese has become a product highly appreciated by consumers due to its interesting nutritional value and unique organoleptic properties (López-Expósito et al., 2012; O'Brien and O'Connor, 2004).

1. Cheese production

More than 1000 different cheese varieties have been described worldwide (Romero del Castillo and Mesters, 2004). Around 36% of the whole milk produced in the European Union countries in 2011 was converted into cheese (European Comission-Eurostat, 2013). In particular, Spain manufactured 302.000 tones of cheese during 2010 (European Comission, 2012).

Cheeses are classified according to different criteria, which include origin, texture or coagulation method among others (McSweeney et al., 2004). However, there are also common features in cheesemaking, as the general combination of four ingredients: milk, rennet, microorganisms and salt. Additionally, a common production process comprising several steps

such as gel formation, whey expulsion, acid production and salt addition, followed by a period of ripening can be found in the majority of cheeses (Beresford et al., 2001), as it is summarized in Figure 1.

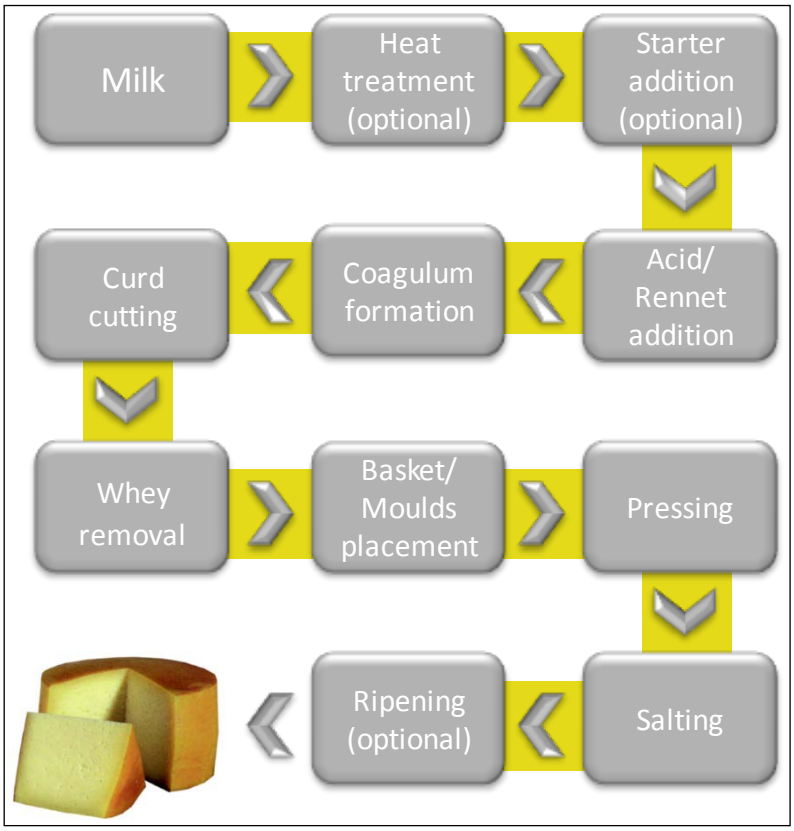

Figure 1. Flow diagram for general cheese production (adapted from Bonet et al. 2009).

After selection and pre-treatment of milk with heat or addition of starter cultures, cheese is coagulated by acidification or enzyme (rennet) addition. Normally, acid-coagulated cheeses are fresh products, where the curds are not subjected to maturation periods. In the case of enzymatic-coagulated cheeses, rennet from different biological origins, such as

microbial (*Rhizomucor mihei* or *Rhizomucor pusillius*), young animal (calves, kids, lamb, buffalo) or plant (cardoon, *Cynara cardunculus*) extracts, is added to the milk (Fox and McSweeney, 2004).

During milk coagulation, proteins precipitate and whey is separated. Several factors determine the stability of the curd. Some of these elements are: coagulum size cut, milk composition, concentration of Ca^{2+} and casein, pH or cooking temperature among others (Fox and McSweeney, 2004). Once pressed, cheeses may be directly salted or immersed into brine (Guinee and Fox, 2004). Afterwards, cheeses are ripened during a variable period of time, ranging from 2 weeks in the case of Mozzarella to 2 years in the Parmigiano variety, under special conditions of humidity and temperature depending on each cheese variety (McSweeney, 2004).

1.1 Traditional Spanish cheeses

Artisanal elaborated cheeses are part of the cultural heritage of many countries. They are historically produced in many parts of Europe, particularly in Portugal and in Mediterranean countries. Traditional cheeses are normally produced with goats', ewes' and cows' milk, and sometimes, raw milk is used (Cogan and Rea, 1996). The elaboration of many artisanal cheeses is regulated by a Protected Designation of Origin (POD), in order to protect high quality products from imitations (Núñez et al., 1989).

In particular, ewes' and goats' cheeses present special flavors and tastes, when compared with cows' products. Six PDO Spanish ewe cheeses are recognized: Manchego, Idiazábal, Zamorano, Roncal, La Serena and Torta del Casar. Besides, six goat cheeses are considered PDO: Majorero, Ibores, Murcia, Murcia al vino, Palmero and Camerano (Medina and Núñez, 2004). Other PDO cheese varieties such as Cabrales or Gamoneu may contain mixtures of ewes', goats' and cows' milk

(Ministerio de Agricultura, Alimentación y Medio Ambiente, 2013). Nevertheless other high quality non-PDO traditional varieties are produced; this is the case of Valencian Community cheeses. Artisanal cheeses from this area are principally produced with ewes' and goats' milk. Mainly fresh products are elaborated, such as *Cassoleta* or *Blanquet*, however, other cheeses including *Servilleta* and *Tronchón* can be also commercialized after different maturation periods (Badia Gutiérrez and Ibáñez i Fuentes, 1987; Associació de Formatgers de la Comunitat Valenciana, 2013).

This thesis focus on cheeses produced in a small factory sited within the borders of the Natural Park *Serra d'Espadà*, in a rural area of Castelló province. Tables 1 and 2 show the main technological and physic-chemical characteristics of these artisanal dairies. The cheeses were made with raw ewes' (EC and EP) or goats' (GC and GP) milk and with the addition of lactic acid bacteria starters. A native ewe's breed called "Guirra" or "Red Levantine" was milked for the production of ewes' cheeses. Coagulation of milk was achieved with the addition of vegetal rennet, composed by the crude aqueous extract of dried flowers of cardoon, or with animal paste rennet. After precipitation of proteins the curd was cut or crumbled manually, and then cheeses were placed in perforated plastic moulds, salted and ripened for 6-8 weeks in wooden shelves at 10-12ºC and at 85-90% of relative humidity.

Table 1. Technological characteristics of the studied cheeses.

Cheese	Milk	Coagulation			Curd cutting	Salting	Ripening days
		Rennet	Temperature (ºC)	Time			
EC	Ewe	Calf	25	18 h	Without cutting	By rubbing both faces	60
EP	Ewe	Plant	30	50 min	1 cm	Brining (24 h)	>60
GC	Goat	Calf	26	16 h	Without cutting	By rubbing both faces	40
GP	Goat	Plant	33	40 min	1 cm	Brining (24 h)	>60

Table 2. Physico-chemical properties of the studied cheeses (data provided by the manufacturer).

Cheese	EC	EP	GC	GP
Dry extract	74.4	56.7	70.6	60.6
Lipids (%)	43.8	33.3	42.8	34.3
Proteins (%)	28.2	18.6	25.3	23.1
Salt (%)	2.8	2	2.8	2.1
pH	5.8	5.1	5.8	5.2
Weight (Kg)	2	2	1	1

2. Biochemical changes during cheese ripening

Relevant biochemical changes take place along cheese ripening, modifying the principal milk components, which are summarized in Table 3. These complex reactions are catalyzed by the following agents: coagulant, indigenous milk enzymes, starter bacteria and secondary microbiota (McSweeney, 2004).

Table 3. Chemical composition (%) of different milks (Jeness, 1974).

Component	Cow	Goat	Ewe
Protein	3.4	2.9	5.5
Casein	2.8	2.5	4.6
Fat	3.7	4.5	7.4
Lactose	4.6	4.1	4.8
Ash	0.7	0.8	1.0

Biochemical reactions, which have a direct impact on texture and aroma of the final product, can be divided into four main categories: citrate, lactose, triglycerides and casein derived reactions (Figure 2). Implication of microorganisms in the development of biochemical reactions will be deeply analyzed in the following section.

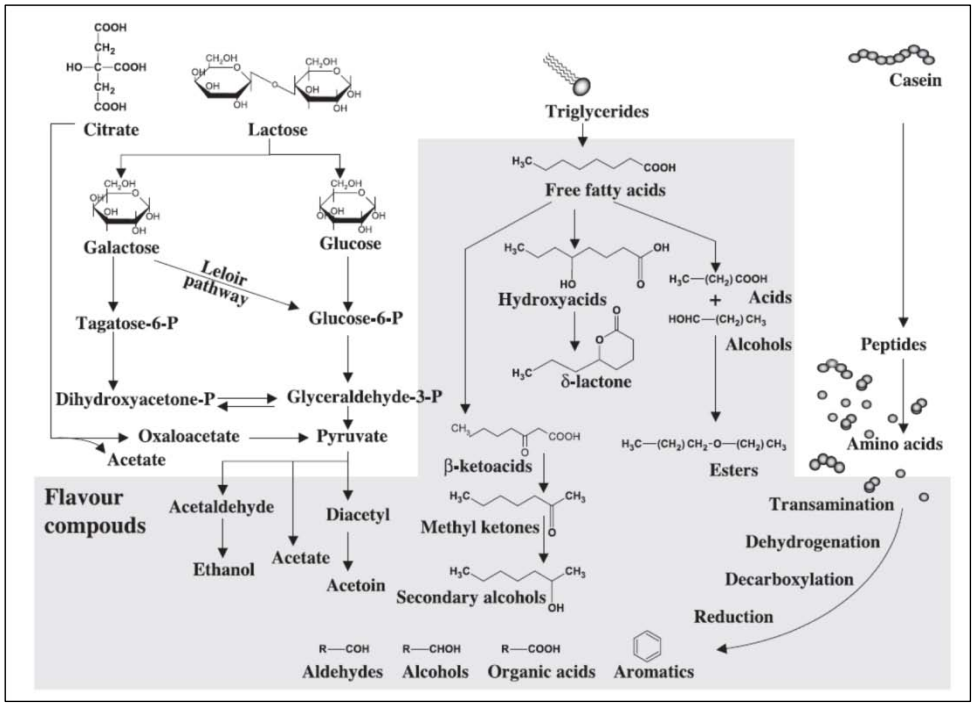

Figure 2. Biochemical pathways leading to the formation of flavor compounds. The grey surface indicates compounds with flavor note (Marilley and Casey, 2004).

2.1 Catabolism of citrate

Citrate is found in milk in a relatively low concentration (8 mmol/L) and can be metabolized by citrate positive strains of *Lactococcus* producing diacetyl, acetate, acetoin and CO_2. The gas generated by citrate metabolism is responsible for the development of some undesirable characteristics such as openness of cheese or floating curd defects. However, positive features such as eyes formation and aroma development due to diacetyl generation may be also attributed to citrate catabolism (McSweeney and Fox, 2004).

2.2 Glycolysis of residual lactose and catabolism of lactate

Fermentation of lactose is highly significant in most cheese varieties. The major part of this disaccharide is removed with the whey, however, the low amount of residual lactose present in the curd is rapidly metabolized to lactate during the first stages of ripening, mainly through the activity of bacteria and *Kluyveromyces* (Fadda et al., 2004). Production of lactate from lactose contributes to the acidification of the medium, which has an impact on the growth of specific microorganisms and on the enzymatic activities involved in cheese ripening. Therefore acidification has an indirect effect on cheese quality, as summarized in Figure 3 (McSweeney, 2004; McSweeney and Sousa, 2000; McSweeney and Fox, 2004).

2.3 Lipolysis and catabolism of free fatty acids

Milk fat is represented by a group of key components, mainly triglycerides and its derived free fatty acids (FFA), which highly determine the quality of cheese (McSweeney and Sousa, 2000). The release of FFA plays a key role in texture and flavor development, since its catabolism produces methyl ketones, lactones, esters and secondary alcohols (Figure 2). Liberation of FFA is caused by two kind of lipolytic enzymes, esterases and lipases from milk, rennet or microorganisms (Collins et al., 2003; Collins et al., 2004).

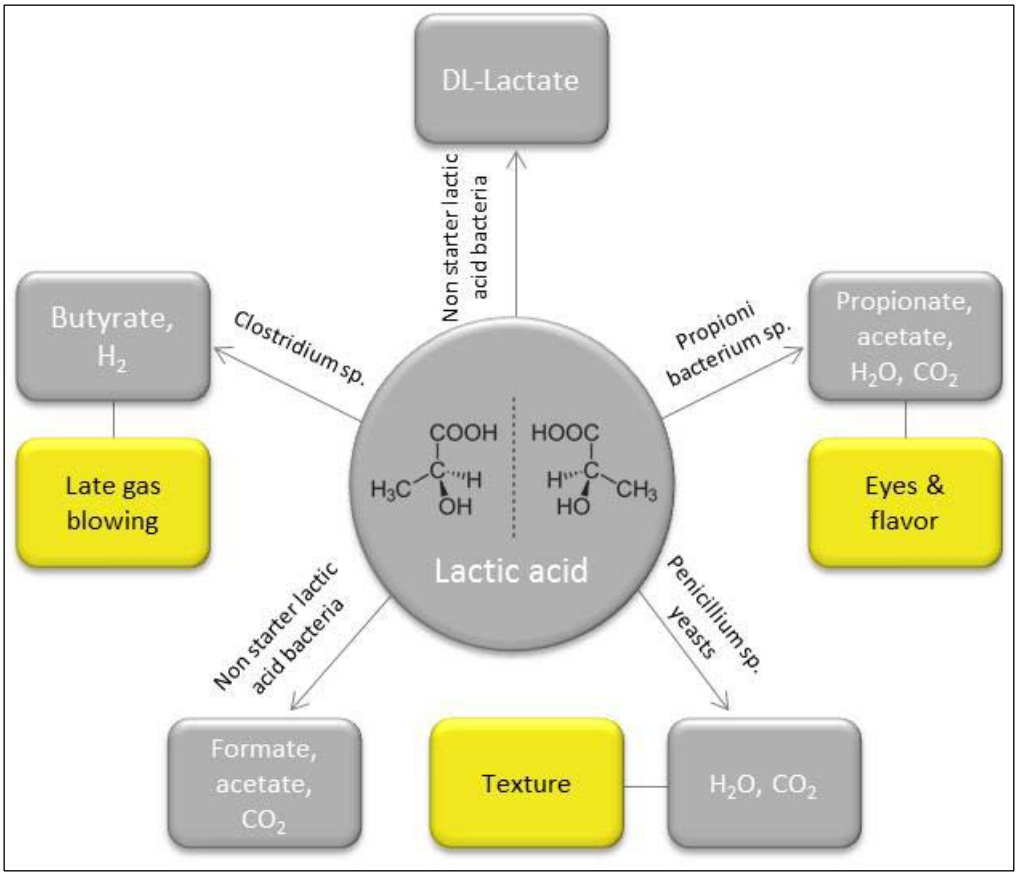

Figure 3. Metabolism of lactate during cheese ripening (adapted from McSweeney and Sousa, 2000).

2.4 Proteolysis and catabolism of amino acids

Proteolysis may be the most complex and in some cheese varieties, the most relevant biochemical process taking place during ripening. It is caused by the hydrolysis of casein, affecting texture and producing free amino acids which act as precursors of many important volatile compounds such as aldehydes, alcohols, organic acids and aromatic compounds (Figure 2). Proteases and peptidases from rennet and microorganisms are the main agents responsible of casein degradation (Upadhyay et al., 2004).

3. Cheese microbiota

Cheese microbiota composed by bacteria, moulds and yeasts plays a major role in the development of the final characteristics of each cheese variety (Beresford et al., 2001), as summarized in Figure 4. Microorganisms found in cheese may come from the milk, in the case of raw milk cheeses, from the added starter cultures or from the cheese factory environment (Beresford and Williams, 2004). Recently, the microbiome from two cheesemaking plants has been deeply analyzed, standing out its importance in the ripening process of the dairy products (Bokulich and Mills, 2013).

Cheese microorganisms may be divided into starter bacteria and secondary microbiota, which is formed by non-starter lactic acid bacteria (NSLAB), propionic acid bacteria (PAB), moulds and yeasts depending on the cheese variety.

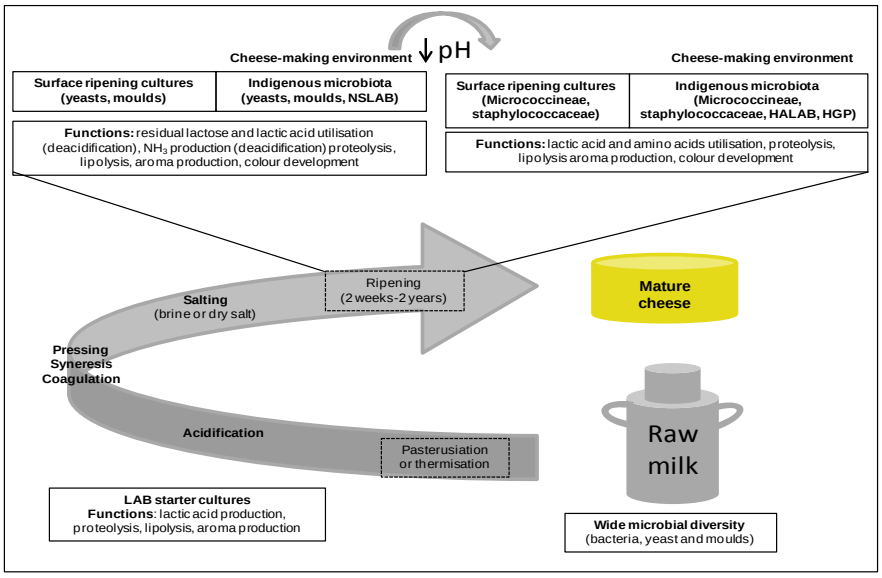

Figure 4. Microbial succession and functions of the different microbial groups during cheese making (Irlinger and Mounier, 2009).

3.1 Starter bacteria

This group of microorganisms comprises lactic acid bacteria (LAB), such as *Lactococcus*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* or *Lactobacillus helveticus*. Starter LAB are fundamental components of cheese microbiota. Their main property is the production of organic acids from lactose during cheese manufacture reducing the pH of milk, and thus creating a selective environment. Moreover, LAB enzymes are involved in lipolysis, proteolysis and conversion of amino acids to flavor compounds. These starter cultures are commonly added to the cheese, although in traditional cheesemaking processes they may come from the cheese factory environment (Parente and Cogan, 2004).

3.2 Non-starter lactic acid bacteria (NSLAB) and propionic acid bacteria (PAB)

Different bacterial groups are considered NSLAB, such as non-starter *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Enterococcus*. Excepting *Leuconostoc*, NSLAB are adventitious contaminants, as they are not added as part of the starter culture. However they are found in most ripened cheese varieties. NSLAB do not decrease milk pH, but they impact on cheese aroma improving cheese quality (Settanni and Moschetti, 2010).

PAB, such as *Propionibacterium freudenreichii* and *Propionibacterium acidipropionici*, are the main responsible of the characteristic flavor and of the eye formation in Swiss-type cheeses due to lactate metabolism (Poonam et al., 2012) as indicated in Figure 3.

3.3 Moulds

Fungal diversity in milk is notable (Delavenne et al., 2011). Moulds contribute to the ripening of several cheese varieties. Particularly, its presence is relevant among blue cheeses such as Cabrales or Gorgonzola, and surface-mould ripened cheeses, as Camembert and Brie, where *Penicillium camemberti* and *Penicillium roqueforti* have been reported. Moulds can be supplemented to the cheese intentionally or they can originate from the cheese factory environment.

Besides being important ripening agents, microorganisms present in cheese can also produce spoilage or generate toxic compounds, such as biogenic amines or mycotoxins (O'Brien et al., 2004). Therefore strains used as starter cultures may be evaluated for different safety issues previous to their industrial application.

4. Yeast species diversity in cheese

Yeasts are widespread eukaryotic microorganisms classified in the kingdom Fungi and are habitually found in cheeses. Several studies have estimated the number of yeasts from 2.7 log10 to 8 log10 (Fleet, 1990; Pereira-Dias et al., 2000). Some physico-chemical properties of this dairy product, such as low pH, low moisture content, elevated salt concentration and refrigerated storage promote yeast occurrence and development (Fleet, 1990). Milk, brine, equipment and hands of manufacturers have been described as different sources of yeast contamination (Delavenne et al., 2011; Seiler and Busse, 1990; Viljoen and Greyling, 1995).

It is important to remark that yeasts may have a positive or negative impact on cheese quality (Fleet and Mian, 1987; Jakobsen and Narvhus, 1996). Desirable effects of yeasts in cheese ripening are related with their

lipolytic and proteolytic activities as well as with volatile compound production, which contributes to aroma formation. Besides, texture development may be also promoted. On the other hand, typical cheese defects associated with yeasts are loss of texture quality, excessive gas formation, increased acidity and yeasty off-flavor (Büchl and Seiler, 2011; Fleet, 1992). For all these reasons is essential to identify and characterize the species and strains involved in cheese ripening.

4.1 Identification and genetic characterization of yeasts

Different phenotypic and molecular methods have been suggested for yeast identification (Vasdinyei and Deák, 2003). Phenotypic techniques are based on morphological, biochemical and physiological characteristics which imply the performance of a high number of tests. Consequently, traditional identification is laborious, complex and time consuming. On the other hand, molecular methods for yeast identification and characterization have been developed. These techniques are focused on the analysis of DNA and RNA molecules and are considered much more sensitive and specific than traditional techniques (Deák, 1995). Among them, DNA-DNA hybridization is a valid identification technique but it presents the inconvenience of being time-consuming (Petersen and Jespersen, 2004). On the contrary, techniques based on polymerase chain reaction (PCR) and in the restriction of DNA are considered appropriate for rapid yeast identification and characterization. The first step to conduct in these techniques is DNA extraction. Different approaches may be applied, as freezing/defrosting, sonication or enzymatic lysis. The application of enzymes is the best approach as it prevents DNA from structural damages. The main molecular techniques employed for yeast identification and characterization are described below.

4.1.1 Identification based on ribosomal DNA analysis

The most used method for yeast identification is based on the heterogeneity in the sequence of the ribosomal DNA. In general, ribosomal regions show low intra-specific polymorphism and high inter-specific variability (Li, 1997), therefore their sequences and RFLPs (Restriction Fragment Length Polymorphism) are a powerful tool for yeast identification (Kurtzman and Robnett, 1998). Moreover, in eukaryotic organisms ribosomal DNA (rDNA) presents the advantage of being hundreds of times copied (White et al., 1990). Figure 5 shows the structure of rDNA, formed by three genes: 5.8S, 18S and 26S, the internal and external transcribed spacers (ITS, ETS), the non- transcribed spacers (NTS) and the intergenic spacers (IGS).

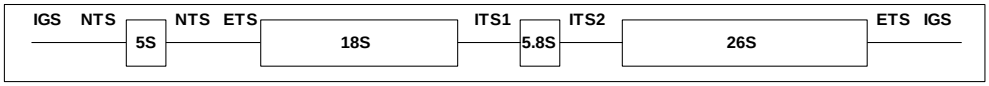

Figure 5. Flow diagram of genes codifying ribosomal RNA in yeast.

Several techniques have been developed based on the study of the different regions of rDNA. The sequencing of rDNA regions is the standard method used to identify yeasts. Once nucleotide sequences from ribosomal regions are obtained and processed, they are compared with those available in different electronic databases such as GenBank (http://www.ncbi.nlm.nih.gov), EMBL (http://www.embl-heidelberg.de) or DDBJ (http://www.ddbj.nig.ac.jp). Three regions are mainly studied: domains 1 and 2 (D1/D2) from 26S gene (Fell et al., 2000; Kurtzman and Robnett, 1998; Lopandic et al., 2006), the 18S gene (Cappa and Cocconcelli, 2001; Roostita et al., 2011) and ITS region (ITS1-5.8S-ITS2) (Martorell et al., 2005).

Additionally, the RFLPs of the ribosomal regions are based on the digestion of the PCR amplified rDNA by several endonucleases. The resulting DNA fragments are separated by electrophoresis and their size compared with a ladder or DNA weight marker. The restriction pattern obtained is specific for each yeast species (Figure 6). This technique provides fast and accurate results for most species and has been used to identify yeasts from different food and beverages, such as wine (Echeverrigaray et al., 2013; Guillamón et al., 1998), candied fruits (Martorell et al., 2005), dairies (El-Sharoud et al., 2009; Giannino et al., 2011) or meat products (Cano-García et al., 2013; Deák et al., 2000; Quirós et al., 2008). Besides, a study conducted by Esteve-Zarzoso et al. (1999) generated a database containing different restrictions patterns belonging to more than 200 yeast species (www.yeast-id.com). Digestion of other rDNA regions has been also proposed for non-*Saccharomyces* yeasts, such as NTS and 18S (Capece et al., 2003), IGS (Quirós et al., 2006), or 26S (Baleiras-Couto et al., 2005).

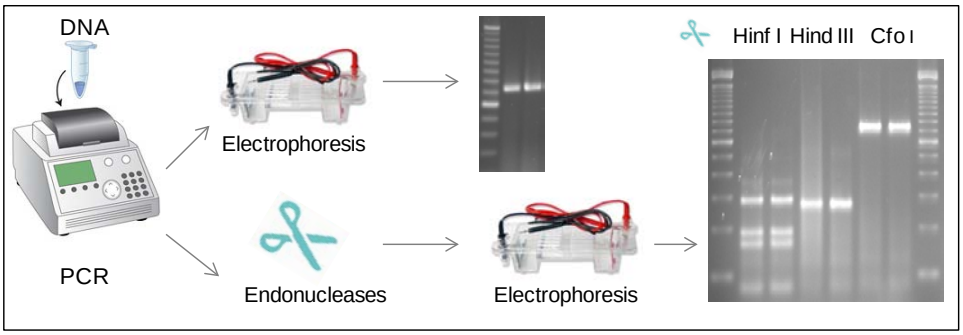

Figure 6. Flow diagram of RFLP of the ribosomal regions.

In the last years, beside culture-dependent methods, identification of microbiota in cheeses by next-generation sequencing technology has been reported (Alegría et al., 2012; Lusk et al., 2012; Quigley et al., 2012).

These studies are focused on elucidating bacterial communities, but there is a lack of information regarding yeast species composition. It would be interesting to conduct this approach to study yeast ecology in fermented foods, and particularly in cheeses, since it might provide novel information about new species present in cheese and useful information regarding microorganism interactions while avoiding culturing bias.

4.1.2 Genetic characterization of yeasts

There are numerous molecular techniques useful for genetic typing of yeasts, but among them, Randomly Amplified Polymorphic DNA (RAPD) and mitochondrial DNA (mtDNA) restriction analysis have been applied to numerous yeast species (Fernández-Espinar et al., 2003). These techniques are relevant as genetically different strains belonging to the same species may have different physiological and biochemical activities. The RAPD-PCR allows detection of DNA polymorphisms through PCR amplification. Different primers may be employed, such as $(GTG)_3$ (5′-GTGGTGGTG-3′) and M13 (5′-GAGGGTGGCGGTTCT-3′) (Senses-Ergul et al., 2012; Williams et al., 1990). Once PCR is performed the different patterns can be visualized using electrophoresis (Figure 7).

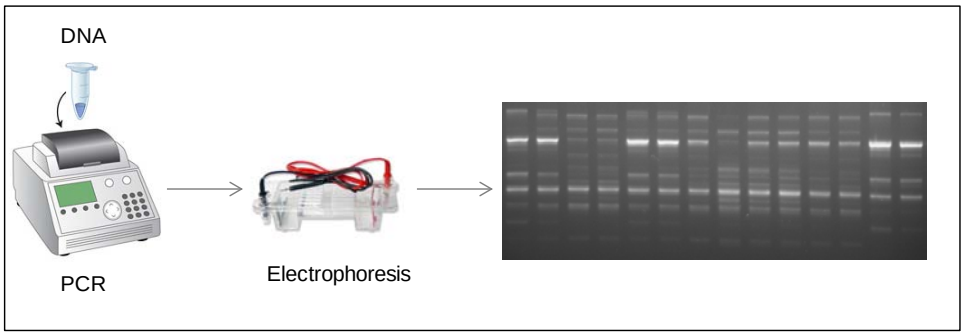

Figure 7. Flow diagram of RAPD analysis.

RAPDs-PCR have been used for typing of yeasts isolated from different food products as wine (Capece et al., 2010; Tofalo et al., 2012), cheese (Binetti et al., 2013; Fadda et al., 2004), table olives (Tofalo et al., 2013) or meat (Andrade et al., 2010; Cocolin et al., 2006).

The heterogeneity in the sequence of yeast mtDNA (De Zamaroczy and Bernardi, 1986) has been used to generate restriction patterns for differentiation of strains within numerous yeast genera such as *Saccharomyces* (Querol et al., 1992a), *Debaryomyces* (Romano et al., 1996), *Zygosaccharomyces* or *Kluyveromyces* (Belloch et al., 1997; Piskur et al., 1995). The RFLPs of mtDNA obtained can be visualized by electrophoresis in agarose gel (Figure 8). Different patterns are generated depending on the endonuclease used for DNA digestion.

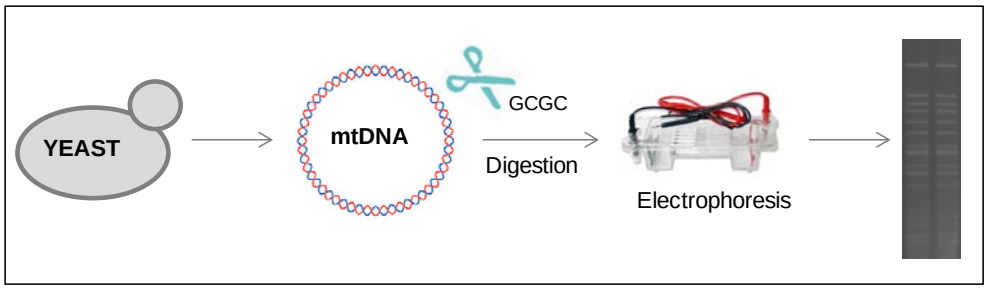

Figure 8. Schematic representation of mtDNA restriction analysis.

