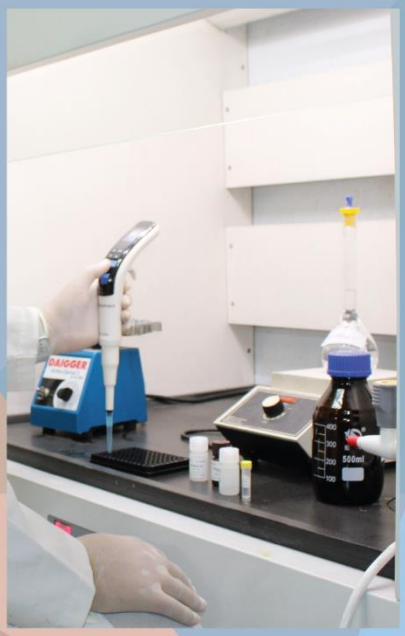
Dirección de Investigación y Proyección Social

Universidad Dr. Andrés Bello

Formando profesionales integrales

Comisión de Acreditación de la Calidad de la Educación Superior
UNIVERSIDAD DR. ANDRÉS BELLO (UNAB)
ACREDITADA
2019 - 2024

RESIDUAL FORMALDEHYDE CONTENTS IN FRESH WHITE CHEESE IN CHALATENANGO, EL SALVADOR: SEASONAL CHANGES ASSOCIATED WITH TEMPERATURE



Residual formaldehyde contents in fresh white cheese in Chalatenango, El Salvador: Seasonal changes associated with temperature

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ARTICLE INFO

Keywords:

Formaldehyde

Unapproved food preservative

Fresh white cheese

Fluorescence intensity

Year-seasons

Average ambient temperature

Central America

Chemical compounds studied in this article:

Formaldehyde (PubChem: 712)

Abbreviations: FA, Formaldehyde; %RSD, Coefficient of variation; HORRAT, Horwitz ratio.

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https://doi.org/10.1016/j.toxrep.2022.08.005

Received 8 June 2022; Received in revised form 1 August 2022; Accepted 16 August 2022

A B S T R A C T

Detection of residual formaldehyde (FA) in dairy products could be explained by direct addition of this preservative to extend the shelf life of raw material or final product at room temperature. FA is not authorized as a preservative by international standards and its addition to dairy products is prohibited due to its potentially harmful effects on consumers. Although the carcinogenicity of FA by oral exposure has not been proven, it is also known it cause histopathological and cytogenic changes in tissues at first contact, so its toxicity by ingestion should not be underestimated.

This research determined both residual FA levels in locally produced fresh white cheese and its variation according to the seasons of the year and its association with ambient temperature. None of the FA levels quantified in cheese exceeded the maximum tolerable concentration (2.6 mg/kg) and although average FA contents did not vary significantly with seasonal changes (0.093-0.181 mg/kg), the number of positive cases did, since the highest prevalence occurred in the dry (60.9 %) and transitional dry-rainy (79.7 %) seasons of 2021, which are characterized by having the highest average ambient temperatures (27.5 °C and 28.3 °C, respectively).

It was also shown that 79.6 % of the variability of FA-positive samples is explained by changes in the average temperature according to the year´s season.

The association between these variables and quantified levels of aldehyde in raw milk sampled at the plant could indicate that FA was used to prevent milk and/or the final product from decomposing due to the effect of high ambient temperature. In addition, residual FA contents decreased in both milk and cheese, depending on added preservative levels, and the time elapsed prior to analysis.

1. Introduction

While formaldehyde (FA) is naturally present as a low concentration metabolic intermediate in the cells of most living organisms [1,2], it is also found in several foods [1-3].

However, the presence of FA in milk, whether for direct consumption or for processing, can be caused by the direct addition of this compound as an "unapproved" preservative to extend its shelf life at room temperature due to its antimicrobial action [4,5] or by the use of Hexamethylenetetramine (HMT, E 239). HMT is a food-grade preservative that breaks down to FA and ammonia under acidic conditions or in the presence of proteins such as cheese [6], therefore it is a proven FA releaser [3,7,8].

Whichever form of FA is used, its addition to milk is adopted by middlemen-collectors and milk traders to prevent economic losses due to deterioration of the milk, mainly during transport [5,9]. In developing countries, deliberate and illicit addition of these chemicals is frequent in dairy processing plants [4], as this practice masks poor hygiene conditions during production, storage, and transport of raw material [10].

Addition of FA to milk is prohibited in several countries due to its potentially harmful effects on consumers' health, including liver and kidney damage [4,5,10,11]. Conversely, levels of FA in dairy products detected up to date (<1 mg/kg) [2,12] do not exceed the maximum tolerable concentration of 2.6 mg/kg orally, established as a global reference value [10,13].

