Novel insights on pink discoloration in cheese: The case of Pecorino Toscano

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Abstract

Pink discoloration in cheese has been the subject of wide research, but the basis for this phenomenon still remain elusive. This defect impacts a wide range of ripened cheeses, resulting in the rejection of cheese and a consequent economic loss for dairy industries. As multiple causes for pink discoloration have been reported for different cheeses in the literature, the aim of this research was to investigate the cause of a pink discoloration found on Pecorino Toscano cheese rind. The results of microbiological analysis revealed the presence of high microbial counts associated to the rind and the nearest inner part. Strains isolated from the colored part were mainly identified as *Serratia liquefaciens*, suggesting that an environmental contamination of the cheese rind by this species could be the cause of the observed defect and expanding the knowledge related to pink discoloration in cheeses.

Keywords: Cheese defects, *Serratia liquefaciens*, environmental contaminations, Pecorino Toscano cheese

1. Introduction

The occurrence of defect in cheeses is of great concern for the dairy industry as it could lead to products recall and thus representing a significant economic loss for dairy companies (Carminati et al., 2019). Defects may have different origin and may occur in diverse step of cheese making process. They can be related to variations in milk quality, milk treatment, hygiene practices and cheese processing parameters (manufacture technology and/or ripening). Defects can also be linked to differences in the starter cultures activity and acidity profiles, as well as to the nature and number of the non-starter microorganisms which come from raw milk or other environmental sources (O'Sullivan et al.,2013).

To date, several studies have been focused on the understanding of cheese discoloration' causes. In particular, the reason for the formation of pink defect in cheeses without colorant addition, in some cases still remains unknown (Quigley et al., 2016). To date, it has been mainly attributed to the metabolism of certain strains of thermophilic lactobacilli, propionic acid bacteria, *Thermus* spp., or *Glutamicibacter arilaitensis* as well as the activity of residual microbial enzymes or to Maillard reactions, particularly related to the presence of unfermented galactose (Cleary et al., 2018; Daly et al., 2012).

During summer 2018, a Tuscan dairy producing Pecorino Toscano, a PDO pasteurized sheep's milk medium ripened Italian cheese, observed an unusual pink discoloration on the rind of several cheese wheels (Personal communication; Figure 1). With the aim of determining the cause of discoloration, microbiological analysis followed by molecular identification of the species recovered in the pink area, were carried out.

2.Materials and Methods

2.1 Sampling

In order to identify the bacteria species potentially responsible for the alteration, the crust and the closer inner part of six representative cheeses were sampled and homogenized 1:10 with sterile Ringer's solution,

by means of a blender (STOMACHER, 400 circulator, UK) (Oxoid, Basingstoke, UK) for 2.30 min at 230 rpm. Each cheese was analyzed separately. The samples dilutions were then plated in triplicated on different growth media. Pseudomonas CFC agar (PS) (Oxoid, UK) was used in order to count *Pseudomonadaceae,* incubating plates at 25°C for 48 hours. Plate Count Agar (PCA) (Oxoid, UK) was used for the determination of total microbial count by incubating the plates at 30°C for 24hours. Yeast and molds were counted on Yeast extract dextrose Chloramphenicol Agar (YEDC). For the determination of aerobic spore-forming bacteria, the homogenized samples were heated to 80°C for 10 min and then cooled before analysis and then plated on Tryptone Soy Agar (TSA, Merck KGaA, Darmstadt, Germany), followed by incubation for 24 h at 30 °C under aerobic condition (Abdelmassih et al., 2011).

2.2 Microbial isolation from pink areas

Fourteen colonies with pinkish or reddish color present only on PS plates were selected and isolated through three purification steps on the same medium. The purity of the strains was checked by verifying the morphology by means of optical microscope Olympus BX51 (Olympus, Waltham, United States) then stored at – 80° C in Tryptic Soy Broth (TSB) (Oxoid, Basingstoke, UK) added with 20% (v/v) glycerol.

2.3. Genotypic identification of isolated strains

The isolated strains were cultured twice at 30 °C for 18 h in TSB (Oxoid) in aerobic conditions and then the DNA was extracted and purified by using DNeasy and Blood Tissue (Qiagen, Germany) and checked on agarose gel with TAE 1X running buffer (1% w/v). Then, the 16S rRNA gene was amplified by PCR (Takahashi et al., 2014).

Primers based on conserved regions of the 16S rRNA gene, 16S 46Fw: CAG GCC TAA CAC ATG CAA GTC and 16S 536Rv: GGG CGG WGT CAA GGC (Marchesi et al., 1998), were used for direct PCR amplification of a 1300-bp portion of the 16S rRNA gene. In brief, samples underwent an initial denaturation of 2 min at 94°C and 30sec at 94°C, then 30 cycles of 30 sec at 55°C, 30 sec at 72°C, followed by 10 min at 72°C. Reaction products were separated by electrophoresis in 1.0% (w/v) agarose gels and the amplification products were sequenced (Macrogen Europe Inc.). Sequences comparison was performed against NCBI database using BLASTN (Basic Local Alignment Tool, BLAST, p://www. ncbi.nlm.nih.gov/blast.cgi). Criteria for identification at the species level were defined as a 16S rDNA sequence similarity of ≥98% (Drancourt et al., 2000) with that of the type strains sequences in GenBank.

