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Study of the Effect of NaCl on Lipolysis in Parmigiano Reggiano Cheese

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ABSTRACT: Lipolysis of PDO Parmigiano Reggiano cheese from different dairies was studied to evaluate the effect of sodium chloride (NaCl) reduction on the lipidic fraction. The total and individual free fatty acid and diglyceride contents were determined for two groups of samples, normal NaCl content (1.50% p/p) and reduced NaCl content (1.37% p/p). In addition, fat, water, NaCl, and cholesterol contents were also determined. The NaCl content was 9.01% higher in the normal NaCl group than in the reduced NaCl group. The most abundant fatty acid was palmitic acid, followed by oleic, myristic, and stearic acid, which altogether accounted for approximately 75% of the total content. Cheese with a normal NaCl content presented concentrations of C8:0 and C18:0 higher than those of reduced NaCl samples, while the latter showed a higher proportion of C10:0, C14:1c, and C16:0. The total free fatty acid and diglyceride contents were higher in the reduced NaCl samples, so a 9.01% reduction in the concentration of this component could accelerate the lipolysis process. On the contrary, the free fatty acid composition profile was similar in both groups.

KEYWORDS: *Parmigiano Reggiano, lipolysis, free fatty acid, diglyceride, NaCl content*

INTRODUCTION

Parmigiano Reggiano (PR) cheese is a hard and brittle cheese with a long ripening time (minimum of 12 months) and a fat content of approximately 30% (w/w).[1] Each wheel weights on average 40 kg after ripening for 12 months. It is produced only in the Italian provinces of Parma, Reggio Emilia, and Modena and part of the provinces of Bologna and Mantua. Parmigiano Reggiano is produced using raw semiskim high-quality milk, without any additive or preservative, so its only ingredients are milk and sodium chloride (NaCl), in conjunction with the natural fermented whey and calf rennet, necessary in the coagulation process. Parmigiano Reggiano is a PDO (protected designation of origin) cheese with distinctive characteristics and a strong link with the production area that is guaranteed by a system of standards and production process rules established by the Consorzio del Formaggio Parmigiano Reggiano.[1]

PR cheese is salted in saturated brine. There are different salting systems; the most frequently used is the traditional one in which the cheese floats in the brine and the wheel has to be turned every day to guarantee the homogeneity of the process, and another is the full immersion in racks system in which the cheese is completely immersed in the brine. The brine time depends on the salting system, the size of the wheel, the temperature of the brine, and the desired characteristic of the final product. The salting process lasts for 21−24 days in the traditional system, while in the full immersion system, the cheese is salted for 18 days. On average, the PR NaCl content after 12 months is 1.40%, and after 24 months, the NaCl content is on average 1.60%[2] as declared by the Consorzio del Formaggio Parmigiano Reggiano.[1]

During the maturation of hard cheeses such as Parmigiano Reggiano, a process known as lipolysis takes place, due to the activity of lipolytic enzymes, esterases and lipases.[3] In lipolysis, triglycerides are hydrolyzed, leading to the formation of free fatty acids (FFA), monoacylglycerol, diacylglycerol (DAG), and glycerol.[4] In these biochemical reactions, the NaCl content plays a key role during cheese ripening; in fact, it affects the microbial growth, enzyme activity, and syneresis.[5] Because of its long maturation time and the natural action of a lipoprotein lipase present in the raw milk used to produce it,[6] in Parmigiano Reggiano an intense lipolysis process takes place and its degree can be measured as the percentage of FFA. Moreover, FFA are important components in this traditional product not only because they contribute directly to its flavor but also because they act as substrates in a series of different reactions producing alcohols, aldehydes, and lactones, among other molecules that also contribute to flavor in the cheese.[4]

In the literature, there are many works devoted to the study of Parmigiano Reggiano cheese, and they are particularly focused on the analysis of volatile components.[7−9] Others are related to the characterization of the milk used in its production,[10−12] while there are only few studies that evaluate the lipolysis phenomenon in PDO Parmigiano Reggiano.[13] Careri and collaborators[14] studied the basic chemical