Although there is no definitive evidence to demonstrate the carcinogenicity of FA by oral exposure [2,6,12], it is known that at low concentrations it can cause histopathological and cytogenetic changes in first contact tissues, therefore, potential toxicity of FA ingestion should not be underestimated [1]. In addition, FA forms peptide adducts that deteriorate the nutritional value of milk [14], for these reasons such insights stress the importance to monitor the levels of FA in dairy products [10].

Evidence of FA adulteration of milk can also be seen in processed products, such as cheese [4,15]. This statement is based on the carry-over effect of FA content (15 mg/kg or 25 mg/kg) added to milk to make Grana Padano cheese, registering residual levels of up to 0.50 mg/kg after-ripening period [3]. A similar effect occurs with FA contained in feed and forages transferred to the milk of cattle in a dose-dependent manner [12].

There are records of the use of FA to preserve cheese destined for rations during World War II, a continuing practice by producers of Grana Padano (Italy) [3,15], despite the fact that FA is not included in the list of preservatives authorized for manufacturing that type of dairy product according to General Standard for Cheese CXS 283-1978 [16,17]. It is also not included in the Salvadoran Standard for Cheese, as well [18].

In Central America, there is little information on the addition of FA to dairy products obtained mainly through short-term random sampling, usually during the dry season [19-21]. Those studies determined an average prevalence of 92.6 % and 44.4 % of artisanal fresh white cheese samples, respectively [20,21], while in samples of hard white cheese of artisanal and industrial production FA presence was detected in 42 % and 33 % respectively [21].

Monitoring levels and prevalence of residual FA in fresh cheese is relevant because the Salvadoran population has an apparent consumption of fresh cheese and hard white cheese that reaches 2.2 and 4.3 million kilograms per year respectively [22]. On the other hand, one of the

key actions to reduce adulteration of dairy products due to the addition of unauthorized preservatives such as FA is to detect them in time, through research and monitoring, mainly in developing countries [23], where production systems are fragmented and cooling and processing of milk is still rudimentary [5,24].

Since the presence residual amounts of FA in cheese is indicative of its illegal use in the preservation of milk for cheese processing. Additionally, FA content is of particular concern to government entities that regulate the quality of locally produced or imported dairy products. [25, 26]. Therefore, this work aimed to measure the residual content of FA in locally produced fresh cheese and determine variations according to the seasons of the year and ambient temperature as a basis for establishing a future monitoring proposal.

2. Material and methods

2.1. *Study type, cheese specimen and sampling*

Locally made fresh white cheese was monitored for 12 months, sampling a range of 34-35 independent retail stores each month. The total number of samples collected and analyzed was 412, each sample
weighed 1 kg and was kept cold during transport to the laboratory. Samples were stored at 2-4 °C in a horizontal refrigerator until processing and analysis.

Fresh white cheese was chosen as the specimen to monitor levels of FA presumably added to milk since it only undergoes lactic fermentation by adding liquid or powder rennet, it is molded by partially draining whey and is ready for consumption [27]. The process of making fresh cheese takes 5-7 h, and it is distributed within 24 h to retailers, where it
has a short shelf life (<8 days at 6 °C) [27].

3. Sample preparation, extraction, and analysis of FA

Samples were homogenized manually in containers with single-use utensils. Five grams aliquot was obtained from each sample, placed in a 50 ml tube, and suspended in 5 ml of distilled water (ratio 1:1). Each tube was vortexed for 1 min to produce a uniform suspension and the tubes were centrifuged at 10,500 RPM for 5 min at 9 °C.

Subsequently, two 100 µl aliquots of the supernatant were removed and poured into separate Eppendorf tubes. The contents of each tube were deproteinized by adding 50 µl of 10 % Trichloroacetic acid (TCA), vortexed for 1 min, and centrifuged at 10,500 RPM for 5 min at 9 °C.

From each deproteinized tube, 100 µl of supernatant was extracted, and poured into other Eppendorf tubes, along with 25 µl of Sodium Hydroxide (NaOH, 7.344% w/v) as a neutralizer was added, proceeding then to vortex for 1 min after technical specifications [28]. Neutralization of 10 % TCA with NaOH was measured and checked using pH paper strips.

4. Validation of the analytical method

This was validated by applying the percentage average recovery criteria and repeatability or

intra-test precision [31]. The procedure used to evaluate the average recovery consisted of spiking the homogenized samples of fresh cheese with aqueous standards of FA Baker® ACS reagent (Avantor™, Mexico) at concentrations of 0.30, 0.60, and 1.80 mg/kg (10, 20 and 60 µM, respectively). The addition of FA was carried out in triplicate for each of the three levels tested and the analysis was carried out according to the method described below using the same type of cheese, reagent kits, instruments, and the laboratory analysts during the four days of the trial, as specified to assess intra-assay precision [31].

