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ORIGINAL ARTICLE



Carbonylation induced by antibiotic and pesticide residues on casein increases its IgE binding and allergenicity

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ABSTRACT

This work aimed to evaluate the effect of carbonylation induced by tetracyclines, β -lactams, fluoroquinolones, and pyrethroids in caseins of bovine origin on their immunoreactivity and allergenicity. Using a spectrophotometric method, ELISA, dot-blot, and an IgE-mediated milk allergy mouse model, we confirmed that antibiotics and pesticides at their maximum residue limit, promoted the *in vitro* carbonylation of caseins (among 5.0 ± 0.01 and 67.5 ± 0.70 nmol of carbonyl/mg of protein); furthermore, carbonylations greater than 19 nmol significantly increase the *in vitro* IgE immunoreactivity of caseins (average OD among 0.63–1.50) regarding the negative control (average OD: 0.56). On the other hand, sensitized mice exposed to oxidized caseins showed increased clinical scores (2–5), positive skin tests, and footpad swelling (0.28–0.59 mm) compared to the negative control (1–2; negative skin tests; 0.1 mm, respectively), denoting increased allergenicity. These results suggest that casein carbonylation increases their IgE immunoreactivity and allergenicity, a fact that could be explained by the resistance to the digestion promoted by carbonylation and by conformational changes in the random coil casein structure, which can expose cryptic epitopes or neoepitopes.

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Introduction

Antibiotics and pesticides are among the substances most consumed in livestock farming systems (LFSs) worldwide [1,2]. The first is generally used for disease treatment in livestock and in subtherapeutic levels as a supplement of concentrated animal feed for growth promotion, improved feed conversion efficiency, and the prevention of the diseases [1]. Molecules belonging to the tetracyclines (TCs), β -Lactams (β -LTs), and fluoroquinolones (FQs) families constitute the most used in livestock farming [3] to treat or prevent bovine mastitis, or as growth promoters [3,4]. On the other hand, pesticides are widely used to control external pests and ectoparasites that affect the animals' health and production [5], being pyrethroids (PTDs) and organophosphates the most consumed currently [5,6].

Nowadays, antibiotic consumption profiles in developing countries are heavily influenced by local

regulatory systems, which are generally very faulty, promoting the heavy abuse and misuse of antibiotics due to their over-the-counter availability through unregulated supply chains, as well as over-the-counter purchase [1]. According to Sachi et al. [3], the global use of antibiotics in veterinary is twice that used in humans, reaching 63.151 ± 1.560 tons by year worldwide only in LFSs, while the global consumption of PTDs follows the same trend, given their less toxicity in non-target organisms compared to organophosphates [7].

The irrational use of antibiotics and pesticides in LFSs promotes the appearance of their residues in tissues and edible fluids of animals such as milk, which can reach the final consumer if their withdrawal times are not respected [8–10].

One of the consequences of consuming milk contaminated with residues of antibiotics and pesticides is the appearance of allergic responses. Food allergies are

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currently recognized as a public health problem that affects up to 10% of infants in some countries, and its incidence has increased over time [11]. Among them, cow's milk allergy (CMA) is one of the most common in early life, with an estimated prevalence in developed countries that varies from 0.5% to 3% at the age of one year, and symptoms range from mild to severe (urticaria, wheezing, acute angioedema and anaphylaxis) [12].

Bovine caseins (CASs), the main protein fraction of cow's milk, represent about 80% of the total milk protein content and have been identified as the main allergens in this food [13–15]. CASs are a heterogeneous group of macromolecules that evolved from calcium ion-secreting phosphoproteins [16] and form colloidal spherical complexes, or micelles made up of 92% protein and 8% inorganic salts (mainly calcium phosphate) [17].

In human nutrition, CASs are significant sources of essential amino acids and bioactive peptides with demonstrated antibacterial, antiviral, antifungal, antioxidant, antihypertensive, antithrombotic, and immunomodulatory properties as β -casomorphins, which have actions pain reliever and tranquilizer like opiates [18]. CASs also have a high added value in the dairy industry and are the most significant milk component, being responsible for the textural, sensory, and nutritional properties of cheese, juices, infant formulas and, yogurt [17–20]. Therefore, any change that affects the structure and physicochemical properties of these proteins could pose a significant risk for industry and health issues of consumers due to the high global demand for dairy products. In this sense, CASs may undergo oxidation reactions, such as carbonylation of amino acids during processing, storage [21–23], and due to the effect of the environment (UV radiation [24], the presence of contaminants [25,26], etc.).

Protein carbonylation (PC) is an irreversible and non-enzymatic post-translational modification induced by oxidative stress and other mechanisms [27], this is defined as the introduction of reactive carbonyl groups (mainly aldehydes and ketones) into the protein structure [28], and due to its high stability and abundance, it is the most accepted biomarker for assess irreversible oxidative stress in food proteins [26–29]. PC includes chemical modifications that occur through different reaction mechanisms, including: (i) direct oxidation of several amino acids by reactive oxygen species (radical hydroxyl ion, superoxide radical ion, hydrogen peroxide, etc.); (ii) Michael addition reactions with cysteine, histidine, and lysine residues on proteins as a product of its interaction with carbonyl species derivate from

lipid peroxidation [4-hydroxynonenal (4-HNE), 2-propanal (acrolein), and malondialdehyde (MDA)], and finally, (iii) glycoxidation reactions between the amino group of lysine with reducing sugars or their oxidative products [29,30].

