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Lysozyme affects the microbial catabolism of free arginine in raw-milk hard cheeses

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ABSTRACT

Lysozyme (LZ) is used in several cheese varieties to prevent late blowing which results from fermentation of lactate by *Clostridium tyrobutyricum*. Side effects of LZ on lactic acid bacteria population and free amino acid pattern were studied in 16 raw-milk hard cheeses produced in eight parallel cheese makings conducted at four different dairies using the same milk with (LZ+) or without (LZ-) addition of LZ. The LZ-cheeses were characterized by higher numbers of cultivable microbial population and lower amount of DNA arising from lysed bacterial cells with respect to LZ + cheeses. At both 9 and 16 months of ripening, *Lactobacillus delbrueckii* and *Lactobacillus fermentum* proved to be the species mostly affected by LZ. The total content of free amino acids indicated the proteolysis extent to be characteristic of the dairy, regardless to the presence of LZ. In contrast, the relative patterns showed the microbial degradation of arginine to be promoted in LZ + cheeses. The data demonstrated that the arginine-deiminase pathway was only partially adopted since citrulline represented the main product and only trace levels of ornithine were found. Differences in arginine degradation were considered for starter and non-starter lactic acid bacteria, at different cheese ripening stages.

1. Introduction

Nowadays hen's egg white lysozyme (EC 3.2.1.17) (LZ) is used in Grana Padano as well as in other hard cheeses to prevent the "late blowing" defect (Brasca et al., 2013; Jiménez-Saiz et al., 2013). In fact, LZ is efficient in lysing the vegetative cells of Clostridia, specifically of *Clostridium tyrobutyricum*, by splitting β (1–4) linkages between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan of the bacterial cell wall (Hughey and Johnson, 1987). These bacteria are capable of producing spores that survive the thermal treatment applied in making hard cheeses and can later germinate and produce gas causing the defect. The origin of the contamination by this bacterium has been identified in the wide use of silage in livestock feeding (Jonsson, 1991; Vissers et al., 2006).

The different sensitivity to LZ of various bacteria, both Gram positive and negative, is due to the different cell wall composition and structure and, thus, to the binding of the enzyme to its specific substrate (Bester and Lombard, 1990; Carini et al., 1985; Hughey and Johnson, 1987). Due to its wide spectrum, LZ activity can also occur against lactic acid bacteria (LAB) involved in curd acidification and cheese ripening. The interference of the enzyme with the acidification process occurring during hard cheese production has been indirectly studied by considering the sensitivity of LAB responsible of curd acidification. For example, LZ inhibitory activity has been extensively evaluated for the main species present in the natural whey starter used to produce Grana Padano, i.e. *Lactobacillus helveticus*. It was found that sensitivity of *L. helveticus* was strain-dependent, and acquisition of resistance can be due to strain adaptation rather than selection of spontaneous mutants (Fortina et al., 1998; Neviani et al., 1991). Resistance to LZ was also reported for *Lactobacillus delbrueckii* (Vinderola et al., 2007). Moreover, a correlation was observed between LZ resistance and bacteriophage sensitivity in *L. helveticus* (Neviani et al., 1992) and the authors suggested the possibility of using LZ as a selective agent to isolate phage-resistant starter strains.

To author's knowledge, few literature data are available on LZ resistance of non-starter LAB (NSLAB). These are part of the raw

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milk cheese microbiota and are not involved in curd acidification but play a relevant role in cheese ripening (Gatti et al., 2014). Carini et al. (1985) reported LZ resistance of *Lactobacillus casei* species. Ugarte et al. (2006) studied NSLAB isolated from soft and semi hard Argentinean cheeses and found most of the species to tolerate 2.5 mg 100 g^{-1} of LZ. More recently, LZ sensitivity of NSLAB was studied as one of the criteria suitable to evaluate their probiotic aptitude (Solieri et al., 2014). A strain-dependent resistance to LZ at the concentration of 10 mg 100 g-1, close to that adopted in Grana Padano, was found for *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, *L. casei*, *Lactobacillus harbinensis*, and *Lactobacillus fermentum*.

The aim of this work was to investigate the effects of LZ in cheese with respect to both the microbial populations and proteolysis pathways responsible of cheese ripening. In particular, the focus was on Grana Padano PDO cheese, usually made with LZ and extensively studied for its microbial and chemical features (Pellegrino et al., 1997; Masotti et al., 2010; Santarelli et al., 2013; Pogacic et al., 2013). Eight cheese makings were therefore conducted at four different dairies, using in parallel, the same milk either added or not with LZ. The 16 derived cheeses were analyzed after 9 months of ripening, i.e. the minimum ripening period for Grana Padano PDO cheese, and after 16 months. The microbial populations were characterized by Length Heterogeneity-PCR (LH-PCR) considering both the intact and lysed cells. Furthermore, the free amino acids (FAA) patterns of the cheeses were evaluated. Since FAA mostly result from the action of intracellular proteinases and peptidases released after the bacterial cell lysis, different patterns could be expected between cheeses produced with and without addition of LZ.