This technique was initially employed to identify and characterize wine isolates of *Saccharomyces cerevisiae* strains (Querol et al., 1992b). However, this method has also been used to characterize yeast isolates present in other foods, such as cheese (Mounier et al., 2005), sourdoughs (Foschino et al., 2004) or cider (Suárez-Valles et al., 2008).

Table 4 summarizes the yeast species identified by several molecular techniques in different varieties of traditional European cheeses.

As can be observed, a wide yeast biodiversity is noticed. Different species have been found in artisanal cheeses. This thesis focuses on the study of *Kluyveromyces* species and *D. hansenii*, which general characteristics are described below.

Table 4. Yeast species isolated from different varieties of artisanal cheeses.

	Traditional cheeses						
	Cabrales	Livarot	Pecorino crotonese	Pecorino siciliano	Salers	Smear-ripened	Taleggio
Milk[A]	R/Cow, ewe, goat	P-R /Cow	P/Ewe	R/Ewe	R/cow	P/Cow	P/Cow
Country	Spain	France	Italy	Italy	France	Ireland	Italy
Reference[B]	1	2	3	4	5	6	7
Yeast species							
Candida catenulata	x						
C. etchellsii							x
C. inconspicua			x				
C. intermedia	x		x		x		
C. parapsilosis					x		
C. pararugosa	x						
C. rugosa					x		
C. sake							x
C. silvae					x		
C. tropicalis					x		
C. zeylanoides					x		
Debaryomyces hansenii	x	x	x		x	x	x

Table 4 (continued).

Geotrichum candidum		x					
Kluyveromyces lactis	x	x	x		x		x
K. marxianus					x		x
Pichia carsonii			x				
P. dubia	x						
P. fermentans	x						
P. guilliermondii					x		x
P. membranifaciens	x			x			
Rhodotorula mucilaginosa	x						
Saccharomyces cerevisiae	x		x		x		
S. unisporus	x				x		
Sporobolomyces ruberrimus	x						
Torulaspora delbrueckii							x
Trichosporon coremiiforme	x						
Yarrowia lipolytica	x	x	x	x		x	x

[A] Treatment of milk: (P) Pasteurized; (R) Raw.
[B] Reference numbers are as follows: (1) Álvarez-Martín et al., 2007; (2) Larpin et al., 2006; (3) Gardini et al., 2006; (4) Todaro, 2011; (5) Callon et al., 2006; (6) Mounier et al., 2005; (7) Giannino et al., 2011.

4.2 Kluyveromyces spp.

Yeasts belonging to the genus *Kluyveromyces* present a high variability in morphological, physiological and molecular features; which explains its ubiquitous nature. This genus is formed by six species, *K. marxianus*, *K. lactis*, *K. dobzhanskii*, *K. aestuarii*, *K. nonfermentans*, and *K. wickerhamii* (Lachance, 2011). *K. lactis* and *K. marxianus* are able to ferment and assimilate lactose and are regularly found in different dairies, such as butter, cheese, milk, yogurt, kefir and in the general dairy environment (Büchl and Seiler, 2011). *K. marxianus* has been isolated from artisanal Italian cheeses during the first 48 hours of cheesemaking whereas *K. lactis* is found until advanced periods of ripening (Fadda et al., 2004; Gardini et al., 2006).

The main metabolic characteristic of dairy *K. lactis* and *K. marxianus* is their lactose-fermenting ability (Belloch et al., 2011; Naumov, 2005). The study of yeast metabolism profiles under different physical and chemical conditions such as nutrients availability, salt content, temperature or pH has become a useful analytical tool to differentiate strains and to predict and describe the behavior of microorganisms during fermentative processes. Evaluation of metabolic profiles has been recently employed to characterize nitrogen requirements of commercial wine yeasts during fermentation of grape must (Gutiérrez et al., 2012), testing of toxic resistance (Warringer and Blomberg, 2003), growth of yeasts at different temperature conditions (Salvadó et al., 2011) and fermentative behavior of different *S. cerevisiae* strains (Liccioli et al., 2011).

K. lactis and K. marxianus play an important role in cheese ripening and fermented milk products as they promote maturation and aroma formation. This contribution is based on lactose metabolism and lipolytic and proteolytic activities produced by *K. lactis* and *K. marxianus* yeasts

(Roostita and Fleet, 1996). *K. lactis* and *K. marxianus* grow at elevated concentration of lactose (4%) due to β-galactosidase activity (Borelli et al., 2006).

The strong proteolytic character of dairy *K. lactis* against casein has been reported by Fadda et al. (2004). On the contrary, Roostita and Fleet (1996) reported a weak proteolytic activity on casein and gelatin of *K. marxianus* isolates, whereas none of the *K. marxianus* strains from Italian ewe's dairy products presented caseinolytic activity (Cosentino et al., 2001). Moreover low lipolytic activity of *K. lactis* and *K. marxianus* on different substrates has been reported (Borelli et al., 2006; Cosentino et al., 2001; Fadda et al., 2004; Gardini et al., 2006).

4.3 Debaryomyces hansenii

Debaryomyces spp. is considered an extremophylic yeast (Breuer and Harms, 2006), as it is capable to tolerate biocides (Ramírez-Orozco et al., 2001), hypersaline habitats (Butinar et al., 2005; Gunde-Cimerman et al., 2009) and low water activity environments, such as sea water, soil and different foods, including milk and meat fermented products, brine and different alcoholic beverages (Lee et al., 2009; Seiler and Busse, 1990; Tamang and Fleet, 2009; Zhang et al., 2012).

Particularly, *D. hansenii* inhabitance in different cheese varieties has been thoroughly documented (Fleet, 1990; Lopandic et al., 2006). This is due to its ability to grow on high salt concentrations, low temperature and to metabolize lactic and citric acids (Capece and Romano, 2009). In different studies, *D. hansenii* has been isolated from cheeses, such as Pecorino, Feta and Cabrales (Álvarez-Martín et al., 2007; Cosentino et al., 2001). Moreover, the investigation on evolution of yeast microbiota during cheesemaking has pointed out that *D. hansenii* dominated the later stages

of maturation in traditional ewes' Italian and Portuguese cheeses (Gardini et al., 2006; Pereira-Dias et al., 2000). However, in products subjected to long ripening periods, such as the Italian ewes' Fiore Sardo, *D. hansenii* is present after half year ripening but it is not detected after 9 months (Fadda et al., 2004; Pisano et al., 2006).

Besides, *D. hansenii* presents proteolytic and lipolytic activities that contribute to the ripening process and highlight the role of this yeast on volatile compound production (Jakobsen and Narvhus, 1996; van den Tempel and Jakobsen, 2000; Yalcin and Ucar, 2009). Leclercq-Perlat et al. (2000) reported the enzyme profile of six *D. hansenii* strains, including C4-esterases, C8-esterase-lipase, leucine arylamidases, valine arylamidases and aminopeptidases.

Regarding proteolytic activity, differences among *D. hansenii* strains have been reported. Capece and Romano (2009) described that 55% of the isolates from an Italian cheese was able to hydrolyze casein. In a study conducted by Gardini et al. (2006) only 17% of the Pecorino isolates presented this enzymatic activity. On the contrary, some other studies reported that none of the dairy *D. hansenii* isolates were clearly caseinolytic (Addis et al., 2001; Borelli et al., 2006; Pereira-Dias et al., 2000; Roostita and Fleet, 1996). Regarding lipolytic activity, relevant strain variations of *D. hansenii* isolates against different substrates have been also reported. According to Addis et al. (2001) and Pereira-Dias et al. (2000) *Debaryomyces* presents activity on tributyrin agar, whereas none of the isolates tested by Borelli et al. (2006), Gardini et al. (2006) and Roostita and Fleet (1996) showed any activity against neither tributyrin nor butter fat agar.

5. Biotechnological applications of non-conventional yeasts

Yeasts have been traditionally employed in the production process of different food and beverages (Deak, 2009). Particularly, *S. cerevisiae* is the main yeast in beer, wine and bread-making. However, in the last decades, other species named non-conventional yeasts have appeared as relevant biotechnological resources not only for food and beverages manufacturing but also for a broad variety of other products made by yeasts or from yeast cells as summarized in Figure 9.

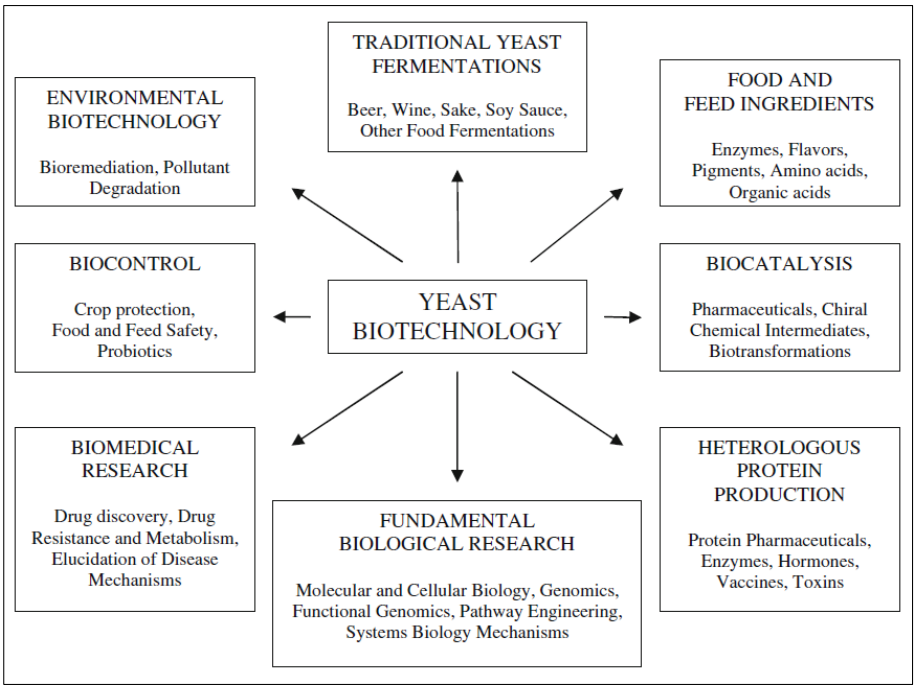

Figure 9. An overview of different areas of yeast biotechnology (Johnson, 2013).

In Table 5, some food industry applications of the dairy yeasts *D. hansenii*, *K. lactis* and *K. marxianus* are presented. As it can be observed these Generally Recognized As Safe (GRAS) yeasts can be used

as cultures, producers of enzymes, aromas, texture as well as bioactive compounds.

Table 5. Biotechnological applications of some dairy yeast species.

Application	Yeast species[A]			Reference[B]
	Km	Kl	Dh	
Enzymes				
α- and β-glucosidases			x	1
β-Galactosidase	x	x	x	1-4
Aminopeptidase	x		x	1, 5
Aramylases			x	1
Carboxypeptidase	x			6
Intrapeptidase			x	7
Inulinase	x			8
Phosphatases	x		x	1, 9
Products				
Antihypertensive hydrolysates	x	x	x	10
Cheese aroma	x	x	x	11-13
Fructooligosaccharides	x			14
Galactooligosaccharides	x	x		15-17
Meat aroma			x	18, 19
Cultures				
Cheese starter culture	x		x	20-22
Kefir starter culture	x			23
Probiotic		x		24

[A] Km: *Kluyveromyces marxianus*; Kl: *Kluyveromyces lactis*; Dh: *Debaryomyces hansenii.*
[B] Reference numbers are as follows: (1) van den Tempel and Jakobsen, 2000; (2) Zhou et al., 2013; (3) Martins et al., 2002; (4) Klein et al., 2013; (5) Ramírez-Zavala et al., 2004a; (6) Ramírez-Zavala et al., 2004b; (7) Leclercq-Perlat et al., 2000, (8) Dilipkumar et al., 2013; (9) Jolivet et al., 2001; (10) García-Tejedor et al., 2013; (11) Leclercq-Perlat et al., 2004; (12) Arfi et al., 2002; (13) De Freitas et al., 2008; (14) Silva et al., 2013; (15) Cheng et al., 2006; (16) Cardelle-Cobas et al., 2011a; (17) Petrova and Kujumdzieva, 2010; (18) Flores et al., 2004; (19) Purriños et al., 2013, (20) Seiler and Busse 1990; (21) Kesenkaş and Akbulut, 2008; (22) Papapostolou et al., 2012; (23) Nambou et al., 2013; (24) Kumura et al., 2004.

In this thesis generation of galactooligosaccharides by *Kluyveromyces* species and production of cheese desirable aromas have been addressed.

5.1 Bioactive compounds: galactooligosaccharides

Prebiotic ingredients are defined as a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health (Gibson and Roberfroid, 1995). Currently, the criteria used for classification of a food component as a prebiotic include: a) resistance to digestion, b) selective fermentation by beneficial bacteria in the colon, c) alteration in the colonic microbiota towards a healthier composition and d) induction of host health benefits (Fooks et al., 1999). Non-digestible carbohydrates that fulfill these criteria are considered as prebiotics. Among them, galactooligosaccharides (GOS) are relevant prebiotics, which promote the growth of different beneficial bacteria, particularly *Bifidobacterium*, *Lactobacillus* and *Streptococcus*. GOS contain from two to ten molecules of galactose and one molecule of glucose linked through glycosidic bonds (Figure 10). Different GOS are represented by various chemical structures, which will determine the prebiotic effect. The position of the glycosidic linkage determines the general preference of bacteria towards β-galactosyl residues with linkages β(1-6) and β(1-1) over those with β(1-4) (Cardelle-Cobas et al., 2011b).

These oligosaccharides are found in natural products such as human and animal milk (Kunz and Rudloff, 1993; Oliveira et al., 2013) and in processed grains and cereals (Biesiekierski, 2011). It is important to remark that human milk is the first source of GOS, which play a key role as prebiotic promoting colonization of beneficial bacteria in the intestine of

breast-fed infants (Boehm and Moro, 2008). Therefore, enrichment of some foods, particularly formula-fed for infants with prebiotic oligosaccharides may be an interesting approach to improve its nutritional value and to promote an intestinal microbiota more similar to that of breast-fed infants. Owing to the scarce availability of human milk, it is necessary to use alternative sources of GOS to supplement infant formulae in order to provide a beneficial prebiotic effect on the gastrointestinal tract microbiota of newborns (Olano and Corzo, 2009). In fact, different commercial mixtures of GOS are available, as for example Elix'or and Vivinal®-GOS (Friesland Food Domo, The Netherlands), Bimuno (Clasado Ldt., UK), Oligomate 55 (Yakult Honsha Co. Ltd., Japan) or Cup Oligo (Nissin Sugar Manufacturing Co. Ltd., Japan).

Different health beneficial effects have been attributed to GOS consumption. GOS administration to healthy elderly subjects improves the immune response and increases the number of beneficial bacteria, especially bifidobacteria (Vulevic et al., 2008). Additionally, GOS reduce adherence of pathogens as *Escherichia coli* or *Cronobacter sakazakii* to tissue culture cells and to the intestinal epithelium respectively, acting as a protective agents against infections (Quintero, 2011; Shoaf, 2006). Moreover, in combination with fructooligosaccharides (FOS), GOS reduce the incidence of allergic manifestations and infections during the first two years of life (Arslanoglu et al., 2008).

Synthesis of GOS can be achieved chemically although enzymatic synthesis present advantages such as stereospecificity and higher final yields (Schwab et al., 2011). Enzymatic synthesis is catalyzed by the hydrolase β-galactosidase (EC 3.2.1.23), which attacks the terminal non-reducing β-D-galactosyl residues of oligosaccharides and transfers the galactosyl moiety to suitable acceptors, performing a reaction called transgalactosylation (Figure 10). This enzyme has different applications in

the food fermentation and dairy industries and, based on its ability to hydrolyze lactose it has attracted the attention of dairy product manufacturers and researchers (Adam et al., 2004).

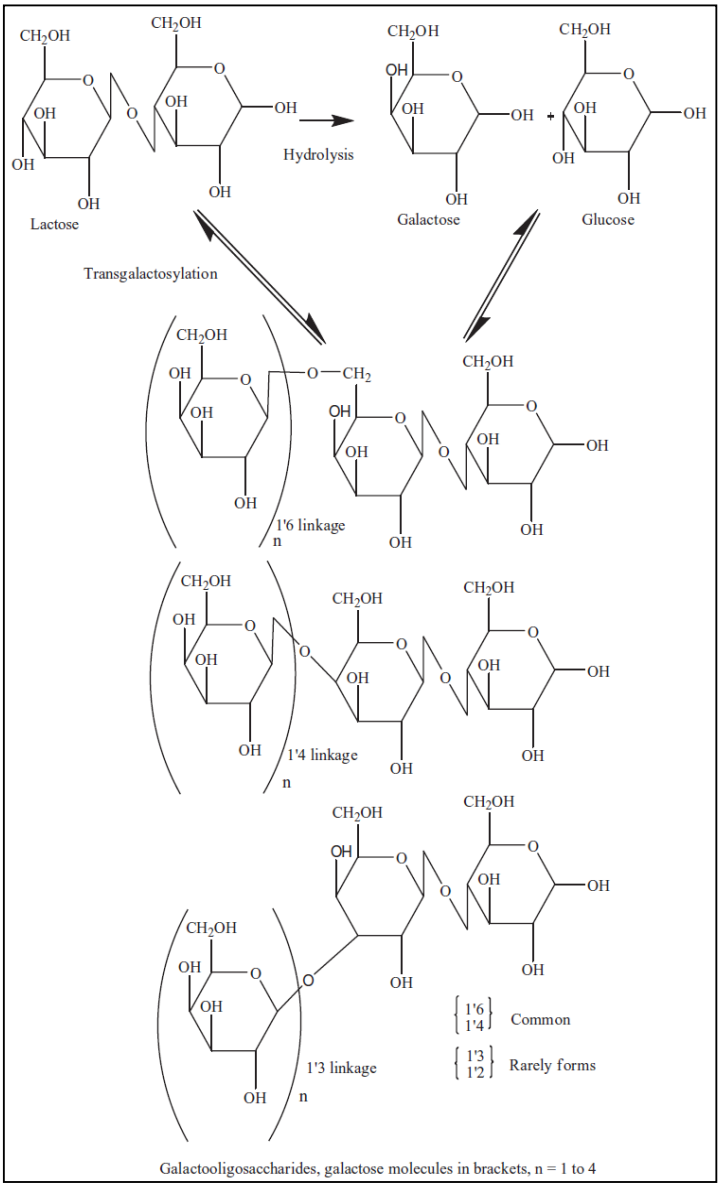

Figure 10. Enzymatic synthesis of different GOS from lactose (Otieno, 2010).

Beside, this enzyme has different microbial origin such as bacterial (*Bacillus circulans*), fungal (*Aspergillus oryzae*, *Aspergillus niger*) or yeast (*Kluyveromyces*, *Sterygmatomyces elviae*). It has been demonstrated that the effectiveness of oligosaccharide synthesis varies depending on the β-galactosidase origin. Moreover, variations in the level and composition of synthesized GOS are observed (Otieno, 2010). Special attention has been paid to the genera *Kluyveromyces*, and particularly to the species *K. lactis* and *K. marxianus,* since both present a good growth yield and a higher β-galactosidase activity compared with other yeasts (Kaur et al., 2009). Thus, both species are relevant industrial sources of β-galactosidase activity, and they have been traditionally used to produce low-lactose products and for the biological treatment of cheese whey waste.

Historically, GOS have been produced from lactose, but recently, other studies have reported the synthesis of prebiotic oligosaccharides using the synthetic disaccharide lactulose, which is produced from lactose during heat treatment of milk and is composed by one molecule of galactose and other of fructose. Lactulose presents by itself prebiotic character; however its consumption may bring some undesirable effects such as excessive gas production. The synthesis of oligosaccharides derived from lactulose may represent an alternative to lactulose consumption and reveals the possibility of developing a new group of compounds with potential new applications (Olano and Corzo, 2009; Tuohy et al., 2002).

Several studies have evaluated the feasibility of *K. lactis* commercial enzymatic preparations regarding oligosaccharide synthesis using lactose and lactulose as substrate (Cardelle-Cobas et al., 2011a; Martínez-Villaluenga et al., 2008; Rodríguez-Fernández et al., 2011; Rodríguez-Colinas et al., 2011). *K. marxianus* β-galactosidases have been only

recently tested for lactose transgalactosylation (Manera et al., 2010; Petrova and Kujumdzieva, 2010). In addition, there is little information about the possibility of food-isolated *Kluyveromyces* strains with potentially different metabolic characteristics to transgalactosylate lactose or lactulose.

5.2 Food production: aroma development

Cheese aroma is one of the most important attributes determining consumer acceptance and preference. Aromatic profile is composed by the interaction of different groups of chemical compounds. Some of them have been described as “cheesy”, but marked differences among the key aromatic compounds of the different cheese varieties have been reported (Urbach, 1993). For instance, acetic and propanoic acid are considered relevant for Cheddar, Gruyere or Emmental (Curioni and Bosset, 2002), sulphur compounds have been reported as an important component from Camembert (Molimard and Spinnler, 1996) while methyl ketones are characteristic of Gorgonzola (Moio et al., 2000).

A relevant number of studies have described many volatile compounds present in cheese and its corresponding aromatic notes, as it is reviewed by Curioni and Bosset (2002). This odorants may be divided in: a) neutral compounds: alcohols, aldehydes, ketones, esters, lactones and furans; b) alkaline components: nitrogen-containing compounds, pyrazines, sulphur compounds and terpenes; c) acidic compounds: phenolic compounds and free fatty acids.

Different combinations of techniques have been employed for isolation, identification and quantification of these components. Gas chromatography coupled with mass spectrometry (GC-MS) is commonly used as the analytical method for cheese flavor, with a prior step involving the extraction and pre-concentration of the volatile fraction, such as purge

and trap or solid phase microextraction (SPME). SPME is a relatively new analytical technique which requires only a small amount of sample, matrices can be both in solid and liquid states and it is fast and easy to perform (Condurso et al., 2008; Delgado et al., 2010).

It is important to highlight that unique aromatic properties have been attributed to traditional ewes' and goats' raw milk cheeses produced in the Iberian Peninsula and Mediterranean countries (Freitas et al., 2000). The aromatic profile of different artisanal ewe's and goats' cheeses such as Torta del Casar and La Serena or Ibores have been analyzed (Carbonell et al., 2002; Delgado et al., 2010; Delgado et al., 2011).

It is well known that yeast contribute to the formation of aromatic compounds in a wide variety of food and beverages, including dairy products (Birch et al., 2013; Chen et al., 2012; Sorrentino et al., 2013). Several studies suggest the use of different yeast species as starter cultures for cheese production (Binetti et al., 2013; Gardini et al., 2006). The application of *D. hansenii* and *Yarrowia lipolytica* during Gouda and Danablu production has been proposed (Ferreira and Viljoen, 2003; van den Tempel and Jakobsen, 2000). Nevertheless it is difficult to evaluate the behavior of the inoculated yeast in the whole cheese ecosystem, since microbial interactions may occur. Moreover, strain selection is necessary to avoid cheese defects due to yeast inoculation. Since this is a complex process, the addition of yeast starter cultures during cheesemaking is still poorly employed in the dairy sector.

Table 6 summarizes the studies focused on elucidating the effect of dairy isolates of *K. lactis*, *K. marxianus* and *D. hansenii* on cheese aroma. These yeast species are able to generate cheese odorants; however, little knowledge is available concerning intra-species variability. Additionally, the impact of native yeasts on final cheese aroma is not well established. This suggests that further investigations concerning the role of different strains of dairy yeast isolates on the key odorants of a specific cheese variety should be evaluated.

Table 6. Production of cheese aromatic compounds by non-conventional yeasts.

Compound	Medium[B]	Yeast[A] Km	Kl	Dh	Odour descriptors[C]
Acids	Cantalet cheese[1, 2]		x		Vinegar[11], rancid[12], cheese[13]
Alcohol	Cantalet cheese[1, 2]		x		Floral[11], mushroom, green[14]
	Cheese curd[3]		x		
	Cheese surface model[4, 5]			x	
	Feta cheese[6]			x	
	Muenster cheese medium[7]			x	
Aldehydes	Cantalet cheese[1, 2]		x		Malty[14], green, nutty[15]
	Cheese curd[3]		x		
	Cheese surface model[4, 5]			x	
	Feta cheese[6]			x	
Esters	Cantalet cheese[1, 2]		x		Sweet[13], floral[14], fruity[15]
	Cheese curd[3]		x		
	Cheese like medium[8]		x		
	Feta cheese[6]			x	
	Muenster cheese medium[7]	x			
Methyl ketones	Muenster cheese medium[7]			x	Musty[12], fruity, blue cheese[14]
Terpenes	Cheese curd[3]		x		Flower, sweet[11], green[13]
Sulphur compounds	Cheese based medium[9]	x	x		Potatoe[11], onion[13], cheese[16]
	Potato dextrose broth + sulphur aminoacids[10]		x	x	

[A] Km: *Kluyveromyces marxianus*; Kl: *Kluyveromyces lactis*; Dh: *Debaryomyces hansenii.*
[B, C] Reference numbers are as follows: (1) Freitas and Malcata, 2000; (2) De Freitas et al., 2009; (3) Martin et al., 2001; (4) Gori et al., 2012; (5) Sørensen et al., 2011; (6) Bintsis and Robinson, 2004; (7) Leclercq-Perlat et al., 2004; (8) Arfi et al., 2002; (9) Kagkli et al., 2006; (10) López del Castillo-Lozano et al., 2007; (11) Suriyaphan et al., 2001; (12) Moio and Addeo, 1998; (13) Christensen and Reineccius, 1995; (14) Moio et al., 2000; (15) Arora et al., 1995; (16) Molimard and Spinnler, 1996.

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Objectives

Nowadays there is increasing interest in the relationship between diet and health. Additionally, consumers are demanding high quality traditional food products, due to its unique organoleptic properties. In this sense, yeasts play a clear role on the production and quality of cheeses, which is closely linked to their ecology and biological activities. Therefore, academic and industrial interest in yeast biodiversity is growing. This doctoral thesis explores the wide yeast composition of Mediterranean ewes' and goats' raw milk artisanal cheeses as well as their potential for prebiotic synthesis and cheese aroma production. Partial objectives are detailed below.

Objective 1. Identification and enzymatic characterization of yeasts isolated during the ripening process of different artisanal ewes' and goats' cheeses made with raw milk. Study of the succession of *Debaryomyces hansenii* strains.

Objective 2. Molecular characterization and physiological profiling of *Kluyveromyces lactis* isolates.

Objective 3. Synthesis of relevant prebiotic oligosaccharides from lactose and lactulose using *Kluyveromyces lactis* and *Kluyveromyces marxianus* β-galactosidases.

Objective 4. Contribution of isolated yeasts to the production of key aroma compounds in cheese.

Objective I

Identification and enzymatic characterization of yeasts isolated during the ripening process of different artisanal ewes' and goats' cheeses made with raw milk. Study of the succession of *Debaryomyces hansenii* strains.

Yeast species and genetic heterogeneity within *Debaryomyces hansenii* along the ripening process of traditional ewes' and goats' cheeses.

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ABSTRACT

The yeasts present during the ripening process of ewes' and goats' cheeses produced in a small traditional dairy in Mediterranean Spain were isolated and identified. Five hundred and thirty strains pertaining to eleven yeast species representing eight genera were identified using molecular methods. *Debaryomyces hansenii* was the yeast species most frequently isolated in all cheeses. Other yeast species commonly found in dairy products were present at the first maturing weeks. Two yeast species *Trichosporon coremiiforme* and *Trichosporon domesticum* have been reported in cheeses for the first time, and *Meyerozyma guilliermondii* has been newly isolated from goats' cheeses. Yeast species composition changed greatly along the process; although, *D. hansenii* dominated at the end of ripening in all cheeses. Most yeast isolates were able to hydrolyze casein and fatty acid esters. One hundred and eighty seven *D. hansenii* isolates were genotyped by PCR amplification of M13 satellites and an UPGMA dendrogram was constructed. The majority of isolates were grouped in 5 clusters while 7 profiles were represented by 1-3 isolates. These results demonstrate the genetic heterogeneity present in *D. hansenii* strains isolated from raw milk cheeses.

Keywords: cheese, yeast identification, enzymatic activities, *Debaryomyces hansenii*, genotyping.

1. Introduction

Mediterranean European countries account for the production of most caprine and ovine milk worldwide. The majority of this production is converted into cheese in small artisanal dairies, using traditional making methods leading to the development of different cheese varieties with unique organoleptic characteristics (Freitas and Malcata, 2000).

The development of flavor and texture distinctive of a cheese variety are the result of complex processes involving microbiological and biochemical changes to the curd during ripening. Cheese microbiota may be divided into lactic acid bacteria and secondary microorganisms (Beresford et al., 2001). Yeasts are an important constituent of the secondary microbiota, which development is favored by the physic-chemical properties of the cheese such as low pH, low moisture content, elevated salt concentration and refrigerated ripening and storage (Fleet, 1990; Viljoen et al., 2003). Regarding the biochemical changes, these yeasts play an important role in proteolysis, lipolysis, fermentation of residual lactose, and assimilation of lactic and citric acid during the ripening of cheese, contributing to aroma development and to the rheological properties of the final dairy product (McSweeney, 2004). Additionally, some cheese yeasts have been recognized by their probiotic character and DNA-bioprotective action against model genotoxins (Kumura et al., 2004; Trotta et al., 2012).

Freitas and Malcata (2000) reviewed the most important aspects of the microbial characteristics of cheeses manufactured from ovine and caprine milk in Spain. These studies focused mainly on the identification and characterization of bacteria; however, there is little knowledge about the yeast population associated with these cheeses.

The impact of yeasts on the production and quality of the cheese is related to their ecology and biological activities (Fleet, 2007). Physico-chemical characteristics of cheese such as low pH, low water activity and high salt content and refrigerated storage favor yeast growth (Fleet, 1990). The number of yeast species frequently isolated from milk and dairy products listed in the Encyclopedia of Dairy Sciences is substantial (Büchl and Seiler, 2011). Species identification and characterization are therefore essential to understand the occurrence and role of yeast in cheeses.

