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Caridi A, 2021. Physiological characterisation of Calabrian dairy yeasts and their possible use as adjunct cultures for cheese making. Acta Alimentaria, Volume 50(3), Pages 341-348, ISSN 0139-3006

which has been published in final doi https://doi.org/10.1556/066.2021.00001

Physiological characterisation of Calabrian dairy yeasts and their possible use as adjunct cultures for cheese making

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ABSTRACT

Seventeen samples of Calabrian ewe's milk, ewe's cheese (*Pecorino del Poro*) made with raw milk, goat's milk, and goat's cheese (*Caprino d'Aspromonte*) made with raw milk were used to obtain 124 yeast isolates. The most abundant species was *Debaryomyces hansenii* (61.3%), followed by *Candida zeylanoides* (32.3%) and *Kluyveromyces marxianus* (3.2%). The enzymatic profile of 25 selected yeast strains was determined. Lastly, they were studied for their interaction with eight dairy lactic acid bacteria - four coccal-shaped and four rod-shaped. The best strains may be used as adjunct cultures for cheese making.

KEYWORDS

adjunct culture, characterisation, cheese microbiology, raw milk, yeasts

1. INTRODUCTION

On the Calabrian mount Poro plateau the production of the artisanal *Pecorino del Poro* cheese is widespread; it is produced in an area of about 665 Km^2 in the province of Vibo Valentia. Each farm

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has 150-180 sheep, all half-wild, and almost exclusively Sarda breed. The annual production of cheese by 40 about farms is approximately 280 t; it is a semi-hard or hard cheese made from raw milk without the addition of lactic acid bacteria. Cheese making is carried out using artisanal methods and traditional tools; all the phases are manual. The rind is thin, yellowish-white; the inner part of the cheese is white, compact, elastic, and with a homogeneous structure. The taste is sweet and acidulous; the aroma is lactic (Caridi, 2003). On the Calabrian Aspromonte massif, in an area overlooking the Ionian coast in the province of Reggio Calabria, milk is almost exclusively produced from native populations of goats. This area, situated prevalently between 300 and 800 m asl, is used almost exclusively for goat production. Each farm has 180-200 goats, all half-wild. The raw goat's milk - mixed with a 20% maximum of ewe's milk - is processed to make the artisanal *Caprino d'Aspromonte* cheese, a semi-hard or hard cheese made without the addition of lactic acid bacteria. The annual production of the cheese is approximatively 300 t, produced by about 200 farms (Caridi et al., 2003).

The numbers and species of yeasts in the different cheeses are variable, but some species are more frequently detected than others. Yeasts, associated with secondary flora of many kinds of cheeses, play an important role in the cheese ripening process (Šuranská et al., 2016). The occurrence of yeasts in cheese may depend on numerous factors (Ferreira and Viljoen, 2003; Gardini et al., 2006). The addition of a cocktail of yeast species to Cantalet cheese in order to modify bacterial survival and aroma compound formation was proposed (De Freitas et al., 2009).

So, the aim of this study was to contribute to the knowledge of the biodiversity of the Calabrian (South Italy) dairy yeasts, which potentially can be used as adjunct cultures for cheese making.

2. MATERIALS AND METHODS

Yeasts were isolated from three samples of ewe's milk, four samples of the ewe cheese *Pecorino del Poro*, three samples of goat's milk, and seven samples of the goat cheese *Caprino d'Aspromonte*. Samples were collected under refrigeration (ca. 5 °C) and analysed within 24 h; cheese samples were taken after ripening for 0, 14, and/or 28 days. The yeast isolation was performed on Yeast Extract Dextrose Chloramphenicol Agar at 25 °C for 4 days. Colonies were grouped according to their morphology. For each sample, 7-8 colonies were taken from plates seeded with the highest sample dilutions at which growth occurred and the yeast isolates were repeatedly streaked onto the same medium to obtain pure cultures. Purified strains were stored at -80 °C using a cryo-preservative bead storage system (Microbank TM, Pro-Lab Diagnostics, Canada).

The yeast isolates were identified at species level by using API ID 32C (BioMérieux, France), following the supplier instructions.

Assimilation profiles of API ID 32C test results were used for the assimilation of glucose, galactose, lactose, and lactic acid (DL-lactate). Assimilation of citric acid was investigated in Yeast Nitrogen Base Agar. Glucose, galactose and lactose fermentations were tested using media with Durham tubes including 2% (w/v) of each tested sugar. Yeast cultures were inoculated into the media and incubated at 28 °C for 7 d. Salt tolerance of the yeast strains was tested using Malt Extract Broth including 5%, 10%, and 15% (w/v) sodium chloride. Malt Extract Broth without salt was used as the control. Yeast cultures were inoculated into Malt Extract Broth with sodium chloride and incubated at 28 °C for 7 d. The hydrogen sulphide production was tested on Bismuth Glycine Glucose Yeast Agar at 25 °C for 48h (Nickerson, 1953).

