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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 a UPGMA dendrogram was constructed. The majority of isolates were grouped in 5 clusters while 7 profiles were represented by 1 to 3 isolates. These results demonstrate the genetic heterogeneity present in *D. hansenii* strains isolated from raw milk cheeses.

Keywords: cheese, yeast identification, enzymatic activities, D. 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 physicochemical 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

73 characterization of bacteria; however, there is little knowledge about the yeast
74 population associated with these cheeses.

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

83 In order to understand the differences between cheese varieties, we
84 need to increase our knowledge on the yeast microbiota leading the ripening
85 process. Several authors have pointed out the main role of *Debaryomyces*
86 *hansenii* leading during cheese ripening (Fleet, 1990; Fox et al., 2000). In most
87 Mediterranean ewes' and goats' cheeses, the yeast species *D. hansenii* seems
88 to be predominant in the ripening process (Capece and Romano, 2009;
89 Cosentino et al., 2001; Fadda et al., 2004; Gardini et al., 2006; Pisano et al.,
90 2006). However, regardless of the importance of *D. hansenii* in the ripening
91 process, very few efforts have been done to investigate changes in the
92 succession of *D. hansenii* population (Capece and Romano, 2009; Petersen
93 and Jespersen, 2004) . Other yeast species such as *Trichosporon cutaneum*
94 (Corbo et al., 2001), *Candida zeylanoides* (Fadda et al., 2010; Pereira-Dias et
95 al., 2000) and *Geotrichum candidum* (Tornadijo et al., 1998) have also been
96 identified as the main yeast in the ripening process of some cheese varieties.

97 This paper reports the identification by molecular methods of yeast
98 isolated along the ripening process of four ewes' and goats' cheeses produced
99 with raw milk using traditional methods in a small dairy sited within the borders
100 of the Natural Park "Sierra de Espadán" (Castellón, Spain). The yeast isolates
101 were also characterised by several technological features. In addition, the
102 genetic heterogeneity within *D. hansenii* strains isolated at different stages of
103 the ripening process was analysed by minisatellite M13 PCR.
104
105 **2. Materials and methods**
106 *2.1. Cheese sample processing*
107 Two goats' (G1 and G2) and two ewes' (E1 and E2) commercial semi-
108 hard cheeses produced in the spring season with raw milk and bacterial starters
109 were sampled during the ripening process. Samples were weekly taken from
110 the first day of ripening after salting and along six weeks of the process.
111 Cylindrical samples (0.5 cm x 5 cm approximately) consisting of rind and
112 cheese interior were taken along the cheese ripening process, collected into
113 sterile flasks and transported refrigerated to the laboratory.
114 Cheese samples of 5 g were aseptically weighted into 10 mL of saline
115 solution in a sterile tube and homogenized using a Polytron PT 2100
116 (Kinematica AG, Switzerland). Microbiological analyses were performed
117 immediately after sample homogenization.
118 *2.2. Yeast content and isolation*
119 Decimal dilutions from 10^{-1} to 10^{-7} of the homogenized samples were
120 carried out for microbiological assays. Samples of 0.1 mL from all dilutions were
121 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 25 ºC 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 -80 ºC.

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′GAGGGTGGCGGTTCT3′) was performed as described in (Fadda et al.,

147 2004) with minor modifications using a PCR (Mastercycler Pro, Eppendorf,
148 Hamburg, Germany). Final volume reaction, 50 µL, contained 0.3 µL rTaq (5U)
149 DNApolymerase, 4 µL dNTP mix (2.5 mM), 5 µL buffer (Takara Bio Inc., Shiga,
150 Japan), 3 µL $MgCl_2$ (1.5 mM) (Sigma, St. Louis, MO, USA), 1 µL M13 primer
151 (Isogen Life Science, PW de Meern, The Netherlands) and 80–100 ng of
152 genomic DNA (Querol et al., 1992). PCR amplification conditions were as
153 follow: 95 °C for 5 min followed by 40 cycles at 93 °C for 45 s, 44.5 °C for 1 min
154 and 72 °C for 1 min with a final extension step at 72 °C for 6 min. The PCR
155 products (10 µL) were resolved by electrophoresis on 2% agarose gel in 1×
156 TAE buffer at 90 V for 3 h, stained with RedSafe (INtRON Biotech., Spain) and
157 visualized under UV light. DNA fragment sizes were determined using a 100-bp
158 DNA ladder (Life Technologies, Carlsbad, CA, USA).