In order to understand the differences between cheese varieties, we need to increase our knowledge on the yeast microbiota leading the ripening process. Several authors have pointed out the main role of *Debaryomyces hansenii* leading during cheese ripening (Fleet, 1990; Fox et al., 2000). In most Mediterranean ewes' and goats' cheeses, the yeast species *D. hansenii* seems to be predominant in the ripening process (Capece and Romano, 2009; Cosentino et al., 2001; Fadda et al., 2004; Gardini et al., 2006; Pisano et al., 2006). However, regardless of the importance of *D. hansenii* in the ripening process, very few efforts have been done to investigate changes in the succession of *D. hansenii* population (Capece and Romano, 2009; Petersen and Jespersen, 2004). Other yeast species such as *Trichosporon cutaneum* (Corbo et al., 2001), *Candida zeylanoides* (Fadda et al., 2010; Pereira-Dias et al., 2000) and *Geotrichum candidum* (Tornadijo et al., 1998) have also been identified as the main yeast in the ripening process of some cheese varieties.

This paper reports the identification by molecular methods of yeast isolated along the ripening process of four ewes' and goats' cheeses produced with raw milk using traditional methods in a small dairy sited within the borders of the Natural Park "Sierra de Espadán" (Castellón, Spain). The yeast isolates were also characterized by several technological features. In addition, the genetic heterogeneity within *D. hansenii* strains

isolated at different stages of the ripening process was analysed by minisatellite M13 PCR.

2. Materials and methods

2.1. Cheese sample processing

Two goats' (G1 and G2) and two ewes' (E1 and E2) commercial semi-hard cheeses produced in the spring season with raw milk and bacterial starters were sampled during the ripening process. Samples were weekly taken from the first day of ripening after salting and along six weeks of the process. Cylindrical samples (0.5 cm x 5 cm approximately) consisting of rind and cheese interior were taken along the cheese ripening process, collected into sterile flasks and transported refrigerated to the laboratory.

Cheese samples of 5 g were aseptically weighted into 10 mL of saline solution in a sterile tube and homogenized using a Polytron PT 2100 (Kinematica AG, Switzerland). Microbiological analyses were performed immediately after sample homogenization.

2.2. Yeast content and isolation

Decimal dilutions from 10^{-1} to 10^{-7} of the homogenized samples were carried out for microbiological assays. Samples of 0.1 mL from all dilutions were spread on GPYA medium plates (glucose 2%, peptone 0.5%, yeast extract 0.5% and agar 2%) supplemented with chloramphenicol (100 mg/L) and incubated at 25ºC for 2 to 3 days. Approximately 20 colonies per sample were picked up randomly. Colonies showing differences in shape, size or color were additionally selected. Yeast colonies were purified on GPYA plates and pure cultures preserved in 15% glycerol at -80ºC.

2.3. Yeast identification: RFLPs of 5.8S ITS rDNA region and sequencing of D1/D2 of 26S rDNA gene

PCR reaction and RFLPs of the 5.8S-ITS rDNA region were performed following the methodology of Esteve-Zarzoso et al. (1999) using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (TCCTCCGCTTATTGATATGC-3') (White et al., 1990) and restriction enzymes *Cfo*I, *Hae*III and *Hin*fI. Band sizes of RFLPs of the 5.8S-ITS rRNA were compared against the Yeast-id database (http://www.yeast-id.com) and the yeast isolate assigned to a known species. Identifications were confirmed by sequencing the D1/D2 domains of 26S rRNA gene. PCR products using the primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') (White et al., 1990) were purified with High Pure PCR Product Purification Kit (Roche, Germany). DNA sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Calif., USA) in an Applied Biosystems (Model 310) automatic DNA sequencer. Sequences were edited using MEGA5 (Tamura et al., 2011) and then subjected to GenBank BLASTN tool.

2.4. Genetic characterization of D. hansenii strains.

Minisatellite PCR amplification using the M13 primer (5′-GAGGGTGGCGGTTCT-3′) was performed as described in Fadda et al. (2004) with minor modifications using a PCR (Mastercycler Pro, Eppendorf, Hamburg, Germany). Final volume reaction, 50 µL, contained 0.3 µL rTaq (5U) DNApolymerase, 4 µL dNTP mix (2.5 mM), 5 µL buffer (Takara Bio Inc., Shiga, Japan), 3 µL $MgCl_2$ (1.5 mM) (Sigma, St. Louis, MO, USA), 1 µL M13 primer (50 pmol/µL) (Isogen Life Science, PW de Meern, The Netherlands) and 80–100 ng of genomic DNA (Querol, 1992). PCR

amplification conditions were as follow: 95°C for 5 min followed by 40 cycles at 93°C for 45 s, 44.5°C for 1 min and 72°C for 1 min with a final extension step at 72°C for 6 min. The PCR products (10 µL) were resolved by electrophoresis on 2% agarose gel in 1x TAE buffer at 90 V for 3 h, stained with RedSafe (INtRON Biotech., Spain) and visualized under UV light. DNA fragment sizes were determined using a 100-bp DNA ladder (Life Technologies, Carlsbad, CA, USA).

2.5. Technological characterization of yeasts: evaluation of proteolytic and lipolytic activities

Proteolytic activity was evaluated in GPYA medium containing 10% of skim milk (Difco, Franklin Lakes, NJ, USA) following the methodology of Gardini et al. (2006). Proteolysis was considered positive when a light halo was visible (1 to 5 mm) after 15 days. Lipolytic activity was assayed on Tween agar medium containing 1% peptone, 0.5% NaCl, 0.01% $CaCl_2$ supplemented with 1% of Tween 40 (palmitic acid ester), Tween 60 (estearic acid ester) or Tween 80 (oleic acid ester) (Sigma) following the methodology of Sierra (1957). Presence of a precipitation ring around the colonies after 15 days of incubation indicated lipolytic activity.

2.6. Data analysis

Graphics were produced using SPSS Statistics v.19.0 (Statistical Package for the Social Sciences, IBM, USA). UPGMA (unweighted pair-group method using arithmetic averages) dendrogram was constructed using the Jaccard Similarity Index in the NTSYS package version 2.21p (NTSYS Numerical Taxonomy and Multivariate Analysis System, Exeter Publishing Ltd., USA). The Similarity Matrix was based on presence (1) and absence (0) of homologous bands in the electrophoretic patterns.

3. Results

3.1. Yeast counts and species succession

Yeast counts increased from day one (24 hours after salting) to the sixth week of the ripening process. Initial counts of ewes' cheeses, E1 and E2, started at 10^4 and 10^5 CFU/g, respectively. These yeast counts increased to 10^7 CFU/g at the third week of the ripening process and they were maintained until the end of the sampling procedure. Goats' cheeses, G1 and G2, initial counts started at 10^4 and 10^5 CFU/g raising to 10^8 and 10^7 CFU/g, respectively, at the sixth sampling week.

A total of 530 yeasts were isolated from the six samples taken from each cheese. Table 1 shows the results from the molecular identification using the RFLPs of ITS-5.8S rDNA and their correlation with the result of the BLASTN sequence comparison for the D1/D2 of 26S rDNA. The identification of isolates from the species *Pichia kudriavzevii*, *Trichosporon coremiiforme* and *Trichosporon domesticum* was done by BLASTN of the D1/D2 26S rDNA gene sequences against GenBank as ITS-5.8S rDNA restriction patterns for these species are not included in the Yeast-id database.

The most abundant yeast species in all cheeses was *D. hansenii* (Figure 1) except in cheese G1, which rendered a higher number of *Kluyveromyces lactis* isolates. Other yeast species were isolated in minor numbers. In all cheeses, yeast diversity decreased along cheese maturation, being *D. hansenii* the most abundant yeast at the end of the process. In cheeses E1 and G1, yeast species *D. hansenii* appeared at the second or third maturation week although concurring with *K. lactis* and other yeast species up to the fifth or sixth week of the process. On the contrary, in cheeses E2 and G2, yeast species *D. hansenii* appeared at the

initial stages of cheese ripening and dominated the process since the fourth and third ripening weeks, respectively.

Table 1. Molecular identification of yeast species by RFLPs of the ITS 5.8S rDNA and sequencing of the D1/D2 of 26S rDNA gene.

RFLPs ITS-5.8S identification	PCR product (bp)	Band sizes (bp)[a]			100% similarity GenBank ACCN[b]	Literature[c]
		Cfo I	HaeIII	HinfI		
Candida mesenterica	390	390	390	195 + 195	U45720	1, 4
C. parapsilosis	550	300 + 240	420 + 110	280 + 260	AF374609	2-5
Debaryomyces hansenii	650	300 + 300	420 + 150 + 90	325 + 325	JQ689041	1-10
Kluyveromyces lactis	740	300 + 300	420 + 150 + 90	325 + 325	U76347	1, 3, 4, 6-10
K. marxianus	740	285 + 185 + 140 + 100	655 + 80	240 + 185 + 120 + 80	CR382124	1, 3, 4, 7-9
Kazachstania unispora	775	350 + 310 + 115	500 + 110	400 + 375	AY048158	1, 9
Meyerozyma guilliermondii	625	300 + 265	400 + 115 + 90	320 + 300	JQ689047	-
Pichia kudriavzevii	550	220 + 190 + 90	400 + 100	230 + 160 + 140	AY048158	3
Trichosporon coremiiforme	550	275 + 275	500	275 + 275	AF139983	-
T. domesticum	550	275 + 275	500	250 + 160 + 100	JN939449	-
Yarrowia lipolytica	380	210 + 170	380	190 + 190	AM268458	1-4, 6-9

[a] Band sizes smaller than 80 bp could not be accurately estimated by comparison with a 100 bp ladder.
[b] The D1/D2 26S rDNA gene sequences determined in this study showed 100% sequence similarity with the GenBank ACCN numbers listed.
[c] Yeast species isolated from ewes' and goats' cheese reported in previous publications: (1) Nahabieh and Schmidt, 1990; (2) Fadda et al., 2010; (3) Cosentino et al., 2001; (4) Corbo et al., 2001; (5) Pereira-Dias et al., 2000; (6) Gardini et al., 2006; (7) Fadda et al., 2004; (8) Pisano et al., 2006; (9) Tornadijo et al., 1998; (10) Capece and Romano 2009.

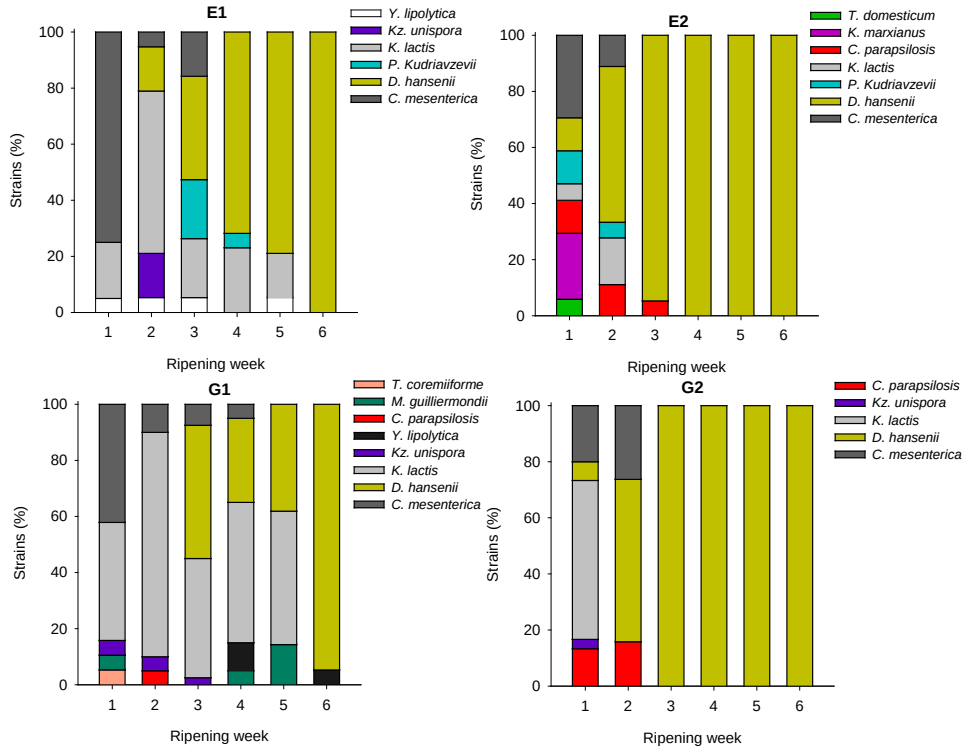

Figure 1. Evolution of yeast species along the ripening weeks of the different types of cheeses. Ewes' cheeses are coded as E1 and E2 and goats' cheeses as G1 and G2.

3.2. Technological characterization

Yeast isolates were evaluated for their proteolytic activity towards casein and lipolytic activity towards palmitic, stearic and oleic acid esters. Table 2 shows the percentage of yeast isolates within each species displaying enzymatic activities. Most yeast isolates showed proteolytic activity (83%). This enzymatic activity was remarkable in *D. hansenii* and *K. lactis*, representing more than 90% of the total activity measured as number of positive isolates. Isolates pertaining to the species *C. parapsilosis* and *Kz. unispora* also showed notable proteolytic activity.

Regarding lipolytic activity, around 60% of the isolates were able to hydrolyze palmitic acid and stearic acid esters whereas this number decreased below 40% in case of oleic acid ester hydrolysis. Most isolates showing lipolytic activity on palmitic acid ester were also able to hydrolyze stearic acid ester; but from these very few isolates could hydrolyze oleic acid ester (data not shown). The exception was *Y. lipolytica* which five isolates displayed lipolytic activity on oleic acid while not showing lipolytic activity on palmitic acid ester and low activity on stearic acid ester. No significant differences were found in the percentage of strains showing proteolytic and lipolytic activities within each type of cheese (data not shown).

Table 2. Percentage of isolates from each yeast species showing enzymatic activities.

Hydrolysis of:	Casein	Palmitic acid ester	Stearic acid ester	Oleic acid ester
Candida mesenterica	8	-[a]	-	-
C. parapsilosis	100	43	43	7
Debaryomyces hansenii	92	86	91	58
Kluyveromyces lactis	97	17	17	8
K. marxianus	50	-	-	-
Kazachstania unispora	89	-	-	11
Meyerozyma guilliermondii	60	100	100	100
Pichia kudriavzevii	44	-	-	-
Trichosporon coremiiforme	100	100	100	100
T. domesticum	100	100	100	100
Yarrowia lipolytica	50	-	17	83

[a] No enzymatic activity was detected.

3.3. Genetic typing of D. hansenii

Minisatellite M13 PCR amplification of 187 *D. hansenii* isolates generated 12 different electrophoretic patterns (Figure 2) labeled A1 to A5, B1 to B5, C1 and D1. The most abundant pattern was A1 (48%) constituted by 6 bands, the heaviest band at 1500 bp and the smallest at 400 bp. Pattern B1 displayed the largest number of bands, 11, the top band at 1400 bp and the bottom one around 300 bp. Patterns A2 to A5 and B2 to B5 displayed minor differences respect to A1 and B1, respectively; these pattern differences, which consist of few additional or absent bands are indicated with arrows on Figure 2.

Band presence or absence in the electrophoretic patterns was used to construct an UPGMA dendrogram (Figure 3). *D. hansenii* isolates were divided into four groups, A to D, at 70% similarity approximately. Groups A and B were subsequently separated into five clusters each.

D. hansenii A patterns were displayed by isolates from all cheeses, whereas patterns B were displayed only by isolates from goats' cheeses. Pattern C1 appeared in one isolate from cheese G2 while D1 was observed in isolates from ewes' cheeses. Within the most populated clusters, A1 and A2, *D. hansenii* isolated from all cheeses could be found while clusters A3, A4 and A5 were constituted solely by *D. hansenii* isolates from cheese G2. Moreover clusters A1, A2 and A3 contained only cheese G2 *D. hansenii* isolates from the first three ripening weeks while clusters A4 and A5 contained only isolates from the last three weeks. Similarly, B patterns were found in *D. hansenii* strains from goats' cheeses isolated from the three last weeks of the ripening process. The overall level of diversity in the M13 patterns was higher in goats' cheeses than in ewes' cheeses. Furthermore, M13 pattern heterogeneity increased as the ripening process of goats' cheeses progressed, indicating that the number of different *D. hansenii*

isolates increased from the first weeks to the last weeks of cheese maturation.

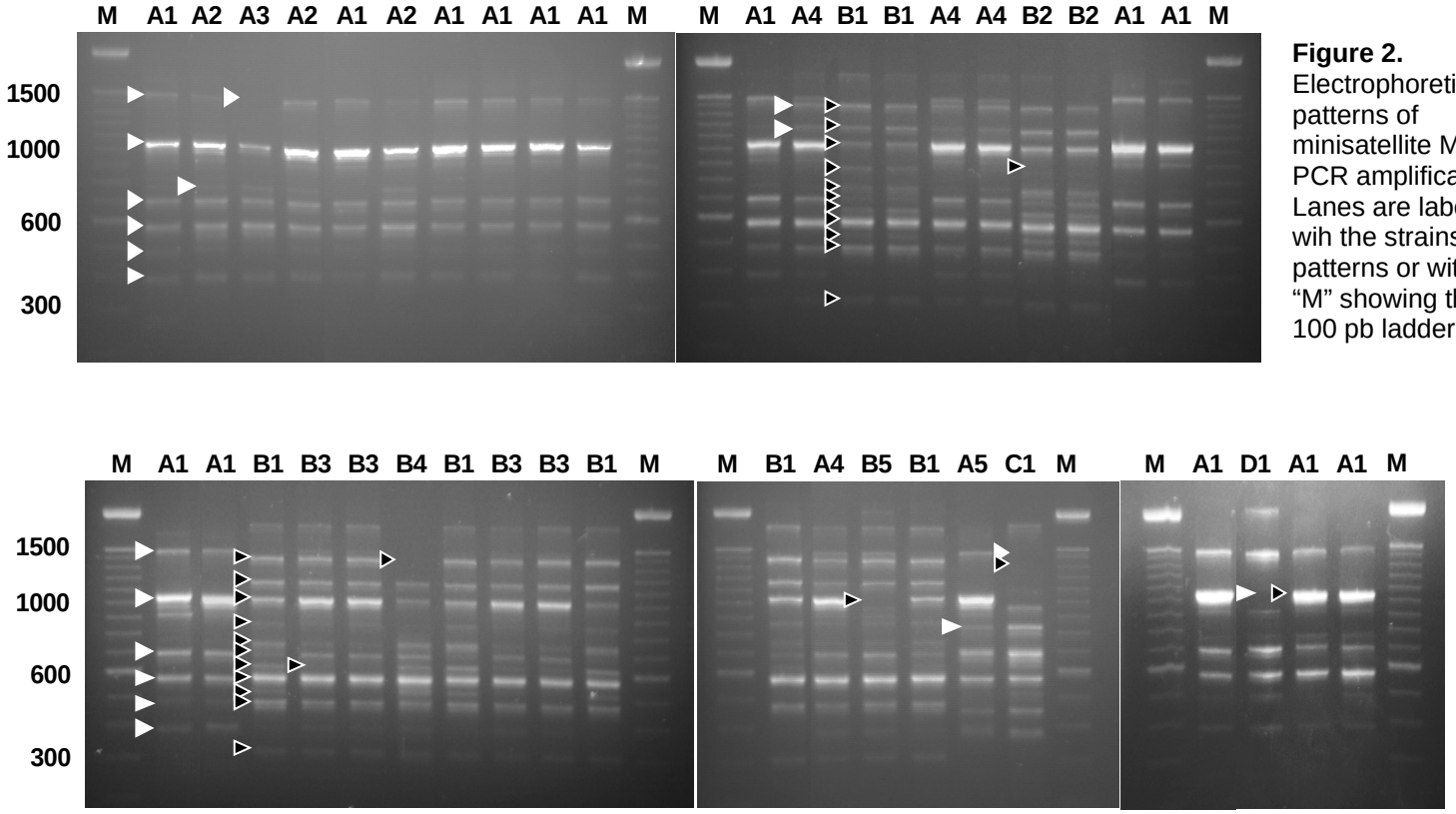

Figure 2. Electrophoretic patterns of minisatellite M13 PCR amplification. Lanes are labeled wih the strains patterns or with an "M" showing the the 100 pb ladder.

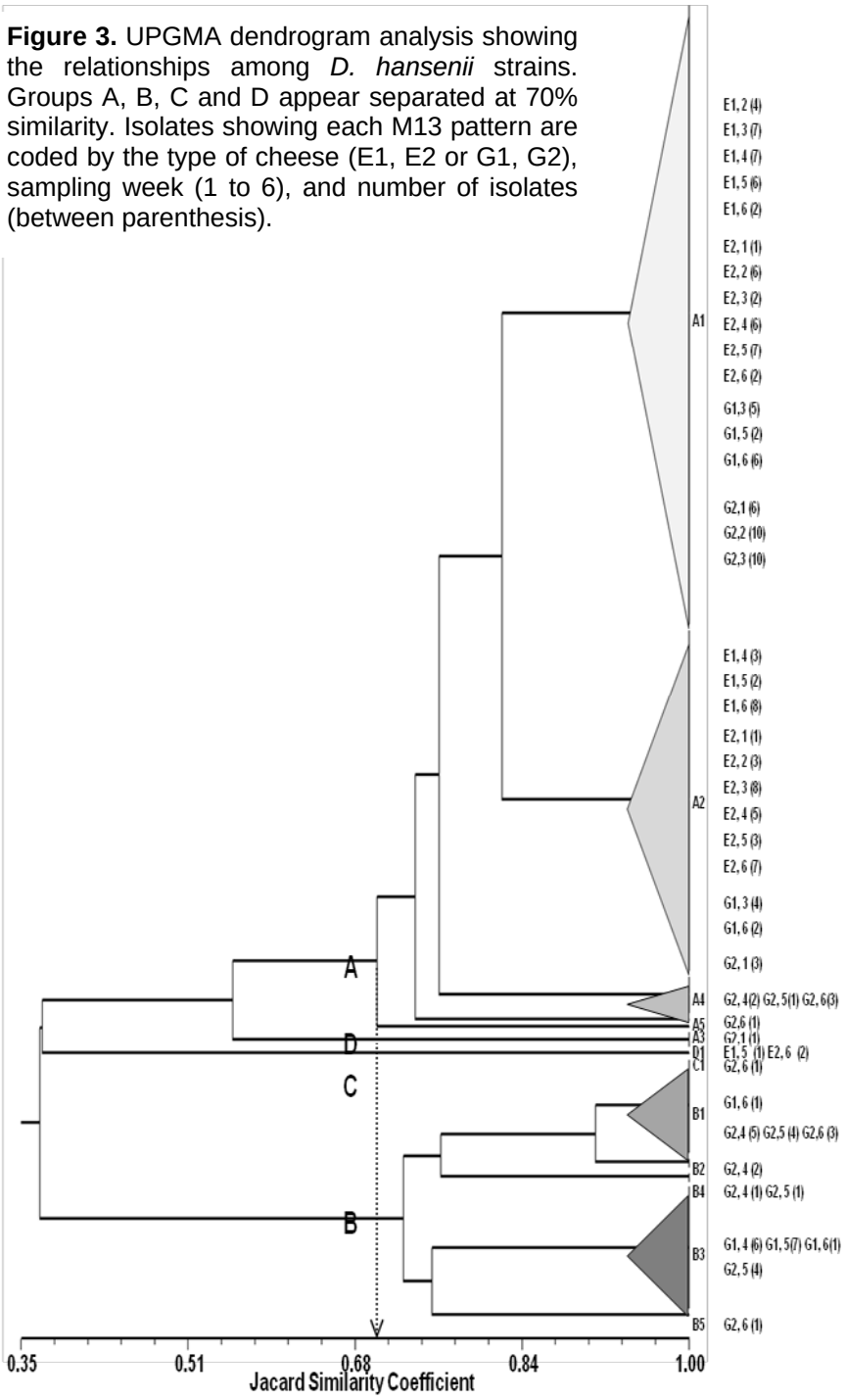

Figure 3. UPGMA dendrogram analysis showing the relationships among *D. hansenii* strains. Groups A, B, C and D appear separated at 70% similarity. Isolates showing each M13 pattern are coded by the type of cheese (E1, E2 or G1, G2), sampling week (1 to 6), and number of isolates (between parenthesis).

4. Discussion

Yeast identification using the RFLPs of the ITS-5.8S rDNA region produced the same results as sequence comparison of the D1/D2 26S rDNA gene, thus confirming the value of the former technique as already mentioned in previous reports (Álvarez-Martín et al., 2007; Dlauchy et al., 1999; Esteve-Zarzoso et al., 1999; Gardini et al., 2006). Our results show that the main yeast species isolated from ewes' and goats' cheeses are common inhabitants of dairy products. However, few yeast species such as *T. coremiiforme* and *T. domesticum* have been isolated from cheeses for the first time. Early reports on yeast microbiota of dairy products point to *T. ovoides*, *T. cutaneum* and *T. capitatum* as the sole species of the genus *Trichosporon* found in dairy products. Similarly, *Mz. guilliermondii* has been isolated from goats' cheeses for the first time although has been previously found in numerous dairy products (Büchl and Seiler, 2011).

Fox et al. (2000) found that *D. hansenii* was by far the dominant yeast found in most cheeses, followed by *K. lactis*, *Y. lipolytica* and *Trichosporon beigelii*. However, the progression in yeast species occurring during ripening is not clear, since in most studies the stage of ripening at which the yeasts were isolated is not defined (Beresford et al., 2001).

The results of our study demonstrate that yeast species composition changes greatly along the cheese ripening process. The first day of cheese maturation several yeast species could be found; however, at the sixth week of the process most yeast species had vanished and *D. hansenii* was the yeast species most frequently isolated. Numerous studies point to the low water activity, acidic environment and high salt content in cheese as the factors favoring the prevalence of *D. hansenii* (Beresford et al., 2001; Büchl and Seiler, 2011; Fleet, 1990). The second yeast species repeatedly found in this study was *K. lactis*. This yeast together with *K. marxianus* is

able to ferment lactose which promotes their growth in the interior of the cheeses, where other dominant yeasts are scarce (Fleet, 1990). *Y. lipolytica* described in numerous studies of dairy microbiota was also found in the cheeses investigated in this study; although it seems not to be a dominant yeast in the ripening process as already reported by other authors (Fadda et al., 2004; Fadda et al., 2010; Gardini et al., 2006; Pereira-Dias et al., 2000; Pisano et al., 2006; Suzzi et al., 2001; Tornadijo et al., 1998).

Only two species of the genus *Candida* were found, *C. parapsilosis* and *C. mesenterica*, both present in appreciable numbers during the first weeks of ripening. Similarly, *Kz. unispora* previously recovered from Spanish and French goats' cheeses (Nahabieh and Schmid, 1990; Tornadijo et al., 1998) has now been also found in ewes' cheeses. Finally, *P. kudriavzevii* has been previously found in Italian ewes' and goats' cheeses (Cosentino et al., 2001; Fadda et al., 2010).

Microbial proteolysis and lipolysis promote complex metabolic changes in the cheese which are vital for proper development of both flavor and texture (Bintsis et al., 2003; Klein et al., 2002; Leclercq-Perlat et al., 2007; Roostita and Fleet, 1996). The evaluation of enzymatic activities conducted in this study shows that most *D. hansenii* isolates were able to hydrolyze casein as well as palmitic and stearic acid esters. Oleic acid ester, probably due to the presence of a double bond, was hydrolyzed by very few isolates with the exception of *Y. lipolytica* and *Mz. guilliermondii*. Although the wide array of substrates and conditions used to test these enzymatic activities hinders comparison among studies (Capece and Romano, 2009; Cosentino et al., 2001; Fadda et al., 2004; Fadda et al., 2010; Gardini et al., 2006; Pereira-Dias et al., 2000) our results show the proteolytic and lipolytic potential of these yeasts. The contribution of these dairy yeast enzymatic activities to cheese quality deserves future studies.

PCR amplification of M13 minisatellites revealed a remarkable diversity within the *D. hansenii* isolates although the similarity between most patterns indicates a notable degree of genetic closeness. Similar studies by Capece and Romano (2009) showed a comparable level of diversity in *D. hansenii* isolated from two dairies in Basilicata region, Italy. By contrast, other studies have found scarce diversity among different dairies using similar techniques (Fadda et al., 2004; Romano et al., 1996). The overall genetic diversity within *D. hansenii* has been analyzed by several authors using different methodologies demonstrating the complexity of taxon *D. hansenii* (Groenewald et al., 2008; Nguyen et al., 2009; Jacques et al., 2009; Lopandic et al., 2013). The analysis by Sohier et al. (2009) revealed that *D. hansenii* strains isolated from the same origin are genetically closely related what would be in agreement with our results. Furthermore, our observations regarding goats' cheeses suggested that more than one strain of *D. hansenii* may be involved in the ripening process as suggested by Petersen et al. (2001). Moreover, we could not find any dominant *D. hansenii* strain at the end of the process.

6. Conclusions

This study provides evidence for the remarkable yeast diversity associated with goats' and ewes' traditional cheeses produced in a small dairy in Spain. The succession of yeast species along the cheese ripening process evidences the complex physico-chemical changes taking place in the cheese eventually restricting yeast growth with the exception of *D. hansenii*. Moreover, the enzymatic characterization revealed the possible contribution of these yeasts to liberation of amino acids and fatty acids from milk. The minisatellite M13 profiles obtained from most *D. hansenii* strains showed little divergence, indicating a close genetic relationship associated with the same origin of isolation. In addition, and based on the distribution by weeks, the diversity of *D. hansenii* isolated from goats' cheeses increased along the ripening weeks.

7. Acknowledgements

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Objective II

Molecular characterization and physiological profiling of *Kluyveromyces lactis* isolates.

Microplate screening of phenotypic profiles of genetically different *Kluyveromyces lactis* strains isolated from goats' and ewes' cheeses.