The main enzymatic activities were studied in the selected yeast strains. According to Karasu-Yalcin and co-workers (2012), biochemical and physiological characteristics of the yeast strains belonging to the same species were considered as selection criteria; so, all strains belonging to the same species with different biochemical and physiological characteristics were enzymatically characterized by using API-ZYM test system (BioMérieux, France), following the supplier instructions to screen 19 different enzymatic activities.

Lastly, the selected yeast isolates were studied for their interaction with 8 dairy lactic acid bacteria - four coccal-shaped (*Enterococcus durans* B45, *E. durans* B46, *E. faecalis* B39, *Pediococcus* spp. B43) and four rod-shaped (*Lactobacillus casei* B3, *L. paracasei* subsp. *paracasei* B1, *L. pentosus*/*paraplantarum* B33, *L. sakei* B29) - isolated from the same raw milk cheeses. The spot-on-lawn assay was used to examine interactions between yeast and bacterial cultures as described by Addis and co-workers (2001).

3. RESULTS AND DISCUSSION

A total of 124 yeast isolates were obtained from the dairy samples; 120 yeast isolates were identified at the species level. Strains were identified only in the presence of excellent or very good identification levels; in a low number of cases, in the presence of worst identification levels the strain remained "not identified". As shown in Table 1, the most abundant species was *Debaryomyces hansenii* (61.3%), followed by *Candida zeylanoides* (32.3%) and *Kluyveromyces marxianus* (3.2%). Obviously, Table 1 shows the percentage incidence of the isolates and not the count of the number of each species in milk or cheese.

← *approximate position of Table 1*

The prevalence of *D. hansenii* is in accordance with data reported in two neighbouring territories (Gardini et al., 2006; Capece and Romano, 2009), where this species dominated in Pecorino Crotonese and Pecorino di Filiano cheeses during the later stages of maturation and was recovered in high numbers from cheeses (10^6-10^9 cfu/g). Among others, *D. hansenii* is a frequent species in the Italian cheese Fiore Sardo (Pisano et al., 2006), in two French traditional cheeses - Cantalet (De Freitas et al., 2009) and Tomme d'Orchies (Ceugniez et al., 2015) - and in the Stilton blue cheese (Gkatzionis et al., 2014). The potential of *D. hansenii* as agent for accelerated ripening of matured Cheddar cheese was evaluated with very interesting results (Ferreira and Viljoen, 2003). In traditional Brazilian Serro Minas cheese, *D. hansenii* was the prevalent species throughout the ripening periods, and its counts increased almost four orders of magnitude from 3 to 15 days of ripening, reaching 8.1 log cfu/g after 15 days (Cardoso et al., 2015). In Turkish Erzincan Tulum cheese, *D. hansenii* was among the most abundant species (Karasu-Yalcin et al., 2012). In Turkish brined Mihalic cheese and in Spanish short-ripened acid curd Cebreiro cheese, *D. hansenii* was the predominant species (Atanassova et al., 2016; Karasu-Yalcin et al., 2017). *D. hansenii* may have potential to be applied as an adjunct culture for contribution to the final cheese flavour by their production of branched-chain aldehydes primarily responsible for nutty/malty flavour notes (Gori et al., 2012).

Concerning *C. zeylanoides*, in Italian Fossa cheese this was the only species found both before and after the cheese ripening period (Biagiotti et al., 2018). *C. zeylanoides* was among the most frequently occurring yeast species in artisanal white pickled cheese of Western Serbia (Šuranská et al., 2016).

Concerning *K. marxianus*, its contribution to the typical flavour of traditional Spanish ewes' and goats' cheeses was assessed (Padilla et al., 2014).

Concerning the biochemical characteristics, all the isolates of *C. zeylanoides* and *D. hansenii* assimilated glucose, galactose, and lactose; all the isolates of *K. marxianus* fermented glucose, galactose, and lactose. All the isolates of *C. zeylanoides*, *D. hansenii* and *K. marxianus* assimilated DL-lactate and citric acid and exhibited growth in the presence of NaCl 5% and 10%. Biodiversity was observed regarding the growth in the presence of NaCl 15% and the hydrogen sulphide production. All strains belonging to the same species, isolated from the same sample and exhibiting comparable biochemical and physiological characteristics were reduced to one.