159 *2.5. Technological characterization of yeasts: evaluation of proteolytic and*
160 *lipolytic activities*

161 Proteolytic activity was evaluated in GPYA medium containing 10% of skim milk
162 (Difco, Franklin Lakes, NJ, USA) following the methodology of (Gardini et al.,
163 2006). Proteolysis was considered positive when a light halo was visible (1 to 5
164 mm) after 15 days. Lipolytic activity was assayed on Tween agar medium
165 containing 1% peptone, 0.5% NaCl, 0.01% $CaCl_2$ supplemented with 1% of
166 Tween 40 (palmitic acid ester), Tween 60 (estearic acid ester) or Tween 80
167 (oleic acid ester) (Sigma, St Louis, MO, USA) following the methodology of
168 (Sierra, 1957). Presence of a precipitation ring around the colonies after 15
169 days of incubation indicated lipolytic activity.

170 *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 5.8S ITS rDNA and their correlation with the result of the BLASTN sequence comparison for the D1/D2 of 26S rDNA gene. 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.

196 The most abundant yeast species in all cheeses was *Debaryomyces*
197 *hansenii* (Figure 1) except in cheese G1, which rendered a higher number of
198 *Kluyveromyces lactis* isolates. Other yeast species were isolated in minor
199 numbers. In all cheeses, yeast diversity decreased along cheese maturation,
200 being *D. hansenii* the most abundant yeast at the end of the process. In
201 cheeses E1 and G1, yeast species *D. hansenii* appeared at the second or third
202 maturation week although concurring with *K. lactis* and other yeast species up
203 to the fifth or sixth week of the process. On the contrary, in cheeses E2 and G2,
204 yeast species *D. hansenii* appeared at the initial stages of cheese ripening and
205 dominated the process since the fourth and third ripening weeks, respectively.

206 *3.2. Technological characterization*

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

215 Regarding lipolytic activity, around 60% of the isolates were able to
216 hydrolyze palmitic acid and stearic acid esters whereas this number decreased
217 below 40% in case of oleic acid ester hydrolysis. Most isolates showing lipolytic
218 activity on palmitic acid ester were also able to hydrolyze stearic acid ester; but
219 from these very few isolates could hydrolyze oleic acid ester (data not shown).
220 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).

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

246 the last three weeks. Similarly, B patterns were found in *D. hansenii* strains from
247 goats' cheeses isolated from the three last weeks of the ripening process. The
248 overall level of diversity in the M13 patterns was higher in goats' cheeses than
249 in ewes' cheeses. Furthermore, M13 pattern heterogeneity increased as the
250 ripening process of goats' cheeses progressed, indicating that the number of
251 different *D. hansenii* isolates increased from the first weeks to the last weeks of
252 cheese maturation.
253
254 **4. Discussion**
255 Yeast identification using the RFLPs of the ITS-5.8S rDNA region
256 produced the same results as sequence comparison of the D1/D2 26S rDNA
257 gene, thus confirming the value of the former technique as already mentioned in
258 previous reports (Álvarez-Martín et al., 2007; Dlauchy et al., 1999; Esteve
259 Zarzoso et al., 1999; Gardini et al., 2006). Our results show that the main yeast
260 species isolated from ewes' and goats' cheeses are common inhabitants of
261 dairy products. However, few yeast species such as *Trichosporon coremiiforme*
262 and *T. domesticum* have been isolated from cheeses for the first time. Early
263 reports on yeast microbiota of dairy products point to *T. ovoides*, *T. cutaneum*
264 and *T. capitatum* as the sole species of the genus *Trichosporon* found in dairy
265 products. Similarly, *Mz. guilliermondii* has been isolated from goats' and ewes'
266 cheeses for the first time although has been previously found in numerous dairy
267 products (Büchl and Seiler, 2011).
268 Fox et al. (2000) found that *D. hansenii* was by far the dominant yeast
269 found in most cheeses, followed by *K. lactis*, *Y. lipolytica* and *T. beigelii*
270 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 (Gardini et al., 2006; Fadda et al., 2004; Fadda et al., 2010; 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).

