Safety assessment of traditional Plaisentif cheese

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Abstract

Traditional foods are gaining more and more market due to consumers' increasing willingness to buy products linked to national cultures: among these products, cheese plays an important role. Plaisentif is a traditional Piedmont cheese, only made during violets blooming season. The aim of this work is to evaluate the safety of this cheese, taking into account the EU Regulations. Microbiological hazards as well chemical, biogenic amines and mycotoxins, analysis were investigated. *Salmonella* spp. and *Listeria monocytogenes* were never detected in cheeses after ripening. Biogenic amines were present in very low quantities. Ochratoxin A was never detected and patulin was detected in over one cheese during the two years of sampling. This is the first attempt to characterize traditional Plaisentif cheese from a safety point of view. All the information acquired can be held as a necessary basis for reinforcing the culture of traditional products, for economic opportunities in mountainous regions and for safeguarding traditions and cultural identities.

Introduction

Traditional products are, according to consumers, food that is handcrafted in a particular way, following a long-established tradition, sourced within a certain local area and among these products, cheese is included (Montel *et al.*, 2014). For this kind of cheese, the producer usually uses raw or thermized instead of pasteurized milk. For this reason, the natural flora persists in the product determining the typical flavor and aroma. On the other side the natural microflora might result in safety issues. Currently information about safety and benefits of many traditional cheeses are missing and for this reason in deep studies are necessary (Montel *et al.*, 2014).

The ancient Italian dairy tradition is expressed in a wide variety of traditional cheeses; in addition to protected designation of origin (PDO), Italian cheesemakers also produce so-called "historical cheeses". These dairy products have made by fewer manufacturers located in a specific area within a region, are produced with non-standardized processes and results in small amount of finals forms, thus representing "niche products". Plaisentif cheese can be considered among them even without being a PDO product, nor a protected geographical indication (PGI). Chronicles from the 1500 already mentioned it, milk and cheese factories are located between 1400 m and 1800 m above the sea level. Another noteworthy characteristic is that this cheese is produced only from June to July, corresponding to violets blooming season, lending it a peculiar flavor. This is the reason why it is called "*formaggio delle viole*".

Plaisentif cheese is produced by mixing the morning milk to the one of the previous evenings, at a temperature of 33-36°C. Following the addition of rennet, the cheese maker evaluates the clotting time visually (normally an hour). The cheese must then age for 60 days after a salting phase (dry salt or brine). Temperature and humidity are not specified in the disciplinary. Finally, the cheese forms are marked (logo, producer, and printing data) and sold (Figure 1).

Raw milk can be a source of pathogens in cheese and traditional cheese-making factories may not always provide good hygienic conditions during the process. For these reasons, the aim of this work, after a previous evaluation of the lactic cheese microbiota (Dalmasso *et al.*, 2016), was to investigate the presence of pathogens in the microflora, quantifying the content of biogenic amines (BAs) and of two common mycotoxins (ochratoxin A and patulin) that could have contaminated the cheese during ripening (Manca *et al.*, 2020; Kokkonen *et al.*, 2007) in not strictly controlled conditions.

Materials and methods

Milk and cheese samples

Samples of milk (n=18) and of the corresponding cheese, after a maturation period of 80 days were collected from nine producers over two years. Milk was collected refrigerated (4°C) and immediately bacteriologically analyzed. At the end of ripening, and in the same temperature conditions, a whole form was taken, refrigerated and immediately analyzed in aseptic conditions in the microbiological laboratory. Cheese samples were collected from the soft edible part and stored at -20°C awaiting chemical analysis.

Aw and pH analysis

For the determination a Medilor Basic 20 pHmeter (Crison Instruments SA, Alella, Barcelona, Spain) and a AquaLab3 TE (Degagon Devices Inc., Pullman, Washington, USA) were used.

