

Original Research Article

Nutritional evaluation of cheeses: cholesterol and its oxide 7- ketocholesterol

ABSTRACT

The present study sought to identify and quantify the contents of cholesterol and 7-ketocholesterol in cheese samples (Mozzarella, Prato and Minas Frescal). Cholesterol and 7-ketocholesterol were identified by comparing the retention time of the sample peaks with the retention time of the standard peaks, and the characteristic wavelength was determined for each substance by using reversed phase high-performance liquid chromatography. The Prato cheese showed a higher cholesterol content, followed by the Mozzarella and Minas Frescal cheeses. Presence of the oxide 7-ketocholesterol was not found, indicating that processing did not result in cholesterol oxidation.

Keywords: *Chromatography, Cholesterol oxidation, Dairy product, Oxides, Processing, HPLC.*

1. INTRODUCTION

In the current stage of economic development, the food industry has been noted for its dynamics. However, certain ambiguities emerge as a result. On the one hand, it preserves foods; on the other hand, it adds substances not naturally present in foods, which cause problems to human health.

Transformations of natural foods, such as those of animal origin, have been studied in this industry to evaluate effects and possible consequences on consumption [1, 2]. In this sense, the dairy industry is particularly relevant because it turns milk into derivatives that require operations for production and marketing. Operations such as heat (above 120 °C), shelf life and light can cause changes to the physical and chemical structure of milk, especially fat [3, 4, 5], non-polar globules comprised of triacylglycerides, cholesteryl esters, retinol esters, phospholipids, proteins, cholesterol and others [6, 7, 8].

The cholesterol content of milk ranges between 12-14 mg 100 g [9, 7]; when milk is processed, it may present alterations, e.g., being degraded into cholesterol oxides, as an auto-oxidation response [10, 1, 2, 5]. Cholesterol in dairy products can undergo oxidations, generating cholesterol oxides.

Many are the products that result from cholesterol oxidation, such as 7-ketocholesterol, 20-hydroxycholesterol, 25-hydroxycholesterol, 7 α -hydroxycholesterol, 7 β -hydroxycholesterol, 5,6-cholesterol-epoxides (α and β) and cholestanetriol among others. However, the product most commonly associated with oxidation is 7-ketocholesterol, formed during heating of the

38 α and β isomers of 7-hydroperoxy-cholesterol, which is quite commonly found in foods and is
39 therefore used as a possible indicator of cholesterol oxidation [11, 12, 13, 14].

40 Fresh milk and fresh dairy products have a low probability of cholesterol oxide formation, as
41 the medium is liquid and the oxygen content is low [13]. Dairy products may have increased
42 resistance to auto-oxidation of cholesterol, even after prolonged periods of adverse
43 conditions. This may be possibly due to the low transitional characteristics of metal content,
44 low cholesterol and saturated fat levels [15, 11].

45 Studies have indicated the formation, although small, of cholesterol oxides in milk heated for
46 different times and to different temperatures, as well as cheeses and butters subjected to
47 high temperatures during processing [16, 17, 18, 5].

48 In the dairy industry, heating is widely used, and it is important for verifying the presence of
49 cholesterol oxides. Studies have shown that these compounds are more hazardous to
50 arterial cells than cholesterol, and they are directly related to atherosclerosis, coronary
51 diseases and mutagenic activity [19, 20, 14, 21].

52 Humans can absorb cholesterol oxides after their consumption in foods. They are found
53 mainly in products such as eggs, meat, dairy and seafood. However, it was observed that
54 fresh foods presented only traces of these compounds or did not exhibit them [11, 19].

55 There have been several studies on cholesterol determination in foods. Colorimetry, gas
56 chromatography (GC) with or without a combination with mass spectrometry and high
57 performance liquid chromatography (HPLC) are some of the most used methods [22, 8].

58 The analysis by gas chromatography has been used for a long time as the best method for
59 determination of cholesterol in foods. This analysis requires the extraction of lipids,
60 saponification and derivatization. However, the steps of extraction and saponification are
61 complex and derivatization reagents are unstable and increase the time of analysis.
62 Additionally, cholesterol may be destroyed thermally as a result of the high temperatures
63 required during the separation of GC, and undesirable compounds may also be formed [23,
64 24, 25]. Thus, cholesterol determination by GC was gradually replaced by HPLC. Although
65 HPLC has the disadvantages of poor absorption of cholesterol in low wavelengths,
66 decreasing detection and increasing difficulty in quantification, as well as the need for large
67 volumes of solvents for analysis, sample preparation for use in HPLC is simpler and can
68 basically use saponification and choice of solvent extraction [23, 25]. Moreover, HPLC uses
69 lower temperatures and shorter times of analysis [24].