296 Microbial proteolysis and lipolysis promote complex metabolic changes in
297 the cheese which are vital for proper development of both flavor and texture
298 (Bintsis et al., 2003; Klein et al., 2002; Leclercq-Perlat et al., 2007; Roostita and
299 Fleet, 1996). The evaluation of enzymatic activities conducted in this study
300 shows that most *D. hansenii* isolates were able to hydrolyze casein as well as
301 palmitic and stearic acid esters. Oleic acid ester, probably due to the presence
302 of a double bond, was hydrolyzed by very few isolates with the exception of *Y.*
303 *lipolytica* and *M. guilliermondii*. Although the wide array of substrates and
304 conditions used to test these enzymatic activities hinders comparison among
305 studies (Capece and Romano, 2009; Cosentino et al., 2001; Fadda et al., 2004;
306 Fadda et al., 2010; Gardini et al., 2006; Pereira-Dias et al., 2000) our results
307 show the proteolytic and lipolytic potential of these yeasts. The contribution of
308 these dairy yeast enzymatic activities to cheese quality deserves future studies.
309 PCR amplification of M13 minisatellites revealed a remarkable diversity
310 within the *D. hansenii* isolates although the similarity between most patterns
311 indicates a notable degree of genetic closeness. Similar studies by Capece and
312 Romano (2009) showed a comparable level of diversity in *D. hansenii* isolated
313 from two dairies in Basilicata region, Italy. By contrast, other studies have found
314 scarce diversity among different dairies using similar techniques (Fadda et al.,
315 2004; Romano et al., 1996). The overall genetic diversity within *D. hansenii* has
316 been analyzed by several authors using different methodologies demonstrating
317 the complexity of taxon D. hansenii (Groenewald et al., 2008; Nguyen et al.,
318 2009; Jacques et al., 2009; Lopandic et al., 2013). The analysis by Sohier et al.
319 (2009) revealed that *D. hansenii* strains isolated from the same origin are
320 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.

5. 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 aminoacids 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.

Acknowledgements

Authors thank grants Consolider Ingenio Fun-C-Food, CSD2007-00063 and INIA RM2007-00010 for financial support. BP thanks CSIC for a JAE Predoc contract. Authors are grateful to Cheese Company "Los Corrales" for kindly supplying the cheese samples.

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476 **Figure Captions**

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

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

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

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] Cfo I	HaeIII	HinfI	100% similarity GenBank ACCN[b]	Literature[c]
C. mesenterica	390	390	390	195 + 195	U45720	1, 4
C. parapsilosis	550	300 + 240	420 + 110	280 + 260	AF374609	2, 3, 4, 5
D. hansenii	650	300 + 300	420 + 150 + 90	325 + 325	JQ689041	1 - 10
K. lactis	740	285 + 190 + 165 + 90	655 + 80	290 + 180 + 120 + 80	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.
Kz. unispora	775	350 + 310 + 115	500 + 110	400 + 375	AY048158	1, 9
Mz. guilliermondii	625	300 + 265	400 + 115 + 90	320 + 300	JQ689047	-
P. kudriavzevii	550	220 + 190 + 90	400 + 100	230 + 160 + 140	AY048158	3
T. coremiiforme	550	275 + 275	500	275 + 275	AF139983	-
T. domesticum	550	275 + 275	500	250 + 160 + 100	JN939449	-
Y. 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.