We recently demonstrated the effect of residues of antibiotics and pesticides such as TCs and PTDs on CASs carbonylation, even at concentrations thought to be safe for the frequent consumer [31,32]. Despite the demonstrated susceptibility of CASs to carbonylation, few and ambiguous studies report the impact of this irreversible oxidative damage on its allergenicity [33].

In this study, we assessed the effect of carbonylation induced by antibiotics and pesticides on the *in vitro* and *in vivo* allergenicity of bovine CASs through immunoenzymatic assays as well as using a validated IgE-mediated food allergy mouse model.

Materials and methods

Milk samples

Raw milk samples were obtained from healthy cows (Holstein-brown) from Arjona town (Bolívar, Colombia). Animals were not administered antibiotics and pesticides for longer than their withdrawal time to guarantee abstinence from assayed these substances in milk. Samples were taken under aseptic conditions, carried to the laboratory, ensuring the cold chain, and stored at -20°C until use. Samples were aliquoted to reduce harmful effects on protein stability and carbonyl group formation due to continuous thawing-freezing steps [31].

In vitro oxidation of milk proteins

CASs carbonylation was induced by spiking the samples with the contaminants; these groups of samples were called treatments, which consisted of raw milk samples individually spiked with four β -LTs [ampicillin (AMP), benzathine penicillin G (PNG), oxacillin (OXA), and dicloxacillin (DCL)], four TCs: oxytetracycline (OTC), chlortetracycline (CTC), tetracycline (TC), and doxycycline (DXC)], two FQs [danofloxacin (DANO) and sarafloxacin (SARA)], three PTDs [cypermethrin (CIP), fenvalerate (FEN), and flumethrin (FLU)], and samples spiked with the following combinations of TCs and PTDs (OTC + FEN; OTC + FLU; TC + FEN; TC + FLU; CTC + FEN; CTC + FLU; DXC + CIP; DXC + FEN; DXC + FLU). The treatments were prepared by adding to 2 mL of raw milk an appropriate aliquot of a stock solution of each contaminant (10 mg/L) to reach their

Table 1. Maximum residue limit concentrations established by the European Commission in bovine milk for the antibiotics and pesticides assayed in this study.

Family	Substance	MRL ($\mu\text{g/L}$)
Antibiotics	OTC	100
	TC	
	CTC	
	DXC	
β -LTs	AMP	4
	PNG	
	OXA	
	DCL	
FQs	DANO	30
	SARA	100
Pesticides		
	CIP	20
	FEN	30
PTDs	FLU	40

maximum residue limit in milk described in Table 1. A total of 22 treatments were assayed by triplicate.

In the assay were used two controls, the first was a milk sample free of contaminants (negative control-NC-), and the second was a sample spiked with 10 mmol/L ferrous sulfate (FeSO_4) in PBS as a free radical generating system (positive control-PC-). We included the vehicle (saline buffer PBS) in the controls. All samples were incubated for 40 min at 4 °C in darkness.

Caseins extraction and quantification

Bovine milk casein fraction was extracted as described in Marrugo et al. (2020). Briefly, 2 mL of milk sample was skimmed by centrifugation ($3000 \times g$, 2 °C, 30 min), and skim milk was adjusted to pH 4.6 for casein precipitation. The sample was centrifuged again ($3000 \times g$, 4 °C, 5 min), and the pellet containing CASs was washed with acetic acid (pH 4.5) to remove co-precipitated impurities. Samples were centrifuged and reconstituted in 6 mL of ELISA coating buffer (carbonate-bicarbonate, pH 9.6).

CASs extracted were quantified by the Bicinchoninic acid (BCA) assay kit using bovine serum albumin (BSA) as standard in a linear dynamic range between 0.0 and 1000 $\mu\text{g/mL}$ (Pierce BCA Protein Assay, Thermofisher, Waltham, MA, USA). For this, BCA solutions were prepared by mixing 50 parts of reagent A (BCA, sodium carbonate, sodium bicarbonate, bicinchoninic acid, and sodium tartrate in 0.1 M sodium hydroxide) with one part of reagent B (CuSO_4 , 4%); 200 μL of BCA solutions were added onto 96-well plate with 25 μL of samples or BSA standard solutions (ratio of 8:1), the mixtures were incubated at 37 °C for 30 min, and then, their absorbance at 562 nm was determined. CASs' concentrations were determined by interpolating the BSA curve [34].

Measurement of carbonyl index

The carbonyl groups induced by contaminants on CASs were determined by a spectrophotometric method, as reported by Mesquita et al. [35]. Briefly, 300 μL of 2,4-dinitrophenylhydrazine DNPH (10 mmol/L) in ortho-phosphoric acid (0.5 mol/L) was added to 300 μL of CASs solution (0.5 $\mu\text{g}/\mu\text{L}$) and incubated in darkness (25 °C, 10 min). Then, 160 μL of the mixtures were added 40 μL of 6 mol/L sodium hydroxide (NaOH) and incubated for 10 min at room temperature in a micro-titer plate. The optical density (OD) was read at 450 nm using a carbonate-bicarbonate buffer as blank. Carbonyl group concentration was expressed as carbonyl index (CI), considering the molar extinction coefficient of DNPH corrected for microplates ($11,154 \text{ mol}^{-1} \text{ cm}^{-1}$). Due to the instability of dinitrophenylhydrazones in an alkaline medium, incubation time after NaOH addition was rigorously controlled.