70 Determining cholesterol and its oxides simultaneously is a complex task, mainly because
71 cholesterol is unstable and the structure of its oxides is very similar to that of cholesterol
72 itself. Thus, methods must be developed carefully for greater detection of compounds and
73 reduced appearance of undesirable compounds. Some studies can be found in the literature
74 for the simultaneous determination of cholesterol and its oxides by HPLC with different
75 extraction alternatives ([24, 26, 25].

76 Thus, the conduction of this study sought to assess cholesterol, as well as the cholesterol
77 oxide 7-ketocholesterol in cheese samples subjected to different processing temperatures.

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80 2. MATERIAL AND METHODS

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82 2.1 Acquisition of the samples

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84 Samples of Prato, Mozzarella and Minas Frescal cheeses were purchased from two dairy
85 farms; they have different production processes (Table 1). Samples of Minas Frescal cheese
86 were purchased in the municipality of Itapetinga-BA, Brazil in the months of September and
87 October 2013, and samples of Mozzarella and Prato cheese were purchased from a dairy
88 farm in Nova Venecia-ES, Brazil in October. The cheese samples were collected already
89 packaged, and then frozen until analysis. The experiments were conducted at the State
90 University of Southwest Bahia, Juvino Oliveira Campus, in Itapetinga-BA, Brazil, in the
91 LAPRON (Natural Products Research Laboratory), CEACROM (Center of Chromatographic
92 Studies and Analysis) and Laboratory of Milk and Dairy Products.

93 **Table 1. Processing conditions of the Prato, Mozzarella and Minas Frescal cheeses**

Variables	Cheeses		
	Prato	Mozzarella	Minas Frescal
Milk temperature	32 °C	33 °C	33 °C
Heating temperature	41 °C	41°C	36 °C
Coagulation time	40 min.	30 min.	30 min.
Mixing time	20 min.	15 min.	15 min.
Cooking time	60 min.	45 min	45 min.
Number of grains	03	02	02
Pressing time	17 min.	15 min.	-
Curd stretching temperature	-	87 °C	-
Curd stretching time	-	3 min.	-
Total time	90 min.	75 min.	60 min.

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96 2.2 Physico-chemical determinations of the cheeses

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98 Physico-chemical analyses were performed of moisture, ash, fat, total nitrogen, fat in the dry
99 extract (FDE) and total dry extract (TDE). All analyses followed the methods described by
100 [27].

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102 2.3 Extraction of cholesterol and 7-ketocholesterol from the cheeses

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104 Acquisition of the unsaponifiable matter was carried out by saponification of cheese fat and
105 subsequent extraction with hexane [28], with modifications [29]. All reagents used in these
106 steps were analytical grade ones.

For this purpose, approximately 0.5 g of the cheese samples was weighed and supplemented with 4 mL of a 50% KOH aqueous solution and 6 mL of ethyl alcohol. The samples were vortexed for 1 minute and left at rest for 22 hours in the dark at room temperature. After this time, 10 mL of distilled water and 10 mL of hexane were added, followed by vortexing for 5 minutes. There occurred phase separation and the hexane phase was collected, which was dried in a rotary evaporator at room temperature. When only the extract remained, it was dissolved in 2 mL of the mobile phase (HPLC purity grade) and filtered through a syringe filter (0.45 μm) and stored in identified eppendorfs until the time of analysis.

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117 **2.4 Chromatographic analysis**

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The experiment used a Shimadzu, LC-20AD Prominence liquid chromatograph (Japan), with a quaternary solvent system, injection valve with 20 μL sample loop, column oven and a diode array detector using a C₁₈ analytical column (Restek C 18, 15 cm $\times$ 6 mm id $\times$ 5 μm).

An isocratic binary solvent consisting of acetonitrile and isopropanol (85:15 v/v) was used as mobile phase. Solvent flow-rate was 2 mL.min⁻¹, where the oven temperature was adjusted to 40 °C aiming to get complete resolution of chromatographic peaks with a total run of 12 min. The solvents in use were chromatographic grade, filtered and degassed before use.

Injections were performed in triplicate and chromatographic data of the cholesterol and 7-ketocholesterol were processed with the LCSolution[®] software (Shimadzu).