Beatriz Padilla, Paloma Manzanares and Carmela Belloch

In preparation

ABSTRACT

An automated screening of phenotypic profiles was used to characterize genetically different *Kluyveromyces lactis* strains isolated from goats' and ewes' cheeses. Genotyping using RAPD M13 showed a rich heterogeneity of patterns within *K. lactis* from both cheese types. Very few patterns were common to both cheese types. On the contrary, the predominant *K. lactis* strains seemed to be different in goats' or ewes' cheeses. Strains representative of the different genotypes were screened for their growth on lactose, lactose supplemented with NaCl and lactate using an automated screening method on microplates. All *K. lactis* strains were able to grow on lactose and lactose supplemented with 2 and 5% of NaCl, whereas an increase of the optical density was not detected after 5 days incubation on lactose supplemented with 8% NaCl and lactate media. All growth parameters were strongly dependent of NaCl content in the medium. Very few strains were not influenced by the presence of 2% salt, whereas all strains were negatively influenced in their growth parameters by the presence of 5% NaCl. Further characterization of *K. lactis* for production of biogenic amines revealed that the slowest strain growing on lactose was the only able to decarboxylate ornithine among the five amino acids tested. Proteolytic activity was exhibited by most of the strains but lipolytic activity was uncommon among the 76 tested strains.

Keywords: *Kluyveromyces lactis*, minisatellite M13, lactose, NaCl, growth parameters, biogenic amines, lipolysis and proteolysis.

1. Introduction

One of the prominent characteristics of *Kluyveromyces lactis* is its ability to assimilate and ferment lactose, the main sugar present in milk fermentations. Although a number of yeasts can aerobically grow on lactose, those that can ferment it are very rare. Besides *K. lactis* only *Kluyveromyces marxianus* is able to ferment lactose (Lachance, 2011). The role of these yeasts in cheese has been thus mainly related to lactose and lactate metabolism (Cholet et al., 2007; Gardini et al., 2006). Both yeast species have been frequently isolated in moderate to significant numbers from a large variety of cheeses and dairy products (Büchl and Seiler, 2011). Despite the unique physiological properties of *K. lactis* and *K. marxianus* influencing fermentation and maturation of cheese, very few studies focus on these species and frequently only *Debaryomyces hansenii* and *Yarrowia lipolytica* have been taken into account (Capece and Romano, 2009; Romano et al., 1996; Suzzi et al., 2001). Only in few instances the prominence of *K. lactis* respect to other yeasts isolated from cheese has caused a second look into strain variability within this species (Fadda et al., 2004). Additionally, the lipolytic and proteolytic activities of *K. lactis* have been considered weak as compared with those of *D. hansenii* and *Y. lipolytica* (Bankar et al., 2009; Breuer and Harms, 2006) and therefore their relevance in cheese maturation has been unnoticed.

Taxonomic studies involving molecular differentiation of *K. lactis* strains have revealed rich population diversity (Belloch et al., 1998; Belloch et al., 1997; Naumova et al., 2004). Moreover, the system of lactose genes enabling *K. lactis* lactose fermentation has been thoroughly studied (Dong and Dickson, 1997; Wiedemuth and Breunig, 2005) and even the origin of the lactose regulon in *K. lactis* has been hinted (Naumov, 2005).

The ability of *K. lactis* to generate desirable aromas in cheese has been also studied. This yeast is known to produce a wide variety and high amounts of volatile sulphur compounds in cheese ecosystems (Kagkli et al., 2006). Moreover, combined systems constituted by *K. lactis* together with *Y. lipolytica* and *Pichia fermentans* revealed the prevalence of *K. lactis* when compared with the other yeasts (De Freitas et al., 2009).

The potential of *K. lactis* to produce metabolites of interest for the food industry has also been explored. Recent studies have surveyed the ability of *K. lactis* β-galactosidase to transglycosylate lactose and lactulose generating prebiotic oligosaccharides relevant as food ingredients (Padilla et al., 2012). Moreover, the ability of this dairy yeast to produce milk protein-derived antihypertensive hydrolysates has also been confirmed (García-Tejedor et al., 2013).

Despite the interest and attention that *K. lactis* has received as both a genetic model and industrial yeast as a source of different metabolites and enzymes (Pariza and Johnson, 2001) very few evidence has been reported on the population diversity of *K. lactis* present in cheese, succession of strains or physiological differences among different strains. The aim of this study is the exploration of strain heterogeneity within *K. lactis* isolated along the ripening process of goats' and ewes' cheeses. Genetic heterogeneity was analysed by RFLPs of mitochondrial DNA and RAPD PCR amplification of minisatellite M13. Physiological profiling of the different genetic types was accomplished by automated screening in microplates of *K. lactis* growth on lactose, lactose supplemented with salt and lactate media. Moreover, production of biogenic amines, and proteolytic and lipolytic activities were also investigated.

2. Materials and methods

2.1 Yeast strains

K. lactis strains were isolated from artisanal goats' and ewes' milk cheeses produced in Castellón, Spain. Table 1 shows the strains isolated from each cheese type at the beginning of the process and along four weeks of ripening. Type strain of *K. lactis* CECT 1961^T (Spanish Type Culture Collection) was included as control in all experiments.

2.2 DNA isolation and mitochondrial DNA restriction

DNA isolation was performed according to Querol et al. (1992). Restriction of mitochondrial DNA (mtDNA RFLPs) was performed as reported in Belloch et al. (1997). DNA was digested with restriction endonuclease *Hin*fI (Roche Applied Science, Mannheim, Germany) according to the supplier's instructions. Restriction fragments were separated on 2% agarose gels in 1x TAE Buffer (40 mM Tris-acetate, 1 mM EDTA pH 8) stained with GelRed^{TM} 3x (Biotium, Hayward, CA, USA) and visualized under UV light. DNA fragments sizes were estimated by comparison against lambda phage DNA digested with PstI.

2.3 Generation of minisatellite M13 patterns

PCR amplification of minisatellite M13 (RAPD M13) was performed using the primer 5'-GAGGGTGGCGGTTCT-3′ as described elsewhere (Fadda et al., 2004) with some minor modifications using a PCR Mastercycler Pro (Eppendorf, Hamburg, Germany). Final volume reaction, 50 µL, contained 0.3 µL rTaq (5U) DNApolymerase, 4 µL dNTP mix (2.5 mM), 5 µL buffer (Takara Bio Inc., Shiga, Japan), 3 µL $MgCl_2$ (1.5 mM)

(Sigma, St. Louis, MO, USA), 1 µL M13 primer (50 pmol/µL) (Isogen Life Science, PW de Meern, The Netherlands) and 80–100 ng of genomic DNA measured using a NanoDrop™ (Thermo Fisher Scientific, Wilmington, DE, USA). PCR amplification conditions were as follow: initial denaturing at 95ºC for 5 min; 40 cycles of denaturation at 93ºC for 45 s, annealing at 44.5ºC for 1 min and extension at 72ºC for 1 min; a final extension step of 6 min for 72ºC. PCR products were separated on 2% agarose gels in 1x TAE Buffer at 90 V for 180 min, stained with GelRed™ 3x (Biotium, Hayward, CA, USA) and visualized under UV light. Sizes were estimated by comparison against a 100 bp DNA ladder (Invitrogen, Carlsbad, CA, USA).

2.4 Growth conditions on lactose, lactose supplemented with salt and lactate media

Strains were inoculated on 5 mL of GPY medium (2% glucose, 0.5% peptone, 0.5% yeast extract) and incubated overnight at 28ºC and 100 rpm/min shaking. Cells were collected by centrifugation at 3000 rpm/min and washed with sterile saline solution (0.9% sodium chloride). Cell growth experiments were conducted using a SPECTROstar Omega microplate reader (BMG Labtech, Ortenberg, Baden-Württemberg, Germany) in 96-well microplates inoculated to approximately optical density (OD) 0.1. The inoculum was always above the detection limit of the apparatus, which was determined by comparison with a previously established calibration curve. Growth assays were done in 250 µl volume and 28ºC temperature. Growth was tested on lactose medium (L4) (4% of lactose 1-hydrate and 0.1% of yeast extract), lactose medium supplemented with NaCl at 2% (L4S2), 5% (L4S5) and 8% (L4S8), and lactate medium (2% of sodium lactate and 0.1% of yeast extract). OD measurements at 600 nm were done every 30 minutes for 3 days in case of L4 and L4S2, and every hour for 5 days in

case of L4S5, L4S8 and lactate medium. Each OD measurement was preceded by a shaking step (double orbital) for 30 seconds at 500 rpm. For each medium one microplate containing triplicates of each strain was used. Blank wells containing media but not inoculated were included in each microplate to subtract the noise signal from culture medium. A total of 285 curves (19 strains x 5 media x 3 replicates) were obtained. Growth data were exported from the microplate reader and further processed in Excel (Microsoft Office 2010) following the methodology of Warringer and Blomberg (2003) for estimation of generation time, maximum OD and duration of lag phase with few modifications. The minimum lag phase observed from visual inspection of L4 and L4S2 curves resulted between 4 and 5 hours, whereas the estimation in case of L4S5 was above 18 hours. Values above 72 hours were discarded in case of L4S5. Estimation of lag phase was based on this observations as in Warringer and Blomberg (2003).

2.5 Evaluation of proteolytic and lipolytic activities

Proteolytic activity was evaluated on GPYA medium (2% glucose, 0.5% peptone, 0.5% yeast extract and 2% agar) containing 10% skim milk (Difco, BD Diagnostics, NJ, USA) (Gardini et al., 2006). Proteolysis was considered positive when a transparent halo was visible (1 to 5 mm) after 15 days incubation at 28ºC. Lipolytic activity was assayed on tween agar medium (1% peptone, 0.5% NaCl, 0.01% $CaCl_2$) supplemented with 1% of Tween 40 (palmitic acid ester), Tween 60 (estearic acid ester) and Tween 80 (oleic acid ester) (Sigma-Aldrich, St. Louis, MO, USA) following the methodology of Sierra (1957). Presence of a precipitation ring around the colonies after 15 days of incubation indicated positive lipolytic activity.

2.6 Production of biogenic amines

The ability of *K. lactis* to decarboxylate the amino acids ornithine, lysine, phenylalanine, tryptophan and histidine (Sigma-Aldrich, St. Louis, MO, USA) was tested following the methodology of Gardini et al. (2006). *Y. lipolytica* was used as positive control. Plates were incubated at 28ºC for 8 days. Experiments were carried out in triplicate.

2.7 Data analysis

Graphics were produced using SPSS Statistics v.19.0 (Statistical Package for the Social Sciences, IBM, USA) and SigmaPlot 12.0 (Systat Software Inc., USA). UPGMA (unweighted pair-group method using arithmetic averages) dendrogram was constructed using the Jaccard Similarity Coefficient in the NTSYS package version 2.21p (NTSYS Numerical Taxonomy and Multivariate Analysis System, Exeter Publishing Ltd., USA). The Similarity Matrix was based on presence (1) and absence (0) of homologous bands in the electrophoretic patterns. Statistical comparisons of growth parameters were made using one-way analysis of variance (ANOVA) with significance among treatment groups evaluated using Fisher's least significant difference (LSD) implemented in Statgraphics Centurion XV (Statpoint Technologies Inc., USA).

3. Results and Discussion

A total of 76 *K. lactis* strains (Table 1) isolated from two different cheese types, goats' or ewes', produced in the same cheese factory were analyzed for genetic diversity using mtDNA RFLP's and RAPD M13. Strains pertaining to different genotyping groups were examined for their growth parameters on lactose, lactose supplemented with NaCl and lactate media using automated monitoring on microplates. Generation of biogenic amines and enzymatic activities, protease and lipase, were also evaluated.

Table 1. List of *K. lactis* strains isolated from goats' or ewes' cheese at different ripening weeks.

	Ripening week				
Cheese	0	1	2	3	4
Goat	69-71	72-82	83-85	30, 31, 47-49, 51, 53, 63	45, 54-61, 64, 66-68
Ewe	26-29, 33-36	20-25, 40, 41	9-19	4-8	1- 3

3.1 Genotyping of K. lactis strains

The mitochondrial DNA restriction profile (mtDNA RFLPs) was identical for all 76 *K. lactis* strains investigated (Figure 1). Previous applications of mtDNA RFLPs to study the strain diversity within dairy yeast species such as *D. hansenii*, *Candida zeylanoides* and *Candida kefyr* isolated from the same or related cheese factories found this technique very discriminating even within a small number of strains (Romano et al., 1996; Suzzi et al., 2000). On the contrary a similar study conducted by Mounier et al. (2006) revealed a low intraspecific diversity with one highly

dominant mtDNA RFLP for *D. hansenii*. Moreover, a systematic exploration of *K. lactis* species and other species within the genus *Kluyveromyces* using mtDNA RFLPs indicated a high level of intraspecific mtDNA divergence (Belloch et al., 1997). In the opposite way as expected, the *K. lactis* strains tested here isolated from cheese show a high degree of homogeneity in their mtDNA. However, this result is in agreement with previous studies of electrophoretic karyotyping and sequence of the 5.8S rRNA gene and the two internal transcribed spacers 1 and 2 which were found identical for lactose positive *K. lactis* strains from dairy origin (Belloch et al., 2002).

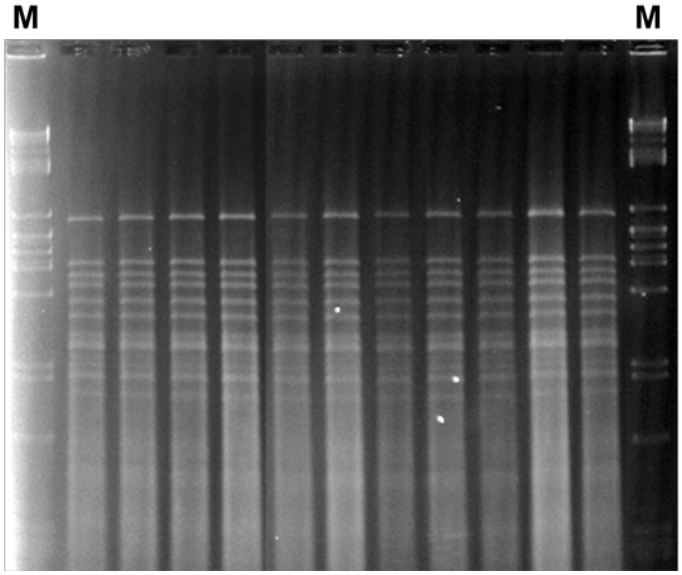
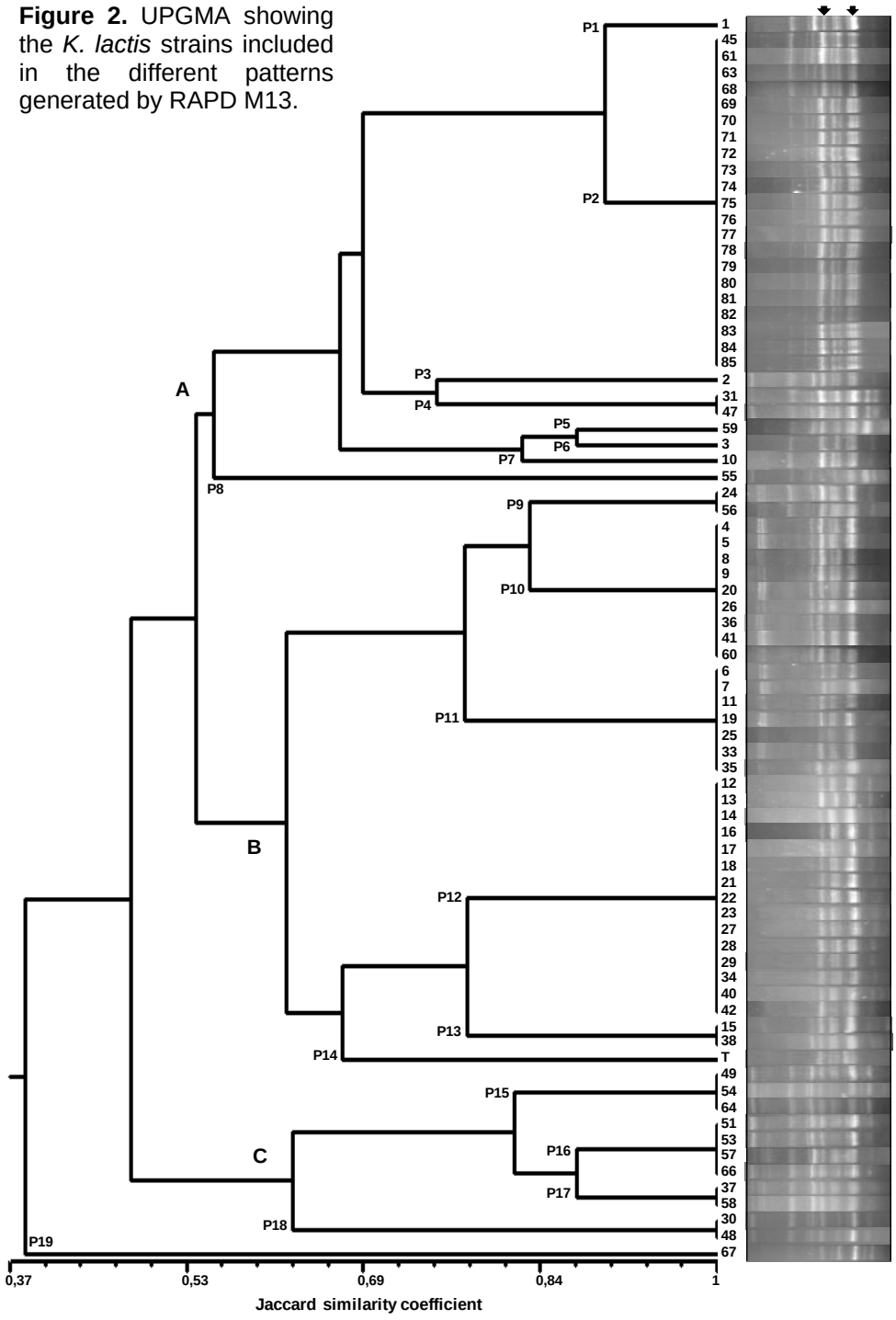


Figure 1. RFLPs of mitochondrial DNA digested with *Hin*fI of several *K. lactis* strains isolated from cheese. M indicates molecular weight marker.

On the contrary, RAPD M13 yielded a large degree of diversity and up to 19 patterns (P1 to P19) were differentiated within the *K. lactis* strains (Figure 2). This technique has been used by several authors to explore the genetic diversity of different yeast species isolated from cheese (Capece and Romano, 2009; Fadda et al., 2004; Fadda et al., 2010; Lopandic et al., 2006) and, in case of *K. lactis* has revealed a similar level of strain diversity (Fadda et al., 2004).

Despite their heterogeneity, all patterns present a common structure consisting of 4 to 5 bands, delimited by black arrows in Figure 2. The UPGMA dendrogram separates three groups (A, B and C) at 55% similarity approximately (Figure 2). Group A is constituted by strains isolated from both types of cheeses, whereas groups B and C include strains isolated mostly from ewes' or goats' cheeses, respectively. No correspondence between RAPD M13 UPGMA groups and cheese ripening weeks was found. Most RAPD M13 patterns were characteristic of *K. lactis* from either goats' or ewes' cheeses, except P9, P10 and P17 which appear in *K. lactis* from both cheese types (Figure 3). Patterns P9 and P17 contain only two strains each, whereas P10 comprises 8 strains from ewes' cheeses and only one strain from goats' cheeses. The patterns containing the largest number of strains are P2 and P12 (Figure 2). Pattern P2 includes solely strains isolated from goats' cheeses along the whole ripening process. Pattern P12 comprises only strains isolated from ewes' cheeses up to the second ripening week (Figure 3).

Figure 2. UPGMA showing the *K. lactis* strains included in the different patterns generated by RAPD M13.



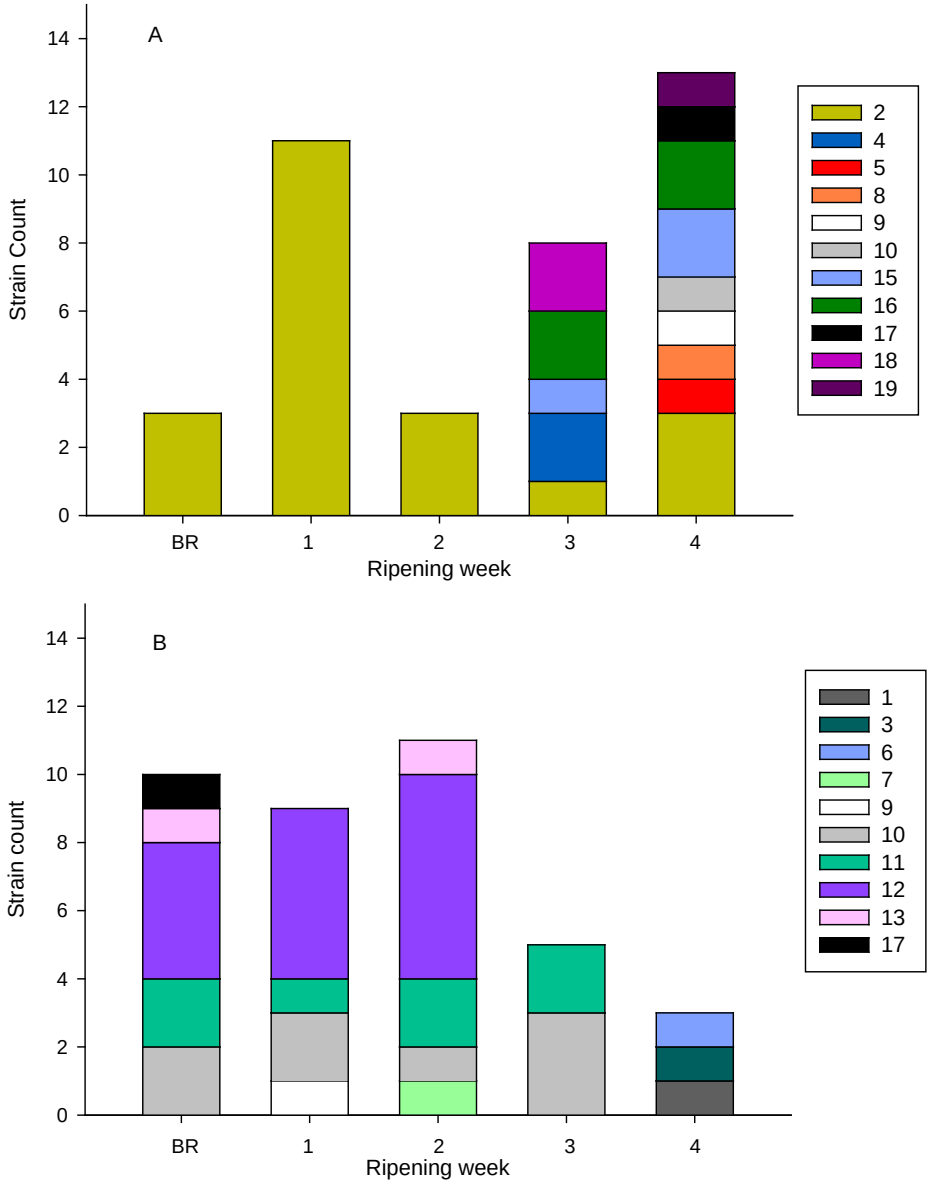


Figure 3. Distribution of RAPD M13 profiles by cheese type and ripening week. BR means before the ripening process started and includes *K. lactis* strains isolated from serum and cheese before the cheese was taken into the maturation chamber.

These results demonstrate the rich heterogeneity of *K. lactis* strains present along the ripening process of either goats' or ewes' cheese. Moreover, some *K. lactis* strains appear at the beginning and remain till the end of the ripening process whereas other genotypes appear in few strains that quickly disappear from the population.

3.2 Lipase and protease activities

Lipolytic and proteolytic activities of yeasts isolated from cheese are considered of technological importance for their positive contribution to cheese fermentation and maturation (Corbo et al., 2001; Roostita and Fleet, 1996; Welthagen and Viljoen, 1998). All *K. lactis* tested were able to hydrolyze casein except strains 24 and 38. On the contrary, very few strains were lipolytic against any of the tested substrates. Strains 7 and 79 were able to hydrolyze Tween 40; whereas strains 85 and, 34 and 35 were able to hydrolyze Tween 60 and 80 respectively. The only *K. lactis* able to hydrolyze all substrates was strain 53. Very few studies have reported proteolysis and lipolysis activities in the species *K. lactis* isolated from cheese. Borelli et al. (2006) and Gardini et al. (2006) tested 6 and 10 strains respectively, but found all of them negative for both enzymatic activities; whereas, Cosentino et al. (2001) reported a low number of strains positive for both activities.

3.3 Production of biogenic amines

Testing of biogenic amines was done solely on 19 strains representing the different genetic groups generated in the RAPD M13 based UPGMA dendrogram. Only strain 69 produced biogenic amines from amino acid ornithine. Several yeasts have been tested for production of

amines from amino acids; however this negative trait has been scarcely found in *K. lactis*, but mostly in strains from the species *D. hansenii* and *Y. lipolytica* as well as several species from the genera *Candida* and *Trichosporon* (Gardini et al., 2006; Suzzi and Gardini, 2003; Wyder et al., 1999).

3.4 Phenotypic profiling of K. lactis

Growth of genetically different *K. lactis* strains was evaluated using a microplate format and automated incubator-reader, in which OD was measured and recorded at selected times during several days. This automated screening of phenotypic profiles has been already proven very useful for large scale screening of *Saccharomyces cerevisiae* and other species within this genus in different cultivation conditions (Liccioli et al., 2011; Salvadó et al., 2011; Warringer and Blomberg, 2003). The slight oxygen limitation affecting the growth of *S. cerevisiae* on microplates described in previous studies might also have a comparable effect on *K. lactis* in spite of being one of the few yeasts able to ferment lactose (Lachance, 2011). The mode of shaking used as well as OD measurements along the recording produced typical growth curves, as observed in a subset of OD data corresponding to *K. lactis* type strain CECT 1961^{T} growing L4, L4S2 and L4S5 represented in Figure 4. Comparison of growth on lactose and NaCl containing lactose media reveals salt dependence; the higher the salinity, the lower the maximum OD and the longer the lag phase (Figure 4) as already reported by other authors (Warringer and Blomberg, 2003). Moreover, the increase of the lag phase duration seemed to overcome our experimental conditions at the highest NaCl concentration, 8%, because no strain growth could be observed after 5 incubation days. Growth on lactate 2% media was recorded for 5 days, but a steady

increase of the OD values was not observed in case of CECT 1961^T or any cheese isolated *K. lactis* strain. Growth of *K. lactis* type strain on lactate has been described as positive when tested on solid media for 21 days incubation (Lachance, 2011). The short incubation time used in our experiments might have prevented observation of strain growth due to longer duration of the lag phase. Additionally, growth on lactate could also be delayed due to limited access to oxygen in the microplates as already observed in previous studies (Liccioli et al., 2011; Warringer and Blomberg, 2003).

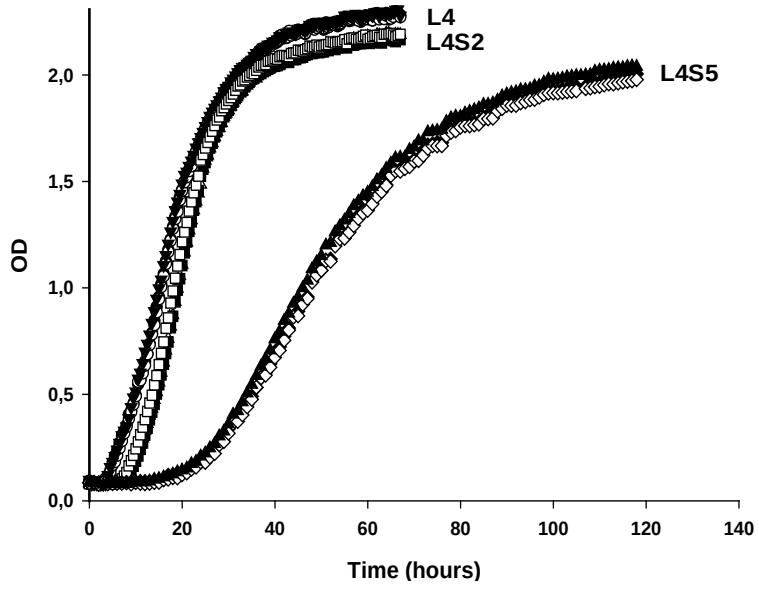


Figure 4. Growth curves of *K. lactis* CECT 1961^T growing on lactose (L4) and lactose supplemented with 2% (L4 2S) and 5% (L4 S5) NaCl.

Table 2 shows growth parameters of *K. lactis* strains. Generation time of *K. lactis* strains growing on L4 medium ranged from 4.90 h for strain 6 to 7.28 h for strain 69. ANOVA analysis indicated that significant differences could be found among the strains ($p<0.05$) and Fisher's LSD analysis separated the strains into 6 different groups. Generation time on L4S2 or L4S5 underwent a significant delay in most strains respect to L4, as indicated by ANOVA analysis ($p<0.05$). The average delay in generation time for strains growing on L4S2 and L4S5 respect to L4 was about 32% and 180%, respectively. Only strains 3, 48, 57 and CECT 1961^T showed no significant differences in generation time when comparing L4 and L4S2. Generation time of *K. lactis* growing on L4S2 ranged from 6.25 h for strain 3 to 13.53 h for strain 69. Similarly on L4S5, generation time stretched from 9.79 h (strain 47) to 25.00 h (strain 69). As can be seen in Table 2, strain 69 displayed the longest generation time growing in any medium.

In NaCl containing media, ANOVA analysis indicated significant differences between averages of generation times of strains. Fisher's LSD test separated the strains into 8 groups in case of L4S2 and 11 groups in case of L4S5. This increase in the number of Fisher's LSD groups respect to L4 indicates that the differences in the generation times increase among the *K. lactis* strains. This might suggest that the *K. lactis* strains are differently affected by the presence of 5% salt in the culture media.

Table 2. Growth data from lactose 4% (L4), lactose 4% and salt 2% (L4 S2) and lactose 4% and salt 5% (L4 S5). Data are the mean of triplicates for each strain under each condition.