Table

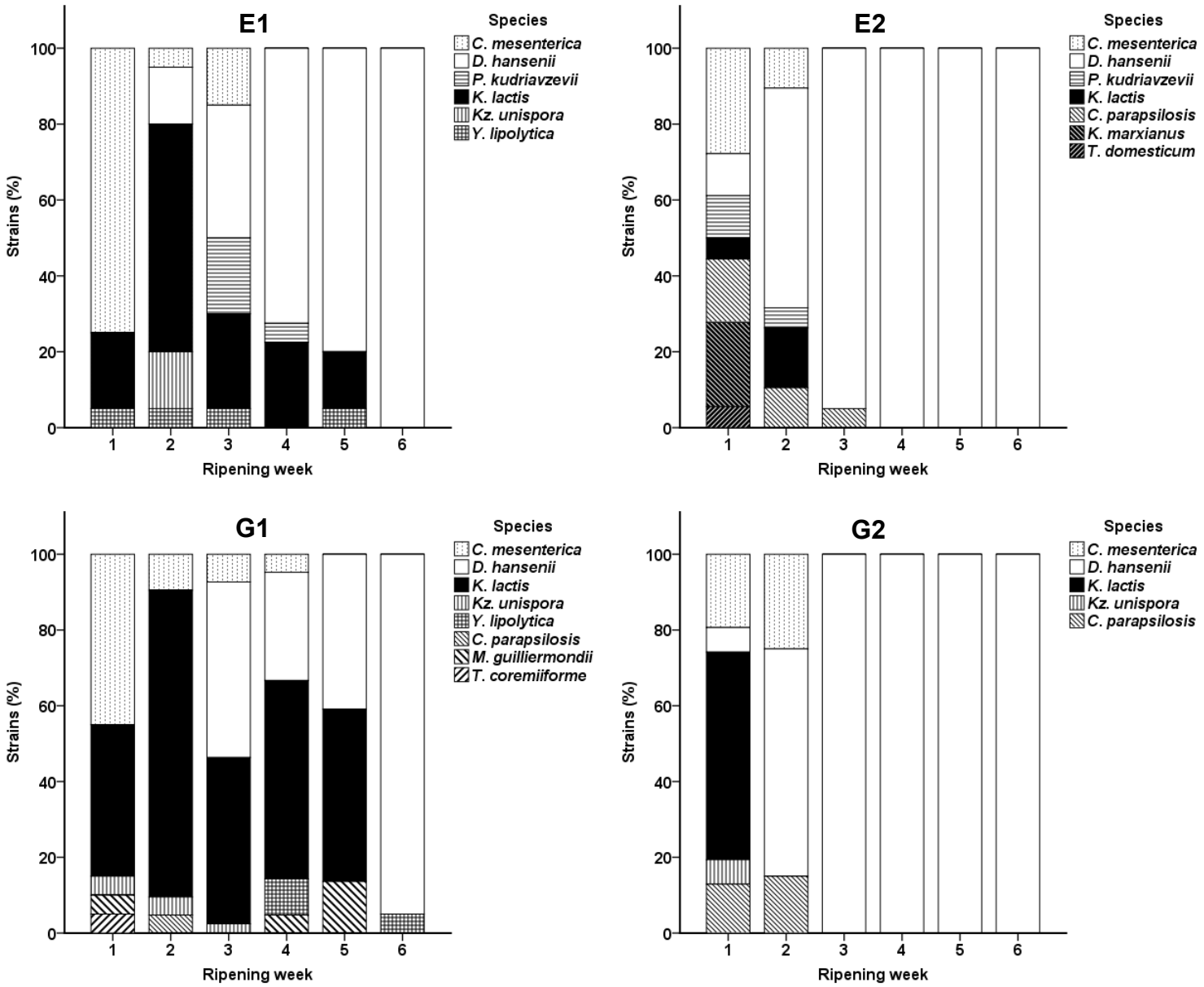
Table 2.- Percentage of isolates from each yeast species showing enzymatic activities.

