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Food Microbiology 23 (2006) 641–648

FOOD MICROBIOLOGY

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Characterization of yeasts involved in the ripening of Pecorino Crotonese cheese

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Received 29 August 2005; accepted 3 December 2005
Available online 23 February 2006

Abstract

The aims of this work were to identify and characterize for some important technological properties the yeast species present throughout the ripening process of Pecorino Crotonese, a traditional cheese produced in a well defined area of Southern Italy. In particular, the strain technological properties considered include fermentation/assimilation of galactose and lactose, assimilation of lactate and citrate in the presence of different NaCl concentrations, hydrolysis of butter fat, skim milk, gelatine and casein, production of brown pigments in cheese agar and ability to produce biogenic amines. High yeast levels were recorded in cheese samples already after 5 h of brining (about 5 log cfu/g) and these concentration remained constant during ripening. The yeast isolates belonged to restrict number of yeast species. While *Kluyveromyces lactis* and *Saccharomyces cerevisiae* were isolated prevalently in the first stages of Pecorino Crotonese production, *Yarrowia lipolytica* and *Debaryomyces hansenii* dominated during the later stages of maturation. Otherwise, the latter two were very NaCl resistant species. In fact, *D. hansenii* strains conserved the ability to assimilate lactose and galactose in the presence of 10% NaCl, while almost all the strains of *Y. lipolytica* isolated assimilated citrate and lactate up to 7.5% NaCl. *Y. lipolytica* isolates evidenced also the highest proteolytic and lipolytic activities and the capability to catabolize tyrosine producing brown pigment. In addition they resulted in the highest aminobiogenic potential decarboxylating ornithine, phenylalanine, tyrosine and lysine. However, they were not able to produce histamine, biogenic amine produced by three strains of *D. hansenii*.

Keywords: Pecorino Crotonese; Cheese ripening; Yeast characterization; Brown pigment production; Biogenic amine

1. Introduction

Pecorino Crotonese is a traditional cheese produced in a well-defined area of Southern Italy and particularly in Calabria Region. According to the traditional protocol, it is produced from pasteurized ewe milk by using caprine rennet and natural whey starter cultures. It is ripened at least for 2 months, and can be commercialized after 1 year. Although the microbiological and physicochemical features of some Italian similar ewes' cheeses have been reported (Albenzio et al., 2001; Cosentino et al., 2001; Caridi et al., 2003; Fadda et al., 2004), no literature data are available on Pecorino Crotonese.

Cheese ripening is a complex phenomenon involving a wide range of biochemical reactions. High microbial counts are present in cheese throughout ripening and the composition of the microbial population has a significant role in the maturation process (Beresford et al., 2001). Although the starter lactic acid bacteria (LAB) are responsible for acid production and contribute to the ripening process, an important contribution to cheese maturation is recognized to the secondary microbiota, mainly constituted by enterococci, micrococci, non-starter LAB and yeasts (Beresford et al., 2001). The precise role of these different microbial groups has not yet been defined. In particular, yeasts are associated with the secondary

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0740-0020/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2005.12.005

microflora of a wide variety of cheeses; however, in most cases, their contribution to cheese ripening is unclear (Jakobsen and Narvhus, 1996; Wyder and Puhan, 1999; Addis et al., 2001). The occurrence in cheese of some species of yeasts with high counts is attributable to their tolerance towards low pH, reduced water activity and high salt concentrations, as well as to their ability to grow at low storage temperature which characterizes the ripening environment (Ferreira and Viljoen, 2003). Other important features able to explain their occurrence in cheese are the assimilation/fermentation of lactose and galactose, the assimilation of succinic, lactic and citric acids. In addition, they are widely distributed in the dairy environments and appear as natural contaminants in raw milk, air, dairy utensils, brine and smear water (Wyder and Puhan, 1999). Also the ability of dairy yeast strains to survive heat treatments and sanitizing agents has been reported (van den Tempel and Jakobsen, 1998, 2000). Yeasts are involved both directly and indirectly in the ripening process (Jakobsen and Narvhus, 1996; Wyder and Puhan, 1999). Consumption of lactate, formation of alkaline metabolites, fermentation of lactose, lipolysis, proteolysis and formation of aroma compounds are some of the yeast activities, important for the typical characteristics of some cheese varieties (Jakobsen and Narvhus, 1996; Rossi et al., 1997). On the other hand, yeasts may also act as spoilage organisms causing typical defects like yeasty off-flavour, loss of texture quality, excessive gas formation, increased acidity due to stimulant effects on LAB and brown surface discoloration (Jakobsen and Narvhus, 1996; Carreira et al., 1998; Wyder et al., 1999). In addition, an increase in biogenic amines in foil wrapped Raclette cheese has been attributed to the activities of *Yarrowia lipolytica*, *Pichia jadinii* and *Debaryomyces hansenii* strains (Wyder et al., 1999).

