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Influence of PDO Ragusano cheese biofilm microbiota on flavour compounds formation

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ABSTRACT

The objectives of the present study were to characterize the biofilm microbiota of 11 different farms (from *A* to *K*), producing PDO Ragusano cheese, and to investigate on its ability to generate volatile organic compounds (VOCs) in milk samples inoculated with biofilm and incubated under Ragusano cheese making conditions. The biofilms were subjected to plate counting and PCR/T/DGGE analysis and the VOCs generated in incubated milk samples were evaluated through SmartNose, GC/O, and GC/MS. *Streptococcus thermophilus* was the dominant species both in biofilms and in incubated milks. *Lactobacillus, Lactococcus, Enterococcus* and *Leuconostoc* were also identified. Low levels of *Pseudomonas* spp. and yeasts counts were detected, whereas coliforms, *Listeria monocytogenes* and *Salmonella* spp., were never found. SmartNose and GC/O analyses were able to differentiate incubated milk samples on the basis of the odour compounds, highlighting that samples *E* and *F* overlapped and sample *C* was clearly separated from the others. These results complied with those acquired by GC/MS analysis, that detected in total 20 VOCs. Principal component analysis showed positive correlations ($r > 0.6$; $P < 0.05$) between some lactic acid bacteria (LAB) and VOCs: such as *Enterococcus hirae* with alcohols, *Lactococcus lactis, Lactobacillus plantarum, Lactobacillus casei* and *Lactobacillus delbrueckii* with aldehydes, and *Lactobacillus fermentum, Lactobacillus helveticus* and *Lactobacillus hilgardii* with ketones. This work demonstrates that biofilm represents an excellent source of LAB biodiversity, which contribute to generate VOCs during the production of PDO Ragusano cheese.

1. Introduction

In a wide variety of cheeses lactic acid bacteria (LAB) contribute to cheese flavour through the action of live cells and, when they lyse, through the release of intracellular materials, including enzymes such as proteinases, peptidases, amino acid catabolic enzymes and esterases, into the cheese matrix (Fox et al., 1995; Garde et al., 2007). Indigenous LAB biodiversity is considered a key factor for the sensorial features of artisanal cheese type. Ragusano cheese is one of the most important Sicilian traditional cheeses, produced in the Hyblean area of Sicily. In 1996 it received the Protected Designation of Origin (PDO) (Regolamento CE n. 1263/1996) and it is appreciated all over the world for its particular sensorial properties (Carpino et al., 2004a). The traditional manufacture of Ragusano, a semi-hard PDO pasta-*filata* cheese, does not require the addition of starter cultures (Licitra et al., 1998), and the fermentation process is driven by indigenous LAB, which arise from raw milk, biofilm present on the inner surface of the vats, and environment (Licitra et al., 2007; Lortal et al., 2009). In particular, traditional wooden vats (*Tina*), used for PDO cheese production, might be covered by a microbial biofilm. The presence of a biofilm, containing LAB, in the *Tina* wood vat, used in PDO Ragusano cheese making process, was already assessed (Licitra et al., 1998, 2007). Recently, Lortal et al. (2009) confirmed that the *Tina* wooden vat is a highly efficient system to deliver thermophilic LAB into the raw milk, contributing to the acidification step. The observation that after few minutes of contact with the *Tinas*, raw milk acidification is accelerated in 80% of the cases clearly supposes the idea of LAB inoculation *via* the *Tina* biofilms (Licitra et al., 2007). Molecular exploration of *Tinas*

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from 5 different farms of the Hyblean region revealed the presence of thermophilic lactobacilli, and lactococci with the dominance of *Streptococcus thermophilus* species (Licitra et al., 2007). The inability of pathogens, such as *Salmonella* spp., *Listeria monocytogenes* and *Eschericia coli O157:H7,* to adhere or to survive in 15 *Tinas,* collected in the Hyblean region, was revealed, demonstrating the safety of this wood vat system (Lortal et al., 2009). Recently, the influence of the wooden vat on microbial composition and biodiversity of traditional Caciocavallo Palermitano type cheese was achieved, demonstrating that the wooden system represents an active source of *S. thermophilus* strains involved in curd acidification (Settanni et al., 2012). Authors also revealed the presence of other non starter LAB (NSLAB), in particular enterococci, that are considered important for the uniqueness of traditional cheeses (Foulquié Moreno et al., 2006), even though the safety status of some species of the *Enterococcus* genus must be considered (Settanni et al., 2012). Moreover, the study of the composition of the biofilms associated with the wooden vats used to produce different French cheeses revealed the presence of several dairy LAB, including the starter (SLAB) species *Lactobacillus helveticus*, *Lactococcus lactis* and *Leuconostoc mesenteroides* and different NSLAB such as *Lactobacillus plantarum* and *Lactobacillus casei* (Didienne et al., 2012), confirming the crucial role of the wooden vat equipment in enriching the milk LAB diversity and its positive contribution to cheese flavor formation (Di Grigoli et al., 2015). Due to the complexity of the dairy products, the detection of odours and VOCs is improved by using Gas Chromatography Mass Spectrometry (GC/MS) and with electronic-noses (E-Noses) and/or GC/MS with olfactometric detection (GC/MS/O) (Brattoli et al., 2013; Cevoli et al., 2011; Gardner and Bartlett, 1994; Wilson and Baietto, 2009).

