Medicine Papers, 2018, 4 (1): 15–20

Quality control of a Sicilian DOP cheese by species-specific molecular targeting

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Summary

Today, the quality of a product is essential for an increasingly experienced consumer. This study was developed in order to evaluate the quality of twelve cheese samples and twelve milk samples from the province of Enna (Sicily, Italy). The quality of milk and cheese partly depends on the amount of somatic cells present in the sample. The objective of the research was therefore to quantify the number of somatic cells in each milk sample and the genomic equivalent for the cheese samples, but also to analyze the genetic traceability of the cheeses using PCR real-time.

Key Words

Milk and cheese derived; Sicilian DOP chesee; DNA extraction.

Received 16.01.2018; accepted 12.02.2018; printed 28.02.2018

Introduction

In order for the animal that produces milk to be considered healthy, and consequently to produce a quality milk, the values of the somatic cells must fall within a certain range, according to the provisions of the EC regulation n. 853/2004 (Gazzetta ufficiale dell'Unione Europea. REGOLAMENTO - CE - N. 853/2004) (see Table 1).

When an increase in the number of somatic cells is observed, this may indicate infection, the presence of a pathogen or a state of defense in which the animal recalls a greater number of leucocytes that are poured into the milk during milking.

The number of somatic cells is an indicator of a lower quality of the raw milk (Houben et al., 1993; Seegers et al., 2003; Geary et al., 2012; Hand et al., 2012). It can be related to the mastitis of the cattle. Several factors are able to increase the number of somatic cells, such as age, stress, breast wounds or indirect causes such as inadequate milking that can facilitate the transmission of infections. It has been shown that the most frequent cause of this increase is a breast infection, like the mastitis, caused by microorganisms that penetrate the breast from the nipple canal and proliferate inside. Therefore, since the number of cells is related to inflammation and breast health, the somatic cell count is internationally accepted as an assessment of the quality of milk.

In addition, mastitis is one of the causes of greater economic loss in dairy farms, due to an increase in veterinarian costs, and a decrease in milk production, for which control measures are increasingly important.

Material and Methods

Samples

A total of twelve samples of cheese, twelve of milk, and five standards, were used, prepared by diluting a sample of milk with a known concentration of somatic cells. The subject of study of the experiment was a DOP cheese (denomination of protected origin), the "*Piacentino Ennese*", a particular hard paste sheep's cheese that owes its peculiarity to the addition of saffron and black pepper grains, that characterize its unmistakable yellow-orange color.

DNA extraction from cheese

The kit used for the extraction is the DNeasy mericon food kit. First, you cut a small piece of cheese that in turn will be chopped into smaller pieces. Once the samples are cut, they are placed in a 50 ml falcon and washed with three rinses of water to eliminate salt, spices, and so on. Now, each sample is ready to be introduced into a PrioCLIP in which T.E. is added (tris of TA) diluted 0.1X. Each PrioCLIP is inserted into the Priogenizer. Two ml of homogenate sample are collected, with a pasteur, and transferred to a 2 ml eppendorf. Once this is done, all the samples are centrifuged at 12,500 rpm for 15 minutes at 4 °C. After centrifugation, two phases will be formed: a higher one consisting of fats and a liquid phase. Five hundred µl of the liquid phase are taken and introduced into a new 2 ml empty eppendorf. Add 25 µl of Proteinase k to each sample and incubate the samples at 50 °C for 30 minutes in Thermomix and then at 95 °C for 15 minutes. Then, add to the samples that have reached a total volume of 525µl of volume, 1/10 of volume of 4 molar NaCl and then 1 volume of Isopropanol. Place the sample in the freezer for at least 2 hours or more over night. When the night is over, the samples are centrifuged at 14,000 rpm for 15 minutes at 4 °C. All the supernatant is removed, leaving only the Pellet and the sample is resuspended in 200 µl of H_2O and it is possible to quantize to the NanoDrop.

DNA extraction from milk

For the extraction of DNA from milk, a commercial DNA kit was employed. Also, in the case of milk, we used twelve different samples which, being already fluids, do not need to be homogenized so that the extraction procedure can be directly started. A volume of 250 µl of milk was employed in a DNA extraction protocol starting with an incubation in lysis buffer containing Proteinase K. The DNA was captured by silica filter, twice washed and eluted in 100 µl of elution buffer. Once these phases were finished, the samples were quantified at the Nanodrop (see also Dąbrowska et al., 2010; Dalmasso et al., 2011; Di Pinto et al., 2017).

Real-time PCR and Amplification plan

The cheese and milk samples were analyzed by real-time PCR, in which specific gene primers for sheep's genes were used.

The calibration curve was based on 10 times serial dilutions standard DNA at 30 ng / µl from milk with a known number of somatic cells.

