Retrospective study on the hygienic quality of fresh ricotta cheeses produced in Sicily, Italy

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Abstract

The present work was carried out to investigate the microbiological profile of Sicilian ewes’ ricotta cheeses during fifteen years of investigations (2002-2016). The samples were collected between those conferred to the Istituto Zooprofilattico Sperimentale della Sicilia (IZSSI) Adelmo Mirri, Palermo (Italy), by the competent authority during official control, by food business operator in HACCP systems and in research projects. Enterobacteriaceae, Escherichia coli and coagulase-positive staphylococci (CPS) were found only in some samples. Bacillus cereus was detected in c.a. 16% of samples but the level of contaminations did not reach the threshold that leads to significant toxin production. Pathogenic bacteria such as Listeria monocytogenes, Salmonella spp. and Brucella spp. were never detected. Furthermore, a total of 47 of lactic acid bacteria (LAB) strains were identified at species level by sequencing the 16S rRNA gene, resulting in the identification of 10 species belonging to four genera Enterococcus, Lactobacillus, Lactococcus and Leuconostoc, commonly employed as starter and non starter cultures in different traditional cheese. Results of this study highlighted an improvement of the hygienic conditions of the Sicilian ewes’ ricotta cheeses during the last ten years of investigation. This observation was confirmed from reduction of undesired microorganisms such as Enterobacteriaceae, E.coli and CPS, used to define the process hygiene criteria. However, in order to improve the final quality of this product are needed further strategy such as the dairy makers training, with the aim to apply a good hygienic practices during the production.

Introduction

Recently, the new trends of life style of the consumer showed an increasing demand of traditional dairy products manufactured at the small dairy level (Gaglioti et al., 2014b). Furthermore, the consumers require foods processed without chemical preservatives and characterized by low-fat and high nutritional values. Ricotta could respond to the demands of consumers because it is characterized by high quantity of proteins, low fat and salt content and other important components, which give this product a high nutritional value (Guatemim et al., 2016).

Ricotta is a fresh dairy product that has soft, grainy, thick, lightly sour taste and presents a white colour. Usually, it can be eaten as a soft cheese but in Sicily is more frequently used as an ingredient in dishes and desserts. The name derived from the Latin word re-cocta, literally recooked or cooked twice. In Sicily it is generally manufactured from the ewes’ whey remaining after the production of hard semi-cooked cheese (Pecorino Siciliano PDO) and fresh cheese (Vastedda della valle del Belice PDO) but can be produced also with goat, cow and buffalo whey milk. Ricotta production technology involves the principle of coagulation and precipitation of the whey proteins such as globulin and albumin favoured by whey acidification at pH<5.6 and by heating at 80-90°C. During the production of Sicilian ewes’ ricotta cheese, the whey is enriched with whole raw ewes’ milk (5-15%) and salt (0.5-1.5%) to increase the yield and improve the organoleptic characteristics. Fresh ricotta is characterized by a short shelflife because of its high moisture level, high concentration of residual sugars and an initial pH above 6.0 which make this product an excellent growth medium for a wide range of microorganisms (Mancuso et al., 2014). The attributed shelflife of ricotta cheese is generally comprised between 7-11 days under refrigerated storage (4°C).

Some of the microorganisms that grow in dairy products are able to produce undesirable reactions that deteriorate the organoleptic characteristics of cheese, while other can potentially cause food-borne diseases (La et al., 2013).

The presence of these microorganisms in dairy productions depends in some factors such as the quality of raw material, the respect of good practices of production and storage conditions (Ledenbach and Marshall, 2009).

In this context the present work was aimed to evaluate the microbiological quality of several samples of Sicilian ewes’ ricotta cheese collected from dairy factories and small and large distribution.

Materials and Methods

Sample collections

A total of 1295 samples of ewes’ ricotta cheeses were collected between those conferred to the Istituto Zooprofilattico Sperimentale della Sicilia (IZSSI) Adelmo Mirri, Palermo (Italy), by the competent authority during official control, by food business operator in HACCP systems and in research projects. The samples were collected from 36 bakeries and pastry shops; from 233 markets and large-scale retail trade and from 365 dairy factories. The samples were collected during fifteen years of investigation (2002-2016). All samples to be microbiologically investigated were transported under refrigeration at the laboratories of the above-mentioned institute.

Microbiological analysis and pH measurement

All samples were analyzed in accordance with international standard or validated method: Total mesophilic count (ISO 4833), sulphite reducing anaerobes (ISO 15213), Enterobacteriaceae (ISO 4832), Escherichia coli (ISO 16649-2); coagulase-positive staphylococci (CPS, ISO 6888-2); Bacillus cereus (ISO 7932); yeasts and

Key words: Ricotta cheese; Microbial community; Lactic acid bacteria; hygienic quality; food safety.