Microbiological analysis

The analysis were conducted according to certified protocols. In particular for the enumeration of *Enterobacteriaceae* we used the AFNOR V08-'54 method; for the enumeration of Coagulase positive Staphylococci, we used the AFNOR V08-14 with Baird Parker Agar added with RPF for highlighting Coagulase Active strains (Liophichem, Italy). For the detection of Salmonella and of *Listeria monocytogenes*

were used respectively the ISO 6579-2002 and the ISO 11290-1/Amd 1:2004E protocols. The reagents were produced by Oxoid (Thermoscientific, Basingstoke, UK).

Mycotoxins analysis

Extraction of mycotoxins

Glassware was treated according to Pattono et al. (2013). In order to prevent loss of Patulin or salt formation Ochratoxin A. Analyses were done in twice.

To extract patulin, a modification of the extraction protocol proposed by Kokkonen et al. (2005) was used. The first phase was an extraction repeated three times with 10 mL of acetonitrile acidified with 0.1% formic acid. Subsequently, the sample was shaked (Ika-Vibramax, Staufen, Germany) for 5 minutes (600 strokes/min). Defattening was achieved by shaking an aliquot (15 ml) of the extracted acetonitrile three times with exane at the condition written above. The final step was the evaporation of one ml at 60°C. Ahead of the chromatographic analysis, 1 ml of mobile phase was used to dissolve the sample.

To extract ochratoxin A the protocol proposed by Pattono et al. (2013) was adopted. Briefly samples were homogenized in acetonitrile and sulphuric acid for acidification, shaked and the defatted always with exane. Both extracted were filtered with cellulose filters and evaporated to dryness until analysis

HPLC analysis

For Ochratoxin A and Patulin the method by Pattono et al. was applied (2013).

The standards were: Patulin in acetonitrile (Fluka, Buchs, Switzerland) and Ochratoxin A in a benzene:acetic acid (99:1) solution (Supelco, Bellefonte, USA) stored at −40°C.

All solvents were HPLC grade (Merck, Darmstadt, Germany). The distilled water (resistivity value = 18.2 mΩ) was produced by a MilliQ system using a (Millipore, Billerica, MA, USA). The HPLC system (Merck, Darmstadt, Germany) was: L-7100 pump, L-7614 vacuum membrane degasser, Rheodyne 7725i injection valve with a 50 µL loop, a column end-capped PuroSpher Star RP-18 endcapped (250 × 4 mm × 5 µm) (Merck, Darmstadt, Germany) and the detectors (Merck, Darmstadt, Germany) were: for Patulin L-7400 UV detector set at 273 nm wavelength, for Ochratoxin A L-7480 fluorescence detector set at 333nm (excitation) and 460 nm (emission). The mobile phases were: for Patulin H_2O:acetonitrile (90:10) at a flow rate of 0.8 mL/min; for Ochratoxin A: H_2O:acetonitrile:acetic acid (49.5:49.5:1) at a flow rate of 1.0 mL/min.

Confirmation of mycotoxins

For Patulin it was used the protocol proposed by Cunha et al. (2009).

Determination of biogenic amines

Extraction and derivatization of biogenic amines

The extraction method was used, as reported for the hard cheese Toma Piemontese (Gennaro et al. 2013). Biogenic amines were extracted with HCl M 0.1 and for the derivatization a saturated solution of $NaHCO_3$ and 1 ml dansylchloride solution (5 mg/ml) were added.

HPLC analysis

For the quantification of Biogenic Amines, it was used the protocol by Moret & Conte (1996). The HPLC apparatus was the same described for patulin. Also, solvents and water were the same previously described. Amines, aminoacids and dansyl-chloride were Sigma (St Louis, MO). The mobile phase was a water/ACN mixture in the following gradient elution: 0–5 min water/ACN 35:65, 5–20 min water/ACN 25/75. The flow-rate was 0.8 ml/min and UV detection at 254 nm.

Results and Discussion

Plaisentif cheese is a very peculiar cheese. It is a semi-hard cheese only manufactured three months a year, when a specific pasture is available.

pH and A_w values of milk and cheese for both years are showed in Table 1. Comparing to other traditional Cheese ("Toma Piemontese DOP" and Montasio cheese), pH of milk was aligned with the values recorded, considering the variability seen within different producers in our and other studies (Astegiano et al., 2014; Maifreni et al., 2013). A_w and pH of cheese forms at the same aging time were aligned with Toma Piemontese but A_w was lower than Montasio Cheese (Astegiano et al., 2014; Maifreni et al., 2013).