Strain	Generation Time (hours)			Maximum OD			Lag phase (hours)		
	L4	L4 S2	L4 S5	L4	L4 S2	L4 S5	L4	L4 S2	L4 S5
1	5.057^{ef}	6.580^{gh}	17.305^{c}	2.425^{g}	2.047^{cdefg}	1.358^{f}	6.75^{cd} *	7.92^{gh} *	83.00^{a}
2	5.657^{cd}	7.115^{defg}	16.034^{cdef}	2.480^{ef}	2.062^{bcdefg}	1.834^{bc}	6.42^{de}	9.08^{defgh}	41.50^{c}
3	5.264^{def}*	6.251^{h}*	14.198^{ghi}	2.504^{def}	2.118^{abc}	1.869^{b}	4.42^{k}	9.08^{defgh}	42.00^{c}
5	5.644^{cd}	7.074^{defg}	16.026^{cdef}	2.528^{bcde}	2.010^{fg}	1.576^{de}	4.75^{jk}	10.42^{d}	43.83^{c}
6	4.899^{f}	6.548^{gh}	15.997^{cdef}	2.423^{g}	2.092^{bcde}	1.590^{de}	5.42^{ghij}	10.25^{de}	73.50^{b}
10	5.508^{cde}	7.367^{cdef}	13.196^{ij}	2.571^{abc}	2.117^{abcd}	1.710^{cd}	5.08^{hijk}	12.50^{c}	41.67^{c}
24	5.741^{c}	6.769^{fgh}	16.795^{cde}	2.535^{abcd}	2.061^{bcdefg}	1.873^{b}	6.92^{bcd} *	8.58^{fgh} *	29.83^{d}
29	5.794^{c}	7.782^{c}	15.321^{efgh}	2.519^{cde}	2.045^{efg}	1.747^{bc}	7.58^{b}	16.42^{b}	47.00^{c}
37	5.744^{c}	7.124^{defg}	16.805^{cde}	2.499^{def}	2.079^{bcdef}	1.780^{bc}	5.25^{ghij}	8.83^{efgh}	44.50^{c}
38	5.061^{ef}	7.017^{efg}	14.280^{ghi}	2.561^{abc}	2.112^{abcde}	1.788^{bc}	5.25^{ghij}	10.08^{def}	25.50^{d}
47	5.478^{cde}	8.511^{b}	9.786^{k}	2.528^{bcde}	2.010^{fg}	1.795^{bc}	7.42^{bc}	19.17^{a}	24.83^{d}
48	5.218^{def}*	6.798^{fgh}*	14.798^{fgh}	2.462^{fg}	2.003^{g}	1.456^{ef}	5.92^{efg}	9.42^{defg}	85.83^{a}
54	5.638^{cd}	7.341^{cdef}	19.470^{b}	2.548^{abcd}	2.045^{defg}	1.812^{bc}	5.58^{fghi}	9.25^{defg}	46.25^{c}
55	5.516^{cde}	7.801^{c}	16.887^{cd}	2.576^{ab}	2.121^{ab}	1.846^{bc}	4.92^{ijk}	13.25^{c}	29.25^{d}
57	6.595^{b}*	6.582^{gh}*	15.185^{fgh}	2.583^{a}	2.086^{bcde}	1.802^{bc}	5.75^{efgh} *	7.58^{h} *	42.50^{c}
59	5.492^{cde}	7.664^{cd}	15.571^{defg}	2.558^{abc}	2.103^{bcde}	1.754^{bc}	5.75^{efgh}	13.42^{c}	83.50^{a}
67	5.638^{cd}	7.514^{cde}	12.582^{j}	2.536^{abcd}	2.116^{abcde}	1.811^{bc}	6.25^{def}	12.67^{c}	28.00^{d}
69	7.279^{a}	13.529^{a}	25.005^{a}	2.066^{i}	0.977^{h}	0.688^{g}	14.25^{a}	15.75^{b}	82.00^{a}
1961 ^{T}	5.921^{c}*	6.319^{h}*	14.002^{hij}	2.312^{h}	2.183^{a}	2.067^{a}	5.25^{ghij}	9.58^{def}	28.17^{d}

Superindexes indicate significant differences between strains ($p<0.05$) growing under the same culture media. Asterisk * indicates not significant differences ($P>0.05$) between strains growing under different culture media. The standard deviation for generation time and maximum OD was inferior to 12% and for lag phase inferior to 20%.

Maximum OD was above 2 for all strains in case of L4 and L4S2 except for strain 69 which maximum OD was below 1 in L4S2. Addition of 5% NaCl generated a broader reduction of the maximum OD which decreased below 2 except in case of CECT 1961^T. ANOVA analysis indicated significant differences among strains between L4 and L4S2 or L4S5. Strains 3, 48, 57 and CECT 1961^T, which had showed no significant differences in their generation time between L4 and L4S2 media, showed a significant decrease in their maximum OD when lactose was supplemented with salt. This would indicate that maximum OD is independent from generation time as already observed by Warringer and Blomberg (2003). Other studies about the effect of salt on the growth rate (directly related to generation time) of yeasts revealed a decrease in this parameter with increasing NaCl levels (Held, 2010).

Estimation of lag phase was not very accurate. The coefficient of variation increased above 15% in most cases, as already observed by Warringer and Blomberg (2003). Duration of lag phase in L4 experiments ranged from 4.92 h for strain 3 to 15.08 h for strain 69. ANOVA analysis indicated that significant differences could be found among the strains ($p<0.05$) and Fisher's LSD analysis separated the strains into 11 different groups. Lag phase on L4S2 and L4S5 underwent a significant delay in most strains as indicated by ANOVA analysis ($p<0.05$). Only strains 1, 24, 57 and 69 showed no significant differences in lag phase when comparing L4 and L4S2. Lag phase of *K. lactis* growing on L4S2 ranged from 7.75 h for strain 37 to 15.25 h for strain 69. Similarly on L4S5 lag phase stretched from 28.50 h (strain 10) to more than 72 h (strains 1, 6, 48, 59 and 69). Strains 55 and 48 showed the greatest increments in lag phase when comparing L4, L4S2 and L4S5.

There are few studies monitoring the growth of yeasts using microplates and in all of them *Saccharomyces* has been the main research subject; nevertheless, all these studies conclude that this methodology is suitable for testing a large number of yeast strains growing in different conditions (Liccioli et al., 2011; Salvadó et al., 2011; Warringer and Blomberg, 2003). In our study this methodology was used to generate phenotypic profiles of genetically different *K. lactis* growing on different medium containing lactose, lactose supplemented with NaCl and lactate. Our results indicate that *K. lactis* was able to grow on microplates generating reproducible and reliable data when using a methodology previously established for *Saccharomyces*. Moreover, the phenotypic analysis displayed strain differences in response to salt suggesting that generation time, maximum OD and duration of lag phase are parameters to take into account for further strain selection. Respect to generation of biogenic amines, only strain 69, the slowest yeast growing at any culture media was able to decarboxylate ornithine. Finally this study also confirms the scarce lipolytic activity of *K. lactis* strains although the proteolytic activity seems to be substantial in most of the strains.

4. Acknowledgements

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Objective III

Synthesis of relevant prebiotic oligosaccharides from lactose and lactulose using *Kluyveromyces lactis* and *Kluyveromyces marxianus* β-galactosidases.

Evaluation of oligosaccharide synthesis from lactose and lactulose using β-galactosidases from *Kluyveromyces* isolated from artisanal cheeses.

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ABSTRACT

The β-galactosidase activity of fifteen *Kluyveromyces* strains isolated from cheese belonging to *K. lactis* and *K. marxianus* species was tested for the production of oligosaccharides derived from lactose (GOS) and lactulose (OsLu). All *Kluyveromyces* crude cell extracts (CCEs) produced GOS such as 6-galactobiose, 3'-, 4'- and 6'-galactosyl-lactose. At 4 h of reaction, the main trisaccharide formed was 6´-galactosyl-lactose (20 g/100g of total carbohydrates). The formation of OsLu was also observed by all CCEs tested, 6-galactobiose, 6'-galactosyl-lactulose and 1- galactosyl-lactulose being found in all the reactions mixtures. The synthesis of trisaccharides predominated over other oligosaccharides. *K. marxianus* strain O3 produced the highest yields of GOS and OsLu after 4 h of reaction reaching 42 g/100g of total carbohydrates (corresponding to 80% lactose hydrolysis) and 45 g/100g of total carbohydrates (corresponding to 87% lactulose hydrolysis), respectively. Therefore, the present study contributes to a better insight into dairy *Kluyveromyces* β-galactosidases and shows the feasibility of these enzymes to transglycosylate lactose and lactulose producing high yields of prebiotic oligosaccharides.

Keywords: *Kluyveromyces lactis*, *Kluyveromyces marxianus*, transgalactosylation, GOS, OsLu, lactose, lactulose.

1. Introduction

β-Galactosidase (EC 3.2.1.23) is a hydrolase that attacks the terminal non-reducing β-D-galactosyl residues of oligosaccharides and transfers the galactosyl moiety to suitable acceptors. These enzymes have several applications in the food fermentation and dairy industries and, mainly due to their ability to hydrolyse lactose they have attracted the attention of researchers and dairy product manufacturers (Adam et al., 2004). Transgalactosylation is favored over hydrolysis in presence of high substrate concentrations, and in the case of lactose, β-galactosidases produce galactooligosaccharides (GOS) (Boon et al., 2000). GOS are mainly disaccharides (allolactose and galactobiose) and trisaccharides (4'- and 6'-galactosyl-lactose), and longer chain oligosaccharides consisting of four or more monosaccharide units (Mussatto and Mancilha, 2007).

Although transgalactosylation of lactose has been known for more than 50 years (Aronson, 1952), GOS production is gaining importance since their recognition as prebiotics (van Loo et al., 1999). Moreover the influence of GOS structure on prebiotic selectivity has been demonstrated (Gosling et al., 2010). Other health benefits such as improvement of mineral absorption, prevention of intestinal infections and enhancement of immune function among others have been described (Arslanoglu et al., 2008; Ebersbach et al., 2010; Pérez-Conesa et al., 2006; Vulevic et al., 2008).

Recently, the synthetic disaccharide lactulose (4-O-*β*-D-galactopiranosyl-D-fructose) has been proposed as an enzymatic substrate for lactulose-derived oligosaccharide (OsLu) production (Cardelle-Cobas et al., 2008b; Cardelle-Cobas et al., 2011a; Martínez-Villaluenga et al., 2008b). Although lactulose has been recognized as prebiotic (Méndez and Olano, 1979; Rycroft et al., 2001), gas production associated with its fermentation in the proximal colon may represent a disadvantage for

lactulose ingestion (Tuohy et al., 2002). In this context, synthesis of OsLu may provide a new group of active compounds with health beneficial effects complementary to those provided by GOS (Olano and Corzo, 2009) and probably, without the inconvenient of lactulose consumption.

Nowadays, microbial β-galactosidases represent a feasible alternative to chemical synthesis of GOS with the benefits of enzymatic stereospecificity and higher final yields. β-Galactosidases have been frequently characterized in lactic acid bacteria and bifidobacteria related to milk, milk products and the intestine of neonates (Osman et al., 2011; Schwab et al., 2011). The genus *Kluyveromyces* and specifically the species *Kluyveromyces lactis* has received considerable attention both as a genetic model and industrial yeast as a source of different metabolites and enzymes (Pariza and Johnson, 2001). Similarly, the species *Kluyveromyces marxianus* has been explored due to its potential biotechnological applications, although the accumulated knowledge on *K. marxianus* is much smaller compared to that on *K. lactis* (Fonseca et al., 2008). Both species, present in dairy products, are considered GRAS (Generally Recognized As Safe) microorganisms and present a good growth yield and a higher β-galactosidase activity compared with other yeasts (Kaur et al., 2009). Thus, both species are relevant industrial sources of β-galactosidase activity, and they have been traditionally used to produce low-lactose products and for the biological treatment of cheese whey waste. With respect to oligosaccharide synthesis, lactose and to a lesser extent lactulose transgalactosylation by *K. lactis* commercial enzymatic preparations has been evaluated (Cardelle-Cobas et al., 2011a; Martínez-Villaluenga et al., 2008a; Rodríguez-Fernández et al., 2011; Rodríguez-Colinas et al., 2011), whereas *K. marxianus* β-galactosidases have been only recently tested for lactose transgalactosylation (Manera et al., 2010; Petrova and Kujumdzieva, 2010).

Previous studies have demonstrated that the transgalactosylation to hydrolysis ratio varies depending on the different sources of β-galactosidase, and that different enzymes can achieve different degrees of transgalactosylation leading to variations in the level and composition of synthesized GOS (Otieno, 2010). However, there is little information about the feasibility of food-isolated *Kluyveromyces* strains with potentially different metabolic characteristics to transgalactosylate different substrates.

The aim of the present work was to evaluate the β-galactosidase activity from different strains of *K. lactis* and *K. marxianus* isolated from artisanal cheeses and to screen their potential to produce GOS and OsLu by hydrolysis and transgalactosylation of lactose and lactulose, respectively.

2. Materials and methods

2.1 Chemicals

Lactose was obtained from Scharlau (Barcelona, Spain). Lactulose, D-glucose, raffinose, D-fructose and o-nitrophenyl β-D-galactopyranoside (oNPG) were purchased from Sigma-Aldrich Co (Steinheim, Germany). D-Galactose was acquired from Fluka (Steinheim, Germany). D-Glucose and lactose for yeast culture media were obtained from Panreac (Barcelona, Spain), bacteriological peptone was purchased from Cultimed (Barcelona, Spain) and yeast extract and agar were acquired from Pronadisa (Madrid, Spain).

2.2 Yeast strains

Fifteen yeast strains belonging to *K. lactis* and *K. marxianus* species were isolated from artisanal ewes' and goats' milk cheeses produced in Cheese Company "Los Corrales" from rural Castelló province (Spain). *K. lactis* CECT 1961^T was obtained from the Spanish Type Culture Collection and was included in the study as control. Isolation sources are shown in Table 1.

2.3 Kluyveromyces crude cell extracts (CCEs)

Yeasts were grown overnight in medium GPY (glucose 2%, peptone 0.5% and yeast extract 0.5%) at 28°C. Afterwards, yeast cells were transferred to LPY medium (lactose 2%, peptone 0.5% and yeast extract 0.5%) and incubated overnight at 28°C. For preparation of CCEs, cells were resuspended in 50 mM potassium phosphate pH 6.5 with 1mM $MgCl_2$ and disrupted with glass beads (0.5 mm) in a Bead-Beater Cell

Disrupter (Model 1107900, Bio Spec Products Inc, Bartlesville, OK). Disruption was achieved at 4°C by subjecting the cells to three bursts of 45 s with resting periods of 5 min. The resulting homogenates were centrifuged at 5000 × g for 20 min at 4°C and the supernatants, considered as CCEs, kept at -20°C until further analysis.

2.4 Determination of β-galactosidase activity and protein content

β-Galactosidase activity from CCEs was quantified using oNPG as substrate according to Martínez-Villaluenga et al. (2008a). One enzyme unit is defined as the amount of enzyme releasing 1 µmol of oNP per mL per minute at 40°C and pH 6.5. Activity against oNPG was used to adjust the activity of the different CCEs (6 U/mL) for transgalactosylation reaction (see below). Protein content of CCEs was determined using the Bradford assay with bovine serum albumin as standard (Bradford, 1976).

2.5 Synthesis of oligosaccharides derived from lactose (GOS) and lactulose (OsLu)

Production of lactose- and lactulose–derived oligosaccharides were carried out using 250 g/L of substrate in 0.1 M phosphate buffer pH 6.5 and 6 U/mL β-galactosidase activity during incubation at 50°C up to 24 h, as described in Cardelle-Cobas et al. (2011a) and Martínez-Villaluenga et al. (2008a). Lactose and lactulose solutions were heated before the enzyme extract was added and were maintained at the required temperature throughout the experiment. Reactions were performed in individual Eppendorf tubes and incubated in an orbital shaker at 400 rpm. Samples of 200 µL were withdrawn from the reaction mixtures at 0, 2, 4, 6 and 24 h and immediately immersed in boiling water for 5 min to inactivate the

enzyme. After appropriate dilution, 20 µL were injected into the chromatograph described below. Control samples were prepared in the same manner except no CCE was added. All experiments were performed in duplicate.

2.6 Chromatographic determination of carbohydrates

GOS and OsLu were determined by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) in a ICS2500 Dionex system (Dionex Corp., Sunnyvale, CA, USA) consisting of a GP50 gradient pump and ED50 electrochemical detector with a gold working electrode and Ag/AgCl reference electrode. Data were acquired and processed with Chromeleon 6.7 software (Dionex Corp.). Separations were performed on a CarboPac PA-1 column (250 × 4 mm) connected to a CarboPac PA-1 (50 × 4 mm) guard column following the method described by Splechtna et al. (2006). Detection time and voltage parameters were set as follows: E_1=0.1V (t_1=400ms), E_2= 2.0V (t_2=10ms), E_3=0.6V, E_4=-0.1V (t_4=60ms); t_t=500ms. Samples and standard solutions were filtered through a nylon Millipore FH membrane (0.22 µm) (Bedford, MA) before injection. Quantification of carbohydrates was performed by external calibration using standard solutions of galactose, fructose, lactose, lactulose and raffinose. The regression coefficients of the curves for each standard were always greater than 0.99. The amount of lactose or lactulose remaining and the yield of GOS and OsLu were expressed as g/100g of the total carbohydrate content in the reaction mixtures.

2.7 Statistical analysis

Bonferroni test was used for mean comparison at 95% confidence level (StatGraphics Plus 5.1, StatPoint, Herndon, VA).

3. Results and Discussion

3.1 β-Galactosidase activity

As shown in Table 1, all CCEs hydrolysed oNPG. *K. lactis* CCEs showed higher oNPG hydrolysis than the two *K. marxianus* CCEs. *K. lactis* BP4 showed the highest β-galactosidase activity (13.1 U/mg), followed by the reference strain CECT 1961^T (11.9 U/mg) whereas *K. marxianus* strains showed values of 1.3 and 1.6 U/mg.

Table 1. β-Galactosidase activity against *o*NPG of yeast species CCEs screened for oligosaccharide production.

Species	Strain[a]	Isolation source	Specific activity (U/mg)
K. lactis	CECT 1961^T	Gassy cheese, UK	11.8
	BP1	Ewes' milk cheese whey	3.5
	BP2	Ewes' milk cheese	5.8
	BP3	Ewes' milk cheese	7.4
	BP4	Ewes' milk cheese	13.1
	BP5	Ewes' milk cheese	3.9
	BP6	Ewes' milk cheese	4.6
	BP7	Ewes' milk cheese	3.3
	BP8	Ewes' milk cheese	4.8
	O1	Ewes' milk cheese	4.2
	O2	Ewes' milk cheese	2.7
	C1	Goats' milk cheese	3.4
	C2	Goats' milk cheese	3.0
K. marxianus	O3	Ewes' milk cheese	1.3
	O4	Ewes' milk cheese whey	1.6

[a]All yeast strains, except CECT 1961^T, were isolated from Spanish cheeses.

3.2 Synthesis of oligosaccharides derived from lactose (GOS)

Lactose transgalactosylation by yeast CCEs was followed by HPAEC-PAD. Under the conditions tested (pH 6.5, 50°C, 250 g/L lactose, 6 U/mL of β-galactosidase activity), which were previously optimized in the laboratory for a *K. lactis* commercial preparation (Martínez-Villaluenga et al., 2008a), GOS production from lactose was compared after 4 h reaction. All yeast CCEs synthesized galactosyl derivatives of lactose showing similar chromatographic profiles of GOS production. A representative chromatogram corresponding to lactose transgalactosylation catalysed by *K. marxianus* O3 CCE after 4h reaction is shown in Figure 1A. The peak 1+2 corresponded to co-eluting galactose and glucose whereas peak 5+6 was assigned to lactose and allolactose. Peak 4 was identified as 6-galactobiose; peak 9 corresponded to trisaccharide 6'-galactosyl-lactose and peaks 12 and 13 corresponded to 4'-galactosyl-lactose and 3'-galactosyl-lactose, respectively. These assignments were made by comparing relative retention times with those found in previous studies (Cardelle-Cobas et al., 2008a). Unidentified di- or trisaccharides as well as high retention time oligosaccharides (peaks marked with asterisk) were also detected. Table 2 summarizes GOS yields after 4 h reaction for all yeast β-galactosidases studied. Total GOS yields ranged approximately from 26 to 42 g/100g of total carbohydrates, in agreement with the range described for other microbial β-galactosidases (Otieno, 2010). Moreover, in a previous work, total GOS yields of approximately 30 g/100g of total carbohydrates were obtained with the commercial *K. lactis* preparation (Martínez-Villaluenga et al., 2008a) what it is in agreement with the total GOS yields found with most of the cheese-isolated yeast strains evaluated in the present work. Recently, a maximum GOS yield of 44 g/100g of total carbohydrates using 400 g/L of lactose and permeabilized *K. lactis* cells has been reported (Rodríguez-Colinas et al., 2011).

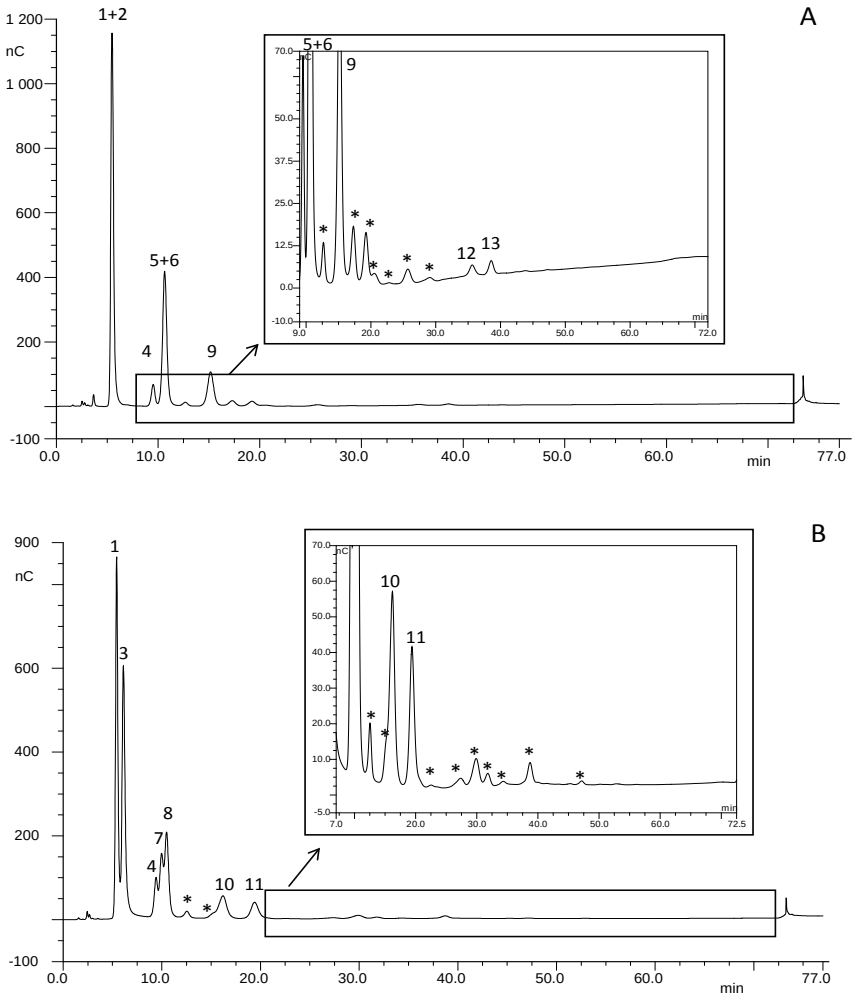

Figure 1. HPAEC-PAD profiles of carbohydrate mixtures obtained by enzymatic hydrolysis of lactose (A) and lactulose (B) by *K. marxianus* O3 β-galactosidase. (A) Compounds: 1, galactose; 2, glucose; 4, 6-galactobiose; 5, allolactose; 6, lactose; 9, 6'-galactosyl-lactose; 12, 4'-galactosyl-lactose; 13, 3'-galactosyl-lactose; *, other oligosaccharides. (B) Compounds: 1, galactose; 3, fructose; 4, 6-galactobiose; 7, allolactulose; 8, lactulose; 10, 6'-galactosyl-lactulose; 11, 1'-galactosyl-lactulose; *, other oligosaccharides.

Table 2. Carbohydrate composition (g/100g of total carbohydrates) of the reaction mixtures during lactose hydrolysis (4 h).

Strain	Monosaccharides	Lactose + allolactose	6-Galactobiose	6'-Galactosyl-lactose	Other GOS*	Total GOS**
CECT 1961^{T}	53.69 ± 2.18 ^{bc}	13.95 ± 0.45 ^{e}	5.63 ± 0.33 ^{c}	16.92 ± 0.84 ^{e}	8.82 ± 0.45 ^{ab}	32.36 ± 1.74 ^{cd}
BP1	59.38 ± 1.86 ^{de}	10.41 ± 0.55 ^{cd}	5.40 ± 0.19 ^{abc}	14.37 ± 0.82 ^{bcd}	9.44 ± 0.30 ^{abcd}	30.21 ± 1.31 ^{bc}
BP2	54.57 ± 0.18 ^{bc}	13.92 ± 0.20 ^{e}	5.29 ± 0.00 ^{abc}	16.01 ± 0.04 ^{de}	9.21 ± 0.09 ^{abc}	31.51 ± 0.02 ^{c}
BP3	55.54 ± 1.03 ^{cd}	13.08 ± 0.38 ^{e}	5.27 ± 0.04 ^{abc}	15.64 ± 0.48 ^{de}	9.48 ± 0.10 ^{abcd}	31.39 ± 0.65 ^{c}
BP4	66.27 ± 0.50 ^{f}	8.12 ± 0.26 ^{a}	4.92 ± 0.07 ^{a}	11.82 ± 0.33 ^{a}	8.03 ± 0.16 ^{a}	25.61 ± 0.24 ^{a}
BP5	63.03 ± 0.95 ^{ef}	9.12 ± 0.17 ^{abcd}	5.09 ± 0.04 ^{abc}	13.53 ± 0.23 ^{abc}	8.33 ± 0.51 ^{a}	27.85 ± 0.78 ^{ab}
BP6	62.68 ± 1.42 ^{ef}	9.90 ± 0.84 ^{bcd}	5.01 ± 0.15 ^{ab}	13.40 ± 0.44 ^{abc}	8.17 ± 0.11 ^{a}	27.41 ± 0.58 ^{ab}
BP7	57.33 ± 0.46 ^{cd}	10.66 ± 0.13 ^{d}	5.57 ± 0.06 ^{bc}	15.14 ± 0.17 ^{cde}	10.29 ± 0.10 ^{bcd}	32.00 ± 0.32 ^{cd}
BP8	59.51 ± 0.88 ^{de}	10.42 ± 0.18 ^{cd}	5.32 ± 0.26 ^{abc}	14.55 ± 0.37 ^{cd}	9.24 ± 0.05 ^{abc}	30.07 ± 0.70 ^{bc}
O1	65.21 ± 0.64 ^{f}	8.52 ± 0.03 ^{ab}	5.32 ± 0.11 ^{ab}	12.01 ± 0.14 ^{a}	8.42 ± 0.40 ^{a}	26.26 ± 0.67 ^{a}
O2	57.67 ± 0.05 ^{cd}	10.59 ± 0.14 ^{d}	5.16 ± 0.09 ^{abc}	15.49 ± 0.40 ^{de}	10.15 ± 0.25 ^{bcd}	31.73 ± 0.19 ^{cd}
C1	57.83 ± 0.66 ^{cd}	10.28 ± 0.02 ^{cd}	5.45 ± 0.07 ^{abc}	14.70 ± 0.25 ^{cd}	10.73 ± 0.30 ^{cd}	31.90 ± 0.64 ^{cd}
C2	63.34 ± 0.48 ^{ef}	8.81 ± 0.13 ^{abc}	5.05 ± 0.03 ^{abc}	12.64 ± 0.15 ^{ab}	9.26 ± 0.15 ^{abc}	27.85 ± 0.35 ^{ab}
O3	40.54 ± 0.14 ^{a}	17.65 ± 0.82 ^{f}	4.90 ± 0.12 ^{a}	20.74 ± 0.39 ^{f}	15.15 ± 0.95 ^{e}	41.81 ± 0.67 ^{e}
O4	50.58 ± 0.56 ^{b}	14.42 ± 0.09 ^{e}	5.48 ± 0.03 ^{abc}	16.57 ± 0.05 ^{e}	10.96 ± 0.46 ^{d}	35.01 ± 0.47 ^{d}

Different letters indicate significant differences for carbohydrate group (Bonferroni test, $p < 0.05$).
* 3'- and 4'-galactosyl-lactose are included.
**These values include 6-galactobiose, 6'-galactosyl-lactose and other GOS.

K. marxianus O3 CCE stood out as the best GOS producer (42 g/100g of total carbohydrates from 250 g/L lactose) but also *K. marxianus* O4 and *K. lactis* CECT 1961^{T}, BP7, O2 and C1 CCEs were good GOS producers as indicated by Bonferroni test. Remarkably the yield obtained with *K. marxianus* O3 β-galactosidase was more than two fold higher than that described using permeabilized cells of *K. marxianus* at an initial lactose concentration of 500 g/L (Manera et al., 2010). Some *K. marxianus* strains were also pointed out by Petrova and Kujumdzieva (2010) as the most effective strains in GOS production among yeast species isolated from dairy products.

For all reactions, the main GOS product was the trisaccharide 6'-galactosyl-lactose (12-21 g/100g of total carbohydrates yield), being the best producers *K. marxianus* O3 and O4 and *K. lactis* CECT 1961^{T} CCEs. Maximum 6-galactobiose yields corresponded to *K. lactis* CECT 1961^{T} and BP7 CCEs whereas the lowest levels were formed by *K. marxianus* O3 CCE. Both oligosaccharides were also the main GOS described for *Aspergillus aculeatus* β-galactosidase (Cardelle-Cobas et al., 2008a) and for *K. lactis* commercial enzyme (Martínez-Villaluenga et al., 2008a). With respect to other GOS (including 4'- and 3'-galactosyl-lactose), yields ranged from 8 to 15 g/100g of total carbohydrates, being *K. marxianus* O3 CCE the best producer. Although *K. marxianus* β-galactosidases with enhanced transgalactosylation activity have been recently described (Manera et al., 2010; Petrova and Kujumdzieva, 2010) individual oligosaccharides formed were not identified.