The aims of this work were to identify and characterize for some important technological properties the yeast species present throughout the ripening process of Pecorino Crotonese. In particular the strain technological properties considered include fermentation/assimilation of galactose and lactose and assimilation of citrate and lactate in the presence of different NaCl concentrations (0%, 2.5%, 7.5% and 10%); hydrolysis of butter fat, skim milk, gelatine and casein, as well as production of brown pigments in cheese agar and ability to decarboxylate aminoacids such as histidine, tyrosine, ornithine, phenylalanine, lysine and producing the relative biogenic amines.

2. Materials and methods

2.1. Enumeration and isolation of yeast strains

A total of nine samples were obtained from different steps during the manufacture and ripening of Pecorino Crotonese. In fact raw milk, rennet, curd, 5 or 24 h brined cheeses, unripened cheese as well as 15, 30 and 60 d ripened cheeses, were considered. The samples were analysed by plating appropriate ten-fold dilutions onto selective media for enterococci (Slanetz and Bartley Medium), lactococci (M17 medium) lactobacilli (MRS agar); total coliforms (Violet Red Bile Agar) and staphylococci (Mannitol Salt Agar). All these media were purchased from Oxoid (Basingstoke, UK) and utilized according to manufacturer's instruction.

Sabouraud dextrose agar (SDA, Oxoid) added with 0.2 g/l chloramfenicol (Merck, Darmstadt, Germany) was used for the enumeration and isolation of yeasts.

Three repetition for each sample were considered.

Yeast colonies were counted and three to five colonies for each sampling point were picked up based on different colony morphology and streaked onto SDA plates. Selected colonies were further purified and maintained in slants with the same medium.

2.2. Molecular identification of isolated strains

The yeast isolates were genotypically characterized by RFLP-PCR. Total genomic DNA was extracted and purified from 7 ml samples of overnight cultures grown in YEPD medium (2% peptone, 1% yeast extract and 2% glucose) as described by Querol et al. (1992). Identification of the isolates was ascertained by PCR-RFLP of 5.8S-ITS region of the rRNA gene as described by Esteve-Zarzoso et al. (1999) and de Llanos Frutos et al. (2004). The PCR fragments were digested with 1 U of *Hae*III, *Hinf*I and *Cfo*I endonucleases (Takara Shuzo Co., Otsu, Shiga, Japan) for 2 h at 37 °C. Restriction products were separated on a 2% agarose gel.

The following type strains were used as reference strains during the molecular characterization of the isolates: *Candida sake* DBVPG 6154^T, (Industrial Yeasts Collection of Perugia, Italy) *C. intermedia* DBVPG 6218^T, *C. stellata* DBVPG 6714^T, *C. incospicua* DBVPG 6901^T. *Pichia kluyveri* DBVPG 6901^T, *P. carsonii* DBVPG 6899^T, *P. norvengensis* DBVPG 6871^T, *Y. lipolytica* DBVPG 6053^T, *Kluyveromyces marxianus* DBVPG 6165^T, *Kl. lactis* DBVPG 6305^T, *Saccharomyces cerevisiae* CBS 1171^T (Centraalbureau Voor Schimmelcultures, Baarn, The Netherlands) and *D. hansenii* CBS 767^T.

2.3. Assimilation/fermentation tests

The yeasts were characterized for their ability to ferment or assimilate lactose galactose, citrate and lactate at different levels of NaCl (0%, 2.5%, 7.5%, 10% w/v) at pH 5.5 and 25 °C according to Kurtzmann and Fell (1998).

2.4. Extracellular lipolytic activity

The medium was prepared according to Tiecco (1992) using yeast extract (Oxoid) 10 g/l, peptone (Merck) 10 g/l, NaCl (Carlo Erba Reagents, Milan, Italy) 5 g/l, agar 20 g/l and commercial butter 50 g/l. Yeasts were precultured in liquid medium for 48 h at 25 °C, streaked onto the surface