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composition of Parmigiano Reggiano and the effect of the geographic zone, season, and manufacturing dairy farm. On the contrary, Malacarne and collaborators[3] studied FFA in samples of Parmigiano Reggiano with different maturation times, from the inner and outer side of the wheels. Finally, Mordenti and co-authors[12] fed cattles with a diet rich in soy and flax and evaluated the effect on the lipid profile in cheese wheels. With regard to cheese composition, the literature showed that most abundant FFA in Parmigiano Reggiano are palmitic, oleic, myristic, and stearic acids.[3,12]

The aim of this work was to study the lipolysis process in PDO Parmigiano Reggiano with a NaCl concentration lower than the traditional value. This was evaluated by determining not only the FFA profile (C4:0−C24:0) but also the DAG one, which are co-products in the generation of FFA. This is important because the determination of FFA alone gives limited information due to the possible neutralization of these components[15] caused by an increasing pH during cheese ripening[16] and a possible enzymatic or microbial degradation.

To the best of our knowledge, this is the first study of lipolysis in Parmigiano Reggiano with reduced NaCl content.

These results would be very useful for evaluating the possibility of developing products matching the needs of both consumers (reduced NaCl products)[17,18] and dairies, the first because of health reasons and the latter due to the fact that a decreasing NaCl concentration means a decrease in brine time, increasing plant/facility productivity.

MATERIALS AND METHODS

Samples. In five Parmigiano Reggiano dairies, using a full immersion brine in racks system, 16 salting trials were performed by comparing the normal brine time (18 days) to a reduced brine time (12 days). Group 1 was formed by cheeses salted for 18 days (normal NaCl content). This group was considered the control group as this is the standard brine time (16 °C). The second group (group 2, reduced NaCl content) was formed by cheeses salted for 12 days in saturated brine at 16 °C. After both groups had been subjected to brine, 32 wheels of cheese were ripened for 15 months at 18 °C and approximately 80% relative humidity (HR). After ripening, they were weighted and sampled. From each wheel, a radial slice sample of ∼1 kg was taken and vacuum-sealed in a plastic bag. Samples were kept at −20 °C until use. Approximately 200 g corresponding to the inner section of the cheese was ground and used for the different tests and/or analysis to study the lipolysis process.

NaCl Content. The NaCl content in the cheese was determined from grated cheese by near-infrared spectroscopy (NIRS) using one replicate (n = 1). The results are expressed as percent (w/w, wet basis).

Water Content. The water content (percent) was evaluated on PR samples in an oven at 105 °C until a constant weight was reached. For each sample, three replicates (3 g) were dried according to the AOAC Official Method.[19]

Lipid Extraction. Soxhlet extraction was performed following the standard protocol according to the AOAC Official Method.[20] Briefly, 10 g of grated Parmigiano Reggiano was placed in a cellulose extraction thimble. The process was performed for 3 h to achieve a complete extraction using 60 mL of n-hexane at 70 °C. The residual solvent was evaporated using a rotary evaporator Laborota 4001-efficient (Heidolph). Each extraction was achieved twice.

Cholesterol Determination. Cholesterol was collected by cold saponification at room temperature[21] after the addition of 500 μL of an internal standard (dihydrocholesterol, 2 mg/mL) to 250 mg of fat. The unsaponifiable fraction was evaporated with a vacuum evaporator (Laborota 4001, Heidolph), silylated,[22] and dried again under a gentle nitrogen flow. After redissolution in 500 μL of n-hexane, 1 μL was injected into a GC 2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan), equipped with a flame ionization detector (FID) and an AOC-20i autosampler, according to the method reported by Marzocchi et al.[23] The cholesterol content was measured in two replicates for each lipid extract (n = 4) and expressed in grams of cholesterol per 100 g of cheese.