The so selected 25 yeast strains were characterized by using API-ZYM test system for detecting general enzyme profiles of the yeast strains; it was possible to observe an interesting biodiversity.

According to Karasu-Yalcin and co-workers (2017), the following enzymatic activities important for cheese ripening were primarily considered: a) acid and alkaline phosphatases activity were common among the isolates; the acid phosphatase was find at the highest grade in strains *C. zeylanoides* 175, *C. zeylanoides* 211 *D. hansenii* 31, and *D. hansenii* 161, - the alkaline phosphatase was find at the highest grade in strain *D. hansenii* 13; b) esterase (C4) and esterase lipase (C8) activities were detected in all the tested strains at low or intermediate (2/5) grade; c) leucine arylamidase activity was find in all the strains, but at the highest grade in strain *C. zeylanoides* 3, *C. zeylanoides* 54, *C. zeylanoides* 112, *C. zeylanoides* 175 (Table 2). β-Galactosidase activity was find only in two strains: at the highest grade for strain *K. marxianus* 181 and at low grade for strain *D. hansenii* 21. α-Glucosidase activity was find only in strain *D. hansenii* 113, at low grade. β-Glucosidase activity was find only in strain *K. marxianus* 181, at low grade. Valine arylamidase activity was find only in two strains: *C. zeylanoides* 3 at low grade and *K. marxianus* 181 at intermediate (2/5) grade. No strain exhibited lipase (C14), trypsin, α-chymotrypsin, and cysteine arylamidase activity.

← *approximate position of Table 2*

Considering the other enzymatic activities studied, naphtol-AS-BI-phosphohydrolase activity was detected in all the tested strains at low or intermediate (2/5) grade (Table 2). α-Galactosidase activity was find at low grade only in strain *D. hansenii* 161. N-acetyl-β-glucoaminidase activity was find at intermediate (2/5) grade only in strain *D. hansenii* 194. α-Fucosidase activity was find at low grade only in strain *D. hansenii* 194. No strain exhibited β-glucuronidase and α-mannosidase activity.

Considering the interactions between yeasts and lactic acid bacteria, yeast strain growth was inhibited in the presence of the following strains of lactic acid bacteria: *E. faecalis* B39, *L. paracasei* subsp. *paracasei* B1, *L. sakei* B29, and *Pediococcus* spp. B43. Only the yeast strain *C. zeylanoides* 211 was inhibited in the presence of the following strains of lactic acid bacteria: *E. durans* B45, *E. durans* B46, and *L. pentosus*/*paraplantarum* B33. Eight out the 25 yeast strains were inhibited in the presence of the strain of lactic acid bacteria *L. casei* B3. No lactic acid bacteria growth was inhibited in the presence of the following strains of yeast: *C. zeylanoides* 3, 112, 192, 201, 221, 238, and *K. marxianus* 181. Often the yeasts and the lactic acid bacteria growth was without apparent interaction (Table 3).

← *approximate position of Table 3*

4. CONCLUSIONS

The present work showed the existence of a high degree of biodiversity among the yeast isolates. This may have potential implications for further technological investigations to use the best strains as adjunct cultures for cheese making. Considering the biochemical profile, the enzymatic activities and the interaction with the lactic acid bacteria, the strain more promising for adjunct starter application appears *C. zeylanoides* 175. Obviously, based on the specific cheese characteristics, may be necessary to choice yeasts with different biochemical profile, different enzymatic activities, able to inhibit the lactic acid bacteria growth or exhibiting different behaviour.

REFERENCES

Addis, E., Fleet, G.H., Cox, J.M., Kolak, D., and Leung, T. (2001). The growth, properties and interactions of yeasts and bacteria associated with the maturation of Camembert and blue-veined cheeses. *International Journal of Food Microbiology,* 69(1-2): 25−36.

Atanassova, M.R., Fernández-Otero, C., Rodríguez-Alonso, P., Fernández-No, I.C., Garabal, J.I., and Centeno, J.A. (2016). Characterization of yeasts isolated from artisanal short-ripened cows' cheeses produced in Galicia (NW Spain). *Food Microbiology,* 53(B): 172−181.

Biagiotti, C., Ciani, M., Canonico, L., and Comitini, F. (2018). Occurrence and involvement of yeast biota in ripening of Italian Fossa cheese. *European Food Research and Technology*, 244(11): 1921−1931.

Capece, A., and Romano, P. (2009). "Pecorino di Filiano" cheese as a selective habitat for the yeast species, *Debaryomyces hansenii*. *International Journal of Food Microbiology,* 132(2-3): 180−184.

