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Recovering traditional raw-milk Tetilla cheese flavour and sensory attributes by using *Kocuria varians* and *Yarrowia lipolytica* adjunct cultures

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ABSTRACT

The rationale of the present study was to evaluate the potential of microbial adjunct cultures including *Kocuria varians* and/or *Yarrowia lipolytica* strains in the recovery of the typical sensory profile of traditional (raw-milk) Tetilla cheese. Four batches of Tetilla cheese, a short ripened cows' milk cheese produced in Galicia (NW Spain), were made in duplicate from pasteurized milk inoculated with different microbial cultures. A control batch was manufactured by adding a mesophilic commercial D-starter only. The other three batches were made with the same starter after a cheese-milk pre-ripening step carried out with (i) an adjunct culture of *K. varians*, (ii) an adjunct culture of *Y. lipolytica*, or (iii) a combination of both adjunct cultures. The highest pH and water activity values, associated with softer textures were determined in the cheeses manufactured with the *Y. lipolytica* adjunct after 21 days of ripening. The contents of the volatile compounds 3-methylbutanol, dimethyl disulfide and dimethyl trisulfide were higher in the cheeses made with only the *K. varians* adjunct than in the cheeses made with the only yeast adjunct and in the control cheeses. The contents of hexanoic and octanoic acids were highest in the cheeses made with the *Y. lipolytica* adjunct, and levels of ethyl hexanoate, ethyl octanoate and ethyl decanoate were higher in the cheeses made with only the yeast adjunct than in the other batches of cheese. The cheeses manufactured with both adjunct cultures were awarded the highest scores for flavour and overall sensory parameters (considering the standards of the traditional product) and were considered very similar to 'good quality' artisanal raw-milk cheeses. We conclude that use of selected *Micrococcaceae* and *Y. lipolytica* strains as adjunct cultures would differentiate the sensory properties and contribute to the quality and typicality of the short-ripened rennet-curd Galician Tetilla and Arzúa-Ulloa cheeses.

Chemical compounds

2,3-Butanediol (PubChem CID: 262)
2-Ethyl-1-hexanol (PubChem CID: 7720)
2-Butanone (PubChem CID: 6569)
2-Heptanone (PubChem CID: 8051)
Acetic acid (PubChem CID: 176)
Butanoic acid (PubChem CID: 264)
Ethyl butanoate (PubChem CID: 7762)
Ethyl hexanoate (PubChem CID: 31265)
Ethyl octanoate (PubChem CID: 7799)
Dimethyl disulfide (PubChem CID: 12232)

1. Introduction

Adjunct cultures may be useful in cheese-making for attaining the typical sensory profile of certain varieties of cheese. Yeasts such as *Yarrowia lipolytica*, *Kluyveromyces lactis* and *Debaryomyces hansenii* have been proposed as adjunct cultures in order to enhance flavour formation during cheese ripening (De Freitas et al., 2009; Ferreira and Viljoen, 2003; Gkatzionis et al., 2014). Yeast adjuncts present at minimal numbers of about 5 log cfu/g have been reported to induce significant changes in the concentrations of volatile aroma compounds in cheese (De Freitas et al., 2009). The fact that such low numbers, relative to lactic acid bacteria (LAB), can influence cheese proteolysis and lipolysis has been related to the strong exocellular hydrolytic activities of some yeast species (Barth and Gaillardin, 1997). In a recent study on the yeast microbiota of Galician short ripened (<1 mo) cheeses (Atanassova et al., 2016), it was concluded that selected *Y. lipolytica*

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strains could be used as adjunct cultures in the manufacture of Arzúa-Ulloa and Tetilla cheeses, in which early lipolysis is a desirable feature involved in the formation of a number of free fatty acids and ester compounds characteristic of traditional products (Rodríguez-Alonso et al., 2009).

In the late 1990s and the early 2000s, different autochthonous cultures of LAB and *Micrococcaceae* strains were tested for their ability to improve the sensory characteristics of short ripened cheeses (Arzúa-Ulloa, Tetilla and Cebreiro) produced in Galicia (NW Spain) (Centeno et al., 1996; Menéndez et al., 2004). Menéndez et al. (2004) concluded that an ideal starter for Tetilla cheese should include *Lactococcus lactis* subsp. *lactis* as well as selected enterococci or micrococci strains, with the aim of achieving more intense lipolysis and specific proteolysis over α_{s1}-casein.

Tetilla cheese is the third most commonly produced cow's milk cheese in Spain (about 1.4 million kg was produced in 2014), with the first place (3.2 million kg) (Anon, 2016) being occupied by the very similar Arzúa-Ulloa cheese variety. Both cheeses represent about 60% of the total annual production of (unmixed) cow's milk cheeses with Protected Designation of Origin (PDO) manufactured in Spain.

Due to (i) the susceptibility of wild lactococcal cells to bacteriophage infection leading to problems related to phage contamination in cheese factories (Garneau and Moineau, 2011), and (ii) the controversial 'beneficial/pathogenic' status of enterococci, in the present work we have used a commercial mesophilic D-type starter together with a selected micrococcal strain for manufacturing experimental Tetilla cheeses. So, the aims of the present study were (i) to investigate the effects of adjunct cultures of one strain of *Micrococcaceae* and one strain of *Y. lipolytica*, both isolated from raw milk Galician cheeses and selected for their proteolytic, and particularly lipolytic activities, on the volatile profile and sensory characteristics of experimental short ripened Tetilla cheeses made with pasteurized milk; and (ii) to compare the volatile and the sensory profiles of the experimental cheeses with those previously described for the traditional 'good quality' raw-milk product.