Hydrolysis of:	Casein	Palmitic acid ester	Stearic acid ester	Oleic acid ester
C. mesenterica	8	- [a]	-	-
C. parapsilosis	100	43	43	7
D. hansenii	92	86	91	58
K. lactis	97	17	17	8
K. marxianus	50	-	-	-
Kz. unispora	89	-	-	11
M. guilliermondii	60	100	100	100
P. kudriavzevii	44	-	-	-
T. coremiiforme	100	100	100	100
T. domesticum	100	100	100	100
Y. lipolytica	50	-	17	83

[a] No enzymatic activity was detected

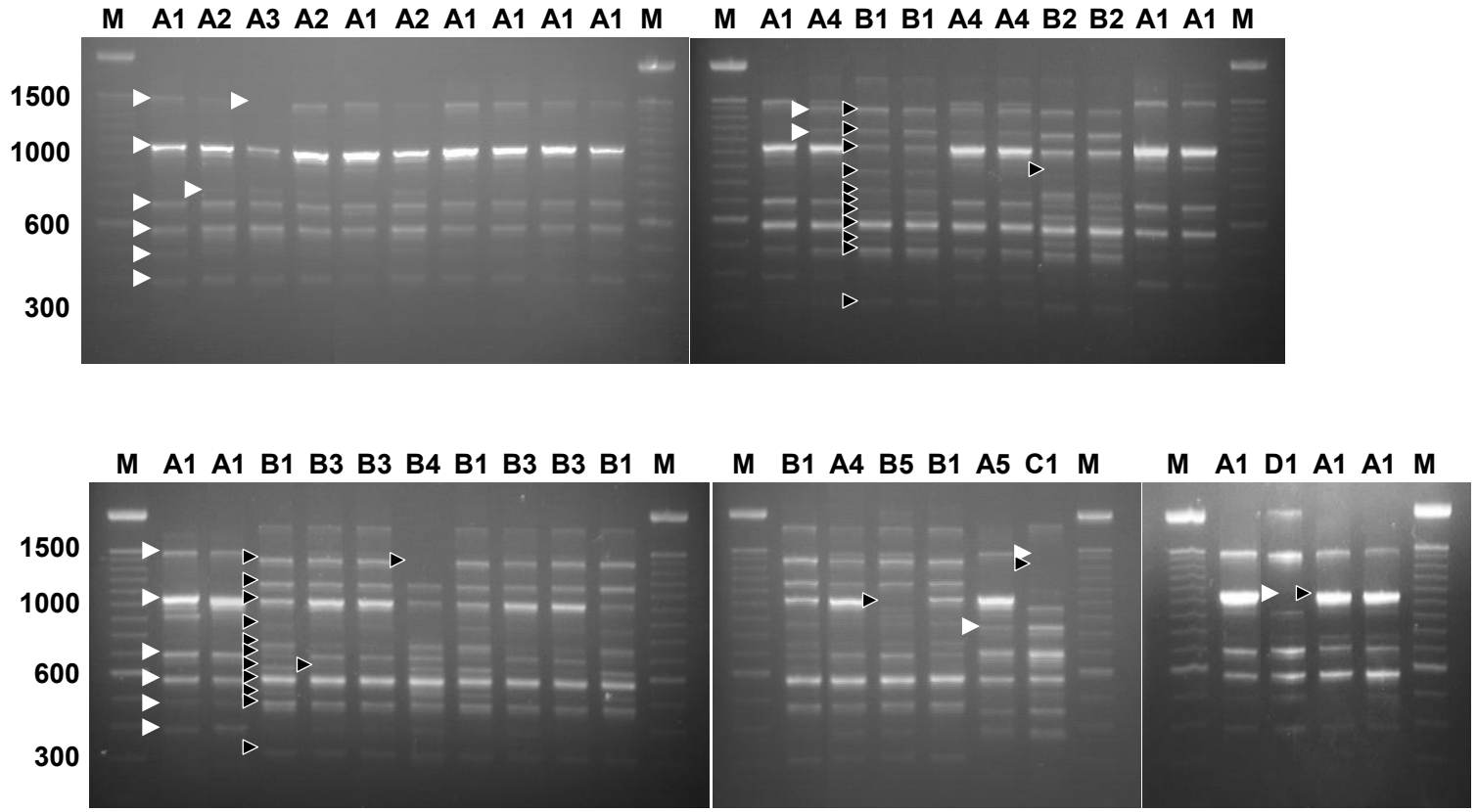
Figure

Figure 1.- Padilla et al. 2013



Figure

Figure 2 Padilla et al.