Salmonella spp and Listeria monocytogenes, were never detected in cheese although Listeria monocytogenes was detected once in milk in the first year of sampling. For the other bacterial species considered, a great variability was noteworthy (Tables 2 and 3). This fact was not surprising, if we consider the differences among the cheesemakers involved. Astegiano et al. (2014) and Maifreni et al.

Table 1. pH and A_w of milk and cheese (mean ± SD).

| Sample | pH | | A_w | |
	First year	Second year	First year	Second year
Milk	6.78±0.05	6.58±0.15	-	-
Cheese	5.39±0.19	5.53±0.22	0.96±0.008	0.95±0.01

Figure 1. Plaisentif cheese at the end of the ripening phase.

(2013) showed similar behavior as traditional cheese Toma Piemontese PDO.

Enterobacteriaceae count ranged between <10 to 3.26×10^4 CFU/ml (mean: 3.7×10^3) in milk and between <10 to 8.1×10^4 CFU/g (mean: 1.8×10^4) in cheese in the first year and between 10 to 1.3×10^7 CFU/ml (mean: 2.5×10^6) in milk and from <10 to 2.25×10^5 CFU/g (mean: 2.9×10^4) in cheese in the second year. Coagulase-positive *Staphylococci* ranged between <100 to 1.4×10^4 CFU/ml (mean 1.8×10^3) for milk and among <100 to 5×10^2 CFU/g (mean: 8.8×10) in cheese in the first year and between <100 to 4.9×10^4 CFU/ml (mean: 6.3×10^3) in milk and from <100 to 2.0×10^4 CFU/g (mean: 3.3×10^3) in cheese in the second year. The hygienic criteria over the process could be considered quite satisfactory, in relation to those permitted by the European Regulations (EC, 2007) and considering both the setting and the low amount of cheese produced every year. The level of 10^5 at which the production of toxins might occur was never reached. However, Coagulase positive Staphylococci were present in high counts: for this reason, control measures are strongly advised.

Even if we did not find any pathogens, a high number of *Enterobacteriaceae* were still present. Other authors reported lower counts, but not for all the samples of milk and cheese in Toma Piemontese (Astegiano *et al.*, 2014); while for Montasio, milk and cheese at the same ripening time had lower counts (Maifreni *et al.*, 2013). The hygienic conditions of some cheese factories, located at high altitude with possible contamination of water, environment, and supplies, could have been the reason for some high microbiological counts (Bhatt *et al.*, 2012; Coton *et al.*, 2012). Actions aimed at improving artisanal establishments conditions and training Food Business Operators (FBO), in order to decrease the microbial counts, have to be proposed.

Biogenic Amines (BAs) have been evaluated as a newly emerging risk in recent years (Ruiz-Capillas & Herrero, 2019; Møller *et al.* 2020; Pluta-Kubica *et al.* 2020). The analysis of BAs was focused on Histamine (HIM) and Tyramine (TYR), as responsible for food intoxication, and on Cadaverine (CAD) and Putrescine (PUT) as responsible for the enhancement of the toxicity of the previous ones. For all of them, we observed a high variability for both years (Table 4). The two BAs responsible for food intoxication, histamine (HIM) and tyramine (TYR), were present in very low levels for both years with the exception of one produced during the first year (38.9 ppm). In the first year, they ranged between 0.2 and 38.9 ppm (mean value 8.3) for HIM, and between 0.1 and 2.6 ppm (mean value 1.5 ppm) for TYR. In the second year, they ranged between 0.3 and 18.4 ppm (mean

Table 2. *Enterobacteriaceae* and Coagulase-Positive Staphylococci enumeration (UFC) in milk.

