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Microbiological, physico-chemical and proteolytic changes in a Spanish blue cheese during ripening (Valdeón cheese)

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ABSTRACT

The aim of this work was to study the microbiological, physico-chemical and proteolytic changes in Valdeón blue-veined cheese during ripening. Eight replicas of cheese were produced and a total of 48 cheeses were analysed. Lactic acid bacteria, mainly lactococci, were the predominant flora during the early stages of ripening, gradually being replaced by moulds and yeasts (8 log units). Enterococci and *Enterobacteriaceae* counts were very low or zero. This variety was characterised by a total solids content of 61.80 g per 100 g^{-1} of cheese, a salt/moisture ratio of 8.92 g salt per 100 g^{-1} moisture, a pH of 6.4–7.6 and a water activity of 0.917. At the end of ripening, primary and secondary proteolysis were very high, which resulted in an almost total degradation of αs1- and β-casein (approximately 90%). The peptide profile of the aqueous soluble extracts at pH 4.6 showed great complexity during ripening.

1. Introduction

Cheese production in Spain has increased by 22% since 2001, currently accounting for 315,710 tonnes. This upward trend is set to continue in the coming years, unlike other European Union countries where both production and consumption of cheese have stabilised. One of the factors behind this increase has been the emergence of numerous artisanal cheese factories, where many producers process milk from their own farms. In some cases, this has enabled the recovery of traditional varieties, but in others, it has contributed to the loss of the original identity of cheeses.

The cheesemaking tradition in Spain is reflected in the existence of more than 100 different varieties of cheese, many of which are certified as being of distinctive quality through either the Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI) certification systems (26 and 2, respectively). These quality designations came into being as strategies to protect original and characteristic regional products from imitation and consequent fraudulent market competition. Differentiation of a cheese granted a quality label involves determination and knowledge of the set of attributes that gives it an original, specific and distinctive product; it is therefore necessary to characterise the cheese. Such characterisation includes several steps, ranging from a study of the raw material from which the cheese is elaborated, through the procedure used to manufacture it, to its main chemical, biochemical and sensory parameters during ripening.

Although various studies have been conducted in recent years to characterise cheeses, encompassing almost all PDOs currently in existence in Europe, some protected cheeses have received very little study. One example of these is the PGI "*Queso de Valdeón*" (Valdeón cheese), for which only a few studies have been reported, related to the microbiota of the artisanal variety (Lopez Diaz, Santos, Gonzalez, Moreno, & Garcia, 1995; Lopez-Diaz, Alonso, Santos, Garcia, & Moreno, 1995). However, to the best of our knowledge, no studies have been conducted of the chemical composition or proteolysis of Valdeón cheese, or of the main biochemical and sensory changes during ripening. Only one study has been published recently about his peptidomic and changes after simulated gastrointestinal digestion (Sánchez-Rivera et al., 2014). This contrasts with other blue-veined cheese varieties, which have been characterised to a greater or lesser extent, such as mould-ripened Civil cheese (Cakmakci et al., 2013), Gorgonzola type-cheese (Seratlic, Miloradovic, Radulovic, & Macej, 2011), Cabrales (Flórez & Mayo, 2006), Picón-Bejes Tresviso (Prieto, Franco, Fresno, Bernardo, & Carballo, 2000) or Gamonedo (González de Llano, Ramos, Rodríguez, Montilla, & Juarez, 1992).

The PGI Valdeón cheese is a blue-veined variety which is manufactured in the municipal region of Valdeón (León, Spain) from a mixture of pasteurised cow's and goat's milk and is ripened for 2 months. It is cylindrical in shape, with a maximum height of 15 cm and a diameter of 25 cm. Although it can weigh between

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0.5 and 3 kg, the most frequent weight is approximately 2.4 kg. The paste is an ivory-white colour with numerous blue-green cavities, evenly distributed due to the growth of moulds of *Penicillium roqueforti*. It has a very characteristic pungent aroma of mould. Unlike the vast majority of Spanish PDO cheeses, PGI Valdeón cheese is mainly marketed in EU countries (75%) and in the United States of America, Malaysia and Japan, providing an indication of its quality and economic potential.

In our opinion, one of the ways to protect these quality products is to conduct a global characterisation in order to determine the main attributes that render them original, specific and distinctive products on the market. Proteolysis plays an important role in the final texture and flavour of the cheese. In blue-veined cheeses, it is associated mainly with the type of fungal flora and its enzymatic activity (Seratlic et al., 2011). In addition, this type of fungal flora would allow differentiate Valdeón cheese from other varieties. Consequently, the aim of our study was to analyse the microbiological, physico-chemical and biochemical changes during the manufacture and ripening of the PGI "Queso de Valdeón", paying particular attention to the degradation of proteins.

2. Materials and methods

2.1. Cheese manufacture and sampling

Eight replicas of Valdeon cheese were manufactured from a mixture of cow's and goat's milk (90% and 10%, respectively) using the standard method established by the Regulatory Board. After it has been filtered, the milk was pasteurised at 74 °C for 20 s. Then, a commercial mesophilic starter culture (FD-DVS CHN-19, Chr. Hansen SL, Madrid, Spain) and a liquid spores suspension (1.6×10^8 spores/ml) of *P. roqueforti* (Biostar, Toledo, Spain) were added to milk at 32 °C; and after 2 h, 30 ml of commercial liquid calf rennet (NATUREN liquid 140 S/S, 90% Chymosin; 140 ± 5 IMCU/ml; Chr. Hansen SL, Madrid, Spain) was added per 100 L of milk. After 60 min, the curd was cut to size of approximately 1 cm^3 cubes. Then the curd was stirred continuously at 32 °C for about 2 h before draining off some of the whey. The curd was placed in cylindrical moulds, but not pressed, where it stayed for 48 h. After this time, the curd was removed from the moulds and dry salt was added to the surface and was left to penetrate for 5 days. Then the cheeses were pierced to promote mould growth. Finally, the cheeses were transferred to a drying room where they remained at 10 °C and 90% relative humidity for 4 months.