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129 **2.5 Identification of the analytes**

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The identification of cholesterol and 7-ketocholesterol was tentatively performed by comparing the retention time of commercial standards of cholesterol (Cholesterol, cod. C8667, Sigma-Aldrich®, Saint Louis, Missouri, USA) and 7-ketocholesterol (5-Cholesten-3 β -ol-7-one, cod.C2394, Sigma-Aldrich®, Saint Louis, Missouri, USA) and also by the characteristic wavelength of each substance. The chromatograms were processed at 202 nm for cholesterol and at 227 nm for 7-ketocholesterol.

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138 **2.6 Validation of the analytical method**

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Validation of the proposed method was demonstrated by means of laboratory assays with standards, performed as routine at the Center of Chromatographic Studies and Analysis [30, 25]. The analytical method was validated for selectivity, linearity and range of application, repeatability, intermediary precision, recovery, limits of detection and quantification, which were carried out according to the [31], which is based on the international norms of IUPAC (International Union of Pure and Applied Chemistry).

Recoveries were evaluated in triplicate by adding to cheese samples aliquot standard solutions of the analytes for reaching the following concentrations: 100, 200 and 300 $\mu\text{g mL}^{-1}$ for 7-ketocholesterol. The injection of control samples was performed without strengthening the standard.

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2.7 Quantification of cholesterol

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The quantification of the cholesterol was performed by external standardization and conducted by integrating the areas of the peaks obtained by injection of standard solutions and calculating the concentrations using the straight-line equation obtained from the standard curve of the cholesterol standard, constructed between 2 and 2000 mg L^{-1} .

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2.8 Experimental design and statistical analysis

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The study used a completely randomized design with three repetitions in triplicate. ANOVA was used for the HPLC results comparing the means by Tukey's test at 5% probability.

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3. RESULTS AND DISCUSSION

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3.1 Physico-chemical analyses

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The treatments exerted effects on the study variables ($P=0.05$), except for FDE and protein content. This result is probably due to differences in cheese production techniques (Table 2).

Table 2. Average concentrations of the physico-chemical variables of Mozzarella, Prato and Minas Frescal cheeses

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Analysis	Cheese		
	Mozzarella (n=9)	Prato (n=9)	Minas Frescal (n=9)
Protein (%)	24.43 ^a ± 1.59	22.67 ^a ± 0.76	24.18 ^a ± 0.78
FDE (%)	46.95 ^a ± 1.58	48.44 ^a ± 1.20	48.67 ^a ± 0.92
Fat (%)	27.08 ^b ± 0.53	28.58 ^a ± 0.34	22.17 ^c ± 0.47
Water content (%)	42.29 ^a ± 0.97	40.97 ^a ± 0.84	54.45 ^b ± 0.95
Ash (%)	4.06 ^a ± 0.34	3.36 ^b ± 0.33	3.07 ^b ± 0.38
TDE (%)	57.71 ^a ± 0.97	59.03 ^a ± 0.84	45.56 ^b ± 0.95

*FDE: Fat in the Dry Extract; TDE: Total Dry Extract. Means followed by different letters in the same line differ at 5% probability by the Tukey Test.

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The cheeses presented FDE percentages ranging from 46.95% to 48.67%. According to legislation, Mozzarella cheese is classified in relation to fat content as extrafatty, fatty to semifatty cheese, with fat percentages ranging from 25 to more than 60% fat; Prato cheese is classified as fatty cheese with fat percentages ranging from 45% to 59.9%. In turn, Minas Frescal is a semifatty cheese (FDE 25%-44.9%). Among the cheeses being studied, Minas Frescal was the only one outside the standards established for fat content [32, 33, 34].

The cheeses presented different classifications in relation to water content, according to legislation. The Minas Frescal cheese is very rich in water and should have a water content higher than 55%. Therefore, this was the only one that did not fit into the established conditions. The Mozzarella and Prato cheeses presented water contents of 42.29% and 40.97%, respectively, classified as cheeses with average water content [35], thus meeting the requirements of legislation.

TDE was determined by subtracting the water content found in the cheeses from the full portion (one hundred percent). The Minas Frescal cheese presented the lowest TDE, differing from the others ($P=.05$). This result was expected, since there was also a significant difference ($P=.05$) for the water content parameter between cheeses; in addition, this cheese has very high water content.