Results and Discussion

As showed in the graph (Fig. 1), all the milk and cheese samples amplified show an exponential kinetics, which increases with increasing cycles (www.qualityitalia.it.). This demonstrates the fact that the specific primers and probe linked to the homologue sequences are directly proportional to the fluorescent signal. The only curve that has no exponential trend is that of the negative control, in which only water was present and in which, therefore, amplification cannot take place.

Product type	Limit somatic cells
Raw cow's milk	≤ 400,000
Raw milk from other species (sheep etc.)	≤ 1,500.000
Raw milk from other species, (sheep etc.), intended for the manufacture of products obtained by a process that does not involve any heat treatment	≤ 500,000

Table 1. Number cell limits permitted in healthy raw milk.

Name	Sequence	
Tanabe sheep Forward	CCTTATTACACCATTAAAGACATCCTAGGT	
Tanabe sheep Revers	GGGTCTCCGAGTAAGTCAGGC	
Tanabe sheep Probe	Fam-ACTAATCCTCATCCTCATGC-(blackhole)	
Temp. °C	**Time**	**Cycles**
95	2'	1
95	15''	40
50	30''	
66	50''	

Table 2. PCR conditions employed.

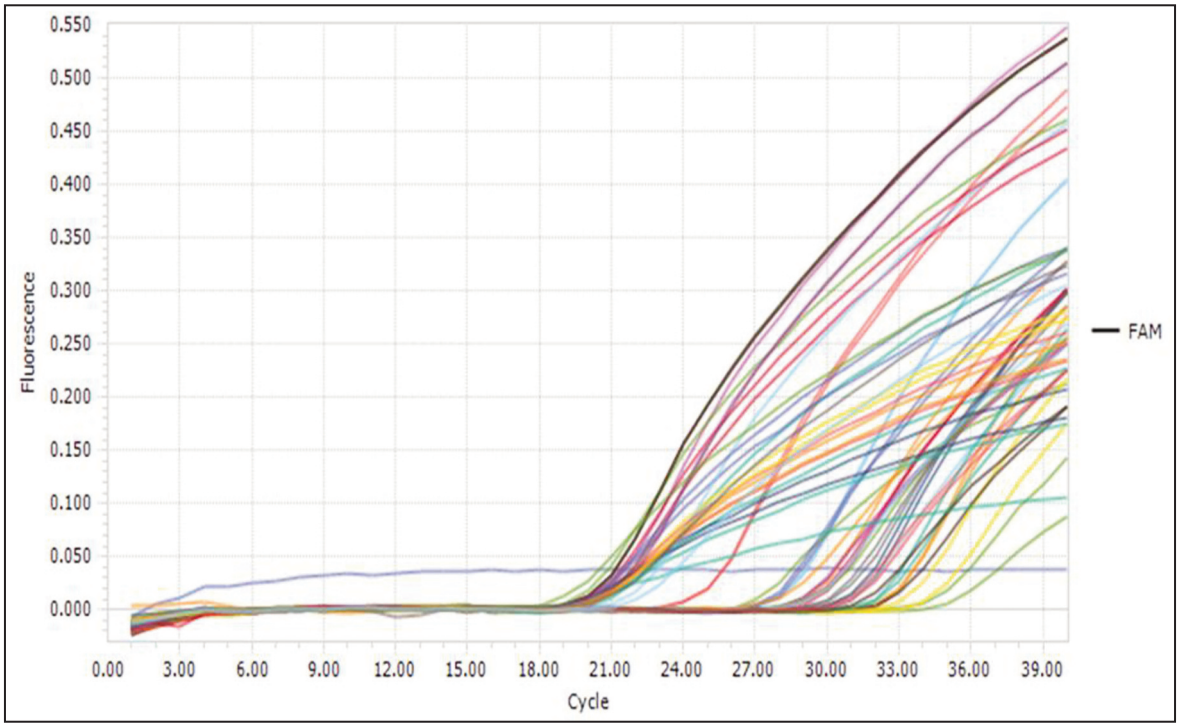

Figure 1. Real time amplication plot.