The evaluation of the intra-run precision was based on the calculations of both the coefficient of variation of the average percentage recovery and the Horwitz Ratio (HORRAT) of spiked samples [31-33]. The acceptable range for the mean percent recovery of an analyte at a concentration equal to or less than 1.00 mg/kg is 80-110 %, as specified by AOAC International [31]. In contrast, the mean percentage recovery is the simple average of recovery values obtained per day and per con- centration of spiking [31]. The intra-assay precision of an analytical method is considered acceptable if it has a Horwitz %RSD not greater than 22.6 for an analyte present in the matrix at concentrations less than 1.0 mg/kg [31,32] and the values of the HORRAT must be between 0.3 and 1.3 [33].

5. Measurement of FA in samples

From each deproteinized and neutralized sample tube, two aliquots of 50 µl each were removed and transferred to two wells of the Corning® flat-bottom black polystyrene plate (Corning, USA), one of which is used as a sample blank.

The preparation of the working reagents and their volumes to be transferred to the wells of standards, samples, and the sample blanks, conform to the DFOR-100 kit manufacturer specifications (Bioassay Systems, Hayward, USA). The assay is based on FA derivatization with acetoacetanilide in the presence of ammonia [29].

The volume of each sample to be tested (50 µl) and the DFOR-100 reagents were mixed in the wells by rotary shaking for 30 min, as specified before [30], at room temperature and protecting the micro- plate from light. The measurement of FA in the samples was carried out by fluorescence intensity at excitation (370 nm) and emission (470 nm) wavelengths, using a Cytation 5 F BioTek® multimodal microplate reader (Winooski, USA). The limit of quantification of the test is 0.045 mg/kg or 1.5 µM [29].

6. Current regulations on presence of FA in dairy products

The regulations with global enforcement, understanding the general standard for food additives CXS 192–1995 [34] and the specific standards for cheeses CXS 283–1978 [16] and unripened cheeses CXS 221–2001 [35] do not authorize the use of FA as a preservative, therefore, this aldehyde should not be found in these dairy products and at detecting it demonstrates its undeclared and illegal use. This same condition is established in current Salvadoran regulations [18,36].

7. FA residuality test in milk and cheese

To carry out the trial, 37.84 liters (50 bottles) of raw milk were obtained from a herd located in Chalatenango, El Salvador. The total volume of milk was divided into 5 fractions of 7.57 liters each (10 bottles), one aliquot was left as a control and reactive grade FA (Avantor™, Mexico) was added to the other four in volumetric proportion to reach concentrations of 1, 5, 10, and 20 mg/kg, respectively. The contents of 10 and 20 mg/kg of FA added to milk are close to the range used in the manufacture of ripened cheeses (15 and 25 mg/kg) [3].

Milk with/without added FA was kept in plastic containers without refrigeration for 4 h until it reached the processing site, to recreate the usual conditions of collection and transport. At the laboratory, 100 ml of milk was extracted from each container and stored in the refrigerator to analyze FA content in duplicate the following day.

The remaining milk from each container was used to make fresh cheese on the same day of collection, according to the procedure described in a previous study [27]. The five batches of the final product were classified according to the amount of FA added: 0 1, 5, 10, and 20 mg/kg. Each 2-kg batch was divided into four 0.5-kg portions to test for FA in duplicate.

Residual FA analyses in cheese were performed on days 1, 2, 6, and 7 after the addition of the preservative, and it was measured in milk on days 1 and 5 after that treatment.

8. Statistical analysis

Statistically significant differences between mean FA levels and prevalence values per month were detected using Student's t and Chi- square tests, respectively. The homogeneity of the variances was demonstrated by applying Levene's test, establishing the level of significance at $p < 0.05$ for all tests. Association between variables was estimated using Pearson's correlation analysis. The statistical analyzes and the elaboration of the figures were carried out with the IBM Statistics v.27 program for Windows.

9. Ethical considerations

Both the consent of informants and the application of an animal experimentation guide were not required for this study, since that no tissues were removed from live animals and no information was extracted from the retailers, only samples of cheese for sale were obtained.

10. Results

10.1. Validation of the method to quantify FA levels in a pool of fresh white cheese samples

Values of the mean recovery percentages and the intra-test precision as validation parameters of the method to analyze FA are shown in Table 1. The average recovery for spiking greater than 0.30 mg/kg, obtained during the four-day trial, was better adjusted to the range settled as acceptable (80 % to 110 %). Mean recovery values did not vary significantly among the four days of the validation test or within the spiking concentrations ($F = 1.762$, 3 df, $p = 0.174$) neither mean FA contents ($F = 0.156$, 3 df, $p = 0.925$).