Determination of FFA. FFA were collected by solid-phase extraction (SPE) after addition of 50 μL of an internal standard (C11:0, 2 mg/mL) to 20 mg of fat. Briefly, after the addition of 1 mL of a chloroform/2-propanol mixture [2:1 (v/v)], the sample was charged on NH_2 cartridges (55 μm, 70 Å, 200 mg/3 mL; Phenomenex, Torrance, CA). After the samples had been conditioned with 3 mL of n-hexane, the polar lipids were eluted with 6 mL of a chloroform/2-propanol mixture [2:1 (v/v)]. The recovery of FFA was performed by using 10 mL of a diethyl ether solution with 2% acetic acid. The recovered fraction was dried under a gentle stream of nitrogen, and 200 μL of diazomethane was added to yield fatty acid methyl esters (FAME). After 2 min at room temperature, the solvent was evaporated under nitrogen and 200 μL of n-hexane was added and centrifuged at 2500 rpm for 2 min. The analysis was performed with a GC 2010 Plus gas chromatograph (Shimadzu) equipped with a flame ionization detector (FID) and an AOC-20s autosampler (Shimadzu) according to the method described by Marzocchi et al.[23] Peak identification was accomplished by comparing the peak retention time with that of the GLC-463 standard mixture from Nu-Check (Elysian, MN) and expressed as the weight percentage of total FAME. The FAME composition was measured in two replicates for each lipid extract (n = 4). The nomenclature for FFA was as follows: C6:0 for caproic acid, C8:0 for caprylic acid, C10:0 for capric acid, C12:0 and C12:1c for lauric acid, C13:0 for tridecanoic acid, C14:0 and C14:1c for myristic acid, C15:0 and C15:1c for pentadecanoic acid, C16:0 for palmitic acid, C16:1c and C16:1t for palmitoleic acid, C17:0 and C17:1 for heptadecanoic acid, C18:3$n6$ for γ-linolenic acid, C18:0 for stearic acid, C18:1$t9$ for elaidic acid, C18:1$t11$ for vaccenic acid, C18:1$c9$ and C18:1$c11$ for oleic acid, C18:2$t9t12$ for linolelaidic acid, C18:2$n6$ for linoleic acid, C18:3$n3$ for α-linolenic acid, C18:2$c9t11$ or CLA for conjugated linoleic acid, C20:1 for paullinic acid, C20:4$n6$ for arachidonic acid, C22:6$n3$ or DHA for docosahexaenoic acid, C20:3$n6$ for dihomo-γ-linolenic acid, C22:0 for docosanoic acid, C20:5$n3$ and C22:5$n3$ for eicosapentaenoic acid, SFA for saturated fatty acids, MUFA for monounsaturated fatty acids, and PUFA for polyunsaturated fatty acids.

Determination of Diacylglycerol. DAG were collected by SPE after addition of 70 μL of an internal standard (dihydrocholesterol, 1 mg/mL) to 100 mg of fat. After the addition of 500 μL of an n-hexane/diethyl ether mixture [4:1 (v/v)], the sample was charged on silica cartridges (55 μm, 70 Å, 500 mg/3 mL; Phenomenex). After being conditioned with 3 mL of n-hexane, the nonpolar lipids were eluted with 5 mL of an n-hexane/diethyl ether mixture [4:1 (v/v)]. The recovery of DAG was performed by using 4 mL of an n-hexane/diethyl ether mixture [1:1 (v/v)] and 3 mL of methanol. The recovered fraction was dried under a gentle stream of nitrogen and silylated[22] and dried again under a nitrogen flow. After redissolution in 200 μL of n-hexane, 1 μL was injected into a GC 2010 Plus gas chromatograph (Shimadzu) equipped with a flame ionization detector (FID) and an AOC-20s autosampler (Shimadzu). DAG separation was performed with a Rtx-65 TG fused silica capillary column (30 m × 0.25 mm × 0.10 μm film thickness) with 35% dimethyl and 65% diphenyl polysiloxane (Restek, Chromatography Products, Superchrom, Milan, Italy). The initial oven temperature of 240 °C was increased to 350 °C at a rate of 5 °C/min and held at 350 °C for 15 min. The injector and detector temperatures were set at 350 °C. The split ratio was 1:100. DAG were identified on the basis of the retention time of a standard mixture injected with the same method and from the comparison with the chromatograms reported in the literature. The total content of DAG was measured in two replicates for each lipid extract (n = 4) and expressed as milligrams per 100 mg of fat.

