International Journal of Food Microbiology 130 (2009) 179–187

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Molecular identification and osmotolerant profile of wine yeasts that ferment a high sugar grape must

Rosanna Tofalo [a], Clemencia Chaves-López [a], Federico Di Fabio [a], Maria Schirone [a], Giovanna E. Felis [b], Sandra Torriani [b], Antonello Paparella [a], Giovanna Suzzi [a,*]

[a] Dipartimento di Scienze degli Alimenti, Università degli Studi di Teramo, Via C.R. Lerici 1, 64023 Mosciano Sant'Angelo, Teramo, Italy
[b] Dipartimento di Scienze, Tecnologie e Mercati della Vite e del Vino, Università degli Studi di Verona, Via delle Pieve 70, 37029 San Floriano, Verona, Italy

ARTICLE INFO

Article history:
Received 17 October 2008
Received in revised form 20 January 2009
Accepted 21 January 2009

Keywords:
Osmotolerance
Yeasts
Ethanol tolerance
Vino cotto

ABSTRACT

The objective of this study was to examine the *Saccharomyces* and non-*Saccharomyces* yeast populations involved in a spontaneous fermentation of a traditional high sugar must (Vino cotto) produced in central Italy. Molecular identification of a total of 78 isolates was achieved by a combination of PCR-RFLP of the 5.8S ITS rRNA region and sequencing of the D1/D2 domain of the 26S rRNA gene. In addition, the isolates were differentiated by RAPD-PCR. Only a restricted number of osmotolerant yeast species, i.e. *Candida apicola*, *Candida zemplinina* and *Zygosaccharomyces bailii*, were found throughout all the fermentation process, while *Saccharomyces cerevisiae* prevailed after 15 days of fermentation. A physiological characterization of isolates was performed in relation to the resistance to osmotic stress and ethanol concentration. The osmotolerant features of *C. apicola*, *C. zemplinina* and *Z. bailii* were confirmed, while *S. cerevisiae* strains showed three patterns of growth in response to different glucose concentrations (2%, 20%, 40% and 60% w/v). The ability of some *C. apicola* and *C. zemplinina* strains to grow at 14% v/v ethanol is noteworthy. The finding that some yeast biotypes with higher multiple stress tolerance can persist in the entire winemaking process suggests possible future candidates as starter for Vino cotto production.

1. Introduction

"Vino cotto" is a wine produced in the Marche and Abruzzo regions (central Italy) according to traditional procedures that involve a prolonged fermentation of cooked grape must. The flow-chart for the traditional manufacture of Vino cotto is showed in Fig. 1. Cooked must derives from white grapes of the *Trebbiano, Passerina or Moscato* cultivars and is obtained by direct heating of the must in copper boilers until its volume is reduced by 10–70%, according to the specific process. During cooking, the must becomes dark and dense, and reaching a very high sugar concentration (up to 55% w/v) (Di Mattia et al., 2007; Piva et al., 2008). Cooking is carried out below boiling temperatures (80–95 °C), and requires long processing times (up to 48 h). To start fermentation, fresh must is added to sterile cooked must, at percentages that depend on the different manufacturing practices. The indigenous yeasts of the added fresh must conduct the alcoholic fermentation that proceeds slowly for about 40 days at room temperature. Stuck or sluggish fermentations often occur during the production of Vino cotto, as observed during the fermentation of Traditional Balsamic Vinegar, another Italian traditional product made with cooked grape must (Solieri and Giudici, 2005, 2008; Solieri et al., 2006). These problems derive from the exposure of yeast cells to high osmotic conditions of cooked must (hyperosmotic shock) that determine a rapid loss of intracellular water (Hohmann, 2002), followed by cytoskeleton collapse (Chowdhury et al., 1992), intracellular damage and subsequent arrest of growth. The majority of yeasts requires a minimum water activity (a_w) of 0.85 for their growth, and requires only xerophilic yeasts can grow, at slow rate, at a_w values between 0.61 and 0.75 (Martorell et al., 2005; Solieri and Giudici, 2008). In general, osmotolerant yeasts are able to retain the ability to synthesize glycerol as a compatible solute or osmoregulator, and some yeasts even have active glycerol uptake pumps (Hohmann, 2002).

There are only few studies dealing on the yeast population involved in the fermentation of cooked grape musts. Recently, Solieri et al. (2006) found a complex yeast population in Traditional Balsamic Vinegar, that includes *S. cerevisiae*, several *Zygosaccharomyces* species, two species belonging to the *Hanseniaspora* genus (*Hanseniaspora osmophila* and *Hanseniaspora valbyensis*), and two *Candida* species (*Candida stellata* and *Candida lactis-condensi*). Also in high sugar grape musts, such as special wines from dried, botrytized grapes or late-harvest grapes, osmotolerant non-*Saccharomyces* yeasts are commonly found (Mills et al., 2002). *Candida zemplinina* has been recently identified as a new osmotolerant and psychrotolerant yeast, fermenting sweet and

* Corresponding author. Dipartimento di Scienze degli Alimenti, via C.R. Lerici 1, 64023 Mosciano S. Angelo (TE), Italy. Tel.: +39 0861 266938; fax: +39 0861 266915.
E-mail address: gsuzzi@unite.it (G. Suzzi).