In vitro antigenicity of carbonylated CASs

IgE binding capacity of spiked and nonspiked CASs with antibiotic and pesticide residues was evaluated by indirect ELISA. Wells were coated with 100 μL of casein samples (0.0125 $\mu\text{g}/\mu\text{L}$) in a carbonate-bicarbonate buffer pH 9.6 for 1 h at 37 °C. Unbound proteins were removed by washing, and blocking solution (5% v/v equine serum in saline phosphate buffer pH 7.4) was added for 2 h at 37 °C. Wells were incubated with IgE-containing sera from milk allergic patients overnight at 4 °C. As a secondary antibody, an anti-human IgE Alkaline Phosphatase conjugated monoclonal antibody (Sigma Aldrich) (1:2000 dilution in 3% v/v equine serum in saline) was used for 2 h at 37 °C. We used p-nitrophenyl phosphate disodium (100 μL , 1 $\mu\text{g}/\mu\text{L}$) as a substrate, and the reaction was stopped with ethylenediaminetetraacetic acid (0.1 mol/L). The OD was measured at 405 nm [36].

Nonoxidized and oxidized CASs with a pro-oxidant medium (10 mmol/L FeSO_4 in PBS) were negative and positive controls, respectively. An additional control consisted of sera from nonatopic patients with no IgE or a history of food allergy.

Fifteen patients sera-containing IgE were previously obtained and thoroughly characterized. Patients were diagnosed as milk allergic according to compatible clinical history, positive skin prick test (SPT) with commercial milk allergen (Allergo Pharma, Buenos Aires, Argentina), elevated total serum IgE, and milk-specific IgE performed by EAST [13], and positive diagnostic elimination diet. These patients showed symptoms associated with milk exposure. The Ethics Committee of

the Children's Hospital of La Plata (Buenos Aires, Argentina) approved the protocols (#389-2014 and #389-2018), and patients or their parents gave written informed consent.

Identification of reactive epitopes on carbonylated CASs

To identify epitopes that could have been modified by the carbonylation induced by TCs, β -LTs, and PTDs, we assessed their IgG recognition by dot-blot, using the methodology described by Curciarello et al. [36]. Briefly, 1.0 μ g of oxidized CASs was spotted by quadruplicate on polyvinylidene fluoride membrane (Immun-Blot® PVDF Membranes for Protein Blotting) and then blocked for 2 h at 37 °C with 3% v/v equine serum in Tris-Buffered Saline pH 8.2 (TBS). An α_{s1} -casein-specific monoclonal antibody 1D5 mAb (1:500) or cow's milk-specific rabbit polyclonal antiserum (1:1000) were used as primary antibodies for 2 h at 37 °C, followed by the appropriate conjugated secondary antibody: biotinylated goat anti-mouse immunoglobulin G (1:1000, Chemicon Int., Temecula, CA, USA) and biotinylated mouse monoclonal antibodies specific to rabbit immunoglobulins (1:1000, Sigma-Aldrich, St Louis, MO, USA), respectively. Then, streptavidin-HRP (1:3000) was added for 1 h at 37 °C. Spots were visualized with 4-chloro-1-naphthol. Image J software was used to compare spot intensities between negative control and spiked samples.

The monoclonal antibody (mAb) specific for α_{s1} -casein (1D5) and the milk-specific antisera were obtained immunizing mice and rabbits, respectively, with skimmed milk [37].

In vivo assessing of allergic reactions promoted by carbonylated CASs

Animals

Six to eight-week-old male mice were obtained from the School of Animal Sciences, University of La Plata (UNLP), Argentina. These were kept under laboratory conditions that guaranteed a pathogen-free environment (20 °C, 70% relative humidity, and a 12-/12-h light/dark cycle), mice were fed with water, and a commercial diet provided *ad libitum*.

Sensitization and treatment

The first step for developing the *in vivo* assay was the sensitization of the mice; a sensitized mouse presents a high clinical score when challenged orally with an allergen (in our case, cow's milk). Mice were sensitized, as

previously described by Candreva et al. [37]. The Institutional Committee for the Care and Use of Laboratory Animals at the School of Sciences UNLP (CICUAL-FCE, Argentina) approved the protocol (protocol # 017–10-15). Briefly, animals received six intragastric (i.g.) doses of 20 mg of cow's milk protein (CMP), one dose per week, administrated as homogenized commercial nonfat dry milk, plus 10 μ g of cholera toxin (CT) (Sigma Aldrich, St. Louis, MO, USA) in a final volume of 200 μ L of 125 mmol/L bicarbonate buffer pH 9.0. Before oral sensitization, mice were fasted (1 h) and administered 200 μ L of ammonium bicarbonate (0.125 mol/L) to increase gastric pH (30 min). Seven days after the final boost, sensitized mice were challenged with 10 mg of CMP to corroborate that they were allergic, evaluating the triggered clinical signs described in section 2.6.3. Next, we carried out challenges with the purified oxidized CASs (1.2 mg of protein in 125 mmol/L ammonium bicarbonate buffer), and we scored the symptoms. Mice were divided into control ($n=4$) and treated groups with oxidized milk proteins ($n=4$ by each protein assayed – 44 mice in total). Finally, a cutaneous test was performed on the last day of the assay.