Statistical Analysis. Results were reported as means ± the global standard deviation. The term "global" indicates that a certain parameter was calculated considering all samples, regardless of the

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group number. In some cases, the global variance coefficient was calculated as the percentage of the global standard deviation divided by the global mean value.

Statistical differences between groups were assessed using a two-sided Student's t test, where a p value of <0.05 corresponds to significant differences between compared means.

In addition, Cohen's index (d) was reported to estimate size effects between the compared means and its probability of superiority (PS) was reported.[24] This parameter was calculated as the absolute value of the numerical difference between the means being compared, divided by the global standard deviation as described by Fritz and co-authors.[24] Values of d near 0.2, 0.5, and 0.8 were taken as a reference to describe small, medium, and large size effects.[25]

RESULTS AND DISCUSSION

The global mean weight of the wheels was 46.38 ± 2.65 kg. In group 1, the PR NaCl content after ripening for 15 months was 1.50 ± 0.13%, while in group 2, it was 1.37 ± 0.13%. The NaCl content in the first group was 9.01% significantly higher than in the second ($p < 0.002$). Cohen's size effect index for the NaCl content was 1.03 (PS ≈ 76), which corresponds to a large size effect according to the references mentioned in Materials and Methods. PS implies that if someone compared two randomly selected cheese samples, the reduced NaCl group will have a lower NaCl content than the traditional group for 76% of the comparisons made.[24,25] According to Bansal and Mishra,[26] the NaCl content is an important parameter that plays a key role in many aspects of cheese manufacturing such as syneresis and final moisture, among several others. In general, a lower NaCl concentration results in cheeses with less water than the traditional varieties, and a reduction in water content is generally associated with a reduction in fat content.[26] Despite this, the work presented here indicates that a 9.01% reduction in NaCl content did not affect the water ($p < 0.76$) or fat ($p < 0.34$) content in Parmigiano Reggiano PDO. The global water mean content was 31.79 ± 0.79%, while the global fat content was 30.53 ± 1.98%. The fat content was in agreement with values reported in other published works.[23−28] Finally, the cholesterol content did not present any significant differences ($p < 0.67$) between the two groups of samples; in fact, it ranged between 0.06 and 0.08 g/100 g of cheese, a range similar to results reported by Manuelian et al.[27]

FFA and DAG Composition. Thirty-two FFA were identified and quantified in the different samples: 11 being saturated fatty acid (SFA), 11 monounsaturated fatty acids (MUFA), and 10 polyunsaturated fatty acids (PUFA). The fatty acid compositions of samples from groups 1 and 2 are listed in Table 1. Fatty acids under 0.1% are not reported in the table but are identified as C12:1*c*, C13:0, C16:1*t*, C18:3*n6*, C20:3*n6*, C22:0, C20:5*n3*, and C22:5*n3*.

On this basis, most abundant fatty acid was palmitic acid (C16:0, 30.2−34.6%) followed by oleic acid (C18:1*cis*9, 20.4−23.1%), myristic acid (C14:0, 10.9−12.7%), and stearic acid (C18:0, 7.8−11.4%), in agreement with other published works.[3,12,30,31] Altogether, they account for approximately 75% of the total FAME present in the samples. The global variance coefficient for the content of most abundant FAME (including all samples, regardless of the groups) was calculated, yielding a value of <10% in all cases. In general terms, this could be used as an acceptable indicator of the global similarity among the FAME profiles of both groups. It is worth mentioning that ∼80% of the FAME reported in Table 1 presented a global coefficient variance of <15%, which is also an acceptable value that reflects the general similarity in the FAME profile of each group, considering the complexity of the matrix and the analytical methods. Variations in the fat composition of the cheese depend on many variables such as the composition of the initial milk fat to make the cheese and the feed of the cattle to produce it,[11] environmental conditions at the dairy farm, the moisture content, the water activity, and the microbial activity of the cheese microflora.[4]

Table 1. Mean Free Fatty Acid (FFA) Composition of Samples from Group 1 (normal NaCl content) and Group 2 (reduced NaCl content)[a]