Regarding the coefficient of variation under repeatability conditions (%RSD), it showed an average range of values from 14.69 to 18.42, depending on the three spiking concentrations used. The %RSD calculated for spiking less than 1.00 mg/kg (1 ppm) did not exceed the limit value of 22.6 % established as acceptable; while the %RSD value for spiking greater than 1.00 mg/kg (1 ppm) did not exceed the acceptance limit value of 16 % (Table 1).

The %RSD values also did not vary significantly among the four days of the validation test or within the spiking concentrations (F 0.443, 3 df, p 0.723). HORRAT presented a range of values from 0.96 to 1.00 (Table 1), coinciding with the limits established between 0.3 and 1.3.

Table 1. Method performance parameters for FA in spiked pooled samples of fresh white cheese.

Spiked level µM (mg/kg)	Day 1 repeatability (n=3 per level)		Day 2 repeatability (n=3 per level)		Day 3 repeatability (n=3 per level)		Day 4 repeatability (n=3 per level)		Average of four-day trial		Predicted coefficient of variation under intermediate precision conditions	Ratio of average trial %RSD to RSD predicted from Horwitz equation (n=12 per level) [32-33]	
	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)[1]	PRSD (%)	HORRAT	Accepted values for HORRAT
10µM (0.30)	131.23	18.43	119.42	18.83	154.75	17.98	129.96	18.44	133.84	18.42	19.18	0.96	0.3 to 1.3
20µM (0.60)	98.32	17.35	116.96	16.93	119.46	16.83	107.45	17.11	110.55	17.05	17.28	0.99	
60µM (1.80)	86.98	15.01	106.65	14.51	112.58	14.39	90.67	14.86	99.22	14.69	14.65	1.00	

[1] Acceptable recovery percentages from 80% to 110%, and acceptable values of %RSD are ≤ 22.6 (for spiking concentrations < 1.0 mg/kg) and ≤ 16 (for spiking concentrations ≥ 1.0 mg/kg) after Horwitz [31-32].

10.2. Change in average content of FA in cheese samples according to annual seasons

Residual FA contents in the cheese samples taken during the study are presented in Table 2. Of the 412 samples collected, 135 (32.8 %) had quantifiable levels of FA (≥ 0.045 mg/kg). However, none of the 135 samples with quantifiable levels of residual FA exceeded the maximum tolerable concentration established at 2.6 mg/kg by oral route. The averages calculated according to the season were 0.137 ± 0.013 mg/kg (n = 42) and a maximum of 0.428 mg/kg for the late dry season, 0.181 ± 0.012 mg/kg and 0.385 mg/kg as a maximum for the transitional dry to rainy, 0.179 ± 0.023 with a maximum of 0.503 mg/kg for the rainy and 0.093 ± 0.036 for the early dry one, with a maximum of 0.129 mg/ kg (Table 2). In the transitional rainy to dry season, there was only one sample with a quantifiable level (0.249 mg/kg). The mean residual FA contents in the samples show an incremental variation from the dry to the rainy season, including the transition between both and another towards the decrease during the early phase of the dry season; however, this variability is not statistically significant (ANOVA, F = 1.768, 4 df, p = 0.139), so it does not seem to be associated with seasonal changes.

Table 2. Occurrence of FA contents in locally-made fresh white cheeses after surveyed year season.

Classification based on FA quantifiable contents	Sampled months by season				
	Late dry season (February to March, 2021)	Dry to rainy transitional season (April to May, 2021)	Rainy season (June to September, 2021)	Rainy to dry transitional season (October to November, 2021)	Early dry season (December, 2021 to January, 2022)
Non-quantified (<0.045 mg/kg)	27 (39.1%)[a]	14 (20.3%)[a]	103 (74.6%)[c]	67 (98.5%)[b]	66 (97.1%)[b]
Quantified (≥ 0.045 mg/kg)	42 (60.9%)[a]	55 (79.7%)[a]	35 (25.4%)[c]	1 (1.5%)[b]	2 (2.9%)[b]
Exceeding the maximum tolerable concentration orally (2.6 mg/kg)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Mean ± S.E.M. (mg/kg)	0.137 ± 0.013	0.181 ± 0.012	0.179 ± 0.023	N.C.	0.093 ± 0.036
Range of quantified contents (mg/kg)	0.047 to 0.428	0.053 to 0.385	0.046 to 0.503	0.249	0.057 to 0.129
Sample size	69	69	138	68	68
FA contents in milk samples from dairy processing plants as a reference.	0.620 (n=1)	0.515 (n=1)	0.523 ± 0.020 (n=3)	0.157 ± 0.064 (n=2)	0.067 ± 0.014 (n=2)
Meteorological parameters					
Average temperature °C	27.5	28.3	26.9	26.7	26.5
Average relative humidity %	64.7	71.9	80.6	76.1	69.3
Average cumulative rainfall (mm)	3.9	115.3	299.9	84.2	3.5