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3.2 Cholesterol

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The Prato cheese presented the highest cholesterol content compared to the other evaluated cheeses, even when showing similar FDE content, followed by Mozzarella, which differed from Minas Frescal. However, the cholesterol/fat ratio showed no difference among the cheeses ($P=.05$) (Table 3).

Table 3. Cholesterol content (mg 100 g⁻¹) and cholesterol/fat ratio (mg g⁻¹) for the Mozzarella, Prato and Minas Frescal cheeses

Variables	Cheese		
	Mozzarella	Prato	Minas Frescal
Cholesterol content (mg 100 g ⁻¹)	98.52 ^b ± 6.46	111.50 ^a ± 7.04	78.75 ^c ± 5.39
Cholesterol/Fat ratio (mg g ⁻¹)	3.15 ^a ± 0.25	3.38 ^a ± 0.26	3.07 ^a ± 0.21

*Means followed by different letters on the line differed at 5% probability by the Tukey test.

200

According to Brazilian institutions, the cholesterol content of Minas Frescal cheese varies from 41-62 mg 100 g⁻¹ [36, 37], and these values are lower than those observed in this study. This is a fresh cheese, typically Brazilian, whose production is not standardized; therefore, it is manufactured in different ways among dairy facilities, sometimes with whole milk and sometimes with semi-skimmed milk, which can change the final cheese water content. Thus, the lack of standardization can modify the fat content of the cheese, which in turn may influence the cholesterol content since it is attached to the membrane of fat globules [38].

209 Reports indicate cholesterol contents between 80-99 mg 100 g⁻¹ for Mozzarella cheese in
210 Brazil ([39, 36, 37], similarly to the findings in this study. In turn, international reports indicate
211 variations between 46.25 and 71.4 mg 100 g⁻¹ [9, 40], which are lower values than those
212 found in the present study. The difference between the cholesterol levels in Mozzarella
213 cheese reported in Brazilian and in international studies is probably due to differences in
214 manufacturing techniques, as well as higher fat content of milk in Brazil.

215 Among the cheeses being studied, Prato cheese showed the highest cholesterol content
216 ($p < 0.05$), 111.50 mg 100 g⁻¹ (Table 3), although it showed similar FDE content and fat
217 content of the raw material (although significantly greater), slightly greater than those of
218 Mozzarella cheese (Table 2). Reports from international literature on cholesterol in Prato
219 cheese are unavailable, probably because it is a typical Brazilian cheese whose production
220 technique was adapted from cheeses like Danbo (Danish) and Gouda (Dutch).

221 Studies indicate that cholesterol positively correlates with fat content in cheeses [41, 38, 40];
222 thus, the higher the fat content the higher the cholesterol content. In this study, Prato cheese
223 showed the highest cholesterol content, although there was no difference ($P = .05$) in FDE
224 content.

225 According to the [42], there is no daily quantity established for cholesterol consumption;
226 however, intake should be minimized. In turn, the [43], by means of the CFR (Code of
227 Federal Regulations Title 21), proposed that the daily value for cholesterol intake in a 2,000
228 calorie diet should be up to 300 mg.

229 The Prato cheese showed more than one third of the recommended amount of cholesterol
230 intake in only 100 grams of cheese. Mozzarella and Minas Frescal cheese showed 98.52
231 and 78.75 mg of cholesterol per 100 g of cheese, respectively. This indicates that intake of
232 the three cheeses must be accompanied by a balanced diet, so as not to exceed the daily
233 recommendation.

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235 3.3 Cholesterol oxides

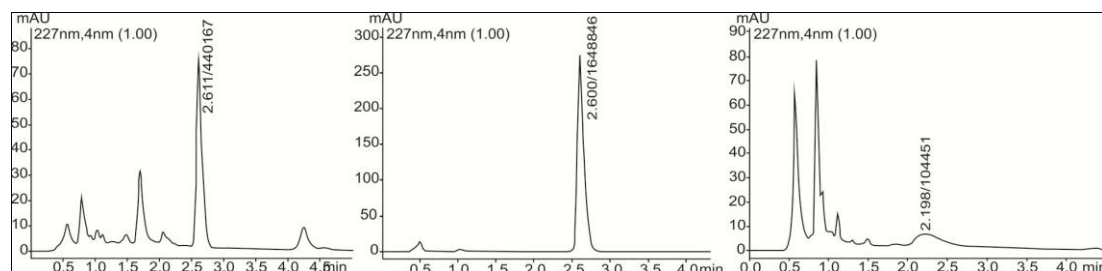
236

237 Cholesterol oxides have many effects on human health [44, 45, 46, 47], which makes the
238 results of this study positive, since the cheeses being studied are not sources of cholesterol
239 oxides.