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moulds (ISO 21527-1), *Pseudomonas* (ISO 11059), enterococci on Kanamycin Aesculin Azide aerobically incubated at 37°C for 24-48 h, rod-shaped lactic acid bacteria (LAB) on de Man-Rogosa-Sharpe (MRS) agar, acidified to pH 5.4 with lactic acid (5 mol·L⁻¹) and incubated anaerobically for 48 h at 37°C; and mesophilic and thermophilic coccus-shaped LAB on M17 agar and incubated aerobically for 48 h at 30 and 44°C, respectively. *Salmonella* spp. was detected using ISO 6579 or AFNOR BIO 12/23-05/07 and *Listeria monocytogenes* using ISO 11290-1 or AFNOR BIO 12/11-03/04. *Brucella* spp. using Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, cap. 2.4.3.:2009.

The pH value of ricotta samples was monitored electrometrically using a portable food and dairy pH meter HI3220-02 (Hanna Instruments, Woonsocket, Rhode Island, USA).

**Isolation, phenotypic characterization and identification of LAB**

Some colonies of various shapes were picked up from count plates used for LAB (MRS and M17) enumeration. The isolates from MRS and M17 were transferred to the corresponding broth media. The isolates were purified by successive sub-culturing and the purity of the isolates, as well as the cell morphology were checked microscopically. Gram-positive and catalase-negative cells were picked up from count plates used for LAB isolation, phenotypic characterization and identification of LAB. Isolation was performed by successive sub-culturing and the purity of the isolates, as well as the cell morphology were checked microscopically. Gram-positive and catalase-negative cells were picked up from count plates used for LAB isolation, phenotypic characterization and identification of LAB.

Lactic acid bacteria isolates were genetically identified by 16S rRNA gene sequencing. The DNA from ricotta LAB isolates was extracted using the InstaGene Matrix kit (Bio-Rad laboratories, Hercules, CA) according to the manufacturer’s instructions. Crude cell extracts were used as templates for the polymerase chain reactions (PCRs). PCRs were performed as described by Weisburg et al. (1991) using the primers rD1 (5′-AAGGAGGTGATCCAGCC-3′) and rD1 (5′-AGAGTTTGATCCTGCTCAG-3′). DNA sequences were determined by using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) and compared using a BLAST search in the GenBank/EMBL/DDBJ database (http://www.ncbi.nlm.nih.gov).

**Statistical analysis**

Microbiological data of ricotta cheeses divided in three 5-year periods of investigation (2002-2006; 2007-2011; 2012-2016) were subjected to statistical analysis. The logarithm values of microbiological parameters were performed using 1-factor ANOVA model with a 5-year periods as fixed factor (SAS, 2010). Least square means were compared using the *t* Student test.

**Results and Discussion**

**Microbiological counts and pH values of Ricotta samples**

The microbial loads of the ricotta samples collected during fifteen years of investigation are reported in Table 1. The results reported in this table are referred only to the microbial group that showed a microbiological grow (positive samples).

The total microbial count (TMC) and LAB concentrations detected in this study were, on average, 1 Log cycle higher than those reported in our previous work (Mancuso et al., 2014), who studied the sensory and microbiological evaluation of traditional ovine ricotta cheese in modified atmosphere packaging (MAP). Furthermore, Spanu and coworkers (2017) always in a study conducted in MAP packed ricotta cheese previously inoculated with commercial biopreservatives and analyzed at different time of refrigerated storage found at 15°C (after 5 h from the production) a lower concentration of TMC and LAB. This may be due to different conditions of packaging and refrigerated storage used in the studies. *Enterobacteriaceae* and *E. coli* were found in c.a. 21% and 13% of samples, respectively as reported by Fadda et al. (2012) with an average values of the *Enterobacteriaceae* between 3 and 4 log CFU/g (Pala et al., 2016). Thirty four samples (43.6% on the total positive samples) showed a concentration of *E. coli* higher of 1000 UFC/g that represents the maximum enumeration limit for cheeses made from milk or whey that has undergone heat treatment according to Reg. (EC) 2073/2005. The highest level of *E. coli* detected in ricotta cheese samples was equal to 6.89 log CFU/g.