Cardoso, V.M., Borelli, B.M., Lara, C.A., Soares, M.A., Pataro, C., Bodevan, E.C., and Rosa, C.A. (2015). The influence of season and ripening time on yeast communities of a traditional Brazilian cheese. *Food Research International,* 69: 331−340.

Caridi, A. (2003). Identification and first characterization of lactic acid bacteria isolated from the artisanal ovine cheese Pecorino del Poro. *International Journal of Dairy Technology,* 56(2): 105−110.

Caridi, A., Micari, P., Foti, F., Ramondino, D., and Sarullo, V. (2003). Ripening and seasonal changes in microbiological and chemical parameters of the artisanal cheese Caprino d'Aspromonte produced from raw or thermized goat's milk. *Food Microbiology,* 20(2): 201−209.

Ceugniez, A., Drider, D., Jacques, P., and Coucheney, F. (2015). Yeast diversity in a traditional French cheese "Tomme d'orchies" reveals infrequent and frequent species with associated benefits. *Food Microbiology,* 52: 177−184.

De Freitas, I., Pinon, N., Maubois, J.L., Lortal, S., and Thierry, A. (2009). The addition of a cocktail of yeast species to Cantalet cheese changes bacterial survival and enhances aroma compound formation. *International Journal of Food Microbiology,* 129(1): 37−42.

Ferreira, A.D., and Viljoen, B.C. (2003). Yeasts as adjunct starters in matured Cheddar cheese. *International Journal of Food Microbiology,* 86(1-2): 131−140.

Gardini, F., Tofalo, R., Belletti, N., Iucci, L., Suzzi, G., Torriani, S., Guerzoni, M.E., and Lanciotti, R. (2006). Characterization of yeasts involved in the ripening of Pecorino Crotonese cheese. *Food Microbiology,* 23(7): 641−648.

Gkatzionis, K., Yunita, D., Linforth, R.S.T., Dickinson, M., and Dodd, C.E.R. (2014). Diversity and activities of yeasts from different parts of a Stilton cheese. *International Journal of Food Microbiology,* 177: 109−116.

Gori, K., Sørensen, L.M., Petersen, M.A., Jespersen, L., and Arneborg, N. (2012). *Debaryomyces hansenii* strains differ in their production of flavour compounds in a cheese-surface model. *Microbiology Open*, 1(2): 161−168.

Karasu-Yalcin, S., Senses-Ergul, S., and Ozbas, Z.Y. (2012). Identification and enzymatic characterization of the yeasts isolated from Erzincan tulum cheese. *Mljekarstvo*, 62: 53−61.

Karasu-Yalcin, S., Senses-Ergul, S., and Ozbas, Z.Y. (2017). Enzymatic characterization of yeast strains originated from traditional Mihalic cheese. *Journal of Microbiology, Biotechnology and Food Sciences*, 6(5): 1152−1156.

Nickerson, W.J. (1953). Reduction of inorganic substances by yeasts. I. Extracellular reduction of sulfite by species of *Candida*. *Journal of Infectious Diseases*, 93(1): 43−56.

Padilla, B., Belloch, C., López-Díez, J.J., Flores, M., and Manzanares, P. (2014). Potential impact of dairy yeasts on the typical flavour of traditional ewes' and goats' cheeses. *International Dairy Journal*, 35(2): 122−129.

Pisano, M.B., Fadda, M.E., Deplano, M., Corda, A., and Cosentino, S. (2006). Microbiological and chemical characterization of Fiore Sardo, a traditional Sardinian cheese made from ewe's milk. *International Journal of Dairy Technology*, 59(3): 171−179.

Šuranská, H., Raspor, P., Uroić, K., Golić, N., Kos, B., Mihajlović, S., Begović, J., Šušković, J., Topisirović, L., and Čadež, N. (2016). Characterisation of the yeast and mould biota in traditional white pickled cheeses by culture-dependent and independent molecular techniques. *Folia Microbiologica*, 61(6): 455−463.