240 The recovery (sample together with the standard) indicates the effectiveness of the results
241 observed in chromatography. It was found that the recovery curve (Figures 1, 2 and 3)
242 overlaps the standard curve, confirming that the sample cheese did not interfere in the
243 retention time of the 7-ketocholesterol peak in all cheeses, while each sample presented
244 peaks at distinct moments for the standard.

245 Presence of the oxide 7-ketocholesterol was not detected in Mozzarella cheese (Figure 1).
246 Among the evaluated cheeses, this cheese was expected to present oxides in function of
247 curd stretching of the mass required for acquiring ideal consistency. This curd stretching
248 occurs in water at 87 °C for approximately 3 minutes.

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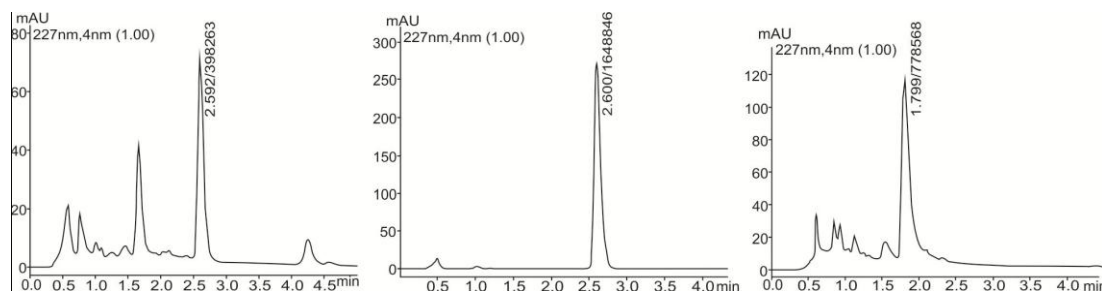


250 **Fig. 1.** From left to right: chromatograms of the Mozzarella cheese samples with the
 251 standard 7-ketocholesterol (concentration $100 \mu\text{g mL}^{-1}$), the standard 7-ketocholesterol and
 252 pure Mozzarella cheese.

253

254 Behavior of the Prato cheese was the same as the one observed in Mozzarella cheese, i.e.,
 255 presence of the oxide 7-ketocholesterol was not detected in the samples, because peaks in
 256 the chromatograms of the pure standard differed from the peak observed in the Prato
 257 cheese samples (Figure 2).

258

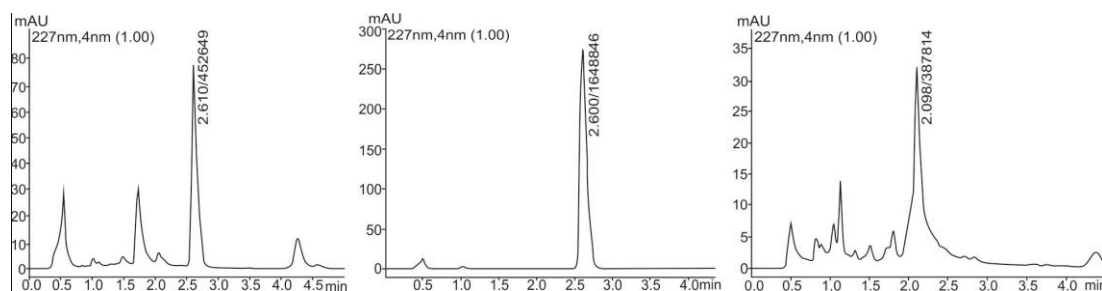


259 **Fig. 2.** From left to right: chromatograms of the Prato cheese samples with the standard 7-
 260 ketocholesterol (concentration $100 \mu\text{g mL}^{-1}$), the standard 7-ketocholesterol and pure Prato
 261 cheese.

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263 Among the study cheeses, Minas Frescal is the only fresh cheese, and it was not expected
 264 to present cholesterol oxides (Figure 3), since fresh dairy products have a low probability of
 265 cholesterol oxide formation ([17, 13]. Thus, the same behavior of the other evaluated
 266 cheeses was observed in this cheese, i.e., no indications were detected of cholesterol
 267 oxides.