Enterobacteriaceae and Coagulase Positive Staphylococci	*Enterobacteriaceae* and Coagulase Positive Staphylococci		*Enterobacteriaceae* and Coagulase Positive Staphylococci	
	First year	Second year	First year	Second year
1	6×10	1.2×10^6	1×10^3	1.1×10^3
2	<10	5.5×10^3	4.9×10^2	4.9×10^4
3	3.26×10^4	10	1.44×10^4	<100
4	1.1×10^3	4×10^2	3.4×10^2	<100
5	<10	1.34×10^7	<100	$3x \pm 10^3$
6	5×10	6.9×10^5	$4.9x \pm 10^2$	<100
7	7×10	3.25×10^5	<100	4×10^3
8	<10	6.9×10^6	1.1×10^2	<100
9	<10	1.09×10^5	4.9×10^2	4.9×10^4

Table 3. *Enterobacteriaceae* and Coagulase-Positive Staphylococci enumeration (UFC) in cheese.

Enterobacteriaceae and Coagulase Positive Staphylococci	*Enterobacteriaceae* and Coagulase Positive Staphylococci		*Enterobacteriaceae* and Coagulase Positive Staphylococci	
	First year	Second year	First year	Second year
1	1.2×10^2	4.22×10^4	$<100^3$	2×10^4
2	8.1×10^4	6.7×10^3	1×10^2	7×10^2
3	1.9×10^3	<10	<100	<100
4	1.09×10^2	3×10^3	5×10^2	1.1×10^3
5	<10	1.5×10^2	1×10^2	$8x \pm 10^3$
6	10	<10	<100	2×10^2
7	2×10	$3.x10$	<100	<100
8	3.43×10^4	2.25×10^5	<100	2×10^2
9	8.1×10^4	2×10^4	1×10^2	<100

Table 4. Biogenic amines content (Mean±SD) of the Plaisentif cheese in the first year.

Sample	Putrescine		Cadaverine		Histamine		Tyramine	
	First year	Second year	First year	Second year	First year	Second year	First year	Second year
1	0.6±0.4c	2.4±0.2bcd	0.3±0.2c	3.5±0.4a	5.4±1.0cd	0.6±0.01c	1.5±0.5abc	8.5±0.01a
2	0.8±0.3c	0.4±0.1d	0.2±0.07c	0.7±0.02c	4.9±1.8cde	0.3±0.1c	2.2±0.6ab	2.0±0.3bc
3	11.8±1.7b	4.3±0.4abc	1.4±0.01c	1.1±0.5bc	15.9±0.2b	7.3±1.7bc	2.6±0.5a	1.4±0.2bc
4	0.8±0.3c	7.2±2.3a	1.1±0.6c	0.2±0.05c	38.9±1.1a	5.2±1.5bc	2.7±0.4a	2.0±0.2bc
5	12.0±0.8b	0.4±0.1d	7.2±3.0b	0.4±0.05c	0.5±0.3f	1.1±0.03c	1.8±0.6abc	0.4±0.01c
6	0.2±0.01c	1.6±0.8bcd	0.2±0.03c	2.1±0.3b	0.2±0.01f	1.2±0.1c	0.1±0.04c	2.2±0.9bc
7	25.0±3.7a	0.8±0.2cd	31.4±2.4a	0.2±0.01c	1.5±0.2def	1.8±0.02c	0.8±0.1bc	0.7±0.07c
8	0.7±0.2c	4.6±0.1ab	0.2±0.01c	1.8±0.3b	1.2±0.04ef	11.3±3.2ab	1.4±0.03	3.7±0.3bc
9	0.7±0.2c	0.4±0.1d	1.7±0.5c	0.6±0.2c	6.1±0.1c	18.4±3.8a	0.10±0.03c	4.2±2.3b

Different letters for different p-values.

value 4.5 ppm) for HIM, and between 0.4 and 8.5 ppm (mean value 2.8 ppm) for TYR. The quantity we found was within the levels considered safe for both BAs (Manca *et al.*, 2020).

Putrescine ranged between 0.2 and 25.0 ppm (5.8 ppm mean value) and between 0.4 and 7.2 ppm (2.4 ppm mean value), respectively. Cadaverine ranged between 0.2 and 31.4 ppm (4.8 ppm mean value) and between 0.2 and 3.5 ppm (1.2 ppm mean value), respectively.