[a, b, c] Counts and percentages with distinct letters differ significantly among the same FA level classification group per season of a year ($p<0.05$, Pearson Chi Square test). Salvadoran fresh white cheese samples, n=412.
Cumulative rainfall, temperature, and relative humidity data are the averages of the sampled months.
N.C. Not calculated

10.3. Seasonal variation of the occurrence of cheese samples with quantifiable levels of FA

The percentage of cheese samples that exceed the quantification limit of FA (> 0.045 mg/kg) and the characteristic meteorological parameters according to the time of year are shown in Table 2. Prevalence of residual FA positivity varied in a statistically significant way according to the year's season (Pearson $\chi 2 = 154.88$, 4 df, $p < 0.001$). The highest values were recorded in the late dry season (60.9 %, n = 42) and in the dry to the rainy season (79.7 %, n = 55), characterized by having the highest temperature records of 27.5 ◦C and 28.3 ◦C, as well as the lowest values of relative humidity (64.7 % and 71.9 %, respectively). Residual FA occurrence decreased to 25.4% in the rainy season (n = 35) and diminished in the rain to dry transition (1.5 %, n = 1) and in the early dry season (2.9 %, n = 2), characterized by temperatures of 26.9 ◦C, 26.7 ◦C, and 26.5 ◦C, respectively, as well as the highest records of relative humidity 80.6 %, 76.1 %, and 69.3 %, respectively (Table 2).

10.4. Association between the occurrence of cheese samples with detectable levels of FA and meteorological parameters A correlation analysis was performed to determine the significance of the coincidence between occurrence values greater than 60% of residual FA-positive samples and the highest temperature records, along with prevalence values below 25.5 % with the lowest average temperatures (Table 2). It was possible to show that the prevalence of samples with detectable levels of FA and temperature are significantly associated ($r2 = 0.796$, $F = 11.681$, 4 df, $p = 0.042$) so that 79.6 % of the variability of the data of positive samples is explained by the change in average temperature (Fig. 1). Conversely, it was not possible to demonstrate the statistically significant association between the number of samples positive for FA with the relative humidity % ($r2 = 0.019$, $F = 0.059$, 4 df, $p = 0.824$) or with the average

accumulated rainfall (r2 = 0.083, F = 0.272, 4 df, p = 0.638).

11. Residual contents of added FA to milk to make cheese

The results of FA residuality test in cheese and milk are shown in Table 3. After adding 20 mg/kg to the milk to be processed, FA could be quantified in the cheese samples up to 6 days after manufacture. In the case of the 10 mg/kg addition, FA was possible to quantify only for the first day. None of the other FA addition levels produced quantifiable values in cheese (Table 3). In the case of milk, FA could be quantified on days 1 and 5 after addition, regardless of the level used (Table 3). Residual FA content presented a statistically significant decrease with respect to the storage time of cheese made with milk treated with 20 mg/kg ($F=1669.462$, 2 df, $p < 0.001$, Table 3). A similar trend in relation to storage time was observed in FA contents of milk added with 1 mg/kg ($t = 16.075$, 2 df, $p < 0.05$) and with 5 mg/kg ($t = 11.784$, 2 df, $p < 0.05$). In contrast, FA levels in milk treated with 10 and 20 mg/ kg did not show significant changes during storage time (Table 3). Data obtained from the validation test of the method, from analytical procedures, and the records of meteorological parameters that support the results presented in this study, are available at the Mendeley Data site: https://data.mendeley.com/datasets/ym29rnzf94/2 [37].

Table 3. Results from FA residually trial in self-making fresh white cheese and raw cow milk.