3 Results and discussion

3.1 Microbial counts

In the present study, the total microbial counts was of 7.82 Log CFU/g (mean value of the samples) (fig.2), which corresponds to the results found by Proroga and colleagues (2009) on Pecorino. Yeast and moulds count was 3 Log CFU/g, which is typical for ripened cheeses such as Pecorino Toscano PDO (Todaro et al., 2011). The presence of spore forming microorganisms was lower than 3 Log CFU/g (Fig. 2), confirming what has been observed in the literature for similar cheeses (Palmas et al., 1999). Microbial counts on Pseudomonas agar were 1 log unit higher than the average data available in literature, reporting counts of no more than 5 Log CFU/g in the same medium (Todaro et al., 2011). In all the analyzed cheese samples, pink colonies were present on PS plates.

3.2 Genotypic identification of isolated strains

Fourteen isolated strains were identified at species level as follows: 10 strains belonging to *Serratia liquefaciens*, 2 *Enterobacter cloacae, 2 Enterobacter hormaechei* (Table 1).

The presence of *Enterobacteriaceae* is not surprising as, despite they are considered as indicative of a poor microbiological quality of cheese, they are also recognized as part of the natural microbiota of many dairy products (Chaves-Lopez et al., 2006).

S. liquefaciens is a psychrotrophic and highly motile organism commonly found in water, soil, plants and more specifically in dairies environment and raw milk (Ikumapayi et al., 2015; Machado et al., 2015) *S. liquefaciens* is a pathogenic microorganism able to cause infections in humans, especially in immunocompromised hosts (Momose et al.,2018; Caneschi et al., 2019). A possible role of this species in discoloration defects had been previously observed, but mainly related to the presence of its hydrolytic heat resistant enzymes, rather that the viable cells (Decimo et al., 2014). In the present study, viable *S. liquefaciens* cells were isolated from the rind and the nearest inner part, where the pink discoloration was present. Furthermore, as no discoloration was found in the deep inner part of the cheeses, despite the species is able to grow both under aerobic and anaerobic conditions (Bergey et al.,2000), an environmental contamination of the rind was hypothesized. Pecorino Toscano is ripened between 5 and 15°C with a relative humidity ranging between 75 and 95%. Such conditions could encourage the growth of *S.liquefaciens* on cheese rind and the production of a red pigment in the later stages of bacterial growth. The production of red pigments such as prodigiosin has in fact been reported to be regulated by a quorum sensing mechanism, by which, the increasing number of cells is the signal to start the production of the pigment (Thomson et al., 2000).

4. Conclusion

The microbial analysis of each sample from the six Pecorino Toscano cheese revealed the presence of *S. liquefaciens*, suggesting that an environmental contamination of the cheese rind could be the cause of the observed defect. In particular, it seems of central importance, when studying this kind of defect, the evaluation of brine and ripening chambers hygiene as well as the overall hygiene of the working areas. Another important aspect is the control of rind humidity, which may promote the development of alterative microorganisms coming from the environment. The result of our study could expand the knowledge related to pink discoloration in cheese, which is still ambiguous in the literature.

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Figure captions

Figure 1. Example of pink discoloration on Pecorino Toscano cheese

Figure 2. Box plot of microbial counts, reported as mean value for each media

Table 1. Identification of isolated strains.

Strain number	Bacterial Genus and Species	Score	Query cover	Accession number
5006	*Serratia liquefaciens*	730	98%	MH669247.1
5007	*Serratia liquefaciens*	819	98%	MT279350.1

5008	*Serratia liquefaciens*	1099	99%	MH669144.1
5009	*Serratia liquefaciens*	926	98%	MH669139.1
5010	*Serratia liquefaciens*	1003	98%	MH668086.1
5011	*Serratia liquefaciens*	1120	99%	MT279350.1
5012	*Enterobacter hormaechei*	1051	98%	KC154051.1
5013	*Enterobacter cloacae*	1280	99%	MT145960.1
5014	*Enterobacter hormaechei*	1253	98%	MT258990.1
5015	*Serratia liquefaciens*	1375	99%	MH668086.1
5016	*Serratia liquefaciens*	1375	99%	MT279350.1
5017	*Enterobacter cloacae*	1373	99%	MT145960.1
5018	*Serratia liquefaciens*	1282	99%	MT279350.1
5019	*Serratia liquefaciens*	1554	99%	MH669139.1