Milk and 2-, 15-, 30-, 60-, 90- and 120-day-old cheese samples were taken from each replica. Each sample was made up one of whole cheese (2.4 kg). All samples were taken to the laboratory under refrigeration (below 5 °C) and then stored below freezing (−30 °C), except when the analyses required fresh samples.

2.2. Microbiological analyses

Fifty grams of each sample were homogenised with 200 ml of a sterile solution at 2% (w/v) sodium citrate for 2 min in a Stomacher 400 Lab Blender (Seward Medical, London, UK). Decimal dilutions were prepared by mixing 10 ml with 90 ml of 0.1% (w/v) of sterile peptone water (Oxoid, Unipath Ltd., Basingstoke, UK), according to standard 122B (FIL-IDF, 1992).

Aerobic mesophilic and psychrotrophic bacteria were enumerated on Standard Plate Count Agar (PCA) (Oxoid) after incubation at 30 °C for 48 h and 7 °C for 10 days, respectively. Lactic acid bacteria were determined on three different media: Lactococci on M17 agar (Biokar, Beauvais, France) after incubation at 30 °C for 18–24 h; *Leuconostoc* on MSE agar (Biokar) after incubation at 22 °C for 4 days, and lactobacilli on Rogosa agar (Oxoid) after incubation at 30 °C for 5 days. *Micrococcaceae* were determined on Mannitol Salt Agar (MSA) (Oxoid) after incubation at 30 °C for 48 h; enterococci on Kanamycin Aesculin Azide (KAA) agar (Oxoid) after incubation at 37 °C for 24 h; *Enterobacteriaceae* on Violet Red Bile Glucose Agar (VRBGA) (Oxoid) after incubation at 37 °C for 18–24 h; and the moulds and yeasts on Oxytetracycline Glucose Yeast Extract (OGYEA) agar (Oxoid) after incubation at 22 °C for 5 days.

2.3. Physico-chemical and compositional analyses

Total solids, protein and fat contents were determined according to standards 004 (FIL-IDF, 2004), 20-1 (FIL-IDF, 2001) and 221 (FIL-IDF, 2008), respectively. NaCl content was determined according to standard 935.43 (AOAC, 1990). Lactose, D-lactic acid and L-lactic acid contents were determined using a Boehringer Mannheim enzymatic kit (R-biopharm, Roche, Germany). pH was analysed according to standard 14.022 (AOAC, 1980). Water activity (aw) was determined instrumentally using an Aqua Lab Dew Point Analyzer CX-2 (Decagon Devices Inc., Pullman, WA, USA).

2.4. Proteolytic parameters

pH 4.6-soluble nitrogen (pH 4.6-SN) extracts were performed according to Kuchroo and Fox (1982). 12% trichloroacetic acid-soluble nitrogen (TCA-SN) was obtained by mixing equal parts of pH 4.6-SN and TCA 24% and filtering through Whatman No. 40 filter paper (Whatman Biosystems, Maidstone, UK). 5% phosphotungstic acid-soluble nitrogen (PTA-SN) was obtained by adding 35 mL of 3.95 M H_2SO_4 and 15 mL of 33% PTA to 50 ml of the pH4.6-SN extract and filtering through a Whatman No. 40 filter paper. The nitrogen content in all fractions was determined by the macro-Kjeldahl method (FIL-IDF 224, 2011) using a Kjeltec System1002 Distilling Unit and a Digestion System 6 1007 Digester (Tecator).

Urea–polyacrylamide gel electrophoresis (urea–PAGE) of the pH 4.6-insoluble fractions of cheeses was studied following the method described by Shalabi and Fox (1987). Densitometric analysis was performed on the scanned image using gel analysis software using gel analysis software (TotalLab 1D, nonlinear Dynamix, Newcastle-upon-Tyne, UK).

The peptide profiles of pH 4.6-soluble nitrogen extracts of Valdeón cheese were determined by Reverse-Phase Ultra Performance Liquid Chromatography (RP-UPLC) using an Acquity UPLC-H Class (Waters Corp) according to the method described by Sousa and McSweeney (2001). The chromatographic profile was processed using the method described by Piraino, Parente, and McSweeney (2004).

Plasmin activity was determined using a modification of the method described by Richardson and Pearce (1981), using N-succinyl-L-Ala-L-Phe-L-Lys 7-amido-4-methyl-coumarin (AMC) as substrate. Plasmin activity was expressed as plasmin units per g^{-1} cheese (where 1 unit was defined as the activity necessary to release 1 nmol AMC per min under standard assay conditions).

2.5. Statistical analysis

Means with a significant difference were compared by ANOVA/MANOVA analysis with a confidence interval set at 95%. Statistical correlations were carried out by the Pearson's correlation coefficient. Both analyses were carried out using Statistica® for Windows version 8.0, StatSoft, Inc. 2007 (Tulsa, OK, USA). Principal component analysis (PCA) was performed by standardising the variables to zero mean and using a covariance matrix. Statistical analysis was performed using Minitab® for Windows version 16.2.2 Minitab, Inc. 2010 (State College, PA, USA).