Added FA level (mg/kg)	Residual FA content in fresh white cheese (mg/kg)				Residual FA content in raw cow milk (mg/kg)	
	Day 1 after FA addition into milk (n=3 per level)	Day 2 after FA addition into milk (n=3 per level)	Day 6 after FA addition into milk (n=3 per level)	Day 7 after FA addition into milk (n=3 per level)	Day 1 after FA addition into milk (n=2 per level)	Day 5 after FA addition into milk (n=2 per level)
0	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.
1	N.Q.	N.Q.	N.Q.	N.Q.	0.727[a]	0.362[b]
5	N.Q.	N.Q.	N.Q.	N.Q.	2.579[a]	1.295[b]
10	0.441	N.Q.	N.Q.	N.Q.	4.057[a]	3.564[a]
20	4.100[a]	1.013[b]	0.101[c]	N.Q.	9.360[a]	9.958[a]

N.Q.: Non-quantified

[a, b, c] Means with distinct letters differ significantly among the same spiked FA level group per day of trial in cheese or milk ($p<0.05$, T-test). Self-made fresh white cheese samples (n=60), and raw cow milk samples (n=20).

12. Discussion

In general terms, the acceptance requirements for the validation of the method to quantify formaldehyde in samples of fresh cheese were met, thus demonstrating its efficiency. For the recovery percentage criteria, averages obtained during the 4-day trial are consistent with those obtained with other methods developed to determine FA in foods, including dairy products [38]. In addition, they adjusted acceptable values for overloads lower (0.60) and higher (1.80) than 1.00 mg/kg (80 %– 110 %) [31]. For the repeatability parameter, the calculated averages of %RSD at the concentrations of 0.30 and 0.60 mg/kg did not exceed the maximum established at 23 % [32], nor did the %RSD set at 16 for the concentration of 1.80 mg/kg [31], denoting the reasonable precision of the method to measure FA in fresh white cheese. Residual FA levels quantified in Salvadoran cheese samples are similar to those reported by other authors from Italy [3] and South Korea [38] (Table 4). There is also similarity between the residual FA contents

in milk quantified in this study and those detected by other authors in Finland [42], Canada [43] and South Korea [38] (Table 4). Regarding the two-thirds of cheese samples negative for FA found in this work, other researchers also found no traces of FA in more than 120 samples of milk and its derivatives from Bangladesh and Egypt [Table 4], even though the analyzes of FA were performed with the High-Performance Liquid Chromatographic method, with detection limits lower than 0.400 mg/kg [44] and 0.020 mg/kg [45], respectively. On the other hand, the quantified levels of residual FA in both cheese and milk in this study, and those reported by most other authors [Table 4] do not exceed the maximum orally tolerable concentration of 2.6 mg/kg [10,13]. However, the potential toxicity of FA ingested by humans through dairy products should not be underestimated [1], nor the deterioration of the nutritional value caused by FA added to milk and its derivatives [14]. The presence of FA in the cheese and in the milk sampled at the plant would indicate its undeclared and illegal addition to prolong its shelf life and prevent economic losses, either during transport, usually without refrigeration [5,9], and/or in processing [4,39]. Detection of residual FA in milk demonstrates non-compliance with the general standard for feed additives CXS 192–1995 [34] which does not authorize the use of FA as a preservative, therefore, this aldehyde should not be found in these dairy products and at detecting it demonstrates its undeclared and illegal use. In the artisanal processing phase of raw material, the reasons for FA addition is that, the milk must rest for 4–6 h to skim it naturally [27]. Additionally, milk coagulation is faster if it is carried out at warm room temperature [27]. This fraudulent practice of adding FA was found in one of every three products sampled and analyzed in this study which does not comply with international [16,35] and local standards for cheese [18,36]. Additionally, the legality as mentioned above is a reason for concern for the Salvadoran government agency that regulates the quality of dairy products consumed by the population [25,26].

Table 4. FA contents in cheese and/or milk as reference values, after report's year and country.

Year and location	No. samples	Product	FA contents (Range and mean ± SD, mg/kg)	Condition of FA contents	Reference
1982, Finland	4	Raw cow milk	0.200 (SD not specified)	Naturally occurring	42
1992, Italy	N.D.	Grana Padano cheese	0.500 (single value)	Residual	3
1993, Canada	18	Fresh cow milk	0.013 up to 0.057, 0.027 ± 0.007	Naturally occurring	43
	12	Processed cow milk	0.075 up to 0.255, 0.164 ± 0.057	Residual	
2015, South Korea	3	Cheese	0.027 ± 0.001	Unspecified	38
	3	Mozzarella cheese	0.057 ± 0.002	Unspecified	
	3	Cheese stick	0.182 ± 0.022	Unspecified	
	3	Cow milk	0.054 ± 0.007	Naturally occurring	
	3	Processed cow milk	0.044 ± 0.005	Unspecified	
2016, Bangladesh	7	Raw cow milk	No detectable (< 0.400)	---	44
	10	Whole cow milk	No detectable (< 0.400)	---	
	14	Processed cow milk	No detectable (< 0.400)	---	
2018, Bangladesh	5	Cow milk	5.200 ± 3.500	Naturally occurring	39
	20	UHT cow milk	58.700 ± 6.600 up to 187.700 ± 3.100	Residual	
2018, Egypt	90	Cow milk, cheese, and yogurt	No detectable (< 0.010 for milk and < 0.020 for diary)	---	45
2021-2022, El Salvador	135	Fresh white cheese	0.046 up to 0.503, 0.166 ± 0.101	Residual	This study
	9	Raw cow milk	0.053 up to 0.620, 0.350 ± 0.233	Residual	