2. Materials and methods

2.1. Microbial cultures

The selected *Micrococcaceae* strain was *Kocuria varians* S157, a strain isolated from a 7-day-old Cebreiro raw-milk cheese assayed in a previous study (Rodríguez-Alonso et al., 2008). This strain was characterized by exocellular proteolytic and lipolytic activities and by production of a predominant rancid odour in pasteurized whole milk (see Supplementary File S1 showing main technological abilities of the two selected microbial strains). The selected yeast strain was *Yarrowia lipolytica* LEV53, a non-haemolytic strain isolated from a 21-day-old Tetilla raw-milk cheese investigated in a previous study (Atanassova et al., 2016). The strain was characterized by strong exocellular proteolytic and lipolytic activities in comparison with other isolates of the same species obtained from artisanal raw-milk Galician cheeses (Atanassova et al., 2016) and by the formation of fatty acids, esters and sulfur compounds in pasteurized whole milk, associated with the production of rancid and cheesy odours.

Cultures of *K. varians* S157 and *Y. lipolytica* LEV53 were grown in sterile (110 °C; 15 min) supplemented milk consisting of reconstituted (10%, w/v) skimmed milk, tryptone (5 g/L) and yeast extract (5 g/L) (all components from Oxoid, Basingstoke, UK), and incubated at 30 °C for 48 h. The supplemented milk cultures were inoculated at 1% (v/v) in 1000 mL pasteurized (100 °C; 15 min) whole (3.8 g/100 mL fat) cow's milk, and incubated at 30 °C under aerobic conditions on a rotary shaker at 150 rpm for 48 h. The pasteurized milk cultures were used to inoculate cheese milk for a pre-ripening step.

The commercial starter used in the manufacture of the Tetilla cheeses was the freeze-dried direct vat set (FD-DVS) CHOOZIT MM100 (Danisco® Food Ingredients, Sassenage, France), a mesophilic D-starter containing *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis*$^{cit+}$ strains. The starter was maintained at −25 °C until use.

The absence of antimicrobial activity of the S157 and LEV53 strains against each other and against the commercial culture MM100 was confirmed by the agar well diffusion assay, as described by Centeno et al. (2002) (data not shown).

2.2. Experimental Tetilla cheesemaking

A total of 160 L whole milk (4.0% fat), obtained in autumn from cows fed higher proportions of pasture relative to feed, was pasteurized (74 °C, 15 s) and divided into four 40 L vats to make four different batches of cheese. A control batch was made with the only MM100 commercial culture added to the pasteurized milk cooled to 32 °C at a ratio of 3.75 direct culture units (DCU) per 100 L of milk (1.5 DCU per 40 L of milk) and acclimatized to this temperature for 20 min before addition of a fermentation-produced chymosin coagulant (CHY-MAX® Plus, Chr. Hansen, ~200 international milk-clotting units or IMCU per mL) at a ratio of 0.25 mL per litre of milk. The other three batches were made with the same starter after a pre-ripening step with (i) the adjunct culture of *K. varians* (S157), (ii) the adjunct culture of *Y. lipolytica* (LEV53), or (iii) the combination of both adjunct cultures (S157 + LEV53).

The adjunct cultures S157 and LEV53 prepared as previously described were added to the cheese milk cooled at 30 °C, and maintained at this temperature for 3 h with gentle agitation (pre-ripening step). The culture of the *K. varians* strain was added at a 0.5% ratio, and the culture of the *Y. lipolytica* strain was added at a 1.5% ratio, in order to achieve an inoculation level of 5.5–6 log cfu/mL. After the pre-ripening step, the milk was heated at 32 °C and inoculated with the MM100 culture, which was acclimatized to this temperature for 20 min before adding the chymosin coagulant, as previously described.

All batches were made as recommended by the Standard Procedures of the Tetilla PDO (MAPA, 1993) and according to Menéndez et al. (2004). Calcium chloride was added (0.1 g/L) to the milk just before addition of rennet. The milk was curdled at 32 °C for 40 min. The curd was cut into 5 to 10 mm pieces and washed (30% over the total volume) with pasteurized water at 36 °C until the Dornic acidity of the whey-water mixture reached 6 °D. The cheese was salted directly in the vat (12 g per L of cheese milk), and the cheeses were moulded and pressed at 2 kg/cm^2 until reaching pH 5.5–5.6 (5–6 h). Each vat yielded a batch of four cheeses of about 1 kg. All cheeses were ripened under the same conditions (7 ± 1 °C, 85 ± 2% relative humidity). Two cheesemaking trials were carried out using milk from the same tank (milk from two milkings maintained at 4 ± 1 °C for up to 18 h).

2.3. Cheese sampling

The cheese batches were sampled on day 21 of ripening. An outer layer of depth 2–3 mm was removed before taking samples for physicochemical and volatile compound analysis. Cheese samples for physicochemical assays were stored at 4 °C until analysis within 24 h. Samples for microbiological analyses (10 g including the cheese surface) were removed aseptically and transferred to sterile blender bags. Volatile compound analysis was carried out on frozen (−30 °C) cheese wedges of about 100 g in weight wrapped into aluminium foil and vacuum-packaged in plastic pouches (Polyskin X, Amcor Flexibles Hispania S.L., Barcelona, Spain).

All physicochemical, microbiological and volatile compound analyses were performed on samples taken from two different cheeses of each batch and each cheesemaking trial (four samples per analysis).

2.4. Physicochemical and microbiological analyses

The pH of the cheeses was measured on 10 g samples, as previously described (Rodríguez-Alonso et al., 2008). Water activity (a_w) was