Time course (up to 24 h) of lactose hydrolysis and GOS production was evaluated for β-galactosidase extracts from the best GOS producers, *K. marxianus* O3 and O4 and *K. lactis* BP7 and CECT 1961^T (Figure 2). Lactose was rapidly hydrolyzed (panel A) to monosaccharides (glucose and galactose) (panel B) which levels increased along the reaction time. *K. marxianus* O3 β-galactosidase hydrolyzed lactose to a lesser extent (90 g/100g of total carbohydrates) than the rest of CCEs which almost completely hydrolysed lactose (around 3 g/100g of total carbohydrates remaining). As a general trend the production of 6'-galactosyl-lactose (panel D) reached a maximum value (15-20 g/100g of total carbohydrates) after 2 h of reaction, the level of remaining lactose being 20-28 g/100g of total carbohydrates. In contrast, the formation of 6-galactobiose (panel C) reached maximum yields after 4 hours (remaining lactose of 10-18 g/100g of total carbohydrates), except for *K. marxianus* O3 β-galactosidase, which reached its optimal time production after 24 h (remaining lactose around 11 g/100g of total carbohydrates). Maximum production of other GOS (panel E) were reached after 2 (*K. lactis* BP7 CCE) or 4 h of reaction (*K. lactis* CECT 1961^T and *K. marxianus* O3 and O4 CCEs). The same pattern was observed for maximum formation of total GOS (panel F). Total GOS production decreased slightly from 4 to 24 h of incubation for *K. marxianus* O3 CCE whereas for the rest of β-galactosidases tested a higher GOS hydrolysis along the incubation time was observed. After 24 h of incubation *K. marxianus* O3 CCE stood out as the best GOS producer (40 g/100g of total carbohydrates).

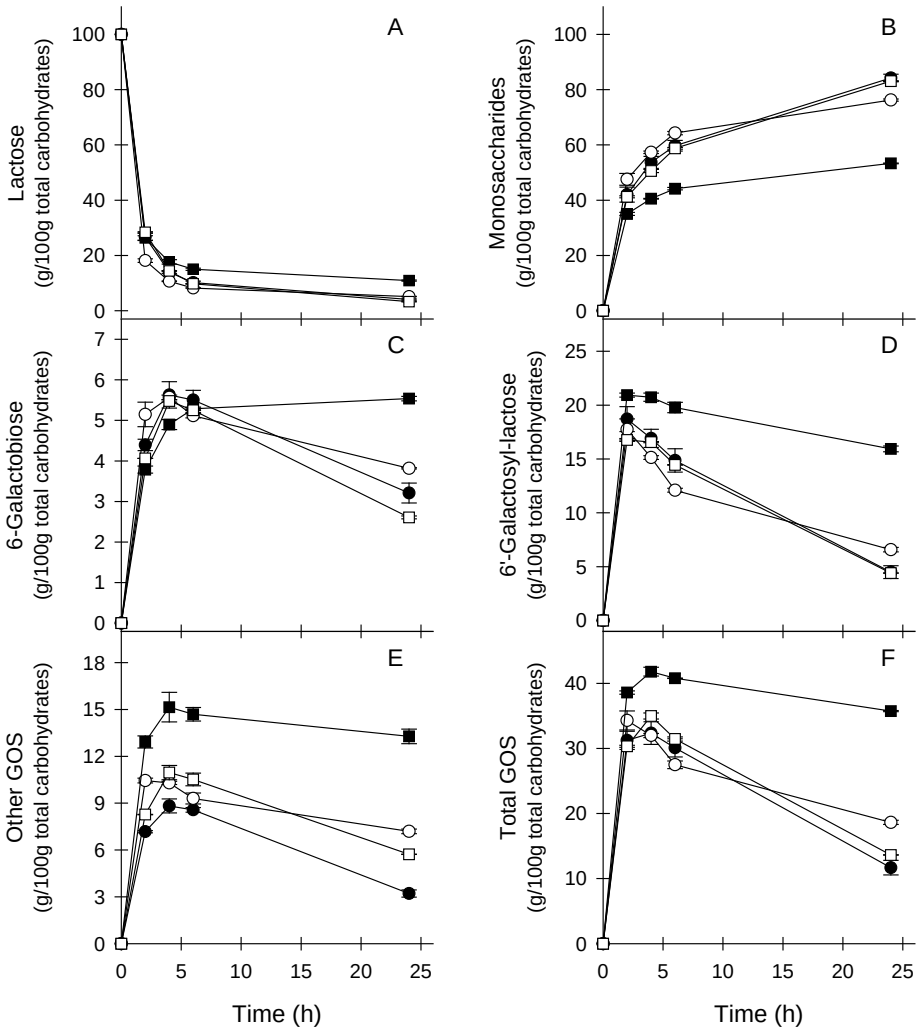

Figure 2. Carbohydrate yields during lactose hydrolysis by selected yeast β-galactosidases. ●, *K. lactis* CECT 1961^{T}; ○, *K. lactis* BP7; ■, *K. marxianus* O3; □, *K. marxianus* O4. Error bars indicate standard deviations.

3.3 Synthesis of oligosaccharides derived from lactulose (OsLu)

Once the ability of CCEs to produce GOS by lactose transgalactosylation was tested, the time course (up to 24 h) of lactulose transgalactosylation by β-galactosidases from CECT 1961^T, BP7, O3 and O4 was also evaluated. In a previous study of our research group, transgalactosylation of lactulose with β-galactosidases from commercial *K. lactis* and *A. aculeatus* preparations was studied and new structures such as the trisaccharides 6'galactosyl-lactulose and 1-galactosyl-lactulose were characterized (Cardelle-Cobas et al., 2008b; Martínez-Villaluenga et al., 2008b). In the present work under the experimental conditions used (pH 6.5, 50°C, 250 g/L lactulose and 6 U/mL of β-galactosidase activity) (Martínez-Villaluenga et al., 2008b), the HPAEC-PAD analysis of reaction mixtures showed that all CCEs produced galactosyl-derivatives from lactulose. Similarly to GOS formation, products obtained from lactulose were the same in all reactions. Figure 1B shows a representative recording of the HPAEC-PAD profiles of the products formed by *K. marxianus* O3 CCE after 24 h. Peaks 1, 3, 7 and 8 were assigned to galactose, fructose, allolactulose, and lactulose, respectively. Peak 4 was identified as the disaccharide 6-galactobiose while peaks 10 and 11 were assigned to the trisaccharides 6'-galactosyl-lactulose and 1-galactosyl-lactulose, respectively (Cardelle-Cobas et al., 2008b). Two unidentified di- or trisaccharides (retention times = 12.5 min and 14.8 min) and other high retention time oligosaccharides (peaks marked with asterisk) were also detected.

Figure 3 shows the time course of lactulose conversion and OsLu synthesis for the yeast β-galactosidases. Lactulose (panel A) was hydrolyzed to galactose (panel B) and fructose (panel C). Comparison of Figures 2 and 3 demonstrates that lactulose hydrolysis was slower than that of lactose. *K. lactis* CECT 1961^T β-galactosidase hydrolyzed lactulose

to a lesser extent (60% hydrolysis) than the rest of CCEs (80-90% hydrolysis). The amount of galactose present in the reaction mixtures was lower than that of fructose in all analyzed samples. As a general trend, trisaccharide formation (6'-galactosyl lactulose and 1-galactosyl-lactulose) predominated over the formation of the disaccharide 6-galactobiose. Synthesis of 6'-galactosyl-lactulose (panel E) and 6-galactobiose (panel D) increased with time, reaching a maximum after 24 h of reaction. Production of 1-galactosyl-lactulose (panel F) increased gradually and attained a maximum value after 2 h reaction in case of *K. marxianus* O3 CCE (remaining value of lactulose 52 g/100g of total carbohydrates) and after 6 h reaction (remaining value of lactulose 47 g/100g of total carbohydrates) for *K. marxianus* O4 β-galactosidase. In contrast, formation of this trisaccharide by *K. lactis* CECT 1961^{T} and BP7 β-galactosidases increased through the reaction time. The formation of other OsLu (including non-identified di- and trisaccharides and high retention time oligosaccharides) followed a similar trend to that of the disaccharide, with maximum yields after 24 h of reaction, and depending on the β-galactosidase extract used, OsLu mixtures with different composition can be achieved. As in the case of lactose, the main OsLu were trisaccharides. Maximum production of 6'-galactosyl-lactulose (13 g/100g of total carbohydrates) at 24 h as well as 1-galactosyl-lactulose (17 g/100g of total carbohydrates) at 2 h was observed for *K. marxianus* O3 CCE. Maximum disaccharide levels (around 5 g/100g of total carbohydrates) were produced by *K. marxianus* CCEs. As in the case of GOS, the best OsLu producer was *K. marxianus* O3 β-galactosidase which yielded 45% total OsLu based on an amount of lactulose consumed of 87.5 g/100g of total carbohydrates.

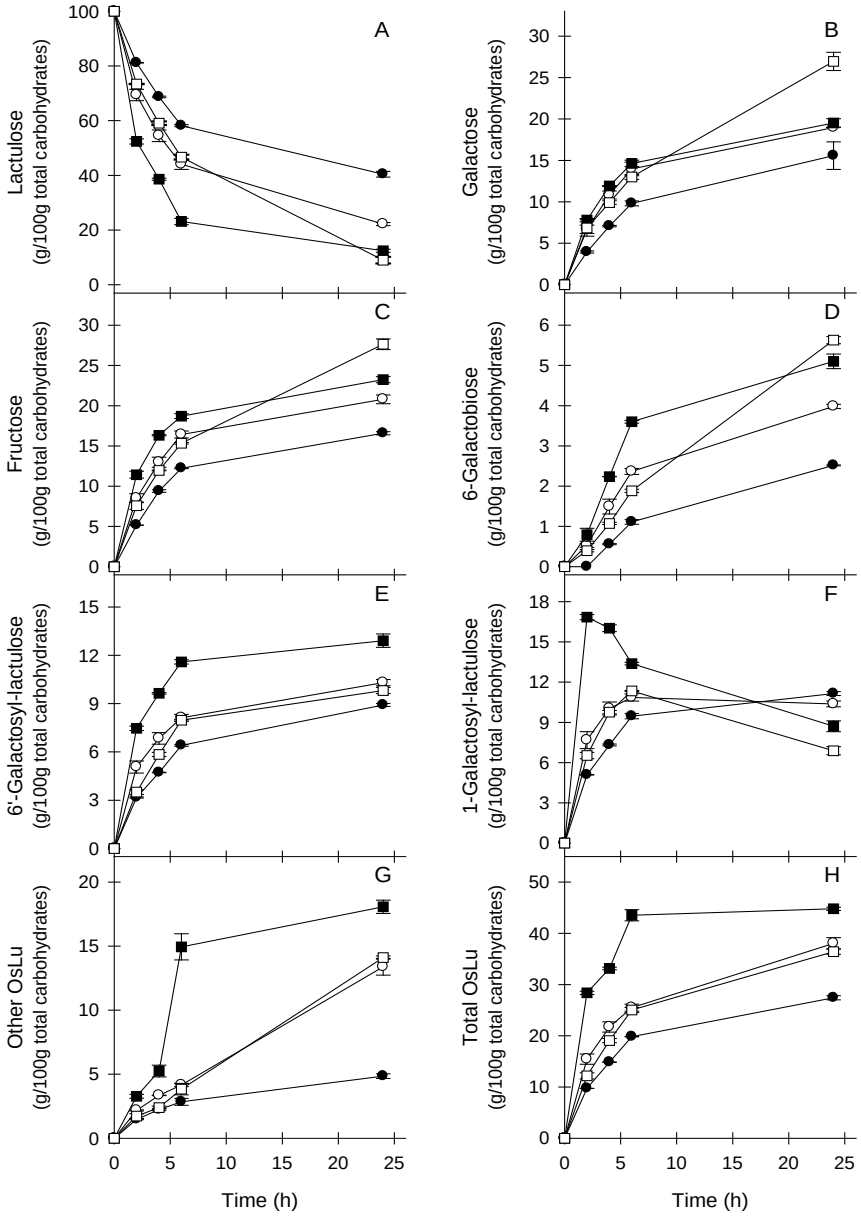

Figure 3. Carbohydrate yields during lactulose hydrolysis by selected yeast β-galactosidases. ●, *K. lactis* CECT 1961^T; ○, *K. lactis* BP7; ■ *K. marxianus* O3; □, *K. marxianus* O4. Error bars indicate standard deviations.

Several studies have demonstrated that glycosidic linkages and molecular weights of carbohydrates contribute toward the selectivity of fermentation by beneficial gut bacteria. Our study demonstrates that the main oligosaccharides produced from lactose or lactulose transgalactosylation using enzyme extracts from cheese isolated yeasts are trisaccharides, which have been reported to show the highest selectivity toward bifidobacteria (Kaneko et al., 1994; Kaplan and Hutkins, 2000). Moreover GOS with β1 → 6 linkages, as those described in this work, can be easily cleaved by β-galactosidases from bifidobacteria (Dumortier et al., 1994; Rowland and Tanaka, 1993) and thus exhibit prebiotic character. Cardelle-Cobas et al. (2011b) reported that the *in vitro* growth of different *Lactobacillus*, *Streptococcus* and *Bifidobacterium* strains was enhanced by β-galactosyl residues β1 → 6 and β1 → 1 linked over those with β1 → 4 linkages. Likewise, Cardelle-Cobas et al. (2012) demonstrated the *in vitro* fermentation of OsLu by mixed fecal microbiota and proposed them as a new generation of prebiotics for improving the composition of gut microbiota.

The present study shows the feasibility of β-galactosidases from *K. lactis* and *K. marxianus* strains isolated from cheese to transgalactosylate lactose and lactulose and produce reaction mixtures with different levels of individual oligosaccharides. To the best of our knowledge this is the first time that *K. marxianus* β-galactosidases are tested for lactulose transgalactosylation. Furthermore *K. marxianus* O3 enzyme yielded the highest total oligosaccharide amount when lactose or lactulose were used as acceptor carbohydrates. Moreover *K. marxianus* is considered as a thermophilic microorganism which suggests the possibility of developing transgalactosyl reactions at higher temperatures than 50°C with the benefit of using higher substrate concentrations for oligosaccharide yield improvement.

4. Acknowledgements

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Objective IV

Contribution of isolated yeasts to the production of key aroma compounds in cheese.

Potential impact of dairy yeasts on the typical flavour of traditional ewes' and goats' cheeses.

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ABSTRACT

The contribution of *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Kluyveromyces marxianus* strains to the typical flavour of traditional ewes' and goats' cheeses was assessed. Fourteen yeast strains were grown in liquid medium mimicking cheese composition and volatile compounds were identified by gas chromatography-mass spectrometry. Yeasts were able to produce key volatile compounds characteristic of the cheeses from which they were isolated. Inter-species and inter-strain variations were observed. Under the conditions tested *D. hansenii* produced the lowest levels of volatile compounds, with large intra-strain variations. *Kluyveromyces* strains primarily produced esters and alcohols. *K. marxianus* strains were associated with the production of acids, ethyl decanoate, 1-propanol and benzaldehyde, whereas *K. lactis* was correlated with the presence of ketones, ethyl acetate and secondary alcohols. In conclusion, this study shows the heterogeneous potential of dairy yeasts to contribute to final cheese flavour.

1. Introduction

Yeasts play an important role in proteolysis, lipolysis, fermentation of residual lactose, and assimilation of lactic and citric acid during the ripening of some cheeses, contributing to aroma development and to the rheological properties of the final dairy product (McSweeney, 2004). Moreover yeasts have been recovered from all stages of cheesemaking, as well as from milk, brine and dairy process equipment among others (Corbo et al., 2001; Delavenne et al., 2011; Gardini et al., 2006; Seiler and Busse, 1990).

Debaryomyces hansenii is the dominant yeast species found in most cheese varieties (Fleet, 1990; Fox et al., 2000). *D. hansenii* possesses the ability to grow at high salt concentrations, low pH and low water activity, as well as metabolising lactic and citric acids, which makes cheese a suitable environment for its proliferation (Breuer and Harms, 2006). Lactose-fermenting yeasts *Kluyveromyces lactis* and *Kluyveromyces marxianus* are also regularly found in dairy products and milk. Their lactose-fermenting ability promotes their growth in the cheese, where other yeasts are scarce. Besides these species, cheeses may often contain other yeast species, such as *Yarrowia lipolytica*, *Geotrichum candidum* and *Saccharomyces cerevisiae* (Fleet, 1990).

Cheese flavour is one of the most relevant attributes influencing consumers' acceptance and preference (Arora et al., 1995), and is the result of a complex balance between various volatile and non-volatile compounds, which individually do not reflect the overall odour and taste (Fox and Wallace, 1997). Many volatile compounds have been implicated in cheese aroma, such as acids, esters, ketones, aldehydes, alcohols or sulphur compounds, and each dairy product has a characteristic and

unique composition of volatile components (Plutowska and Wardencki, 2007).

The contribution of yeasts to development of cheese aroma is considered positive in some instances, creating commercial interest in using selected strains as ripening cultures (Frohlich-Wyder, 2003; Romano et al., 2006). Several studies have shown that, in different cheeses, relevant yeast species contribute differently to volatile production. *G. candidum* and *Y. lipolytica* are known to produce considerable amounts of various volatile sulphur compounds; *K. lactis*, *K. marxianus* and *S. cerevisiae* have been found to produce primarily esters; and *D. hansenii* mainly produced branched-chain aldehydes and alcohols (Arfi et al., 2002; Leclercq-Perlat et al., 2004; Martin et al., 2001; Sørensen et al., 2011; Spinnler et al., 2001). However, these studies emphasised inter-species aroma production, with few surveys focusing on strain variation. Berger et al. (1999) reported the production of different yields of sulphur compounds by *G. candidum*, depending on the strain selected, and Gori et al. (2012) recently showed large strain variations in the production of flavour compounds by *D. hansenii.*

Iberian traditional cheeses made from ewes' and goats' milk have high intrinsic value, arising from their unique sensory characteristics, which makes them highly appreciated by consumers (Freitas and Malcata, 2000). In previous studies, yeasts present during the ripening process of ewes' and goats' raw milk cheeses produced in a small traditional dairy in the Mediterranean area of Spain were identified (Padilla et al., 2014). *D. hansenii* and *K. lactis* were the yeast species most frequently isolated from both kind of cheeses, and the former predominated at the end of ripening period. *K. marxianus*, although less frequent, was present during the first weeks of maturing. Moreover, results demonstrated genetic heterogeneity present in the isolates (Padilla et al., 2014), and their strain-

dependent ability to generate bioactive compounds (García-Tejedor et al., 2013; Padilla et al., 2012). However, there is little knowledge about the impact of the yeast isolates on the final quality of the cheeses.

The objective of the present study was to further characterize both the aforementioned raw milk cheeses and their yeast microbiota, to gain a better understanding of the relationship between yeast ripening strains and cheese flavour. For this purpose, the volatile profile of the cheeses was characterized. Volatile compounds were extracted by solid phase micro extraction (SPME) and analysed by gas chromatography-mass spectrometry (CG-MS). Moreover, the ability of 14 yeast strains belonging to *D. hansenii*, *K. lactis* and *K. marxianus* species to grow in a defined medium and produce volatile compounds also present in ripened cheeses was assessed.

2. Materials and methods

2.1 Cheese samples

Commercial semi-hard ewes' and goats' cheeses produced in an artisanal dairy farm sited in the rural Castelló province (Spain) were analysed for volatile compounds. The cheeses were made from raw milk coagulated with the addition of mesophilic lactic acid bacteria starters and plant (*Cynara cardunculus*) rennet (Abiasa Company, Pontevedra, Spain). After precipitation of proteins, the curd was cut with vertical and horizontal knives and crumbled manually. The remaining whey was removed first manually and afterwards using a press. After salting, cheeses were air-dried until the rind was formed and ripened in wooden shelves at 10-12ºC and a relative humidity of 85-90% for 60 days.

Three cheeses from the same batch and from ewes' milk and goats' milk were analysed at the end of ripening period (3 batches x 2 cheeses = 6 samples). After the rind was removed, cheeses were cut in pieces and ground with 0.75 mg butylated hydroxytoluene/20 g sample, wrapped in aluminium foil, vacuum-packed and stored at –20ºC until GC analysis.

2.2 Yeast strains

Fourteen yeast strains belonging to the species *K. marxianus* (Km1-Km4), *K. lactis* (Kl1-Kl5) and *D. hansenii* (Dh1-Dh5) isolated during the ripening process from the artisanal cheeses described above and with different genetic characteristics were used in this study (Padilla et al., 2014). Yeast strains were maintained on GPYA medium (2% glucose, 0.5% peptone, 0.5% yeast extract and 2% agar, pH 5.5).

2.3 Culture conditions and media

Cheese-like medium (CLM; casamino acids 15 g L^{-1}, sodium lactate 19 ml L^{-1}, yeast extract 1 g L^{-1}, $CaCl_2$ 0.1 g L^{-1}, $MgSO_4$ 0.5 g L^{-1}, KH_2PO_4 6.8 g L^{-1}, NaCl 10 g L^{-1} and lactose 28 g L^{-1}) was prepared according to Kagkli et al. (2006) without addition of L-methionine. Flasks (100-mL) containing 50 mL of CLM were inoculated with 10^6 cells mL^{-1} from overnight pre-cultures grown in GPY medium (GPYA without agar) at 28ºC and 150 rpm. CLM cultures were incubated over 48h at 28ºC and 150 rpm. At the end of the incubation period, samples were taken for OD_{600} measurement. Yeast cells were removed by centrifugation (3220 x g, 10 min) and culture pH was measured. Lactose and L-lactic acid were quantified in the supernatants using Roche enzymatic kits (Darmstadt, Germany). For each strain, three replicate cultures were analysed and a control without yeast inoculation was also included.

2.4 Analysis of headspace volatile compounds by SPME GC–MS

An Agilent HP 7890 series II GC (Hewlett- Packard, Palo Alto, CA, USA) with an HP 5975C mass selective detector (Hewlett-Packard) equipped with Gerstel MPS2 multipurpose sampler (Gerstel, Mülheim an der Ruhr, Germany) was used in all experiments. The volatile components of the samples were extracted by SPME. All extractions were carried out using a DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane) fibre of 50/30 mm film thickness (Supelco, Bellefonte, PA, USA). The fiber was conditioned as indicated by the manufacturer prior to use in order to remove any possible contaminants. For cheeses, 5 g of product was placed in a 20 mL headspace vial sealed with a PTFE-faced silicone septum. The vial was maintained at 50ºC for 15 min to equilibrate the headspace, and then the fiber was exposed over 30 min at the same temperature. Before

each injection, the fiber was baked at 250ºC for 10 min. Each sample was analysed in triplicate. For CLM yeast cultures, 7 mL of supernatant plus 1.4 g of NaCl were added to a 20 mL headspace vial sealed with a PTFE-faced silicone septum. The vial was kept at 50ºC for 15 min to equilibrate the headspace. The SPME fiber was then exposed to the headspace while maintaining the sample at 30ºC for 15 min. During extraction, the sample was agitated continuously in pulses of 10 sec at 250 rpm. Before and after each injection, the fiber was baked at 250ºC for 10 and 5 min, respectively. Each sample was analysed twice.

After the extraction step, the analytes were thermally desorbed for 5 min from the fiber into the injector port of the GC-MS operating at 240ºC in splitless mode. The compounds were then separated using a DB-624 capillary column J & W Scientific (Agilent Technologies, Santa Clara, CA, USA) (30m, 0.25mm i.d., film thickness 1.4 µm). For volatile analysis, the GC oven temperature program began at 40ºC, where it was held for 5 min, then ramped to 100ºC at 3ºC min^{-1} and maintained for 5 min, then to 150ºC at 3ºC min^{-1} and to 210ºC at 4ºC min^{-1}, and, finally, held at 210ºC for 5 min. Mass spectra were obtained by electron impact at 70 eV, and data were acquired across the range 29–400 amu (scan mode).

Compounds were identified by comparison with mass spectra from the library database (Nist'05), Kovats retention index (Kovats, 1965) and by comparison with authentic standards. The quantification of volatile compounds was done in SCAN mode using total ion chromatograms (TIC). The results were expressed as abundance units (AU x 10^{-6}). Volatile compounds quantitated from CLM control were subtracted from each yeast-inoculated medium.

2.5 Statistical evaluation

The effect of yeasts on the generation of volatile compounds in CLM was tested by one-way analysis of variance (ANOVA). Differences between sample means were analysed according to Fisher's least significant difference (LSD) test. Principal component analysis (PCA) was used to test relationships among yeast species, pH, lactose and lactate consumption and main volatile compounds. Statistical analysis was performed using the statistic software XLSTAT, 2009.4.03 (Addinsoft, Barcelona, Spain).

3. Results

3.1 Volatile compounds in ewes´and goats´milk cheeses

Sixty-five volatile compounds were quantitied in the headspace of Mediterranean ewes' and goats' cheeses (Table 1). They were classified into acids (14), esters (18), ketones (9), aldehydes (5), alcohols (17), terpenes (1) and sulphur compounds (1). Four of the sixty-five compounds were not present in the ewe's cheese, while eight of them were not present in the headspace of goat's cheese. As expected, most of the volatiles found in these cheeses have been previously reported in other varieties of ewes' and goats' raw milk cheeses (Table 1).

Esters and alcohols were the most abundant chemical families identified in the headspace of the Mediterranean cheeses studied, whereas, quantitatively, carboxylic acids were the most abundant volatiles. Among short and medium-chain carboxylic acids, the most abundant were acetic, butanoic, hexanoic, octanoic and decanoic acids, although branched-chain fatty acids such as 3-methylbutanoic and 2-methylbutanoic acids were also found in both cheeses. Among esters, ethyl esters were the most abundant, although propyl- and branched-chain esters were also identified. Methyl ketones were the most abundant ketones detected in these products while aldehydes were not major components in these cheeses.

Table 1. Abundance of volatile compounds (expressed as AU×10^6 extracted by SPME) in the headspace of the raw milk cheeses[a].

Compound	LRI[b]	RI[c]	Goats' cheese	Ewes' cheese	Previously reported[d]
Acids					
Acetic acid	709	A	196.2 ± 13.6	224.6 ± 31.6	1-7
Propanoic acid	815	A	3.2 ± 0.1	9.5 ± 1.6	3-7
2-Methylpropanoic acid	852	A	27.3 ± 5.3	12.5 ± 0.1	1,3-6
Butanoic acid	891	A	482.7 ± 15.0	216.3 ± 15.6	1-7
3-Methylbutanoic acid	932	A	30.2 ± 7.1	20.8 ± 1.6	1,3,5-7
2-Methylbutanoic acid	939	A	31.8 ± 7.8	15.1 ± 2.0	7
Pentanoic acid	971	A	2.7 ± 0.3	1.3 ± 0.1	3,4,7
Hexanoic acid	1080	A	676.6 ± 19.5	219.5 ± 9.6	1-4,6,7
Heptanoic acid	1165	A	4.7 ± 0.2	2.0 ± 0.1	3,4
Octanoic acid	1264	A	271.4 ± 7.9	67.1 ± 1.7	2-4
Benzenecarboxylic acid	1283	A	2.0 ± 0.3	1.4 ± 0.1	2
Nonanoic acid	1357	A	2.3 ± 0.1	nd	2,4
Decanoic acid	1453	A	128.2 ± 9.5	33.0 ± 1.8	2-4
Dodecanoic acid	1646	B	3.1 ± 0.4	0.9 ± 0.1	2,3
Esters					
Ethyl acetate	641	A	7.3 ± 2.1	25.4 ± 5.2	1-7
Propyl acetate	743	A	1.1 ± 0.1	4.1 ± 2.0	1,4,5,7
1-Methylpropyl acetate	787	B	nd	10.8 ± 7.4	1,7
Ethyl butanoate	828	A	18.9 ± 6.3	11.2 ± 2.5	1-5,7
Butyl acetate	844	A	nd	0.6 ± 0.1	1,5,7
3-Methyl-1-butanol acetate	907	A	5.6 ± 0.7	3.8 ± 0.3	1,7
Propyl butanoate	923	A	4.6 ± 0.4	2.8 ± 0.7	3-5,7
1-Methylpropyl butanoate	960	B	4.9 ± 0.6	7.5 ± 0.3	-
2-Methylpropyl 2-methyl butanoate	979	B	0.6 ± 0.1	nd	-
Ethyl hexanoate	1027	A	28.9 ± 13.5	15.6 ± 1.0	1-7
3-Methylbutyl butanoate	1084	B	6.2 ± 0.1	nd	-
2-Propenyl hexanoate	1111	B	0.5 ± 0.0	nd	-
Propyl hexanoate	1123	A	6.3 ± 0.1	2.1 ± 0.4	5, 7
1-Methylbutyl butanoate	1175	B	1.4 ± 0.0	0.9 ± 0.1	-
Ethyl 2-methyl-propanoate	1182	B	1.4 ± 0.1	nd	1
Ethyl octanoate	1225	A	16.8 ± 1.4	3.9 ± 0.4	1,3-7

Table 1 (continued).