N.D. Not determined

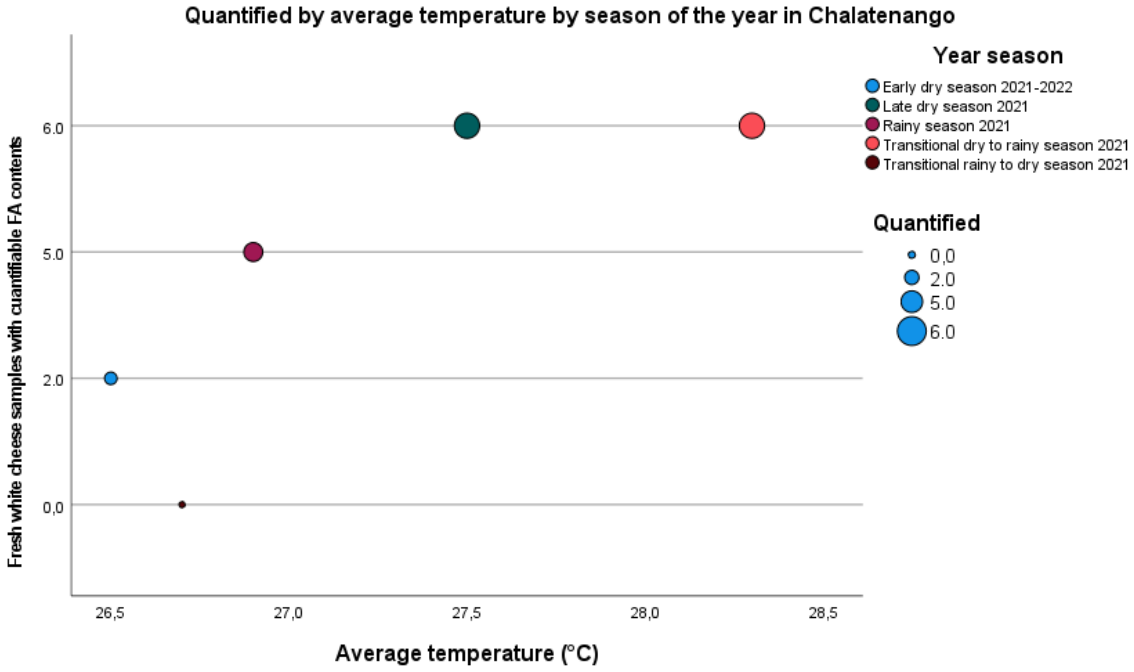
Accumulated percentage of samples with quantifiable levels of residual FA in the 12 months of monitoring, calculated at 32.8 % is lower than the averages obtained in samples collected from fresh cheese in dairy processing plants in the eastern zone of Honduras (44.4 %) and in resale stalls in markets of the Pacific region of Nicaragua (92.6 %) during the dry and transitional dry to rainy seasons [20,21]. Specifically, local prevalence of samples positive for FA in the same periods is also high (> 60 % and 79 %, respectively) close to the value reported for the Nicaraguan product [20]. Other authors found that the use and quantity of FA to preserve dairy change with the season of the year [3], especially in the summer [40] when the ambient temperature is higher.

Both residual FA levels quantified in collected milk from processing plants, and the largest number of positive samples during the late dry season and its transition to the rainy season, as well as the significant association with ambient temperature, would indicate that the presumed addition of FA aims to prevent milk deterioration due to the prevailing high temperatures. This fraudulent practice has been reported by other authors [9] and FA is detected in the dairy product due to its residual effect [3,40]. The decreased tendency of FA contents added with storage time observed in this work has been previously described for both fresh [40] and matured [3] cheeses. In this particular case, the addition of FA to milk could be detected in the manufactured cheese up to a maximum of 1 day when 10 mg/kg was used and up to 6 days when 20 mg/kg was added. A previous trial described that FA added to milk used to make Domiatti cheese was difficult to detect after a period between 2- and 4-days post-production [40]. The decrease in residual FA content in milk used to make fresh cheese, after 5 days of being added with the illegal preservative, provides more evidence of the trend described in cheese, specifically at levels equal to or less than 10 mg/kg (Table 3). Based on residuality results, it is safe to assume that the levels of FA added for milk preservation would be between 10 and 20 mg/kg. These levels are equivalent to 1 or 2 tablespoons per 160 kg barrel (5–10 ml of FA 37% v/v), similar to the volumes added to milk by cheese producers in Italy and Egypt, presented in two previous studies [3,40].

