J. Dairy Sci. 98:1–5
http://dx.doi.org/10.3168/jds.2015-9844


# Identification of peptides in functional Scamorza ovine milk cheese

**M. Albenzio,[1] A. Santillo, R. Marino, A. Della Malva, M. Caroprese, and A. Sevi**
Department of the Sciences of Agriculture, Food and Environment (SAFE), University of Foggia, Via Napoli, 25, 71100 Foggia, Italy

## ABSTRACT

Ovine bulk milk was used to produce Scamorza cheese with probiotics: either a mix of *Bifidobacterium longum* and *Bifidobacterium lactis* or *Lactobacillus acidophilus* as the probiotic strains. Peptides obtained from reverse phase-HPLC water-soluble extract of Scamorza cheeses were analyzed using a quadrupole time-of-flight liquid chromatography-mass spectrometry system. Identified fragments were derived from casein hydrolysis or probiotic bacterial enzymes; some of the fragments showed encrypted peptide sequences that shared structural homology with previously described bioactive peptides in ovine milk and dairy products. *Bifidobacterium longum* and *B. lactis* showed greater proteolytic potential both in terms of level of pH 4.6 water-soluble nitrogen extract and ability to generate peptides with potential biofunctionality. Fragments deriving from microbial enzymes may be regarded as tracing fragments useful for monitoring probiotic activity in functional Scamorza cheese.

**Key words:** *pasta filata*, ovine cheese, probiotic, peptide

## INTRODUCTION

Today, many cheeses are available on the market, varying in composition, texture, appearance, and taste. Cheese is a nutritious food, being a rich source of essential nutrients such as fat, fatty acids, proteins, peptides, amino acids, vitamins, and minerals; thus, it can play an essential role in meeting nutritional requirements. Furthermore, cheese represents a good choice as a probiotic food carrier, showing potential advantages for human health over other dairy fermented products; however, the development of probiotic cheese also represents a technological challenge (Castro et al., 2015). *Pasta filata* cheese varieties are mainly produced from cow and buffalo milks; the principal phases of *pasta filata* cheese production are acid development, rennet-catalyzed coagulation, and heating and stretching of the curd mass. Albenzio et al. (2013a,b) developed a production protocol and evaluated the characteristics of Scamorza ovine cheese containing probiotics and verified that *Bifidobacterium longum*, *Bifidobacterium lactis*, and *Lactobacillus acidophilus* could survive the heat stress during the stretching phase and persist at high levels in the cheese matrix, influencing the proteolytic, lipolytic, and sensorial properties of the cheese.

Milk proteins are currently the main source of a range of biologically active peptides, which have gained special interest because they may influence numerous physiological responses in the organism. Many bioactivities in milk are encrypted within the primary structure of milk proteins, requiring proteolysis for their release from precursors (Gobbetti et al., 2004).

In Manchego ovine cheese ripened for 8 mo, several peptides with angiotensin-converting enzyme (**ACE**)-inhibitory activity were identified (Gómez-Ruiz et al., 2004), and antibacterial peptides were obtained in Pecorino Romano ovine cheese (Rizzello et al., 2005). Peptide formation is associated with cheese ripening, thus biologically active peptides can be ingested as naturally occurring components of food (Gómez-Ruiz et al., 2002).

The present study was undertaken to evaluate the peptide profile of *pasta filata* Scamorza ovine cheeses ripened for 15 d and containing either a mix of *Bifidobacterium longum* and *Bifidobacterium lactis* or *Lactobacillus acidophilus*.

## MATERIALS AND METHODS

Scamorza cheese production was performed according to the protocol reported in Albenzio et al. (2013a). Briefly, ovine milk was thermized and inoculated with 1% commercial starter (Lyofast, ST044, Sacco, Como, Italy) and with 2% heat-adapted cells of *Lactobacillus acidophilus* or a mix of *Bifidobacterium longum* and *Bifidobacterium lactis*. Lyophilized cultures of *Lactobacillus acidophilus*, *Bifidobacterium longum*, and *Bifidobacterium lactis* were supplied by Mediterranea Biotecnologie S.r.l. (Termoli, Italy). After the milk was cooled, liquid rennet (Chr. Hansen S.p.A., Parma,

Received May 21, 2015.
Accepted June 15, 2015.
[1]Corresponding author: m.albenzio@unifg.it

Italy) was added, and the curd reached a final pH value of 4.9; then, the curd was stretched mechanically in hot water and molded into a pear shape. Scamorza cheese was brine salted and ripened for 15 d. Cheeses were designated **S-CO** for control Scamorza cheese without probiotics added, **S-BB** for Scamorza cheese made using a mix of *B. longum* and *B. lactis*, and **S-LA** for Scamorza cheese made using *L. acidophilus* as the probiotic strain. Cheeses were analyzed at 15 d of ripening.