*Enterobacteriaceae* and *E. coli* are not able to survive at the thermal treatment of the whey, to this purpose their presence in

| Microorganisms* | Number of samples | Positive samples | % positive samples | Mean | Min. | Max. | SD |
|-----------------|-------------------|------------------|------------------|------|------|------|----|
| TMC             | 371               | 350              | 94.34            | 5.11 | 2    | 8.26 | 1.63 |
| Rod LAB (37°C)  | 98                | 74               | 75.51            | 3.6  | 1.85 | 5.58 | 1.16 |
| Coccus LAB (30°C) | 98            | 82               | 83.67            | 3.73 | 2.09 | 6.83 | 1.38 |
| Coccus LAB (44°C) | 98          | 69               | 70.41            | 3.09 | 1.78 | 4.85 | 0.92 |
| Enterococci     | 106               | 40               | 37.74            | 3.67 | 1.77 | 6.20 | 1.18 |
| *Enterobacteriaceae* | 371      | 78               | 21.02            | 3.68 | 1.43 | 7.00 | 1.26 |
| *E. coli*       | 598               | 78               | 13.04            | 3.26 | 1.3  | 6.89 | 1.29 |
| CPS             | 639               | 14               | 2.19             | 3.11 | 1.56 | 5.38 | 1.27 |
| Yeasts and moulds | 88            | 12               | 13.64            | 2.68 | 1.00 | 4.00 | 0.97 |
| *B. cereus*     | 157               | 25               | 15.92            | 3.79 | 1.70 | 6.00 | 0.95 |
| *Pseudomonas*   | 91                | 2                | 2.20             | 1.98 | 1.96 | 2    | 0.03 |
| SRA             | 194               | 1                | 0.52             |      |      |      |     |
| *L. monocytogenes* | 1156          | n.d.             | n.d.             |      |      |      |     |
| *Salmonella* spp. | 998           | n.d.             | n.d.             |      |      |      |     |
| *Brucella* spp. | 721               | n.d.             | n.d.             |      |      |      |     |

*Units are log CFU/g. TMC, total mesophilic microorganisms; CPS, coagulase-positive staphylococci; SRA, Sulphite reducing anaerobe; n.d. not detected; SD, standard deviation.*

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ricotta cheese is usually attributed to secondary contamination that depend exclusively to the low hygienic conditions during production. The level of enterococci ranged between 1.78 and 6.20 Log CFU/g with an average value of 3.67 Log CFU/g as reported by Pala et al. (2016). The presence of these bacteria in dairy products is usually associated with inadequate hygiene practices as a consequence of fecal contamination. However, several authors (Fouliqué Moreno et al., 2006; Gaglio et al., 2016b) suggest that Enterococcus strains are involved in the development of the organoleptic characteristics especially in long ripened traditional cheeses and contribute to extend their shelf life. On the other hand, enterococci have assumed a major importance in clinical microbiology and their presence needs to be validated by the absence of risks for consumer in term of antibiotic resistance and virulence as well as cellular toxicity.

Yeasts and moulds between 1 and 4 Log CFU/g as reported by Pintado and Malcata (2000). Bacillus cereus was found in ca. 16% of the samples analysed with a viable count of 3.79±0.95 Log CFU/g. This results was similar to previously reported by Cosentino et al. (1997) who studied the incidence and biochemical characteristics of Bacillus flora in Sardinian dairy products, but lower than those reported by Spanu et al. (2016) in ricotta salata cheese analyzed after 24 h from production (t). However, the level of contaminations did not reach the threshold that leads to significant toxin production (EFSA 2016). Sulphite reducing anaerobe was found just in a one sample of 194 analyzed. The presence of B. cereus and SRA in ricotta samples depend from contamination of milk during transformation and to the ability of the spores to survive at the thermal treatment of the whey.

CPS were detected in a very low percentage of samples (2.19%) and their level ranged between 1.56 and 5.38 Log CFU/g. Only two samples showed a final concentration above 5.00 Log CFU/g that represents the threshold that leads to staphylococcal enterotoxins production (European Commission, 2005). Furthermore, the production of enterotoxins were never detected in both samples.

Spoilage psychrotrophic bacteria such as Pseudomonas spp. were detected just in two samples with showed an mean values of 1.98±0.03 Log CFU/g lower than those reported by Spanu et al. (2017). This may be due to that the 91 samples tested for the presence of this microbial group were referred to the IZSSI in a higher percentage from large-scale retail trade and in some cases from dairy factories characterized by high hygienic standards. Indeed, usually the presence of this microbial group is a result of a secondary contaminations and their level increase during refrigeration storage. Their presence in ricotta cheeses can overgrow the other microflora and cause an spoilage of the final quality and the sensory profile of the products (Carrascosa et al., 2015; Pala et al., 2016).