Figure

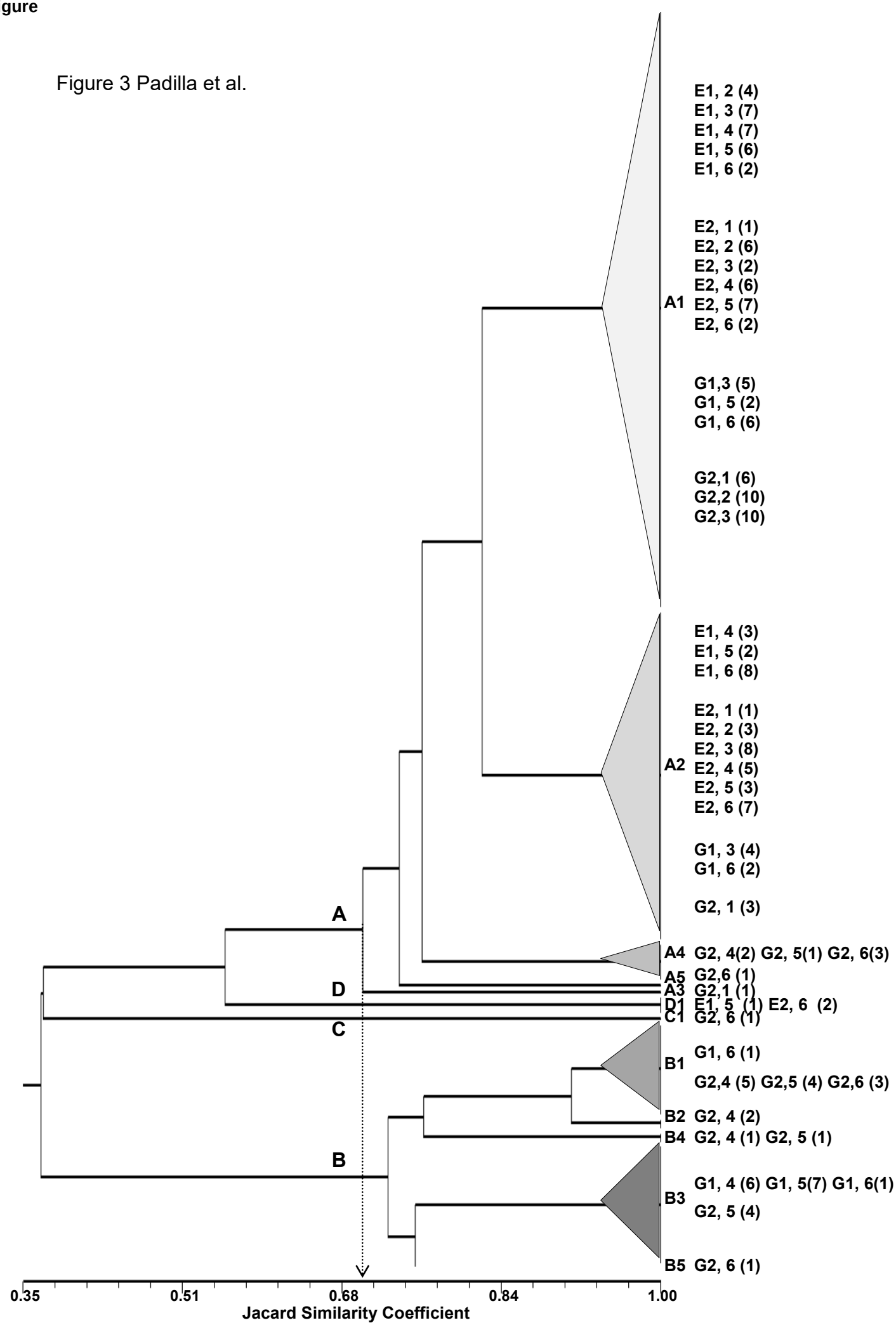



Figure 3 Padilla et al.