Clinical signs

In the following 30 to 45 min to the oral challenge, clinic signs were observed in a blinded fashion by two independent researchers. Clinical scores were assigned according to the table shown in Figure 3(a).

Cutaneous test

Mice were intradermally injected with CMP (20 μ g in 20 μ L of sterile saline) or oxidized CASs (10 μ g in 20 μ L of carbonate-bicarbonate buffer pH 9.6) in one footpad, and nonoxidized CASs in the contralateral footpad as a negative control, followed by an intravenous injection of 100 μ L of 0.1% w/v Evans blue dye (Anedra, Buenos Aires, Argentina). A few minutes after the injection, the blue color in the footpad was considered a positive cutaneous test. The footpad swelling was measured with a digital micrometer with a minimum increment of 0.01 mm [38].

Statistical analysis

The data were expressed as the mean $\pm$ SD. Statistical analyses were performed using the GraphPad Prism 8 software and ANOVA, followed by multiple comparisons between spiked samples and the blank, using Dunnett post-test. In all tests, $p < 0.05$ was considered

statistically significant. A simple linear regression ($y = b + mx$) model was used to evaluate carbonylation (fixed effect) on *in vitro* and *in vivo* allergic reactions.

Results

Casein carbonylation

The oxidation promoted by the contaminants assayed on CASs was evaluated as total carbonylation and expressed as carbonyl index (CI) in nmol of carbonyls/mg of protein. These results are shown in Figure 1(a) and were calculated considering the non-carbonylated protein or negative control CI (NC) (4.0 ± 0.06 nmol of carbonyls/mg of protein). All residues of antibiotics and pesticides assayed induced an increase of the CI on caseins. Nonetheless, none of the treatments induced a CI greater than the positive control (PC) (94.0 ± 4.00 nmol of carbonyls/mg of protein).

We found a heterogeneous oxidative behavior in all groups of the contaminants, or even within members of the same family, except for FQs, which showed a similar oxidant power, reaching a CI around 13.83 ± 0.23 nmol of carbonyls/mg of protein.

Our finding showed that PTDs pesticides and their combination with TCs produced the highest increases on CI, with maximum values of 67.0 ± 0.60 and 45.3 ± 1.45 nmol of carbonyl/mg of protein, corresponding to CASs treated with FLU and FLU + CTC, respectively. These values account for 17- and 11-fold higher carbonylation compared to the untreated CASs. By contrast, CTC showed the lowest oxidative effect on this fraction with a CI of 5.1 ± 0.07 nmol of carbonyl/mg of protein.

In all contaminant families, the carbonylation level promoted on CASs increased as a function of their degree of lipophilicity, being those with the highest partition coefficient (logP) into each family (apolar