Listeria monocytogenes, Salmonella spp. and Brucella spp. were never detected; this may be due to heat treatment of the whey used for the ricotta production that represent a killing step of these microorganisms and to the absence of secondary contamination from the processing environment. These results confirmed previous investigations performed in other Sicilian dairy environment and products where this pathogenic microorganisms were never detected (Cruciata et al., 2018; Gaglio et al., 2016a; Scatassa et al., 2015). Regarding the pH, the ricotta samples showed a mean values of 6.37±0.33 as reported by (Mucchetti and Neviani, 2006) but during the study were registered values ranged between 6.04 and 6.74.

Isolation, phenotypic characterization and identification of LAB

A total of 98 cultures were collected from 30 ricotta cheese representative of the three level of sampling. All of the cultures were inspected microscopically and classified as cocci (69) or rods (29). Gram determination and the catalase test indicated that 64 cocci and 27 rods could be considered putative LAB cultures.

The 91 strains were identified by sequencing of the 16S rRNA gene. The strains were allotted into 10 species within the genera Enterococcus, Lactobacillus, Lactococcus and Leuconostoc. The species with the highest number of strains were Lactococcus lactis (n=23) e Lactobacillus casei (n=22). Other strains were identified as Enterococcus faecium (n=19), Leuconostoc lactis (n=7), Lactococcus lactis sub. lactis (n=6), Leuconostoc mesenteroides (n=6), Lactobacillus sakei (n=5). Enterococcus durans (n=1), Enterococcus faecalis (n=1) and Enterococcus gallinarum (n=1). Except L. lactis, all species identified are commonly reported to be part of the non starter LAB (NSLAB) population in several cheeses (Gatti et al., 2014). Furthermore, all these species were found in other traditional ewes’ Sicilian cheeses (Gaglio et al., 2014a; Guarcello et al., 2016) and wooden equipment employed for cheese manufacture (Scatassa et al., 2015). Some works showed the ability of these LAB species to produce bacteriocin-like inhibitory substances (BLIS) active against Listeria and other pathogenic bacteria and this allows to improve the safety, control the fermentation microbiota, speed maturation and increase the shelf life of the final cheeses (Macaluso et al., 2016; Scatassa et al., 2017).

Statistical analysis

The statistical analysis of the microbiological counts conducted on the ricotta samples during three 5-year periods of investigation (2002-2006; 2007-2011 and 2012-2016) are reported in Table 2.

According to t Student test, significant statistical differences were found for the levels of Enterococci, Enterobacteriaceae, yeasts and moulds, E. coli and CPS between the ricotta samples collected during the first 5-years periods of investigation in comparison with the last two periods. A possible explanation to these observations is that in the last decade food business operators adopted the good hygiene and manufacturing practices, in compliance with the Commission Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs (European Commission, 2005).

Table 2. Statistical analysis of microbial loads.

| Microorganisms* | 2002-2006 | 2007-2011 | 2012-2016 |
|-----------------|-----------|-----------|-----------|
| TMC             | 5.33±1.13 | 6.45±1.12 | 4.78±0.75 |
| Enterococci     | 2.58±0.28* | 0.01±0.70* | 0.81±2.22* |
| Enterobacteriaceae | 1.79±0.24* | 0.50±0.17* | 0.68±1.00* |
| E. coli         | 1.33±0.09* | 0.24±0.08* | 0.19±0.07* |
| CPS             | 0.35±0.05* | 0.01±0.05* | 0.01±0.06* |
| Yeasts and moulds | 1.94±0.40* | 0.15±0.12* | 0.49±0.17* |
| B. cereus       | -         | 0.67±0.11 | 0.91±0.17 |

*Units are Log CFU/g. TMC, total mesophilic microorganisms; CPS, coagulase-positive staphylococci. On the row different letters are significant at P≤0.01.
Conclusions

The results reported in this study showed that from a hygienic point of view, the Sicilian ewes’ ricotta represents a safe cheese production. In particular, during the entire periods of investigation the pathogenic bacteria (L. monocytogenes, Salmonella spp. and Brucella spp.) were never detected while the levels of undesired microorganisms such as Enterobacteriaceae, E. coli and CPS, during the last two 5-year periods of investigation were reduced in number in comparison with the first 5-year period of investigation. The last findings are not satisfactory since in that period the structural and hygienic conditions of dairies improved consistently. However, in order to improve the final quality of this product are needed further strategy such as the dairy makers training, with the aim to apply a good hygienic practices during the production.

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