Compound	LRI[b]	RI[c]	Goats' cheese	Ewes' cheese	Previously reported[d]
Propyl octanoate	1322	B	1.0 ± 0.0	nd	-
Ethyl decanoate	1425	A	7.5 ± 0.8	1.5 ± 0.1	1,2,4-6
Ketones					
Acetone	529	A	1.2 ± 0.1	1.1 ± 0.3	2
2-Butanone	635	A	43.2 ± 10.7	529.3 ± 26.3	1,3-7
2-Pentanone	729	A	41.7 ± 2.8	14.0 ± 6.2	1,4-7
3-Hydroxy-2-butanone	779	A	3.1 ± 0.6	31.8 ± 7.9	5,6
2-Hexanone	833	A	1.6 ± 0.0	nd	5-7
2-Heptanone	932	A	42.1 ± 3.2	3.7 ± 1.6	1,2,4-7
8-Nonen-2-one	1135	B	3.4 ± 0.5	nd	-
2-Nonanone	1139	A	147.4 ± 22.2	7.8 ± 2.9	1,2,4-7
2-Undecanone	1344	A	3.2 ± 0.6	0.6 ± 0.1	-
Aldehydes					
2-Propenal	519	A	1.4 ± 0.1	0.5 ± 0.1	1, 5, 7
3-Methylbutanal	691	A	1.2 ± 0.2	1.0 ± 0.1	1-7
Hexanal	838	A	0.8 ± 0.1	nd	1, 2, 5, 7
Benzaldehyde	1017	A	1.1 ± 0.1	1.6 ± 0.2	2,3
Benzeneacetaldehyde	1107	A	1.4 ± 0.2	0.8 ± 0.2	3
Alcohols					
Ethyl alcohol	511	A	62.4 ± 9.2	32.5 ± 6.5	1,5-7
Isopropyl alcohol	538	A	3.0 ± 0.5	1.6 ± 0.3	5,7
2-Propen-1-ol	610	B	3.8 ± 0.4	2.7 ± 1.0	1,5-7
1-Propanol	615	A	16.7 ± 1.5	20.6 ± 6.8	1,4-6
2-Butanol	647	A	133.2 ± 13.4	461.4 ± 32.5	1,3-5
2-Methyl-1-propanol	682	A	1.0 ± 0.1	0.4 ± 0.1	1,5,6
1-Methoxy-2-propanol	718	B	1.3 ± 0.2	1.4 ± 0.3	3, 5, 6
1-Butanol	719	A	3.2 ± 0.5	3.8 ± 0.7	1,2,4-7
2-Pentanol	747	A	68.2 ± 5.3	10.7 ± 3.9	1,4-7
3-Methyl-1-butanol	793	A	18.3 ± 0.8	6.6 ± 0.9	1,3-7
2-Methyl-1-butanol	796	A	2.8 ± 0.2	1.2 ± 0.1	7
2,3-Butanediol	879	A	37.9 ± 3.6	59.4 ± 6.4	-
1-Hexanol	920	A	4.9 ± 0.3	2.8 ± 0.7	1,2,5,6
2-Heptanol	944	A	71.2 ± 6.9	14.2 ± 5.9	1,4-7
1-Heptanol	1022	A	nd	0.8 ± 0.1	1,7

Table 1 (continued).

Compound	LRI[b]	RI[c]	Goats' cheese	Ewes' cheese	Previously reported[d]
2-Nonanol	1147	A	12.1 ± 1.1	2.7 ± 0.7	1, 4
Phenylethyl alcohol	1193	A	5.8 ± 0.6	2.3 ± 0.3	-
Terpenes					
D-Limonene	1043	A	nd	39.2 ± 1.4	1, 2, 4, 5, 7
Sulphur compounds					
Dimethyl sulfone	1057	A	3.1 ± 0.5	1.9 ± 0.3	-

AU: Abundance units, the result of counting the total ion chromatogram (TIC) for each compound.
[a]Values are mean ± SD (n=3).
[b]Linear retention indices (LRI) of the compounds eluted from the GC-MS using a DB-624 capillary column (J&W Scientific 30 m x 0.25 mm i.d. x 1.4 film thickness).
[c]Reliability of identification (RI): A, mass spectrum and retention time identical with an authentic standard; B, tentative identification by mass spectrum.
[d]Compounds previously reported in ewes' and goats' raw milk cheeses. Reference numbers are as follows: (1) Carbonell et al., 2002; (2) Condurso et al., 2008; (3) Delgado et al., 2010; (4) Delgado et al., 2011; (5) Fernández-García et al., 2004; (6) Izco and Torre, 2000 and (7) Larráyoz et al., 2001.
nd: Not detected.

3.2 Yeast growth in CLM and production of volatile compounds

Growth and aromatic profile from pure cultures of yeast strains belonging to *D. hansenii*, *K. lactis* and *K. marxianus* were determined. All yeast strains were able to grow in a liquid medium mimicking cheese composition (CLM). Lactose and lactate concentrations and pH values after 48 h of growing in CLM were determined. *Kluyveromyces* strains depleted the available lactose almost completely. *K. lactis* consumed around 5% lactate, while lactate consumption by *K. marxianus* strains was around 16%. When grown in CLM, *Kluyveromyces* strains increased the pH from 5 to 5.2-5.8. *D. hansenii* strains grew in CLM, consumed around 25% of lactose and 5% of lactate, and the pH value increased to 5.6-6.6.

Volatile compounds detected in the headspace of CLM are summarised in Tables 2 and 3. Only compounds which were also found in the Mediterranean cheeses (Table 1) are listed in Table 2, while Table 3 shows other volatile compounds detected in the headspace of CLM.

As observed in this study, yeasts were able to produce 27 compounds of those compounds found in the cheeses, including 6 acids, 7 esters, 3 ketones, 2 aldehydes and 9 alcohols (Table 2). Interestingly, the volatile composition of the headspace of CLM showed inter-species and inter-strain variations. General variations can be seen in Figure 1, which shows volatile compounds classified by chemical groups and yeast species. *K. marxianus* and *K. lactis* were the best producers of esters and alcohols, without significant differences between the two species. Similar production of aldehydes was found for *K. marxianus* and *D. hansenii*, while the former was the best acid producer. In general, *D. hansenii* produced the lowest levels of volatile compounds in the conditions tested. Moreover, standard deviations indicated large strain variations for *D. hansenii*.

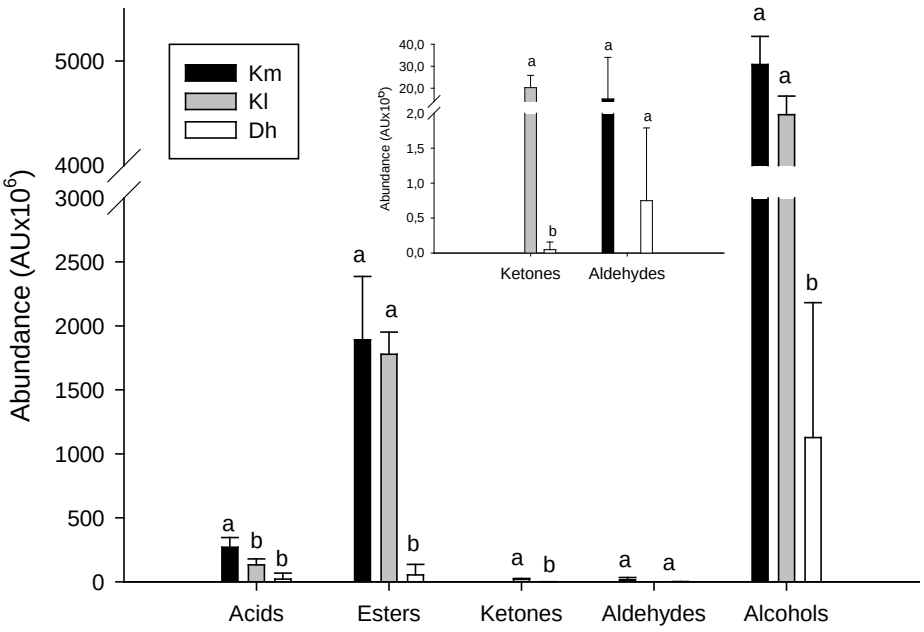

Figure 1. Total volatile compounds abundance by chemical group (expressed as AU x 10^6) in the headspace of CLM supernatants after yeast growth: Km: *Kluyveromyces marxianus*; Kl: *Kluyveromyces lactis*; Dh: *Debaryomyces hansenii*. Data are mean ± SD of levels of volatile compounds produced by the different strains tested. Different letters in the same chemical group indicate significant differences ($p < 0.05$) among yeast species.

Table 2. Volatile compounds (expressed as AU×10^6 extracted by HS-SPME) identified in the headspace of CLM after yeast growth[a].

	Kluyveromyces marxianus				*Kluyveromyces lactis*					*Debaryomyces hansenii*				
Compound	Km1	Km2	Km3	Km4	Kl1	Kl2	Kl3	Kl4	Kl5	Dh1	Dh2	Dh3	Dh4	Dh5
Acids														
Acetic acid	214.81^{a}	206.69^{a}	247.29^{a}	97.49^{b}	5.57^{c}	nd	4.19^{c}	49.31^{bc}	17.97^{c}	nd	nd	22.70^{c}	nd	nd
Propanoic acid	nd	nd	nd	6.24^{a}	nd	nd	nd	nd	nd	2.71^{a}	nd	nd	nd	nd
2-Methylpropanoic acid	21.33^{f}	19.76^{f}	39.16^{de}	25.23^{ef}	68.89^{b}	59.98^{bc}	55.01^{bcd}	91.20^{a}	104.68^{a}	23.03^{ef}	0.11^{h}	45.83^{cd}	0.56^{h}	0.93^{gh}
3-Methylbutanoic acid	11.49^{a}	12.52^{a}	1.41^{bc}	2.53^{b}	nd	nd	nd	nd	nd	1.51^{bc}	nd	2.46^{b}	0.60^{c}	0.50^{c}
2-Methylbutanoic acid	52.52^{a}	54.62^{a}	35.17^{c}	29.28^{cd}	38.15^{bc}	34.90^{c}	34.82^{c}	51.31^{a}	50.65^{a}	20.70^{d}	2.93^{e}	46.13^{ab}	0.88^{e}	2.28^{e}
Octanoic acid	1.32^{b}	nd	3.18^{a}	0.50^{b}	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Total acids	301.47^{a}	293.59^{a}	326.21^{a}	161.27^{b}	112.61^{cd}	94.88^{de}	94.02^{de}	191.82^{b}	173.30^{b}	47.95^{ef}	3.04^{f}	117.12^{cd}	2.04^{f}	3.71^{f}
Esters														
Ethyl acetate	770.82^{d}	914.69^{d}	909.30^{d}	904.95^{d}	1418.8^{c}	1612.9^{abc}	1519.0^{bc}	1702.7^{ab}	1795.4^{a}	185.98^{e}	1.86^{e}	58.67^{e}	10.29^{e}	0.80^{e}
Propyl acetate	15.06^{cde}	22.94^{a}	19.76^{ab}	12.86^{de}	10.35^{e}	16.25^{bcd}	11.84^{de}	18.41^{abc}	16.00^{bcd}	0.68^{f}	nd	nd	nd	nd
Butyl acetate	nd	nd	11.88^{a}	11.33^{a}	0.53^{b}	0.84^{b}	0.34^{b}	0.55^{b}	0.49^{b}	nd	nd	nd	nd	nd
3-Methyl-1-butanol acetate	1290.6^{b}	1561.4^{a}	545.40^{c}	550.74^{c}	120.26^{de}	148.23^{de}	144.69^{de}	188.40^{d}	163.81^{de}	4.96^{e}	0.30^{e}	0.91^{e}	nd	nd
3-Methylbutyl butanoate	nd	0.79^{a}	nd	0.74^{a}	nd	0.74^{a}	0.68^{a}	0.65^{a}	0.79^{a}	nd	nd	nd	nd	nd
Ethyl octanoate	2.23^{a}	3.19^{a}	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

Table 2 (continued).

Compound	Km1	Km2	Km3	Km4	Kl1	Kl2	Kl3	Kl4	Kl5	Dh1	Dh2	Dh3	Dh4	Dh5
Ethyl decanoate	2.00^{bc}	1.38^{cd}	2.85 ^{b}	5.78^{a}	nd	nd	nd	nd	nd	2.13^{bc}	nd	0.91^{d}	nd	nd
Total esters	2080.7^{b}	2504.4^{a}	1489.2^{d}	1486.4^{d}	1549.9^{d}	1778.9^{bcd}	1676.6^{cd}	1910.7^{bc}	1976.5^{bc}	193.75^{e}	2.16^{e}	60.49^{e}	10.29^{e}	0.80^{e}
Ketones														
2-Pentanone	nd	nd	nd	nd	1.24^{b}	0.95^{bc}	0.52^{de}	0.76^{cd}	1.95^{a}	nd	nd	nd	0.24^{e}	nd
2-Heptanone	nd	nd	nd	nd	8.22^{d}	11.28^{c}	10.97^{c}	15.90^{a}	13.51^{b}	nd	nd	nd	nd	nd
2-Nonanone	nd	nd	nd	nd	4.54^{c}	6.36^{c}	5.26^{c}	11.02^{a}	8.80^{b}	nd	nd	nd	nd	nd
Total ketones	nd	nd	nd	nd	14.00^{d}	18.59^{c}	16.75^{c}	27.68^{a}	24.26^{b}	nd	nd	nd	0.24^{e}	nd
Aldehydes														
3-Methylbutanal	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.62^{a}	nd	nd	nd	2.13^{a}
Benzaldehyde	nd	nd	21.59^{b}	38.96^{a}	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Total aldehydes	nd	nd	21.59^{b}	38.96^{a}	nd	nd	nd	nd	nd	1.62^{c}	nd	nd	nd	2.13^{c}
Alcohols														
Ethyl alcohol	1075^{cde}	1054.3^{de}	1273.6^{a}	1134.1^{bcd}	1215.5^{ab}	1011.8^{e}	994.4^{e}	1157.9^{bc}	1140.8^{bcd}	543.92^{f}	nd	252.55^{g}	10.85^{h}	12.37^{h}
1-Propanol	29.90^{c}	26.35^{c}	55.70^{b}	66.05^{a}	16.34^{d}	14.92^{de}	16.13^{d}	12.09^{de}	10.85^{ef}	7.15^{fg}	0.29^{h}	2.95^{gh}	0.52^{h}	0.33^{h}
2-Methyl-1-propanol	193.25^{d}	193.23^{d}	187.82^{d}	156.24^{e}	283.99^{b}	277.63^{bc}	256.03^{c}	285.64^{b}	337.74^{a}	193.06^{d}	10.53^{g}	66.77^{f}	9.97^{g}	3.02^{g}
3-Methyl-1-butanol	1570.1^{cd}	1517.2^{cd}	1619.7^{bc}	1440.6^{d}	1748.1^{ab}	1658.9^{abc}	1633.5^{abc}	1578.4^{cd}	1789.1^{a}	591.30^{ef}	702.24^{e}	458.81^{f}	87.48^{g}	31.85^{g}
2-Methyl-1-butanol	942.22^{e}	932.17^{e}	1228.1^{a}	1181.0^{abc}	1144.8^{bcd}	1130.8^{cd}	1110.8^{d}	1111.0^{d}	1110.0^{d}	1213.1^{ab}	191.44^{g}	825.22^{f}	28.01^{h}	22.46^{h}
2,3-Butanediol	13.98^{b}	11.58^{bc}	31.72^{a}	14.40^{b}	8.91^{cd}	2.03^{e}	4.06^{e}	7.66^{d}	7.88^{d}	nd	nd	nd	nd	nd
2-Heptanol	nd	nd	nd	nd	6.11^{b}	6.61^{b}	5.78^{b}	7.64^{a}	8.44^{a}	nd	nd	nd	nd	nd

Table 2 (continued).

Compound	Km1	Km2	Km3	Km4	Kl1	Kl2	Kl3	Kl4	Kl5	Dh1	Dh2	Dh3	Dh4	Dh5
2-Nonanol	nd	nd	nd	nd	8.37^{b}	8.34^{b}	7.65^{b}	11.72^{a}	13.13^{a}	nd	nd	nd	nd	nd
Phenylethyl alcohol	1301.5^{a}	1291.7^{a}	745.37^{b}	578.62^{c}	282.76^{de}	251.70^{de}	286.85^{d}	230.59^{de}	218.21^{e}	47.08^{f}	230.40^{de}	49.93^{f}	15.12^{f}	28.15^{f}
Total alcohols	5126.1^{a}	5026.5^{ab}	5142.0^{a}	4571.0^{cd}	4714.9^{bc}	4362.7^{d}	4315.1^{d}	4402.6^{cd}	4636.1^{cd}	2595.6^{e}	1134.9^{g}	1656.2^{f}	151.95^{h}	98.18^{h}

AU: Abundance units, the result of counting the total ion chromatogram (TIC) for each compound.
[a]Values are mean from n=3. Volatile compounds from CLM control were subtracted to each yeast CLM. Means followed by different letters in the same row indicate significant differences among yeast strains ($p<0.05$; one-way ANOVA with Fisher's LSD test).
nd: Not detected.

Table 3. Generation of volatile compounds (not found in the Mediterranean cheeses) in the headspace of CLM inoculated with yeasts.

			Yeast[a]		
Compound	LRI[b]	RI[c]	*Km*	*Kl*	*Dh*
Esters					
Ethyl propanoate	738	A	100	100	80
Ethyl 2-methyl-propanoate	785	A	100	100	40
2-Methylpropyl acetate	804	A	100	100	20
Propyl propanoate	837	A	50	100	0
Ethyl 2-methyl-butanoate	876	A	100	100	40
Ethyl 3-methyl-butanoate	879	A	0	0	40
2-Methylpropyl propanoate	895	A	75	100	20
2-Methyl-1-butanol acetate	909	A	100	100	40
3-Methyl-1-butanol propanoate	995	A	100	100	20
2-Methyl-1-butanol propanoate	999	A	100	80	20
2-Methylbutyl 2-methyl-propanoate	1044	A	50	100	40
3-Methylbutyl 2-methyl-butanoate	1128	A	0	0	40
2-Methylbutyl 2-methyl-butanoate	1133	A	0	0	40
2-Phenylethyl acetate	1317	A	25	100	20
2-Phenylethyl propanoate	1407	A	100	100	0
Phenylethyl butyrate	1451	A	100	100	20
Ketones					
Methyl isobutyl ketone	781	B	0	0	80
Aldehydes					
Acetaldehyde	469	A	0	100	60
2-Methylpropanal	595	A	0	0	60
2-Methylbutanal	700	A	0	0	60
Alcohols					
3-Methyl-pentanol	899	A	0	100	80
3,7-Dimethyl-6-octen-1-ol	1285	B	100	100	20
Sulphur compounds					
Methionol	1060	A	100	100	20

[a] Percentage of strains producing volatile compound in CLM media.
[b] Refer to footnote [a] in Table 1.
[c] Refer to footnote [b] in Table 1.

Among *D. hansenii* strains (Table 2), Dh1 produced the highest levels of total esters and alcohols. *K. marxianus* species was prominent due to the production of acetic acid. Production of octanoic acid was restricted to *K. marxianus* species, with Km3 being the best producer strain. *K. marxianus* Km2 stood out as the leading producer of total esters, due to the high production of 3-methyl-1-butanol acetate, the level of which was almost ten-fold higher than that produced by *K. lactis* strains. Among esters, *K. lactis* strains produced primarily ethyl acetate. Interestingly, ethyl octanoate production was restricted to *K. marxianus* Km1 and Km2 under the conditions tested. With the exception of 2-pentanone production by Dh4, ketone production was restricted to *K. lactis* strains. In contrast, none of the *K. lactis* strains were able to produce aldehydes. Only two aldehydes were detected after yeast growth in CLM: benzaldehyde produced by *K. marxianus* Km3 and Km4 and 3-methylbutanal produced by *D. hansenii* Dh1 and Dh5. Regarding alcohol production, *K. lactis* produced 9 different volatiles, although *K. marxianus* strains Km1, Km2 and Km3 stood out as the best total alcohol producers, given the production of phenylethyl alcohol. Neither *K. marxianus* nor *D. hansenii* species were able to produce 2-heptanol and 2-nonanol. Moreover 2,3-butanediol was not detected after growth of *D. hansenii* in CLM, while *K. marxianus* strains were the best producers of such compounds (Table 2).

Apart from these compounds, 23 more volatiles compounds were identified in the headspace of CLM after yeast growth (Table 3). Those compounds mainly comprised esters (16), although 1 ketone, 3 aldehydes, 2 alcohols and methionol were also detected; Table 3 also shows the percentage of yeast strains able to produce each compound. Some of these, 3 esters, 2 aldehydes and methyl isobutyl ketone, were only detected in CLM after growth of *D. hansenii*. In contrast, none of the *D. hansenii* strains tested was able to produce propyl and 2-phenylethyl

propanoate. It is also worthwhile to note that none of the *K. marxianus* strains were producers of aldehydes or 3-methyl-pentanol. Although these compounds were not detected in the cheeses, most of them have been described as typical cheese volatiles (Curioni and Bosset, 2002).

3.3 Principal component analysis

Finally, a PCA model was developed using a dataset with 14 yeast strains and 30 variables, comprising 27 volatiles (those present in cheeses and CLM, Table 2), lactose and lactate consumption and pH of the medium (Figure 2). Two principal components were able to explain 70.5% of the total variance observed. Principal component 1 (PC1) accounted for 39.6% of the variance while PC2 accounted for 30.9%. PC1 differentiated the incubations by the yeast genera inoculated. *Kluyveromyces* strains appeared in the positive part of PC1, while *Debaryomyces* was situated in the negative side. *Kluyveromyces* strains were related to the maximum production of volatile compounds and to the highest lactose consumption. On the other hand, growth of *D. hansenii* was associated with the highest increase in pH value, and with the presence of 3-methylbutanal. PC2 differentiated the inoculations within *Kluyveromyces* strains. *K. lactis* was related to the presence of volatiles compounds such as ketones, ethyl acetate and secondary alcohols; on the other side, *K. marxianus* strains were associated with the highest consumption of lactate and with the production of acids (acetic, propanoic and octanoic acids), ethyl decanoate, 1-propanol and benzaldehyde, among others.

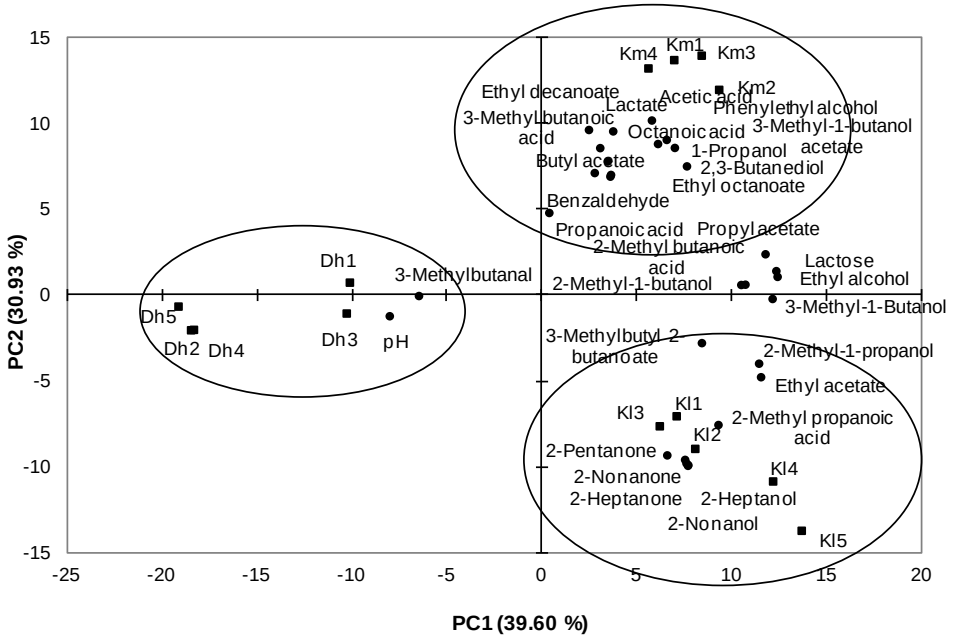

Figure 2. Loadings of the first two principal components (PC1-PC2) of the analysed parameters (pH, percentage of lactose and lactate consumption and volatile compounds) of CLM after growth of different yeast strains: Km1-Km4 (*Kluyveromyces marxianus*), Kl1-Kl5 (*Kluyveromyces lactis*), Dh1-Dh5 (*Debaryomyces hansenii*).

4. Discussion

This study provides a characteristic fingerprint of volatiles present in Mediterranean cheeses and indicates the metabolic potential of ripening yeast strains to impact on cheese flavour. The proportion of volatile compounds depends on the extraction method used, and in this case a SPME technique with DVB/CAR/PDMS fibres was employed. The method used allowed comparisons among the different yeast strains, since the volatile compounds were obtained on a semi-quantitative basis.

The present research demonstrates the ability of *K. marxianus* and *K. lactis,* and to a lesser extent *D. hansenii* strains, to produce key volatile compounds characteristic of the cheeses from which they were isolated (Table 1 and 2). All the strains tested in this study were able to grow in a defined cheese-like medium (CLM) containing lactose, lactate and casamino acids and generate volatile compounds. Although these conditions differ from real cheese, this medium has been successfully used for screening purposes of yeast species and strains with potential use in cheese ripening (Kagkli et al., 2006; Spinnler et al., 2001).

As expected, *D. hansenii* strains consumed less lactose than *Kluyveromyces*, and this might account for the lower production of aroma compounds in CLM. The prevalence of *D. hansenii* during ripening in different kind of cheeses has been reported by several authors (Fleet, 1990; Fox and Wallace, 1997; Fox et al., 2000) and it is considered as an obvious candidate for starter cultures (Bockelmann, 2002). Recently, Gori et al. (2012) reported the potential of *D. hansenii* strains to increase the nutty/malty flavour of cheese due to the production of aldehydes, although large strain variations were found. In this study and under the conditions tested, 3 branched-chain aldehydes (2-methylpropanal, 3-methylbutanal and 2-methylbutanal) were only produced by *D. hansenii*, with a large inter-

strain variation. 2-Methylpropanal and 2-methylbutanal derived from the catabolism of valine and isoleucine, respectively, were only detected in CLM, whereas 3-methylbutanal derived from leucine was also detected in cheeses characterized here. Aldehydes are potent odorants in several cheese varieties, although they are considered transitory compounds because they are quickly reduced to primary alcohols (Curioni and Bosset, 2002). In fact, the corresponding alcohols derived from the three branched-aldehydes (2-methyl-1-propanol, 3-methyl-1-butanol and 2-methyl-1-butanol) were detected in the cheeses and in *D. hansenii* CLM, as also reported by Sørensen et al. (2011).

Ester formation in cheese is mainly related to yeast metabolism (Molimard and Spinnler, 1996) although some lactic acid bacteria and *Micrococcaceae*, as well as chemical reactions, can be responsible (Gripon et al., 1991). Esters come from a reaction between an alcohol, derived from lactose fermentation or amino-acid catabolism, and a fatty acid or amino acid catabolite intermediate. Most esters detected in cheese are described as having sweet, fruity and floral notes. Although a fruity flavour is traditionally regarded as a defect in cheese varieties such as Cheddar (Horwood et al., 1987), it is a positive attribute of other cheese varieties such as Parmigiano Reggiano (Meinhart and Schreier, 1986). Ester production by *Kluyveromyces* strains has been reported by several authors (Arfi et al., 2002; Jiang, 1993; Leclercq-Perlat et al., 2004; Martin et al., 2001). Ethyl acetate was the main ester produced, although ethyl propanoate, propyl acetate, butyl acetate, ethyl butanoate and ethyl octanoate were also detected after growth of *Kluyveromyces* (Arfi et al., 2002; Leclercq-Perlat et al., 2004). Moreover, 2-phenylethyl acetate, 3-methylbutyl ethanoate and 2-methylpropyl ethanoate are also produced by *Kluyveromyces* strains (Jiang, 1993; Leclercq-Perlat et al., 2004), but strain-specific variations were not addressed. The present results confirm

ethyl acetate as one of the primarily esters formed by *Kluyveromyces* strains, together with the production of 3-methyl-1-butanol acetate by two strains of *K. marxianus*. In total, *K. marxianus* and *K. lactis* strains produced 20 and 16 different kinds of esters, respectively, (Table 2 and 3) highlighting the capability of the genus *Kluyveromyces* for ester production. Production of ethyl octanoate, restricted to *K. marxianus* strains under the conditions tested, was also reported by Leclercq-Perlat et al. (2004). These authors also observed that the ester production efficiency of *K. marxianus* was higher than that of *D. hansenii*, in agreement with the results obtained here. With the exception of two ester compounds, the five *K. lactis* strains tested produced the same ester profile, whereas *K. marxianus* strains differed in seven esters. These results suggest a lower inter-strain variation in *K. lactis* than in *K. marxianus*.

Several of the potential alcohols which may be precursors of the aforementioned esters were also identified in yeast CLM. Those alcohols were also detected after the growth of *D. hansenii* strains, where production of esters was negligible. It has been suggested that a highly hydrolytic activity towards esters in *D. hansenii* strains might be the reason for the limited accumulation of ester compounds (Besancon et al., 1995). The *D. hansenii* strains tested in this study have also been characterized as having hydrolytic activity towards fatty acid esters (Padilla et al., 2014).

Interestingly this study shows that only *K. lactis* strains were able to produce 2-pentanone, 2-heptanone and 2-nonanone, which were characteristic compounds of those cheeses from which they were isolated (Table 1 and 2). A previous study has shown the ability of *K. lactis* to produce other kinds of ketones, such as 3-hydroxy-2-butanone and 1-hydroxy-2-propanone, from a medium containing glucose, yeast extract and vitamins (Jiang, 1993). However, to the best of our knowledge, ketones generation by yeasts in a medium mimicking cheese composition has not

been reported. Methyl ketones are associated with fruity, floral and musty notes, and their synthesis has been related to the enzymatic activity of moulds in surface-ripened cheeses (Curioni and Bosset, 2002).

Short-chain free fatty acids, predominant components of the flavour of many cheeses such as those described here, were mainly characteristics of *K. marxianus* CLM. As milk fat was not present in CLM, those acids may originate from the degradation of lactose and free amino acids or by oxidation of ketones, esters and aldehydes (Molimard and Spinnler, 1996). Branched-chain fatty acids, such as 2-methylpropanoic, 2- and 3-methylbutanoic acids, are characteristic compounds of goat and ewe cheeses, and they are probably derived from valine, isoleucine and leucine respectively (Kuzdzal-Savoie, 1980). The potential of selected yeast strains to produce fatty acids when grown in a fat-containing medium deserves further study.

Sulphur compounds were not abundant volatiles either in cheeses or CLM. López del Castillo-Lozano et al. (2007) reported the necessity of methionine supplementation in culture media for the production of volatile sulphur compounds by yeasts. Since only one sulphur compound was detected in cheeses under the conditions tested, we did not consider supplementing casamino acids present in CLM with methionine. The only sulphur compound generated in CLM was methionol (Table 3), a stable end product of methionine metabolism by yeasts (Liu and Crow, 2010). In the conditions tested, methionol was produced by *Kluyveromyces* strains, and only to a small extent by *Debaryomyces* strains.

5. Conclusions

This study has confirmed the potential of dairy yeasts to contribute to the final cheese flavour. Moreover, species and strain variations were significant, indicating a heterogeneous contribution to volatile compound production and the feasibility of strain selection to modulate cheese flavour and aroma. However, the development of suitable yeast starters requires further studies, since complex interactions among cheese microbiota should be taken into account. Characterization of enzyme activities involved in flavour formation by dairy yeasts is in progress.