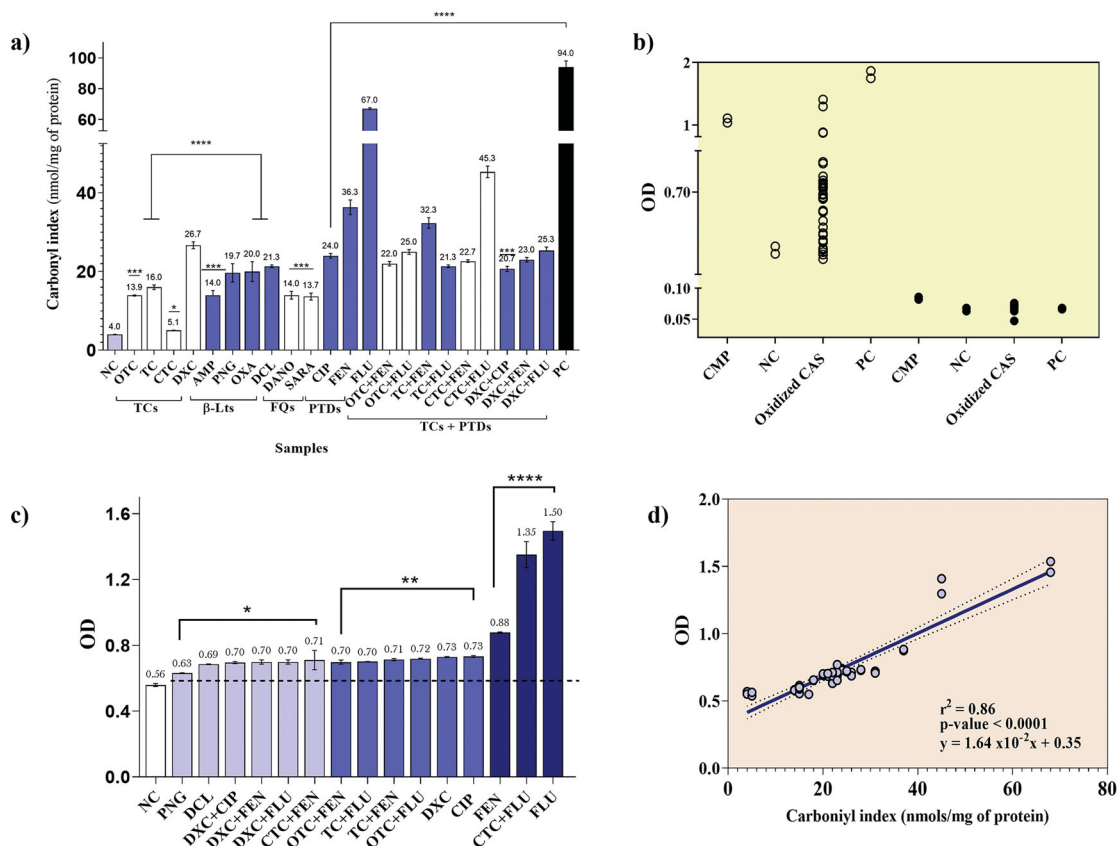


Figure 1. (a) Carbonyl index corresponding to oxidized caseins. Samples were grouped according to the nature of the residue used to conjugate to caseins. Data are expressed as the mean in nmol of carbonyls/mg of protein $\pm$ SD ($n = 2$). (b) IgE binding of oxidized caseins: Optical density corresponding to IgE recognition assessed by indirect ELISA. Wells were coated with caseins and incubated with sera from allergic (open circles) and nonallergic patients (dark circles). NC and PC denote negative and positive controls of nonoxidized and oxidized casein, respectively; (c) Optical density corresponding to IgE recognition of individual casein fractions. **** $p < 0.0001$, *** $p < 0.005$, ** $p < 0.01$ and * $p < 0.05$ denote statistically significant differences; (d) Linear correlation analysis between carbonyl index and OD corresponding to IgE binding.

molecules) exhibited the strongest oxidant effect. In this sense, DXC (logP -0.72), DCL (logP 3.19) and FLU (logP 7.3), with the highest lipophilicity from TCs, β -LTs, and PTDs, respectively, exhibited the highest carbonylation, with CIs values of 26.7 ± 0.88 ; 21.3 ± 0.33 and 67.0 ± 0.58 nmol of carbonyl/mg of protein, respectively (Figure 1(a)).

The correlation between the logP and the carbonylation effect can be better appreciated in spiked samples with PTDs: CIP, FEN, and FLU, with logP values of 6.0 , 6.2 , and 7.3 , respectively, which exhibited CI of 24.0 , 36.3 , and 67.0 nmol of carbonyl/mg of protein. In turn, TCs, with logP between -0.13 and -0.99 , and β -LTs, with logP between 0.88 and 3.19 , showed reduced carbonylation induction on CASs, compared to PTDs.

Finally, we found that PTDs induced higher carbonylation on CASs than TCs, β -LTs, and FQs. Nonetheless, the combination of PTDs with TCs (TCs + PTDs) reduced the individual carbonylation effect of PTDs. Among all combinations, CTC + FLU produced the highest CI on CASs (45.3 ± 1.45 nmol of carbonyl/mg of protein). This attenuation of PTDs-induced CASs carbonylation when are combined with TCs might be explained by a masking effect or competition for amino acids susceptible to carbonylation CASs, likely due to higher MRL values of TCs ($100 \mu\text{g/L}$) compared with PTDs ($20\text{--}40 \mu\text{g/L}$) (Table 1).

In vitro antigenicity of carbonylated CASs

The differential IgE reactivity of nonoxidized and oxidized CASs was evaluated with sera from patients allergic to milk proteins and sera from healthy nonallergic donors. We found that carbonylated CASs had an increased recognition of IgE (average OD among $0.63\text{--}150$), compared with non-treated CASs or negative control (CASs not oxidized) (average OD: 0.53) (Figure 1(b)). The controls: CMP and CASs treated with FeSO_4 (PC) rendered high OD, whereas basal OD was obtained with sera from nonatopic patients. Fifteen out of the 22 treatments that were assessed (68.2%) induced CIs greater than 19 nmol of carbonyls/mg of protein, and these modified CASs showed a significantly increased IgE recognition (Figure 1(c)). Our findings showed that treatments that provoked the highest CIs values (FEN, CTC + FLU, and FLU) induced oxidized caseins with the greatest IgE recognition ($p < 0.0001$) (Figure 2(b)). Furthermore, we found a strong correlation between the CIs and IgE recognition ($r^2=85.97$, $p < 0.0001$) (Figure 1(d)).

Identification of reactive epitopes on carbonylated CASs

To get more insight into the epitopes that could be modified by carbonylation induced by TCs, β -LTs, and PTDs, we assessed the IgG recognition of these oxidized caseins by dot blot, using a specific bovine α_{s1} -casein monoclonal antibody (1D5 mAb) (Figure 2(a)) and a cow's milk-specific rabbit polyclonal antiserum (Figure 2(b)). CASs fractions with the highest levels of carbonylation and IgE binding from each group (PNG, DXC + FLU, TC + FEN, OTC + FLU, DXC, CIP, FEN, CTC + FLU, and FLU) were selected for this experiment; we compared the intensity of the spots corresponding to the oxidized and nonoxidized CASs (ratio). The results obtained in this assay showed that the recognition of epitopes on α_{s1} -casein by 1D5 mAb was enhanced in CASs spiked with FEN, CTC + FLU, and FLU (Figure 2(a)), and we found a statistically significant correlation of CI with ratio ($r^2=67.65$, $p < 0.0001$) (Figure 2(c)).