The pH 4.6-soluble N fraction was obtained according to Kuchroo and Fox (1982). The peptide profiles of the pH 4.6-soluble N fractions were determined by reverse-phase (**RP**)-HPLC by using the Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA). The column used was a Zorbax 300 SB-C18 (250 mm × 4.6 mm × 5 μm; Agilent Technologies). The mobile phase comprised water (solvent A) and acetonitrile (solvent B), both containing 0.1% trifluoroacetic acid, and the solvent flow rate was 1 mL/min. The eluate was monitored at 220 nm; all solvents were of chromatography grade (Mallinckrodt Baker Inc., Phillipsburg, NJ). Peptides eluting between 1 and 20 min were selected and collected using a fraction collector automatic sampler (G1364C, Agilent Technologies) from each Scamorza cheese sample for subsequent identification.

Peptide aliquots were analyzed using a 6520 Accurate-Mass Quadrupole Time-of-Flight (Q-Tof) liquid chromatography mass spectrometry (LC/MS) system (Agilent Technologies) equipped with a 1200 HPLC System and chip cube (Agilent Technologies). After loading, the peptide mixture was first concentrated and washed on a 40-nL enrichment column (Agilent Technologies chip), with 0.1% formic acid in 2% acetonitrile as the eluent. The sample was then fractionated on a C18 reverse-phase capillary column (Agilent Technologies chip) at a flow rate of 400 nL/min, with a linear gradient of eluent B (0.1% formic acid in 95% acetonitrile) in A (0.1% formic acid in 2% acetonitrile) from 7 to 80% in 50 min. Peptide analysis was performed using data-dependent acquisition of one MS scan (mass range from 300 to 1,800 $m/z$) followed by a tandem MS (MS/MS) scan of the 5 most abundant ions in each MS scan. The MS/MS spectra were measured automatically when the MS signal surpassed the threshold of 50,000 counts.

Double and triple charged ions were preferably isolated and fragmented over single charged ions. The acquired MS/MS spectra were transformed in *mzData* (.XML) format and used for protein identification with a licensed version of MASCOT software (version 2.4.0; www.matrixscience.com). Raw data from nano LC-MS/MS analysis were used to query the National Center for Biotechnology Information nonredundant sequence database (NCBInr database, 20121120; 21,582,400 sequences; 7,401,135,489 residues). The MASCOT search parameters were: trypsin as enzyme; 3 as allowed number of missed cleavage; carboamidomethyl as fixed modification; oxidation of methionine; phosphorylation of serine/threonine/tyrosine; pyro-Glu N-term Q as variable modifications; 10 ppm MS tolerance and 0.6 Da MS/MS tolerance; peptide charge from +2 to +3. The eptide score threshold provided from MASCOT software to evaluate quality of matches for MS/MS data was 25. Spectra with MASCOT score of <25 having low quality were rejected.

## RESULTS AND DISCUSSION

The RP-HPLC chromatograms of the water-soluble N fraction of Scamorza cheeses at 15 d of ripening are shown in Figure 1. The percentage of pH 4.6-soluble N fraction was highest ($P < 0.01$) in S-BB, reaching a value of 0.93 ± 0.02% compared with 0.74 ± 0.02% and 0.79 ± 0.02% in S-CO and S-LA, respectively, at 15 d of ripening. The RP-HPLC was focused on early-eluting peaks characterized by high hydrophobicity with a retention time lower than 20 min because this zone showed the major differences among peptides profiles of the Scamorza cheeses in terms of number of peaks and area under the curve. The sum of peak area shown in the chromatograms was 26,497 ± 650 arbitrary units (**AU**) for S-BB, 24,777 ± 680 AU for S-LA, and 8,780 ± 750 AU for S-CO. In the present study, Scamorza cheeses containing probiotics displayed a profile with higher total area than control cheese, confirming the major effect of probiotics on secondary proteolysis, with the liberation of low-molecular-weight peptides collected in the soluble N fraction of Scamorza cheese after 15 d of ripening (Albenzio et al., 2013a). Within functional Scamorza cheeses, S-BB cheese showed the highest total area, confirming the greater ability of the selected bifidobacteria to control the proteolytic process, as previously observed in Pecorino ovine cheese (Santillo et al., 2009; Albenzio et al., 2010).

A qualitative evaluation of the RP-HPLC profiles was performed: 2 peaks were chosen for each cheese type based on major peak area and comparable retention time among Scamorza cheeses. In addition, an additional peak was selected and identified for S-BB cheese because it was unique to the RP-HPLC profile of that Scamorza cheese.

Peptides identified in the fraction collected by RP-HPLC from the pH 4.6-soluble N fraction of Scamorza cheese at 15 d of ripening are reported in Table 1. The selected peak 1 in S-BB cheese showed co-elution of