Table 1. Yeast frequency in dairy products

	Milk		Cheese		**Total**
	Ewe	Goat	Ewe	Goat	
			Percentages		
Candida zeylanoides	39.1	75.0	25.0	12.2	**32.3**
Debaryomyces hansenii	60.9	4.2	67.9	85.8	**61.3**
Kluyveromyces marxianus	0.0	16.6	0.0	0.0	**3.2**
Not identified	0.0	4.2	7.1	2.0	**3.2**
Total	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**

Table 2. Enzymatic activities of 25 yeast strains isolated in the present study

Identification	Origin*	1	2	3	4	5	6
C. zeylanoides 3	gc	0	1	2	5	1	1
D. hansenii 6	gc	3	1	1	3	3	2
D. hansenii 13	gc	5	1	1	2	2	2
D. hansenii 21	gc	2	1	1	2	4	2
D. hansenii 31	gc	2	2	1	2	5	1
D. hansenii 42	gc	3	1	1	3	3	2
D. hansenii 52	gc	2	1	1	2	4	2
C. zeylanoides 54	gc	1	1	1	5	2	2
D. hansenii 75	gc	3	2	2	2	3	2
C. zeylanoides 112	ec	2	1	2	5	4	2
D. hansenii 113	ec	3	2	1	2	4	2
D. hansenii 141	ec	3	1	1	2	3	2
D. hansenii 161	ec	3	1	1	2	5	1
C. zeylanoides 175	ec	1	2	1	5	5	2
D. hansenii 176	ec	3	1	1	1	1	1
K. marxianus 181	gm	1	1	1	4	1	1
C. zeylanoides 190	gm	0	2	1	4	3	2
C. zeylanoides 192	gm	2	1	1	3	2	2
D. hansenii 194	gm	3	1	1	1	2	1
C. zeylanoides 201	gm	1	2	1	3	2	1
C. zeylanoides 211	em	1	2	2	3	5	1
D. hansenii 216	em	2	1	1	3	3	1
D. hansenii 224	em	2	1	1	3	4	1
D. hansenii 231	em	2	2	1	1	1	1
C. zeylanoides 238	em	1	2	1	4	2	2

*Origin: gc=goat's cheese; ec=ewe's cheese; gm=goat's milk; em=ewe's milk. Identification and names of the enzymatic activities - 1: Alkaline phosphatase, 2: Esterase (C4), 3: Esterase lipase (C8), 4: Leucine arylamidase,5: Acid phosphatase, 6: Naphtol-AS-BI-phosphohydrolase. Legend: no activity (0), low activity (1), intermediate activity (2-3) and high activity (4-5).

Table 3. Interaction among 25 yeast strains and 8 lactic acid bacteria

Dairy yeasts	Origin*	Dairy lactic acid bacteria**							
		1	2	3	4	5	6	7	8
C. zeylanoides 3	gc	0	0	2	0	0	0	0	0
D. hansenii 6	gc	1	1	1	1	1	1	1	1
D. hansenii 13	gc	1	1	0	1	1	1	1	1
D. hansenii 21	gc	1	1	1	1	1	1	1	1
D. hansenii 31	gc	1	1	1	1	1	1	1	1
D. hansenii 42	gc	1	1	1	1	1	1	1	1
D. hansenii 52	gc	1	1	1	1	1	1	1	1
C. zeylanoides 54	gc	0	0	2	0	0	1	0	0
D. hansenii 75	gc	1	1	1	1	1	1	1	1
C. zeylanoides 112	ec	0	0	2	0	0	0	0	0
D. hansenii 113	ec	1	1	1	1	1	1	1	1
D. hansenii 141	ec	1	1	1	1	1	1	1	1
D. hansenii 161	ec	1	1	2	1	1	1	1	1
C. zeylanoides 175	ec	0	0	0	0	0	0	0	1
D. hansenii 176	ec	1	1	1	1	1	1	1	1
K. marxianus 181	gm	0	0	0	0	0	0	0	0
C. zeylanoides 190	gm	0	0	2	0	0	0	0	1
C. zeylanoides 192	gm	0	0	2	0	0	0	0	0
D. hansenii 194	gm	1	1	1	1	1	1	1	1
C. zeylanoides 201	gm	0	0	0	0	0	0	0	0
C. zeylanoides 211	em	2	0	2	0	0	0	2	2
D. hansenii 216	em	1	1	1	1	1	1	1	1
D. hansenii 224	em	1	1	1	1	1	1	1	1
D. hansenii 231	em	1	1	1	1	1	1	1	1
C. zeylanoides 238	em	0	0	2	0	0	0	0	0

*Origin: gc=goat's cheese; ec=ewe's cheese; gm=goat's milk; em=ewe's milk. **Dairy lactic acid bacteria: 1. *Enterococcus durans* B45; 2. *Enterococcus faecalis* B39; 3. *Lactobacillus casei* B3; 4. *Lactobacillus paracasei subsp. paracasei* B1; 5. *Lactobacillus sakei* B29; 6. *Pediococcus spp.* B43; 7. *Enterococcus durans* B46; 8. *Lactobacillus pentosus/ paraplantarum* B33. Legend: no interaction (0), the LAB's growth is inhibited (1), the yeast's growth is inhibited (2).