The spot analysis using the cow's milk-specific rabbit polyclonal antiserum showed that only CASs spiked with a CTC + FLU and FLU, which induced a CI higher than 45 nmol of carbonyls/mg of proteins, significantly increased the signal corresponding to IgG recognition of casein epitopes, compared to the nonoxidized CASs (Figure 2(b)). The linear regression analysis between the CI and ratio of spot intensities also showed a positive and significant correlation with antibody binding ($r^2=74.80$, $p < 0.0001$) (Figure 2(d)).

In vivo assessing of allergic reactions induced by CASs

To evaluate the allergenicity of the oxidized caseins, we assessed their *in vivo* recognition by cell-bound IgE. Mice were sensitized and then orally challenged with CMP (Figure 3(a)). Sensitized animals exhibited high clinical scores according to symptoms elicited following the oral challenge (see table included in Figure 3(a)), positive skin tests, and increased swelling of footpads injected with CMP compared to unsensitized or naïve mice (Figure 3(c)). Then, allergic mice with increased cell-bound IgE were exposed to the different oxidized and nonoxidized CASs. We first challenged mice with CASs fractions with low (DANO, OTC, TC, and AMP), intermediate (DXC, DXC + CIP, and OTC + FEN), and high CIs (CIP, FEN, FLU, and CTC + FLU). As depicted in Figure 3(d), mice orally exposed to CASs with CIs greater than 24 nmol of carbonyls/mg of proteins showed higher clinical scores than other fractions. The most intense signs were observed in mice exposed to