0168-1605/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2009.01.024

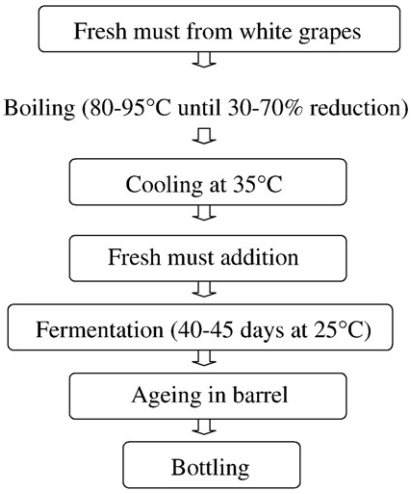

Fig. 1. Vino cotto flow chart.

botrytized musts (Sipiczki, 2003). Various authors have mentioned that indigenous yeast species, such as *Kloeckera apiculata*, *C. stellata* and *Torulaspora delbrueckii*, may have better ability than *S. cerevisiae* to grow during fermentations conducted at high sugar concentrations (e.g., >200 g/L) (Benda, 1982; Lafon-Lafourcade, 1983). Malacrinò et al. (2005) reported that strains of osmosensitive species, such as *S. cerevisiae*, may possess appreciable capability to overcome osmotic stress and to yield ethanol by fermentation of grape musts with high sugar concentration in winemaking conditions. These authors suggested to use these strains as starter for winemaking of partially dried grapes.

Very little information is available about the yeast microbiota of Vino cotto, as previous researches on this peculiar wine have been focused more on the technological aspects of the winemaking process. Therefore, the aims of this study were to monitor the *Saccharomyces* and non-*Saccharomyces* yeast populations during Vino cotto manufacturing and to characterize the yeast isolates for their tolerance to osmotic stress and ethanol. Initially, yeast isolates were presumptively identified using morphological identification on Wallerstein Laboratory Nutrient (WLN) agar and speciation of the new isolates was carried out by applying two well-recognized yeast identification procedures based on ribosomal RNA (rRNA) gene analysis, i.e. restriction fragment length polymorphism (RFLP) analysis of the ITS1–ITS2 region (Esteve-Zarzoso et al., 1999) and sequencing of the genes encoding the D1/D2 domain of the large (26S) subunit of rRNA (Kurtzman and Robnett, 1998). A combination of the two procedures was required, since the fast and not expensive ITS-RFLP approach is not as discriminatory as 26S rRNA gene sequence (Arias et al., 2002). In addition, differentiation of the isolates at the subspecies level was acquired by applying the random amplification of polymorphic DNA (RAPD)-PCR technique. This whole genome PCR sampling method works by priming at arbitrary sites and results in strain-specific fingerprints from which differentiation of strains belonging to different yeast species was possible (Quesada and Cenis, 1995; Torriani et al., 1999; Bujdoso et al., 2001; Urso et al., 2008). From the results obtained in the present study, it is anticipated that the traditional and region-dependent handling makes Vino cotto a wide genetic source of diverse yeast species and strains suitable for following starter selection programmes.

2. Materials and methods

2.1. Wine production

Cooked must was prepared according to Piva et al. (2008). White grapes of Trebbiano d'Abruzzo were removed from stalks and pressed. The fresh Trebbiano must (FTM) (100 L) was concentrated in copper boilers of 120 L capacity to about 70%. The masses were rapidly heated to boiling point, then the temperature was set at 95 °C for the whole process (up to 18 h).

2.2. Analytical determinations

Chemical analyses were conducted using the official EU Community methods for the analyses of wines (EEC, 1990). The total polyphenols were determined by the colorimetric reaction with the Folin Ciocalteau reagent as reported by Di Mattia et al. (2007).

2.3. Fermentation characteristics and yeast population dynamics

Vino cotto must (VCM), obtained by mixing 70% cooked must (CM) added with 30% FTM, was spontaneously fermented by indigenous yeasts in tanks (120 L) at room temperature (approximately 18 °C) for 45 days. Samples were taken from FTM, VCM and during the fermentation process. For all samples, decimal dilutions in sterile physiological solution (NaCl 8.5 g/L) were made and 0.1 mL of each dilution was spread on three different media: Wallerstein Laboratory Nutrient agar (WLN; Oxoid, Milan, Italy), Lysine-medium (LM; Oxoid) and Yeast Peptone Dextrose agar (YPD; 1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose and 2% w/v agar). All media were supplemented with chloramphenicol (150 ppm). Plates were incubated at 25 °C for 3–5 days. According to Pallman et al. (2001), the WLN medium plating was used to monitor yeast population diversity during fermentations. The colony morphologies are characteristic enough to identify the corresponding yeast's genus and species. After counting, a total of 20 colonies of yeasts with different colour and morphology was isolated from WLN plates. The isolates were purified by repetitive streaking on YPD and then stored at −20 °C in YPD broth supplemented with glycerol (25% final concentration).

2.4. Molecular identification of the isolates

Yeast cells were grown aerobically in YPD at 28 °C. DNA was isolated according to Querol et al. (1992). The 5.8 internal transcribed

Table 1
Chemical composition of the musts and Vino cotto

Sample	Reducing sugars (g/L)[a]	Tartaric acid (g/L)	Total acidity (g/L of tartaric acid)	Volatile acidity (g/L of acetic acid)	Ethanol (%)	pH	Total phenolics (ppm GAE[b])
Fresh Trebbiano must[c]	172.5±21.4	4.81±0.08	7.41±0.28	–	–	3.08±0.03	348±12.34
Cooked must	554.5±28.5	13.32±0.35	17.64±0.15	–	–	2.75±0.02	1754±56.72
Vino cotto must[d]	304.5±18.7	7.35±0.21	9.42±0.26	–	–	2.94±0.02	1200±31.21
Vino cotto[e]	64.5±2.21	3.61±0.11	8.59±0.37	0.76±0.05	15.35±0.27	3.03±0.02	569±16.28

[a] Data are the means of 3 repetitions with the relative standard deviations.
[b] GAE: galic acid equivalent.
[c] Trebbiano d'Abruzzo must.
[d] Cooked must added with fresh must.
[e] Vino cotto must after 40 days of fermentation.