FFA	group 1	group 2	GSD
C6:0	1.99 A	1.93 A	0.09
C8:0	1.80 A	1.64 B	0.23
C10:0	2.99 A	3.16 B	0.25
C12:0	3.45 A	3.52 A	0.19
C14:0	11.54 A	11.83 A	0.47
C14:1*c*	1.24 A	1.38 B	0.15
C15:0	1.28 A	1.31 A	0.09
C15:1*c*	0.50 A	0.28 A	0.48
C16:0	32.03 A	32.97 B	1.18
C16:1*c*	1.93 A	2.02 A	0.17
C17:0	0.66 A	0.66 A	0.05
C17:1	0.31 A	0.33 A	0.05
C18:0	10.14 A	9.38 B	0.92
C18:1*t9*	0.36 A	0.38 A	0.06
C18:1*t11*	1.29 A	1.29 A	0.31
C18:1*c9*	21.99 A	21.74 A	0.65
C18:1*c11*	0.36 A	0.36 A	0.06
C18:2*t9t12*	0.45 A	0.41 A	0.06
C18:2*n6*	2.91 A	2.79 A	0.28
C18:3*n3*	0.60 A	0.63 A	0.08
C18:2*c9t11* (CLA)	0.69 A	0.71 A	0.14
C20:1	0.20 A	0.20 A	0.00
C20:4*n6*	0.20 A	0.21 A	0.02
C22:6*n3* (DHA)	0.34 A	0.24 B	0.10
SFA	66.71 A	67.07 A	0.92
MUFA	28.44 A	28.26 A	0.78
PUFA	4.87 A	4.64 A	0.38

[a]Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Fatty acids under 0.1% were not reported but are included in the corresponding category. FAME (means ± GSD) are expressed in milligrams of FA per 100 mg of FAME (percent), where GSD stands for global standard deviation. Means with different superscript letters within a row indicate significant differences ($p < 0.05$).

A more detailed comparison between individual FFA of the two groups showed significant differences ($p < 0.05$) only for a few FAME. Group 1 presented a higher content of C8:0, C18:0, and DHA than group 2, while group 2 presented a higher content of C10:0, C14:1*c*, and C16:0. All differences in individual FAME content between groups were <10%, with the exception of that of DHA, which was 30% higher in group 2. This last result may not be very important because the DHA concentration was very low in both groups (0.24−0.34 mg of FA/100 mg of FAME). It is worth mentioning that C8:0, C10:0, and C14:0 are short and medium FFA, which implies that they contribute directly to the cheese flavor.[4] Despite this, a sensory analysis would be recommended to evaluate if these differences between individual FFA are sufficient for the consumers to detect differences in flavor.[29]

Despite this, and taking into consideration the ranges between the lowest and highest content of FAME reported by

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Table 2. Mean Contents of Individual Diacylglycerol (milligrams of DAG per 100 mg of DAG) in Group 1 (normal NaCl content) and Group 2 (reduced NaCl content)[a]

	D26	D28	D30	D32	D34	D36
group 1	8.84 A	3.93 A	8.88 A	20.85 A	28.14 A	29.39 A
group 2	7.28 A	4.48 A	10.80 A	24.16 A	28.89 A	24.39 A
GSD	6.30	0.94	2.84	6.02	2.84	7.70

[a]Means with the same superscript letter within a column indicate that no significant differences were found ($p > 0.05$). GSD stands for global standard deviation.

other authors,[3,12,30,31] global means obtained in this study were within that expected range, with the exception of that of C8:0, which was approximately 20% higher than the highest C8:0 content reported in the consulted bibliography.

These are favorable results that in general terms show that a reduction of 9.01% in NaCl content may not interfere with the FFA profile compared to the traditional variety.

On the contrary, SFA was the preponderant class in both groups, which could be explained by the high concentrations of C14:0, C16:0, and C18:0 that represented approximately 80% of the total SFA. No significant differences were found between the groups ($p > 0.27$), and the global mean content of SFA was 67 mg/100 mg of FAME. MUFA were present in a range between 27% and 30%, and C18:1*c9* represented almost 77% of this class; the other MUFA identified in the samples were C14:1*c* (∼1.3%), C16:1*c* (∼2−3%), and C18:1*t11* (∼1−2%). Finally, PUFA represented between 4% and 6% of the total FAME, linoleic acid (C18:2*n6*) being the major FAME of that class (∼3%).