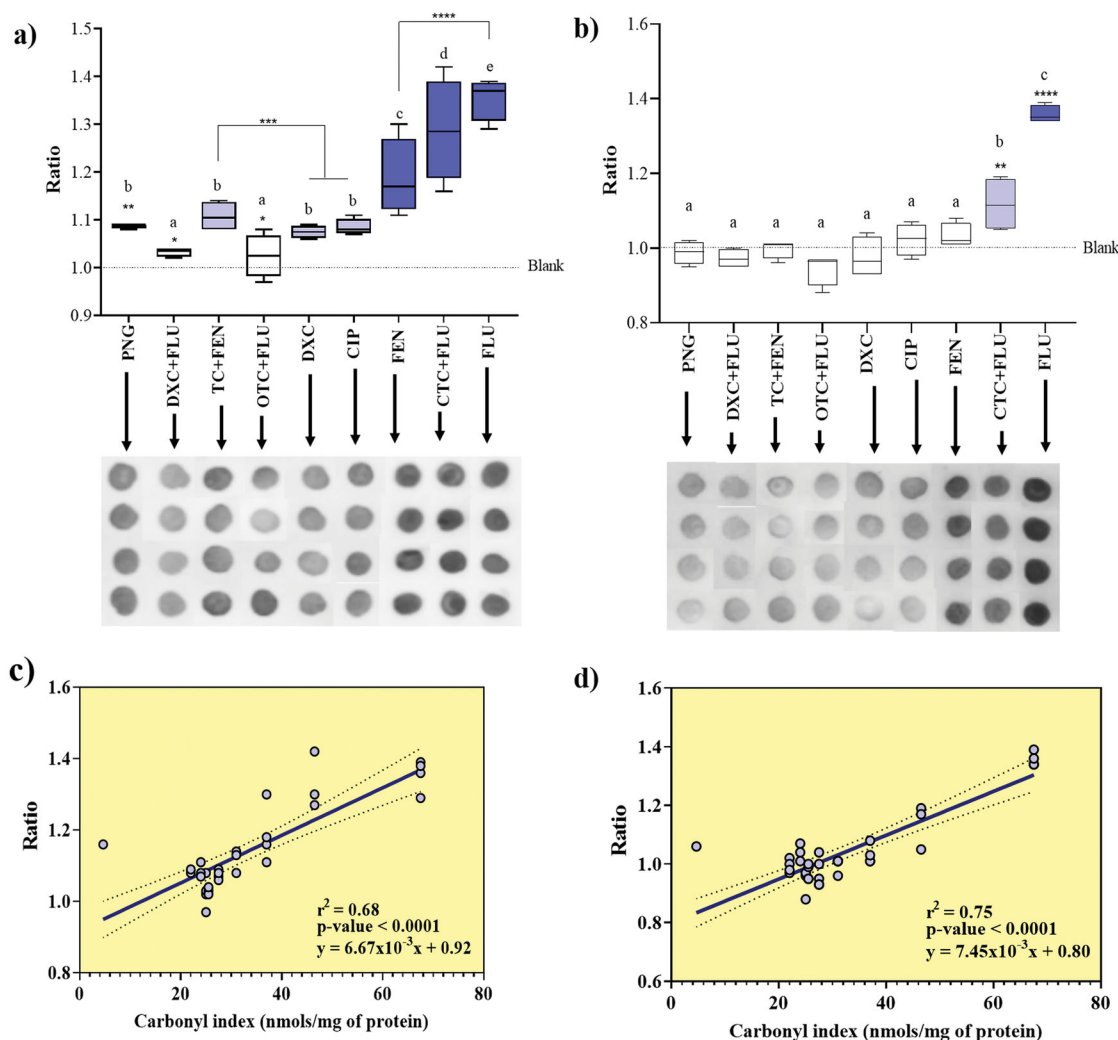


Figure 2. IgG binding to oxidized caseins. (a) Ratio of intensities of spots corresponding to immunochemical reactivity of carbonylated CAS incubated with the α S1-casein-specific monoclonal antibody 1D5 mAb; (b) Ratio of intensities of spots corresponding to immunochemical reactivity of carbonylated CAS incubated with the milk-specific rabbit antiserum; (c) Linear correlation analysis between carbonyl index and ratio signals ($p < 0.0001$); (d) Linear correlation analysis between carbonyl index and ratio signals ($p < 0.0001$). Data are expressed as Box and Whisker plot showing the maxim and minimum ($n = 4$). The model was significant to **** $p < 0.0001$, *** $p < 0.005$, ** $p < 0.01$ and * $p < 0.05$ denote statistically significant differences. OTC: Oxytetracycline; TC: Tetracycline; CTC: Chlortetracycline; DXC: Doxycycline; AMP: Ampicillin; PNG: Benzylpenicillin; OXA: Oxacillin; DCL: Dicloxacin; DANO: Danofloxacin; SARA: Sarafloxacin; CIP: Cypermethrin; FEN: Fenvalerate, FLU: Flumethrin, PC: Positive control.

oxidized CASs with the greatest CIs values (CASs spiked with CIP, FEN, FLU, and CTC + FLU). Similar scores were developed in mice challenged with CMP and PC (CI of 94 nmol of carbonyls/mg of proteins). Most of the clinical signs found were related to moderate-to-severe symptoms (respiratory distress, cyanosis around the mouth and tail, and a reduction in the general activity of the mice). However, no deaths were observed.

Sensitized mice challenged with the noncarbonylated CASs (NC) showed lower clinical scores than those challenged with CMP or carbonylated CASs (scratching the nose and head, and a slight redness around the nose, eyes, and head). Furthermore, we found a positive and significant correlation between CASs carbonylation

and clinical scores ($r^2 = 37.43$, $p < 0.0001$), suggesting that 37.43% of the observed increase in clinical score was due to the carbonylated caseins induced by the pollutants assayed (Figure 3(e)).

Cutaneous test

The skin test analysis showed visible extravasation of the Evans blue dye and increased footpad swelling in mice injected with 10 μ g of CTC + FLU and FLU (0.29 ± 0.005 and 0.59 ± 0.005 mm, respectively). Control skin tests showed that CMP induced a footpad swelling of 0.64 ± 0.03 mm, whereas saline, 0.13 ± 0.03 mm (Figure 4). These *in vivo* findings suggest that the level