2. Materials and methods

2.1. Cheese manufacture

Grana Padano cheeses were manufactured at four dairies belonging to the Consorzio Tutela Grana Padano, following the traditional manufacturing process (European Parliament and Council, 2012). At each dairy, the cheese makings were carried out on two different days for a total of eight trials. For each cheese making trial, raw bulk milk was partially skimmed (fat content: 2.2–2.3%) by natural creaming, divided into two vats (1000 L each), one of which was added with 20 g LZ (Sacco, Cadorago, Italy) carefully dispersed in 200 mL water, and the two vats were worked in parallel. The natural whey starter (titratable acidity: 30–32°SH/50 mL), obtained from the residual whey of the previous days' cheese-making, and the calf rennet were added to coagulate the vat milk at 32 °C in 8–10 min. The curd was gently cut into small granules while progressively heated up to 52–54 °C, then it was allowed to compact at the bottom of the vat for 60 min before extraction. The cheeses (two wheels per vat) were molded for 48 h to allow lactic acid fermentation (pH was measured in the core of the wheels) and then salted in brine for 18–20 days. During ripening, all the cheeses were regularly inspected by X-ray tomography (Philips CT Brilliance 16P, Zürich, Switzerland) to evidence possible development of defects. The twin cheeses obtained from each vat were cut after 9 and 16 months of ripening respectively. A portion representative of the whole wheel was taken from each, grated and deep-frozen until analysis. Samples for microbiological analyses were kept at 4 °C until arrival at the laboratory and immediately analyzed. Cheese samples either containing or not LZ were coded as LZ+ and LZ-respectively, whereas numbering from 1 to 8 identifies the cheese making trial they come from.

2.2. Bacterial counts

Bacterial counts were determined on de Man, Rogosa and Sharpe (MRS) agar (Oxoid, Basingstoke, United Kingdom). Representative samples (10 g) of the grated cheese were suspended in 90 mL of 20 g L^{-1} tri-sodium citrate (pH 7.5) (Sigma–Aldrich, St. Louis, USA) and homogenised for 2 min in a blender (Seward, London, United Kingdom). For enumerating mesophilic lactobacilli, as main microbiota of cheese during ripening, decimal dilutions of cheese homogenates were made in quarter-strength Ringer solution (Oxoid, Basingstoke, United Kingdom) and spread plated in triplicate on MRS. The plates were incubated at 30 °C for 72 h under anaerobic conditions.

2.3. DNA extraction

Bacterial genomic DNA was extracted directly from samples by using a General Rapid Easy Extraction System (GERES) DNA kit (InCura S.r.l., Cremona, Italy) according to the manufacturer's instructions. Cheese samples were pre-treated in order to discriminate the DNA from whole and lysed cells as described by Gatti et al. (2008). Briefly, cheese samples resulted in two fractions, the free-cell fraction was obtained by filtration and the whole-cell fraction was obtained by treating samples with DNase to digest free DNA arising from lysed cells. DNA was extracted from 1 mL of the filtered untreated fraction (lysed cells) and from 1 mL of the treated fraction (whole cells).

2.4. Length heterogeneity (LH)-PCR

LH-PCR was used in order to determine the microbial community composition. V1 and V2 16S rDNA gene regions were amplified with primers 63F and 355R (Lazzi et al., 2004). The forward primer was 5′-end labeled with a 6-carboxyfluorescein (6-FAM) dye. Amplicons were then separated by capillary electrophoresis in an automated sequencer (Applied Biosystems, Foster City, USA). PCR and capillary electrophoresis conditions were as described by Bottari et al. (2010). The fragment sizes (base pairs) were determined with GeneMapper software version 4.0 (Applied Biosystems), local Southern method to generate a sizing curve from the fragment migration of the internal size standard (GS500 LIZ®; Applied Biosystems) and a threshold of 150 fluorescence units. The fragment analysis software converted fluorescence data into electropherograms. The peaks represent fragments of different sizes and the areas under the peaks are the amount of the fragments. Total area were considered to directly correlate to the total amount of the DNA arising from whole or lysed cells depending on the two fractions previously described. Each peak, corresponding to amplicon of specific length on the electropherogram profile, was attributed to bacterial species according to published databases (Lazzi et al., 2004; Gatti et al., 2008) and the areas under the recognized peaks were used to estimate the amount of the assigned species in the samples. Total area under all the peaks (sum of attributed and unattributed peaks) of the LH-PCR electropherograms was used for measuring total amount of DNA arising from both intact and lysed cells.

2.5. Determination of free amino acids by ion-exchange chromatography

2.5.1. Free amino acid extraction

The grated cheese was weighted (1.5 g) in a 100 mL beaker, added with 40 mL 0.2 N tri-sodium citrate buffer at pH 2.2 (SCB), kept under magnetic stirring for 15 min then carefully homogenized with Ultra-Turrax (5 min at low speed). The extract was