6. Acknowledgements

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General discussion

Cheesemaking is an intensely investigated process in which milk is acidified by lactose fermentation and casein is coagulated by addition of enzymes. Microorganisms are key components during cheesemaking as long as they perform the fermentation, and influence the quality of the final dairy product by proteolysis and lipolysis. Besides the role of starter lactic acid bacteria, an important contribution is recognized to the secondary microbiota (Beresford et al., 2001). Particularly, yeasts have been described as responsible for the production of specific volatile compounds (Fleet, 2007). Moreover, biotechnological advances highlight these microorganisms and their enzymes as producers of functional ingredients increasing the interest on the study of yeast composition in food and beverages (Deak, 2009).

Frequently, physiological and technological properties of microorganisms are strain dependent, therefore studies involving a wide number of isolates are essential to succeed while screening technological applications. In this sense, traditional fermented foods account for an important microbial diversity when compared with industrial goods. On the other hand, the heterogeneity of microorganisms present in natural fermentations influences the unique sensorial properties of artisanal products (Capozzi and Spano, 2011). In the case of traditional raw milk cheeses, its special aroma is highly appreciated and determines consumer preferences. Hence, the study of the influence of native yeasts on cheese volatilome attracts de attention of researchers (Marilley and Casey, 2004).

Artisanal production of food and beverages has a strong impact in local economies and rural development. Moreover there is an increasing consumer pressure for a larger variety of distinctive quality food products (Renting et al., 2003). However, severe EU food safety legislation has resulted in lower flexibility in food production and will eventually lead to the disappearance of a number of geographical and artisanal dairy products

and their native microorganisms (Golić et al., 2013). The evaluation of yeast diversity conducted in this thesis remarks the influence of unique production processes, such as the use of unpasteurized milk, the specific environmental conditions or the use of homemade brine on the microbial ecology of goats' and ewes' cheeses, and thus on the final product quality.

The aim of this thesis was to explore yeast biodiversity of artisanal goats' and ewes' raw milk cheeses produced within the borders of Natural Park "Sierra de Espadán" and to study their technological applications, in particular galactooligosaccharide (GOS) synthesis and volatile compound production.

In order to screen molecular and technological traits of yeasts and to study microbial population dynamics, a total of 530 yeast strains were isolated and identified from artisanal goats' and ewes' cheeses along the ripening period. Yeast species composition changed greatly along the process; although at the end of the ripening only *Debaryomyces hansenii* could be isolated. Other yeast species commonly found in dairy products were present at the first maturing weeks and disappeared at different times of the process. The most abundant yeast species *D. hansenii*, was followed in number by *Kluyveromyces lactis* and *Candida mesenterica*. These species codominated the cheese environment up to the third week in some cheeses. *D. hansenii* has been described as the predominant yeast in Pecorino di Filano, Gouda and Portuguese ewes' cheese (Capece and Romano, 2009; Pereira-Dias et al., 2000; Welthagen and Viljoen, 1998). However, in a Brazilian cheese, together with *D. hansenii, Torulaspora delbrueckii* and *Candida catenulata* were the prevalent yeasts after five days of ripening (Borelli et al., 2006). Similarly, *D. hansenii*, *K. lactis*, *Geotrichum candidum*, *Candida zeylanoides* and *Candida lambica* were the prevalent species during Fiore Sardo ripening (Fadda et al., 2004).

Other minoritary species were also isolated from the Mediterranean cheeses such as *Kluyveromyces marxianus* or *Yarrowia lipolytica*. All these species have been previously described as typical from dairy environments as summarized in the Introduction (Table 4) (Andrighetto et al., 2000; Corbo et al., 2001; Vasdinyei and Deák, 2003). However, to the best of our knowledge, the species *Trichosporon coremiiforme* and *Trichosporon domesticum* have been found for the first time in cheese. Other species belonging to *Trichosporon* genus, in particular *T. beigelii*, *T. ovoides* and *T. capitatum* have been isolated from milk, butter or yogurt (Büchl and Seiler, 2011). Moreover, *Meyerozyma guilliermondii* has been for the first time isolated from goats' cheeses, although it was previously described in other dairies such as butter milk, yogurt and in the dairy environment (Büchl and Seiler, 2011).

Cheese yeasts may come from various origins such as milk, the dairy factory environment or brine, as different scientific publications have shown for strains isolated from other cheese varieties (Mounier et al., 2005). Giannino et al. (2011) found *D. hansenii* and *T. delbrueckii* in the wooden boxes where Taleggio cheese was dried salted. Likewise, an assessment of yeast diversity associated with Gouda and Cheddar making found *Y. lipolytica*, *C. intermedia* and *S. cerevisiae* (Viljoen and Greyling, 1995). Moreover, Seiler and Busse (1990) revealed the presence of different yeast species including *D. hansenii*, *S. cerevisiae*, *K. marxianus* and *C. versatilis* in German cheese brines. Additionally, a study conducted by Corbo et al. (2001) found yeasts belonging to *D. hansenii*, *Candida*, *Cryptococcus* and *Pichia* genera in milks from different animal origin. The presence and survival of yeasts in cheese seems to be correlated to their degree of tolerance to low pH, salt, low water activity and low storage temperature (Ferreira and Viljoen, 2003). The high tolerance of *D. hansenii* to these physico-chemical factors might favor its dominance at most stages of the cheese ripening process. However, the low tolerance of other yeasts

to these conditions would imply their faster disappearance from the cheese environment (Ferreira and Viljoen, 2003).

Following identification, a typing method to characterize strains from the same species was performed. As proposed by other authors the presence of diverse strains of *D. hansenii* and *K. lactis* in ewes' and goats' cheeses was revealed by RAPD analysis (Capece and Romano, 2009; Fadda et al., 2004; Lopandic et al., 2006). Twelve and nineteen M13 profiles were found for *D. hansenii* and *K. lactis*, respectively. Moreover, in *K. lactis* some patterns were found specific for ewes' or goats' cheese, which suggests the type of milk as the most probable origin of these particular strains. The diversity of genotypes within both yeast species isolated from cheese is in agreement with previous studies of other authors who found different but closely related groups of these species in cheeses (Fadda et al., 2004; Romano et al., 1996). It is important to highlight that the presence of different genetic patterns could indicate that more than one strain of *D. hansenii* and *K. lactis* may be involved in the ripening process as suggested by Petersen et al. (2001). Additionally, in the case of *D. hansenii*, different strains are present at the end of the process suggesting the lack of a single strain imposition.

The evaluation of the enzymatic activities showed differences between yeast species but also within isolates from the same species. A total of 83% of the isolates presented proteolytic activity against casein, the main protein present in milk, while in a study conducted by Cosentino et al. (2001) only around 30% of the isolates presented such enzymatic activity. Proteolysis is one of the most important biochemical changes occurring during the ripening of cheese which influences both texture and flavour development through peptides and amino acids formation (Gonzáles de Llano et al., 1991). Likewise, cheese ripening is also associated with lipolysis of milk fat, an essential element of the development of cheese

aroma (Molkentin, 2013). Different esters from dairy fatty acids (palmitic, steraic and oleic acid esters) were selected to screen esterase activity of yeast isolates. Around 60% of total strains presented activity against palmitic and stearic acid ester, but less than 40% of the strains could hydrolysate oleic acid ester, probably due to the double bound present on its chemical structure. Cosentino et al. (2001) reported similar percentage of positive strains for esterase activity against esters. Moreover, 57% of the isolates presented both caseinolytic and esterase activity. This outcome highlights the contribution of dairy yeasts to milk proteolysis and lipolysis in agreement with literature findings (Capece and Romano, 2009; Fadda et al., 2010).

Additionally to lipolysis and proteolysis, consumption of lactate and lactose by yeasts is determinant for the typical characteristics of some cheese varieties (Jakobsen and Narvhus, 1996), therefore these parameters should be evaluated. Moreover, cheese is a product containing a notable amount of salt, and the presence of this compound in the cheese environment may affect microbial growth (Roostita and Fleet, 1999). Thus, different media composed by lactate or lactose and various concentrations of salt were used to evaluate the growth of genetically different *K. lactis* strains. Yeast growth was evaluated using a microplate format and automated incubator-reader, in which OD was measured during several days. The yeast strains showed slight differences in their growth behaviour and a general trend could be observed. All *K. lactis* were able to grow on media containing lactose, and lactose supplemented with 2% and 5% NaCl although the addition of 8% NaCl seemed to prevent their growth. Additionally, comparison of growth on lactose and NaCl containing lactose media revealed salt dependence as has been previously found in *Saccharomyces* by other authors (Warringer and Blomberg, 2003). Nevertheless, *K. lactis* strains are likely to have a positive impact on cheese quality as the percentage of salt in cheeses varies between 2 and

4%. However, under the conditions tested, *K. lactis* strains were unable to grow on lactate, contrarily to other studies performed using longer incubation time (Lachance, 2011).

Yeasts may also negatively affect food quality, producing spoilage or generating toxic compounds, such as biogenic amines (Fleet, 1992; Wyder et al., 1999). It is well known that fermented foods and particularly cheeses may contain biogenic amines, therefore an evaluation of microbial strains is essential to ensure a selection of safe starters (Komprda et al., 2007; Schirone et al., 2011). *K. lactis* examination for biogenic amine production, revealed that only one from the nineteen strains tested was positive for ornithine decarboxylation, indicating the weak contribution of this yeast to biogenic amine formation, in agreement with previous published reports (Gardini et al., 2006).

The identification and subsequent technological and genetic characterization of yeasts isolated from a natural dairy environment points out the relevance of these microorganisms in artisanal cheese production and their impact on its quality. Nevertheless, it would be interesting to know the origin of the yeasts found along the ripening process. Thus further studies should be addressed to study representative samples from the milk and from the cheese factory environment, such as brine solution, wood shelves or producers' hands in order to broaden the information related to yeast origin. Moreover, different cheese batches produced along the year or from diverse periods should be sampled to determine the seasonal diversity of yeasts, as suggested by other authors (Rea et al., 2007; Viljoen and Greyling, 1995).

From all the dairy isolates belonging to *K. lactis*, *K. marxianus* and *D. hansenii* species, several strains were selected for prebiotic synthesis and volatile compound production. Strains isolated from different kind of

cheeses, several ripening weeks and exhibiting different M13 profiles were chosen.

In this thesis, the use of crude cell extracts (CCEs) from different *K. lactis* and *K. marxianus* strains containing β-galactosidases was assessed for transglycosilation reactions using lactose and lactulose as substrate. The β-galactosidases produced a mixture of different prebiotic galactooligosaccharides (GOS) from lactose, containing mainly the disaccharide 6-galactobiose, and the trisaccharides 3'-, 4'- and 6'-galactosyl-lactose among other minor lactose derived compounds. The synthesis of oligosaccharides from lactulose (OsLu) resulted in mixtures composed mainly by 6-galactobiose, 6'-galactosyl-lactulose and 1-galactosyl-lactulose. It is important to highlight that from all the strains tested, *K. marxianus* strains produced the highest yield of total GOS and OsLu.

In our experiments, oligosaccharide mixtures were formed mainly by trisaccharides, although compounds with a higher degree of polymerization were also detected but not identified. Notably, trisaccharides show the highest selectivity towards bifidobacteria (Kaneko et al., 1994). Similarly, commercial GOS preparations contain mixtures of tri, tetra, penta, and hexagalactooligosaccharides (Cardelle-Cobas, 2009). Moreover, the mixtures obtained in our experiments were mainly formed by linkages β1→6 and to a lesser extent by β1→3 and β1→4. Glycosidic linkages β1→6 and β1→1 seemed to be more effective than those β1→4 (Cardelle-Cobas et al., 2011). Cup-Oligo and VivinalGOS produced by *Cryptococcus laurentii* and *Bacillus circulans* β-galactosidases, respectively, contain GOS with glycosidic linkages β1→3 and β1→4. On the contrary, Bimuno which is produced by *Bifidobacterium bifidum,* contains mainly linkages β1→3 (Cardelle-Cobas, 2009). Besides, a product manufactured combining *Aspergillus oryzae* and *Streptococcus thermophilus* β-galactosidases

named Oligomate contains mainly structures with linkages β1→6 and β1→4 (Ito et al., 1990). The difference in composition found among different commercial preparations and the mixtures obtained in this thesis with *Kluyveromyces* β-galactosidases may be due to the different microbial origins of the enzymes and to variations in physico-chemical conditions such as substrate concentration, time, pH or temperature (Otieno, 2010).

The industrial production of prebiotics faces several challenges, including the search for abundant and economical raw materials (Figueroa-González et al., 2011). An approach in food and beverage industry is to profit byproducts to generate value-added goods. This strategy has been widely employed with cheese whey, because its worldwide production is estimated around 180 to 190×10^6 ton/year, only 50% is processed and displays serious environmental problems (Baldasso et al., 2011; Gänzle et al., 2008). In this context, production of prebiotic oligosaccharides is a feasible option (Rustom et al., 1998). In fact, several studies report GOS and OsLu production from cheese whey permeate (Adamczak et al., 2009; Corzo-Martínez et al., 2013). Thus the use of dairy *Kluyveromyces* regarding prebiotic synthesis from cheese whey would be an interesting approach for further studies.

Although GOS are currently added to infant formulas, fruit juices, yoghurts, milk or buttermilk powder (Čurda et al., 2006; Torres et al., 2010), some of the applications of the health claims based on their bifidogenic properties have been recently rejected by European regulatory bodies (EFSA Panel on Dietetic Products, 2011). Further studies should be addressed to unravel the specific mechanism of action through which prebiotics exert a beneficial effect to the gut of the host (van Loveren et al., 2012).

Some of the microorganisms frequently isolated from traditional dairies have been declared as probiotics indicating the possible health-

promoting role of the artisanal products (Golić et al., 2013). Other health related activities have been attributed to non-conventional yeasts such the improvement of bioavailability of minerals through the hydrolysis of phytate and folate biofortification (Moslehi-Jenabian et al., 2010). In the particular case of *Kluyveromyces*, it has been proven that *K. lactis* is able to produce phytases (Nakamura et al., 2000) and that *K. marxianus* increases the content of folate in a fermented African product (Hjortmo et al., 2008) and has been characterized as probiotic (Maccaferri et al., 2012). Hence, coming investigations with food isolates may be addressed to evaluate health beneficial properties of non-conventional yeast, as has been suggested by recent scientific publications (Binetti et al., 2013; Diosma et al., 2013; Pedersen et al., 2012).

Finally, the aromatic profile of Mediterranean ewes' and goats' cheeses was analyzed. In order to study how native yeasts influence cheese aroma, different strains isolated from the studied cheeses were inoculated in a synthetic cheese-like medium. This simple method has been previously validated by other authors and has been applied in this thesis as a first approach to know which volatile compounds may be generated due to yeast inoculation (Kagkli et al., 2006). Since the volatile compounds were extracted by SPME, comparisons can be only done among the different yeast strains used in the present study.

Different strains belonging to three different species were studied: *D. hansenii* and *K. lactis* were selected since both were the predominant yeast species in the studied cheeses and *K. marxianus* because it was the yeast yielding the highest amount of GOS and OsLu, and could therefore add interest to the evaluation. We have shown that native yeasts are likely to have an impact on raw milk cheese volatilome. Principal chemical groups detected were: alcohols, aldehydes, ketones, esters and acids. Distinct flavour profiles were obtained, since marked differences at quantitative and

qualitative level among strains and species were detected. *Kluyveromyces* strains were related to the main production of volatile compounds. *K. lactis* produced compounds such as ketones, esters such as ethyl acetate and secondary alcohols, whereas *K. marxianus* produced acids (acetic, propanoic and octanoic acids), ethyl decanoate, 1-propanol or benzaldehyde among others.

In this thesis, the effect of a single yeast strain inoculation in the volatile compound production was evaluated, while in cheese, different yeast species and strains coexist along the ripening process (Fadda et al., 2004). Besides coexisting, interactions are likely to occur, affecting growth parameters of the strains involved. In this sense, some strains of *D. hansenii* isolated from Camembert and blue-veined cheeses enhanced the growth of *Y. lipolytica* and *K. marxianus* (Addis et al., 2001). Therefore, further experiments should evaluate the effect of longer co-inoculation of a combination of different native yeasts in order to understand how microbial population dynamics affects production of volatile compounds (Fleet, 2007). Mixed cultures of bacteria and yeast should also be addressed. Martin et al. (2001) studied the aroma compound formation in cheese curd by growing *K. lactis* with bacteria, and high production of aldehydes and esters was found. Moreover the overall aroma compound production of *K. lactis* was enhanced in comparison with pure cultures. On the contrary, *Y. lipolytica* produced unremarkable changes when associated with bacteria. Similarly, De Freitas et al. (2008) studied the impact of inoculating yeast to real Cantalet cheese, where *K. lactis* but not *Pichia fermentans* promoted flavour development. Thus, both studies have shown frequent interactions between species and that aromatic profiles may vary when grown in mixed cultures. Therefore, the influence of coculturing lactic acid bacteria with yeasts should be also taken into account in future experiments since both kinds of microorganisms are present in the cheese ecosystem. Moreover, research should be also addressed to test the volatile production ability in a

medium composed by milk, cheese or directly in a cheese model (De Freitas et al., 2008; Leclercq-Perlat et al., 2004; Price et al., 2013; Sørensen et al., 2011). In these cases, results might be much closer to the real cheese context.

Even though only four *K. marxianus* strains were isolated along the ripening process of the studied cheeses, this species presented the most promising results concerning both generation of key volatile compounds and synthesis of prebiotic oligosaccharides. Contrarily to our results, surveys of other dairies revealed a widest population of this yeast, as it is the case of kefir or French cheeses (Gao et al., 2012; Sohier et al., 2009; Vardjan et al., 2013). Therefore it would be interesting to explore a large variety of *K. marxianus* strains obtained from other sources to test their potential technological abilities.

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Conclusions

1. Yeast diversity associated with raw milk artisanal ewes' and goats' cheeses produced in Mediterranean Spain has been shown, although at the end of the ripening process only *Debaryomyces hansenii* was found. Moreover, the enzymatic characterization pointed out the potential contribution of isolated yeasts to the release of fatty acids and amino acids from milk components.

2. Population analysis of *D. hansenii* showed limited genetic divergence, indicating a close relationship which could be associated with the same origin of isolation. The diversity of *D. hansenii* and *Kluyveromyces lactis* isolated from goats' cheeses increased along the cheese ripening process. *K. lactis* strain patterns were characteristic of either goats' or ewes' milk cheeses.

3. *K. lactis* strains were able to grow on lactose media but not on lactate medium. The growth on lactose media was affected by salt content. Production of biogenic amines was not a frequent characteristic of *K. lactis* strains.

4. The feasibility of β-galactosidases from *K. lactis* and *K. marxianus* to transgalactosylate lactose and lactulose has been shown. Reaction mixtures with different concentrations of individual oligosaccharides were obtained. *K. marxianus* enzyme yielded the highest total oligosaccharide amount when lactose or lactulose were used as acceptor carbohydrates.

5. Dairy yeasts were capable to generate cheese key volatile compounds, which suggests their influence on cheese aroma. Quantitative and qualitative differences in volatile compounds were found among yeast species and among strains. In the conditions tested, *Kluyveromyces* species stood out for volatile production whereas *D. hansenii* contribution was not relevant.

6. This study has confirmed the potential of dairy yeasts to produce prebiotic ingredients and to contribute to the final cheese flavor. Since marked differences were found among strains belonging to the same species, strain selection is highly advisable for biotechnological applications.

7. This thesis highlights the biotechnological potential of *K. marxianus*, since this species was the best prebiotic oligosaccharide and volatile compound producer.

Annexes

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Yeast species and genetic heterogeneity within *Debaryomyces hansenii* along the ripening process of traditional ewes' and goats' cheeses

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ABSTRACT

The yeasts present during the ripening process of ewes' and goats' cheeses produced in a small traditional dairy in Mediterranean Spain were isolated and identified. Five hundred and thirty strains pertaining to eleven yeast species representing eight genera were identified using molecular methods. *Debaryomyces hansenii* was the yeast species most frequently isolated in all cheeses. Other yeast species commonly found in dairy products were present at the first maturing weeks. Two yeast species *Trichosporon coremiiforme* and *Trichosporon domesticum* have been reported in cheeses for the first time, and *Meyerozyma guilliermondii* has been newly isolated from goats' cheeses. Yeast species composition changed greatly along the process; although, *D. hansenii* dominated at the end of ripening in all cheeses. Most yeast isolates were able to hydrolyze casein and fatty acid esters. One hundred and eighty seven *D. hansenii* isolates were genotyped by PCR amplification of M13 satellites and an UPGMA dendrogram was constructed. The majority of isolates were grouped in 5 clusters while 7 profiles were represented by 1–3 isolates. These results demonstrate the genetic heterogeneity present in *D. hansenii* strains isolated from raw milk cheeses.

1. Introduction

Mediterranean European countries account for the production of most caprine and ovine milk worldwide. The majority of this production is converted into cheese in small artisanal dairies, using traditional making methods leading to the development of different cheese varieties with unique organoleptic characteristics (Freitas and Malcata, 2000).

The development of flavor and texture distinctive of a cheese variety are the result of complex processes involving microbiological and biochemical changes to the curd during ripening. Cheese microbiota may be divided into lactic acid bacteria and secondary microorganisms (Beresford et al., 2001). Yeasts are an important constituent of the secondary microbiota, which development is favored by the physico-chemical properties of the cheese such as low pH, low moisture content, elevated salt concentration and refrigerated ripening and storage (Fleet, 1990; Viljoen et al., 2003). Regarding the biochemical changes, these yeasts play an important role in proteolysis, lipolysis, fermentation of residual lactose, and assimilation of lactic and citric acid during the ripening of cheese, contributing to aroma development and to the rheological properties of the final dairy product (McSweeney, 2004). Additionally, some cheese yeasts have been recognized by their probiotic character and DNA-bioprotective action against model genotoxins (Kumura et al., 2004; Trotta et al., 2012).

Freitas and Malcata (2000) reviewed the most important aspects of the microbial characteristics of cheeses manufactured from ovine and caprine milk in Spain. These studies have focused mainly on the identification and characterization of bacteria; however, there is little knowledge about the yeast population associated with these cheeses.

The impact of yeasts on the production and quality of the cheese is related to their ecology and biological activities (Fleet, 2007). Physico-chemical characteristics of cheese such as low pH, low water activity and high salt content and refrigerated storage favor yeast growth (Fleet, 1990). The number of yeast species frequently isolated from milk and dairy products listed in the Encyclopedia of Dairy Sciences is substantial (Büchl and Seiler, 2011). Species identification and characterization are therefore essential to understand the occurrence and role of yeast in cheeses.

In order to understand the differences between cheese varieties, we need to increase our knowledge on the yeast microbiota leading the ripening process. Several authors have pointed out the main role of *Debaryomyces hansenii* leading during cheese ripening (Fleet, 1990; Fox et al., 2000). In most Mediterranean ewes' and

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Evaluation of Oligosaccharide Synthesis from Lactose and Lactulose Using β-Galactosidases from *Kluyveromyces* Isolated from Artisanal Cheeses

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ABSTRACT: The β-galactosidase activity of 15 *Kluyveromyces* strains isolated from cheese belonging to *Kluyveromyces lactis* and *Kluyveromyces marxianus* species was tested for the production of oligosaccharides derived from lactose (GOS) and lactulose (OsLu). All *Kluyveromyces* crude cell extracts (CEEs) produced GOS, such as 6-galactobiose and 3′-, 4′-, and 6′-galactosyl-lactose. At 4 h of reaction, the main trisaccharide formed was 6′-galactosyl-lactose (20 g/100 g of total carbohydrates). The formation of OsLu was also observed by all CEEs tested, with 6-galactobiose, 6′-galactosyl-lactulose, and 1-galactosyl-lactulose being found in all of the reaction mixtures. The synthesis of trisaccharides predominated over other oligosaccharides. *K. marxianus* strain O3 produced the highest yields of GOS and OsLu after 4 h of reaction, reaching 42 g/100 g of total carbohydrates (corresponding to 80% lactose hydrolysis) and 45 g/100 g of total carbohydrates (corresponding to 87% lactulose hydrolysis), respectively. Therefore, the present study contributes to a better insight into dairy *Kluyveromyces* β-galactosidases and shows the feasibility of these enzymes to transglycosylate lactose and lactulose, producing high yields of prebiotic oligosaccharides.

KEYWORDS: *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *transgalactosylation*, *GOS*, *OsLu*, *lactose*, *lactulose*

INTRODUCTION

β-Galactosidase (EC 3.2.1.23) is a hydrolase that attacks the terminal non-reducing β-D-galactosyl residues of oligosaccharides and transfers the galactosyl moiety to suitable acceptors. These enzymes have several applications in the food fermentation and dairy industries, and mainly because of their ability to hydrolyze lactose, they have attracted the attention of researchers and dairy product manufacturers.[1] Transgalactosylation is favored over hydrolysis in the presence of high substrate concentrations, and in the case of lactose, β-galactosidases produce galacto-oligosaccharides (GOS).[2] GOS are mainly disaccharides (allolactose and galactobiose), trisaccharides (4′- and 6′-galactosyl-lactose), and longer chain oligosaccharides consisting of four or more monosaccharide units.[3]

Although transgalactosylation of lactose has been known for more than 50 years,[4] GOS production is gaining importance because of their recognition as prebiotics.[5] Moreover, the influence of the GOS structure on prebiotic selectivity has been demonstrated.[6] Other health benefits, such as the improvement of mineral absorption, prevention of intestinal infections, and enhancement of immune function, among others, have been described.[7−10]

Recently, the synthetic disaccharide lactulose (4-*O*-β-D-galactopiranosyl-D-fructose) has been proposed as an enzymatic substrate for lactulose-derived oligosaccharide (OsLu) production.[11−13] Although lactulose has been recognized as prebiotic,[14,15] gas production associated with its fermentation in the proximal colon may represent a disadvantage for lactulose ingestion.[16] In this context, synthesis of OsLu may provide a new group of active compounds with health beneficial effects complementary to those provided by GOS[17] and probably without the inconvenience of lactulose consumption.

Nowadays, microbial β-galactosidases represent a feasible alternative to the chemical synthesis of GOS, with the benefits of enzymatic stereospecificity and higher final yields. β-Galactosidases have been frequently characterized in lactic acid bacteria and bifidobacteria related to milk, milk products, and the intestine of neonates.[18,19] The genus *Kluyveromyces* and specifically the species *Kluyveromyces lactis* have received considerable attention as both a genetic model and industrial yeast as a source of different metabolites and enzymes.[20] Similarly, the species *Kluyveromyces marxianus* has been explored because of its potential biotechnological applications, although the accumulated knowledge on *K. marxianus* is much smaller compared to that on *K. lactis*.[21] Both species, present in dairy products, are considered generally recognized as safe (GRAS) microorganisms and present a good growth yield and a higher β-galactosidase activity compared to other yeasts.[22] Thus, both species are relevant industrial sources of β-galactosidase activity, and they have been traditionally used

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Potential impact of dairy yeasts on the typical flavour of traditional ewes' and goats' cheeses

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ABSTRACT

The contribution of *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Kluyveromyces marxianus* strains to the typical flavour of traditional ewes' and goats' cheeses was assessed. Fourteen yeast strains were grown in liquid medium mimicking cheese composition and volatile compounds were identified by gas chromatography-mass spectrometry. Yeasts were able to produce key volatile compounds characteristic of the cheeses from which they were isolated. Inter-species and inter-strain variations were observed. Under the conditions tested, *D. hansenii* produced the lowest levels of volatile compounds, with large intra-strain variations. *Kluyveromyces* strains primarily produced esters and alcohols. *K. marxianus* strains were associated with the production of acids, ethyl decanoate, 1-propanol and benzaldehyde, whereas *K. lactis* was correlated with the presence of ketones, ethyl acetate and secondary alcohols. In conclusion, this study shows the heterogeneous potential of dairy yeasts to contribute to final cheese flavour.

1. Introduction

Yeasts play an important role in proteolysis, lipolysis, fermentation of residual lactose, and assimilation of lactic and citric acid during the ripening of some cheeses, contributing to aroma development and to the rheological properties of the final dairy product (McSweeney, 2004). Moreover, yeasts have been recovered from all stages of cheesemaking, as well as from milk, brine and dairy process equipment among others (Corbo, Lanciotti, Albenzio, & Sinigaglia, 2001; Delavenne et al., 2011; Gardini et al., 2006; Seiler & Busse, 1990).

Debaryomyces hansenii is the dominant yeast species found in most cheese varieties (Fleet, 1990; Fox, Guinee, Cogan, & McSweeny, 2000). *D. hansenii* possesses the ability to grow at high salt concentrations, low pH and low water activity, as well as metabolising lactic and citric acids, which makes cheese a suitable environment for its proliferation (Breuer & Harms, 2006). The lactose-fermenting yeasts *Kluyveromyces lactis* and *Kluyveromyces marxianus* are also regularly found in dairy products and milk. Their lactose-fermenting ability promotes their growth in the cheese, where other yeasts are scarce. Besides these species, cheeses may often contain other yeast species, such as *Yarrowia lipolytica*, *Geotrichum candidum* and *Saccharomyces cerevisiae* (Fleet, 1990).

Cheese flavour is one of the most relevant attributes influencing consumers' acceptance and preference (Arora, Cormier, & Lee, 1995), and is the result of a complex balance between various volatile and non-volatile compounds, which individually do not reflect the overall odour and taste (Fox & Wallace, 1997). Many volatile compounds have been implicated in cheese aroma, such as acids, esters, ketones, aldehydes, alcohols or sulphur compounds, and each dairy product has a characteristic and unique composition of volatile components (Plutowska & Wardencki, 2007).

The contribution of yeasts to development of cheese aroma is considered positive in some instances, creating commercial interest in using selected strains as ripening cultures (Frohlich-Wyder, 2003; Romano, Capace, & Jespersen, 2006). Several studies have shown that, in different cheeses, relevant yeast species contribute differently to volatile production. *G. candidum* and *Y. lipolytica* are known to produce considerable amounts of various volatile sulphur compounds; *K. lactis*, *K. marxianus* and *S. cerevisiae* have been found to produce primarily esters; and *D. hansenii* mainly produced branched-chain aldehydes and alcohols (Arfi, Spinnler, Tache, & Bonnarme, 2002; Leclercq-Perlat, Corrieu, & Spinnler, 2004; Martin, Berger, Le Du, & Spinnler, 2001; Sørensen, Gori, Petersen,

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