The aims of the present study were: (i) to explore the microbial community of biofilms collected from 11 different *Tinas* of Hyblean area of Sicily and of milk samples both inoculated and incubated with biofilm; (ii) to identify the VOCs generated in the incubated milk samples; and (iii) to correlate the VOCs formation with the detection f LAB species.

2. Materials and methods

2.1. Biofilm sampling

Tina-biofilm (TB) samples were collected from the inner surface of the wooden vats from 10 different farms (from *A* to *J*). Moreover, wooden *Tina* of CoRFiLaC Experimental Dairy Plant (*K*) was also investigated. Cells from the 11 TB were withdrawn from 5 different areas of 100 cm^2, using sterile swabs suspended in 10 mL of peptone water, pooled and used for plating counts or stored at −70 °C for culture-independent analyses (Lortal et al., 2009). In addition, swabs from 5 different areas of 100 cm^2 of *Tinas* were suspended in 10 mL UHT milk and immediately used to make inoculated milk samples (IM1).

2.2. Biofilm inoculation in UHT milk and incubation conditions

Each of the 11 TB samples (swabs from 5 × 100 cm^2) suspended in 10 ml UHT milk were used to inoculated 5 l of UHT milk in a sterilized glass vessel (IM1 samples). The IM1 samples were subsequently incubated at different temperatures, to obtain the related incubated milk samples (IM2). Temperatures and time intervals used to incubate the IM1 samples were chosen in order to mimic the applied thermal cycles during PDO Ragusano cheese manufacture as descried previously (Licitra et al., 2007). In details, IM1 samples were incubated under the following conditions: 65 min at 35 °C, 45 min at 40° C (first cooking), 120 min at 45 °C (second cooking), 24 h at 15 °C (time before brining) to obtain the incubated milk samples (IM2). UHT milk not inoculated and incubated at the same conditions was used as control (C).

2.3. Microbiological analyses

Microbiological analyses of TB, IM1 and IM2 samples were performed in duplicate. Samples (1 ml) were aseptically transferred to 9 mL of sterile quarter-strength Ringer's solution (QRS). Decimal dilutions in QRS were prepared and plated into following agar media (all from Oxoid, Milan, Italy): Plate Count Agar (PCA) plus 0.1% of Skim milk, for mesophilic aerobic bacteria counting, incubated at 32 °C for 24–48 h; de Man-Rogosa-Sharp (MRS) adjusted to pH 5.4 containing cycloheximide (100 mg/l), for termophilic and mesophilic lactobacilli, incubated under anaerobic conditions, using Anaerocult® A (Merck, Millipore, Italy), at 45 °C and 15 °C for 48–72 h and 10 days, respectively; LM17 agar, for lactococci and streptococci incubated under anaerobic conditions at 15 °C and 45 °C for 10 days and 24–48 h, respectively; Kanamycin Aesculin Azide agar (KAA), containing the kanamycin supplement, for enterococci, incubated under anaerobic conditions at 37 °C for 24–48 h; violet red bile glucose agar (VRBGA) aerobically incubated at 37 °C for 24 h for coliforms; *Pseudomonas* agar base (PAB), supplemented with 10 mg/ml of cetrimide fucidin, aerobically incubated at 20 °C for 48 h for *Pseudomonas* spp.; dichloran rose bengal chloramphenicol (DRBC) agar, aerobically incubated at 25 °C for 48 h for yeasts counting. Moreover, the pathogenic bacteria *Salmonella* spp. and *Listeria monocytogenes* were investigated following the procedure described by Mucchetti et al. (2008) applying the pre-enrichment on the cotton swabs streaked onto the vat surface.

2.4. Bacterial DNA extraction, PCR amplification and T/DGGE analysis and sequencing of the 16S rRNA gene

Bacterial DNA extraction was carried out as previously described (Licitra et al., 2007). The amount of DNA was verified using the NanoDrop ND1000 Spectrophotometer (Thermo Fisher). To investigate the dominant bacterial communities of TB samples through TGGE analysis, PCR amplification of the V3 region of the 16S rRNA gene was performed as described by Licitra et al. (2007). In order to identify bands in the profiles from each farm, a reference strains collection with known sequences was used as marker in the TGGE gel.

Microbial community of IM2 samples was investigate through DGGE analysis and the PCR products were obtained using primers U968-GC and L1401-r, to amplify the V6 to V8 region of eubacterial 16S rDNA (Nubel et al., 1996) as previous described (Randazzo et al., 2002).

Clone libraries of the 16S rRNA gene amplicons from IM1 and IM2 samples, obtained using TB from farms *C, D, E*, and *I* (selected for the number and the position of the bands in the DGGE gel) were constructed as described previously (Randazzo et al., 2002). The clones that produced a single DGGE amplicon with a melting position identical to that one of the dominant bands in the cheese samples DNA patterns were sequenced by BMR Genomics (Padova, Italy) company. To determine the closest known relatives of the isolates, partial 16S rRNA gene sequences were compared to those in the GenBank database (http://ncbi.nlm.nih.gov/BLAST/) and the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp) using BLAST program. Sequences with a percentage of identity of 99% or greater were considered to belong to the same species.