As reported in Table 2 for groups 1 and 2, six classes of DAG were identified and quantified in all samples, corresponding to DAG with carbon numbers (CN) of 26−36. All of the samples showed a high content of long-chain DAG (CN32, CN34, and CN36), with CN34 and CN36 being the most abundant classes, representing ∼30% of the total DAG, followed by DAG with CN32 (19−29%) and in decreasing amounts the CN30 (8−17%), CN26 (3−19%), and CN28 (2−6%) classes. These results reflect a close relationship among the FFA and DAG contents of samples. Indeed, a high content in CN34 and CN36 was coincident with high levels of C18:1, C18:0, C16:0, and C14:0.

Individual DAG content did not show significant differences among the samples of each group ($p < 0.05$). High variability was observed due to the complex matrix, which is Parmigiano Reggiano.

Assessment of Lipolysis by Total FFA and DAG Content. With regard to DAG content, group 1 presented a range between 0.37 and 1.11 mg/100 mg of fat while Group 2 a range between 0.83 and 4.10 mg/100 mg of fat. Highly significant differences ($p < 0.01$) were found between the means of the groups, and the total DAG content of group 1 (0.61 mg/100 mg of fat) was approximately 70% lower than that of group 2 (2.12 mg/100 mg of fat). The *d* index for total FFA and DAG content was 1.5 (PS ≈ 85), which corresponds to a large size effect.

FFA analysis showed behavior similar to that of DAG content. In fact, values ranged from 0.24 to 0.53 mg/100 mg of fat and from 0.35 to 1.07 mg/100 mg of fat in groups 1 and 2, respectively. Once more, highly significant differences were found between the total FFA content ($p < 0.01$), with mean values of 0.37 and 0.70 mg of FAME/100 mg of fat for groups 1 and 2, respectively. The total FFA content in group 1 was approximately 50% lower than in group 2. This could be explained by the different concentration of NaCl, which was lower in the samples of group 2.

The higher FFA and DAG content presented in group 2 could possibly indicate that the lipolysis process was faster than that of the cheeses from group 1. In other words, an increase in NaCl concentration could have a "delaying effect" on the lipolysis process. According to Bansal and Mishra,[26] a reduction in NaCl content increased water activity in cheddar cheese and an increase in this parameter correlates negatively with the NaCl:moisture ratio. On this basis, the fact that lipolysis was faster in the reduced NaCl group could possibly be explained because by the decrease in the NaCl:moisture ratio, which could be increasing the amount of water available for the lipolysis reactions. In addition, decreasing the NaCl:moisture ratio may lead to uncontrolled enzymatic activity.[26]

In general terms, total FFA contents were higher than those reported by Malacarne et al.[3] They also reported large standard deviations, which confirms the high, but natural, sample heterogeneity. In addition to the variables mentioned above, this also could be explained by the different origins of the lipases involved in the cheese production process, which are milk, rennet paste, starter bacteria, the secondary starter microorganism, and the exogenous lipase preparation.[32]

This study provides information about the characterization of reduced NaCl PDO Parmigiano Reggiano cheese with regard to the lipolysis process.

According to the results obtained in this study, even though a decrease in NaCl concentration could have increased the extent of the lipolysis process, the FFA as well as the DAG profiles were very similar. On this basis, the possibility of a 9% reduction in NaCl content in Parmigiano Reggiano cheese seems possible. This is an important achievement as it would allow an increase in the amount of sales of the product to consumers interested in nutritional aspects. In addition, a 33% reduction in the length of the salting process would allow dairy farms to work under more flexible conditions and may lead to an increase in their productivity.

Finally, this study sets the first precedent to continue the investigation of the reduced NaCl variety of Parmigiano Reggiano PDO to explore the effect of shorter salting times, not only in terms of composition but also in terms of a sensory point of view, such as texture and consumer acceptability.

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Notes

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