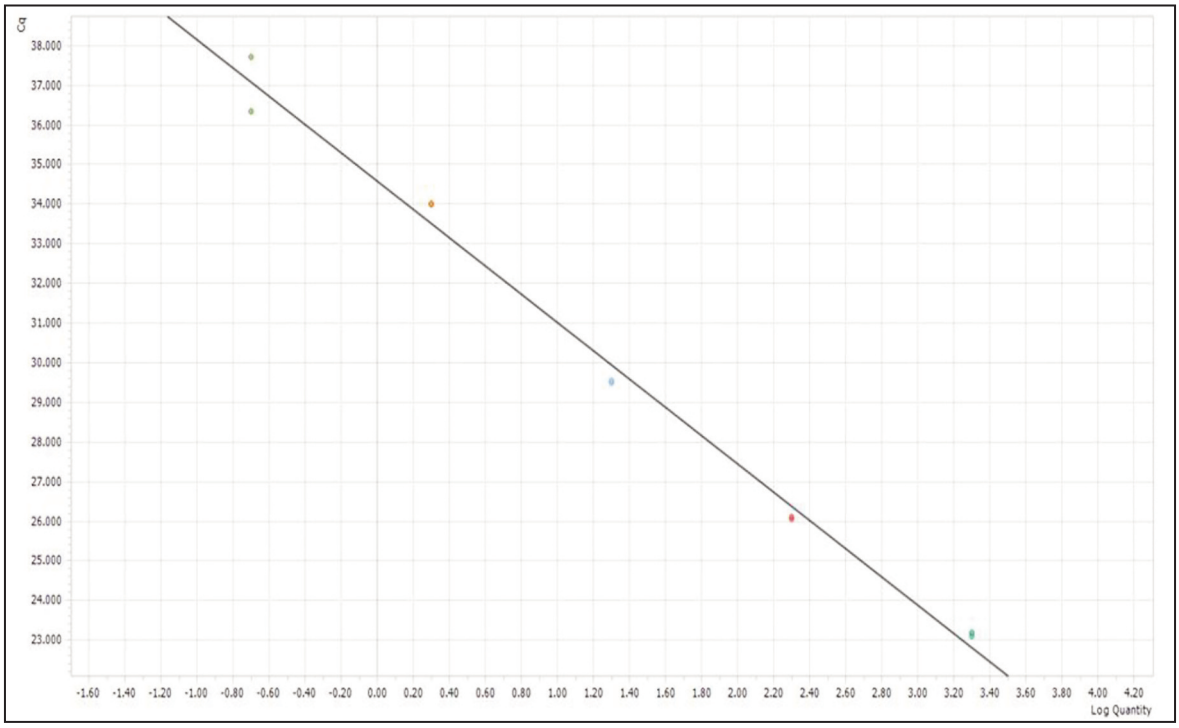

Figure 2. Standard curve obtained by employng serial DNA dilutions.

By amplifying the standards, in which we know the concentration at different dilutions, in parallel to the samples with unknown concentration, it was possible to construct a calibration curve (Fig. 2). Using the Ct values of the standards, it is possible to identify the amount of somatic cells in milk samples and the genomic equivalent in cheese samples.

Imagining to trace a median line (Fig. 3) that combines the values of dispersion of Ct of the cheeses and one that combines those of milk, it is denoted that the difference between milk samples and the corresponding cheeses is about 9 cycles of PCR, corresponding to a reduction of DNA of 1000 times.

The relation between the average of the Ct values obtained can be developed as follows:

$$\Sigma \text{ Ct milk} / \Sigma \text{ Ct cheeses} = 1000$$

Conclusions

In conclusion, from the observation of the graphs and the analysis of the Ct values, it is highlighted that the test allows, on one hand, to evaluate the number of somatic cells in the milks that make up the raw material and, on the other hand, the genomic equivalents in the derived cheeses. Moreover, the test allows to evaluate the relationship between the genomic equivalents in the two materials with a quantitative reduction equal to 1000 times.

This is probably due to the ripening or maturing phases, where innumerable bacterial and mold species intervene, depending on the metabolites produced and the changing pH conditions.

The transformations of carbohydrates (lactose), proteins, and lipids, due to the action of lytic enzymes released by the bacterial species, as well as the bacteriocins, contribute to the degradation of the DNA and, therefore, to the decrease of its concentration. This methodology allows to evaluate the animal species relative to the raw milk and the traceability of the product. Moreover, it allows to set a cut off value in terms of genomic equivalents, related to the number of original somatic cells of milk, above which the cheese would not have optimal qualities.

Practically, we focused on a correspondence between the quality of milk and that of cheese. It was related to the DNA and cell content, contributing to the ripening of the cheese. This parameter could also be recognized as official (Di Domenico et al., 2017; www.qualityitalia.it.; www.piacentinuennese.it.), and could be proposed as a quality assessment tool to guarantee the consumer in the field of food safety