268



269 **Fig. 3.** From left to right: chromatograms of the Minas Frescal cheese samples with the
270 standard 7-ketocholesterol (concentration $100 \mu\text{g mL}^{-1}$), the standard 7-ketocholesterol and
271 pure Minas Frescal cheese.

272

273 When studying the effect of processing on cheeses under similar conditions to those of this
274 study, [1] observed cholesterol oxides in them.

275 The effects on cholesterol oxidation to form cholesterol oxides in dairy products is indicated
276 at temperatures exceeding 120°C [3, 18]; however, the exposure time to temperatures may
277 elevate cholesterol oxide levels as well [3, 2]. Of the cheeses evaluated, only the Mozzarella
278 mass is exposed to temperatures higher than that of pasteurization (87°C), which could
279 exert some effects on cholesterol content. However, this was not demonstrated under the
280 conditions of the present study. On the other hand, it was suspected that the cholesterol
281 observed in the milk used for producing the Prato cheese would also suffer some effect
282 because of the exposure time of 60 minutes to the cooking temperature of 41°C . In light of
283 the data, this hypothesis was not confirmed. In turn, the temperature of milk processing to
284 produce Minas Frescal cheese was 36°C , a low temperature compared to the temperature
285 at which an effect on cholesterol is observed, above 120°C [3], therefore, cholesterol
286 oxidation was not expected as a function of temperature.

287 Generally, cholesterol-containing foods, when exposed to heat, air or light during processing
288 and storage, may present cholesterol oxides as a response to auto-oxidation [10, 1, 2, 5];
289 however, in dairy products, auto-oxidation of cholesterol occurs moderately even in adverse
290 conditions. Factors such as the modest cholesterol level and high level of saturated fat can
291 explain this behavior [15, 17].

292 It is important to highlight that cholesterol cannot be the primary cause of atherosclerosis
293 because it has low reactivity [21]. Lesions to blood vessel walls can be caused by oxidation
294 products formed during processing, preparation and storage of food products. When these
295 lesions are already formed, cholesterol is deposited on the walls of blood vessels, causing
296 atherosclerosis [47].

297 The absence of 7-ketocholesterol can therefore be considered as a positive result. Although
298 it is considered the least toxic cholesterol oxide, its presence in food is a reference for
299 evaluation of cholesterol oxidation [11, 48] and these oxides cause undesirable reactions in
300 humans, including cytotoxicity, apoptosis and proinflammatory effects, and they are also
301 associated with chronic diseases, such as atherosclerosis and neurodegenerative processes
302 [47].

303 The cheeses used in this work were submitted to processing temperatures below 80°C and
304 the presence of 7-ketocholesterol was not observed, corroborating the reports of [49].

305 Compared to non-oxidized cholesterol, cholesterol oxides have greater pathogenic and toxic
306 effects as a result of their ability to overcome the lipophilic membrane [44, 45, 46], and they
307 can cause imbalance in the ratio of oxidation and redox biochemical reactions, working at all
308 levels of the body, from signaling of diseases to regulation of inflammation, apoptosis and
309 fibrosis [47].

310 Consumer products that might contain cholesterol oxides in their composition should be
311 observed more carefully. As mentioned, the cheeses have different methods of processing
312 and different manufacturing temperatures, which can promote the formation of these

compounds. Cholesterol oxides can be absorbed by the human diet, and they may also be formed endogenously in the body [17, 18] and perform different functions, including obligatory intermediates in of bile acid synthesis, and they can be considered a transport means for cholesterol. However, the binding of cholesterol oxides in atherosclerosis is still uncertain, hence the level of cholesterol oxides in food products should be kept to a minimum [18].

The results of this study indicated that consumption of the Mozzarella, Minas Frescal and Prato cheeses did not result in intake of cholesterol oxides, helping to clarify that dairy products should not be treated as enemies of health, but due to their complexity should be viewed holistically and not in a segmented manner, as they have been.

323

324 **4. CONCLUSION**

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326 Pasteurization of milk increased cholesterol content; however, further studies are needed to
327 clarify this effect.

328 Presence of the oxide 7-ketocholesterol was not detected in the analyzed cheese samples,
329 indicating that processing did not result in cholesterol oxidation.

330 Consumption of Minas Frescal, Prato and Mozzarella cheeses did not lead to intake of
331 cholesterol oxides.

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335 **COMPETING INTERESTS**

336

337 Authors have declared that no competing interests exist.

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339

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