Overall, the total amount of Bas, ranged between 0.7 to 58 ppm (20.4 ppm mean value) in the first year, and from 2.3 to 21.4 ppm (10.9 ppm mean value) in the second year. Even in this case, as stated above for the two BAs separately, the total amount of BAs never reached the warning amount set for food (Manca *et al.*, 2020); this threshold may be precautionary and, as stressed by many authors, lacks in reliability given the few data available about food consumption (Combarros-Fuertes *et al.*, 2016; EFSA, 2011). This threshold was stated at 900 mg/kg, in absence of co-factors, and 100 mg/Kg if this consumption was associated to co-factors, such as pharmacological treatments with amine oxidase inhibiting substances, pathological status of gastrointestinal apparatus or alcohol (Manca *et al.*, 2020; Reinholds *et al.*, 2020).

Cheese has been considered suitable for the presence of mycotoxins, due to the presence of aflatoxins in milk, and the possibility of developing toxigenic molds in storage rooms and on the cheese crust (Decontardi *et al.*, 2018; Kalinina *et al.*, 2018; Møller *et al.*, 2020; Kadakal *et al.*, 2020). Another way of contamination was identified in the dairy tools used in the cheese-making process (Casquete *et al.*, 2018). Among them, Penicilli and Aspergilli were the most detected (Montel *et al.*, 2014; Casquete *et al.*, 2018; Decontardi *et al.*, 2018).

We carried analysis regarding the presence of Ochratoxin A and Patulin (Pattono *et al.* 2013; Ioi *et al.* 2017; Camaldo Leggieri *et al.* 2020).

Ochratoxin A was never detected in the examined samples, while patulin was detected in one sample at level of 10.0 ± 1.2 µgr/kg. This situation was also confirmed by other studies, considering other traditional cheeses (Anelli *et al.*, 2019; Kadakal *et al.*, 2020)

For this reason, we might say that safety is assured for the considered mycotoxin (Reinholds *et al.*, 2020). More investigation has to be performed to confirm the present results, and to detect other mycotoxins, even if regarding Aflatoxin M1 its presence is unlikely, given the way grazing animals are fed during the production period (June-July) (Rojas-Marín *et al.* 2018; Ráduly *et al.*, 2020).

The detected levels were far from the toxic dose established for patulin by the European Union (25 µgr/Kg), even if considering the one settled for solid products (Ioi *et al.*, 2017).

The reason for such low quantities could be explained by many factors, influencing molds growth and mycotoxins productions: small quantity of carbohydrates, available in these ripened cheeses, strong bacteria antagonism, and a not optimal extrinsic factors for the mycotoxins production (Casquete *et al.*, 2018; Camaldo Leggieri *et al.*, 2020). In our opinion, temperature might have been a very important factor for the low quantity of this mycotoxin we observed (Camaldo Leggieri *et al.*, 2020).

Conclusions

Traditional food answers to many consumers' needs at multiple levels. First, it has been recognized as an important tool to create an added value production among the EU member states (Balogh *et al.*, 2016); the EU itself encourages this policy, recognizing specific regulations such as PGI and PDO, or the corresponding national specifications. Second, it provides an answer to consumers in order to hinder globalization, promoting the local food culture and being an alternative to the intensive production which are less sustainable from the environmental point of view (Fernández–Ferrín *et al.* 2018). Lastly but not least, local food production represents a strong connection to tradition, local identity, culture, and an improved employment opportunity for rural areas, where the economy is relatively weak. Traditional food producers and, in particular traditional cheese producers must manage safety issues following a day-by-day empirically based (Montel *et al.*, 2014). Nonetheless the results of this study did not show significant risk for human health due to consumption of Plaisentif cheese.

This was the first attempt to evaluate and assess the food safety of Plaisentif. All data will be a valuable starting point for the implementation of a hygienic criteria based production in mountainous cheese-making factories. All the improvements will lead to a new identity of such products and a valuable help in order to provide effective certification and regulatory systems that are vital to achieve higher standards of quality while protecting the integrity of traditional food products.

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