Name	Ct	Average Ct	Somatic cells	Average C.Som.
milk 1	24.22	23.805	801	1084.5
milk 1	23.39		1368	
milk 2	23.1	22.735	1649	2145
milk 2	22.37		2641	
milk 3	22.88	22.82	1901	1977.5
milk 3	22.76		2054	
milk 4	22.86	22.705	1925	2138
milk 4	22.55		2351	
milk 5	21.05	21.18	6183	5706
milk 5	21.31		5229	
milk 6	22.49	22.24	2444	2908.5
milk 6	21.99		3373	
milk 7	22.05	22.125	3245	3095.5
milk 7	22.2		2946	
milk 8	21.88	22.085	3621	3200.5
milk 8	22.29		2780	
milk 9	25.89	24.72	273	753.5
milk 9	23.55		1234	
milk 10	22.98	22.935	1782	1835
milk 10	22.89		1888	
milk 11	22.6	22.7	2277	2139
milk 11	22.8		2001	
milk 12	21.6	21.585	4338	4380
milk 12	21.57		4422	
Name	Ct	Average Ct	Somatic cells	Average C.Som.
Cheese1	32.84	32.69	3	3.35
Cheese1	32.54		3.7	
Cheese2	33.48	33.935	2	1.57
Cheese2	34.39		1.14	
Cheese3	31.14	31.92	9.25	6.31
Cheese3	32.7		3.37	
Cheese4	31.98	31.86	5.39	5.84
Cheese4	31.74		6.29	
Cheese5	30.1	30.44	18	14.85
Cheese5	30.78		11.7	
Cheese6	35.89	35.325	0.4	0.6
Cheese6	34.76		0.8	
Cheese7	32.32	30.67	8.24	22.275
Cheese7	29.02		36.31	
Cheese8	30.79	30.735	11.6	12.025
Cheese8	30.68		12.45	
Cheese9	29.17	29.28	32.96	30.78
Cheese9	29.39		28.6	
Cheese10	31.05	31.11	9.8	9.4
Cheese10	31.17		9	
Cheese11	33.47	33.615	2	1.85
Cheese11	33.76		1.7	
Cheese12	32.31	32.435	4.35	4.025
Cheese12	32.56		3.7	

Table 3. Obtained data showing threshold cicles and corresponding cell numbers for each sample.

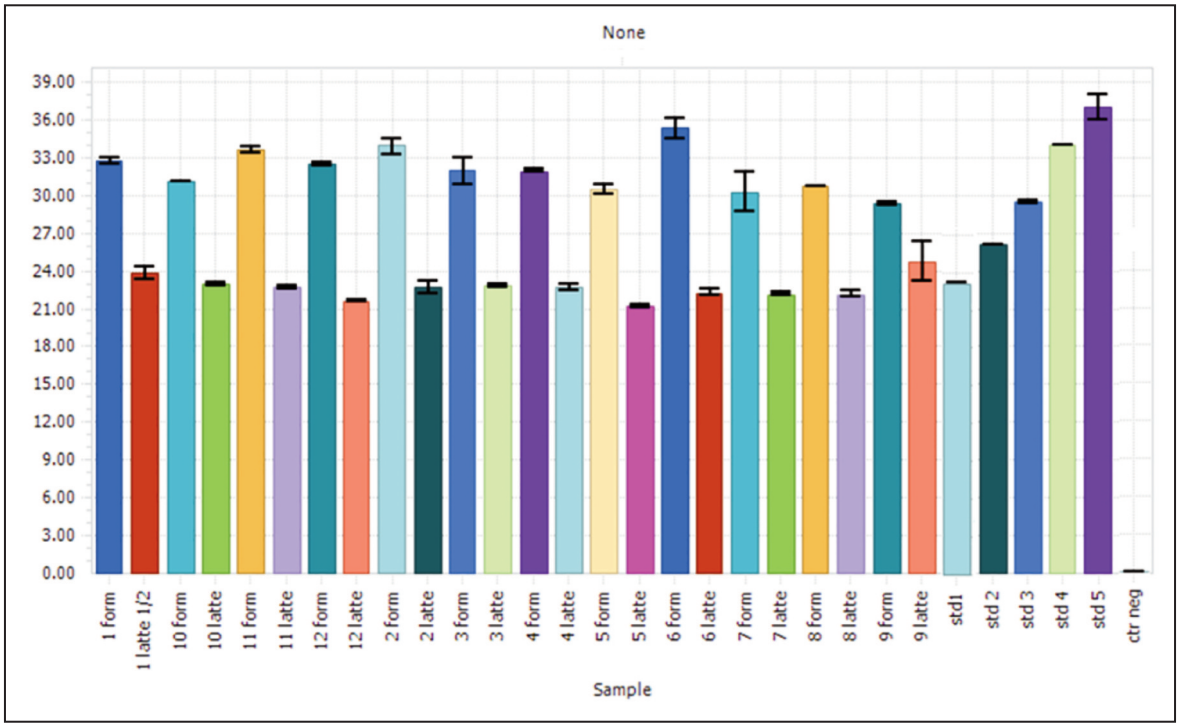

Figure 2. Data analisys graphic showing the obtained data for each milk and cheese sample. Std columns 1-5 indicate the data obtained on standard DNA.

(see also European Commission, 2002, van Hengel A.J., 2007, Zhang et al., 2007).

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