		Location			
		Chalatenango - El Salvador			
		Quantified			
	Average Temperature (°C)	0,0 Count	2,0 Count	5,0 Count	6,0 Count
Early dry season 2021-2022	26,5	0	1	0	0
Late dry season 2021	27,5	0	0	0	1
Rainy season 2021	26,9	0	0	1	0
Transitional dry to rainy season 2021	28,3	0	0	0	1
Transitional rainy to dry season 2021	26,7	1	0	0	0

1: Marks the count of FA quantified in a give season

Another plausible assumption is that the proportion of cheeses with quantifiable levels of residual FA is greater than the third part found in this monitoring, because it could not be detected, either because of the amount added or because of the time elapsed prior of analysis. Illegal use of FA as a preservative during the transport and processing of milk to make cheese is likely more widespread than it seems. Considering that El Salvador has a fragmented dairy production system and the conservation by cooling of the raw material is rudimentary, it seems likely that FA is being used illegally as a preservative, given these previously identified conditions of vulnerability [5,24]. The intensification of government monitoring of imported and national products as of January 2022 [41] seems to be deterring those responsible for the illegal addition of FA to milk and its derivatives, since during the additional samplings in February and March no product, nor raw material, detected positive for FA (Data not shown but available at https://data.mendeley.com/datasets/ym29rnzf94/2) [37].



13. Conclusions

The analytical method adapted for the detection and quantification of FA in cheese samples is efficient, based on the values obtained for recovery and precision of the assay. On average, a third of the fresh cheeses sampled on the shelf have quantifiable levels of residual FA, presenting the highest prevalence during the two seasons of the year with the highest temperature records, similar to the results obtained in other Central American countries. The quantified levels of residual FA in Salvadoran cheese and milk are comparable to those found in similar products from other countries, although none of the quantified levels of FA exceeded the maximum tolerable concentration of 2.6 mg/kg by oral route, established by the World Health Organization. Residual FA levels found in raw milk sampled at the processing plant and

the significant association between the proportion of positive cases and ambient temperature constitute evidence that FA was used to prevent heat deterioration, either of the raw material and/or of the product available on the shelves. Under artisanal dairy processing conditions, the extension of milk's shelf life by adding FA would be required to complete the skimming and coagulation stages of the milk at warm room temperature. Following the previous argument, it was shown that residual FA contents decrease in both milk and fresh cheese. This tendency to decrease appears to depend on the levels of preservative used and the time elapsed from addition to analysis. Considering the experimental evidence, it is estimated that levels between 10 and 20 mg/kg of FA are added to preserve milk destined for the manufacture of fresh cheese. In any case, the mere presence of quantifiable FA in the analyzed samples violates international and local standards for fresh cheese, with the connotation that it is a product frequently consumed by the Salvadoran population.

14. Funding The study was financed exclusively with research funds from Universidad Doctor Andrés Bello.

CRediT authorship contribution statement O. Pena-Rodas: ˜ M. Pineda-Rivas: Conception and design of study, Acquisition of data (laboratory or clinical). M. Guzman-Rodriguez: Acquisition of data (laboratory or clinical). R. Martinez-Lopez: Conception and design of study, Acquisition of data (laboratory or clinical). R. Hernandez-Rauda: Conception and design of study, Data analysis and/or interpretation, Drafting of manuscript and/or critical revision, Approval of final version of manuscript. Declaration of Competing Interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
Data availability Datasets generated and analyzed during the current study are available at the Mendeley Data site: https://data.mendeley. com/datasets/ym29rnzf94/2.

Acknowledgments This work is dedicated to the memory of Mr. Arturo Perla Ferrufino, former Mayor and renowned dairy producer of Jocoro (Province of Morazan), who encouraged the authors to carry out this research. The authors express their deep gratitude to Ms. Tania Gonz´ alez, Mrs. Marcela Doradea, Mrs. Alejandra Varela-Aparicio, Mr. Josue Monterroza, Mr. Juan Escuintla and Mr. Samuel Cano for their assistance in collecting cheese samples. They would also like to thank Mr. Mario Rivas and Ms. Rosa María Araujo for their valuable support in the collection and statistical processing of the meteorological parameters of the databases of the Observatory of Threats and Natural Resources of the Ministry of Environment of El Salvador.

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