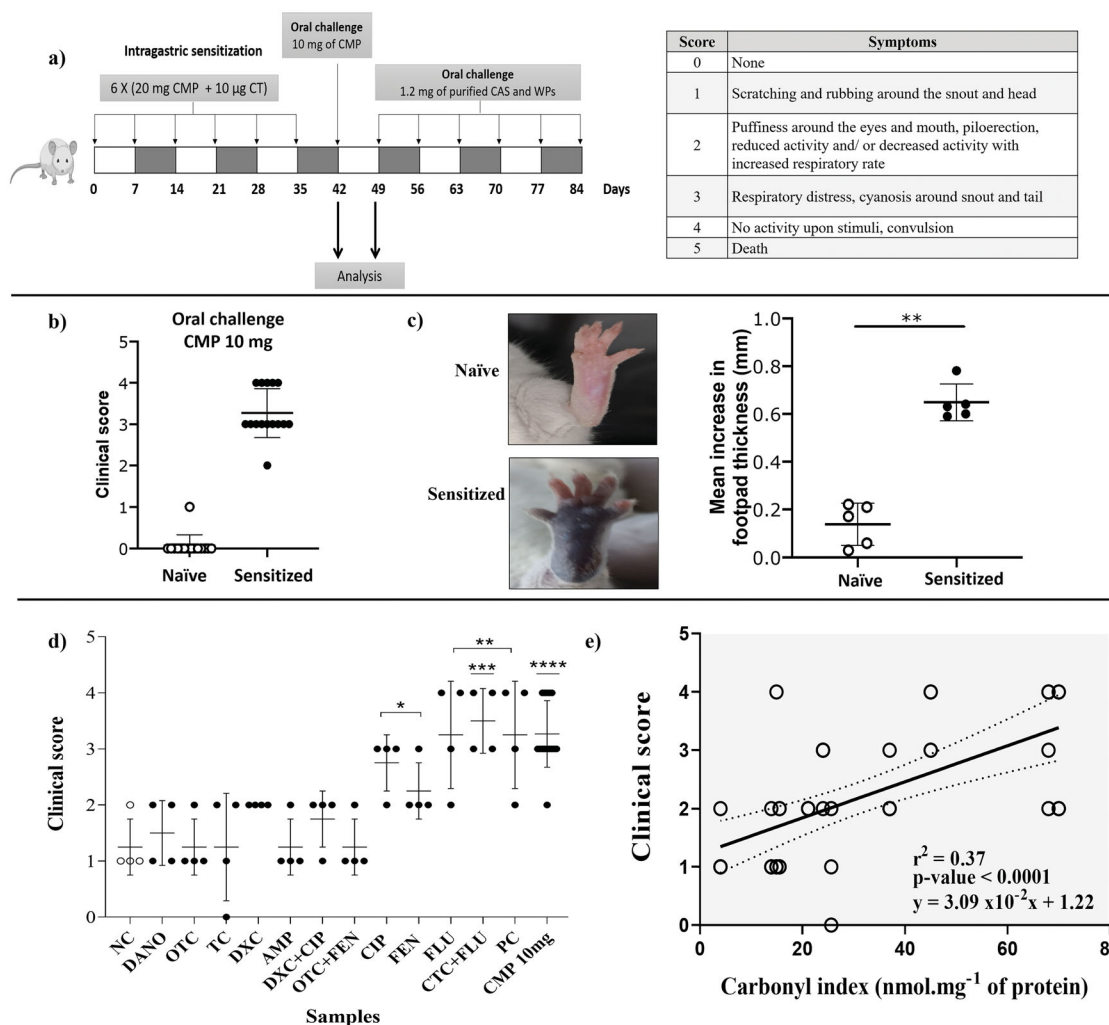


Figure 3. Allergenicity of casein fractions. (a) Schematic representation of the experimental protocol followed to sensitize Balb/c mice. The table indicates the clinical scores corresponding to symptoms elicited immediately after the oral challenge with the proteins; (b) Clinical scores for sensitized and naïve mice challenged with milk proteins; (c) Skin tests performed in naïve and sensitive mice with milk proteins (representative images of the footpads) and footpad swelling (difference in thickness between the footpad injected with milk protein and saline). Data are expressed as the mean ± SD; (d) Clinical scores for sensitized mice challenged with casein fractions exposed to residues; (e) Linear correlation analysis between the clinical score and the carbonyl index ($p < 0.0001$).

of carbonylation of the CASs fractions increased the allergenicity of CASs depending on the pollutant assessed. Notably, the results found in the oral challenges and the skin tests were consistent with the *in vitro* findings using milk-specific IgE-containing human sera.

Discussion

This work demonstrated that TCs, β-LTs, and PTDs could induce oxidative alterations in CASs, increasing its carbonylation level *in vitro* even at concentrations established as safe for the consumer (MRLs). Our previous studies showed that these proteins are prone to TCs and PTDs-induced carbonylation through different

mechanisms [31,32], including the generation of reactive oxygen species [39], production of reactive aldehydes in mediated lipid peroxidation process (4-hydroxy-2-nonenal, malondialdehyde, 2-propenal) by the promotion of glycation and glycoxidation reactions with reduced sugar [30], and by covalent binding of these residues to CASs amino acids. In fact, the mechanism of action of these contaminants requires the binding to proteins, such as the 30S subunit of the bacterial ribosome, penicillin-binding proteins [40], and the sodium channels of nerve cells from insects [41] for the case of TCs, β-LTs, and PTDs, respectively.

We also demonstrated that the carbonylation induced by TCs, β-LTs, and PTDs on CASs increased the *in vitro* protein recognition by the IgE (Figure 1(b)).

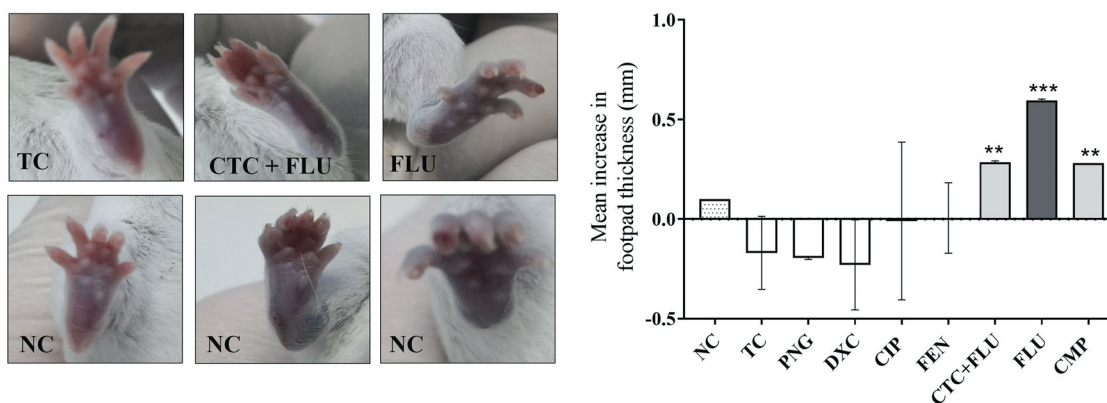


Figure 4. Skin tests performed in sensitive mice with casein fractions (representative images of the footpads) and footpad swelling (difference in thickness between the footpad injected with the oxidized-casein and the casein). Data are expressed as the mean $\pm$ SD. Statistically significant difference by ANOVA: *** $p < 0.01$, * $p < 0.05$.

Cow's milk allergy is increasingly recognized as a significant burden on public health problems, being the second most incident allergic epidemic worldwide after asthma [11]. Although caseins have been identified as the main allergens from milk by several authors, the molecular and structural bases of this phenomenon are not fully understood [14,15,36,37]. Several works have shown that the presence of antibiotics (TCs and β -LTs) and PTDs pesticides in animal food, such as milk, may trigger allergic reactions in sensitive subjects, suggesting that residues of these contaminants represent a significant risk to the consumer's health [42–44].

Although it is known that food protein oxidation impairs its functional properties, including solubility and digestibility [21,23,25], few articles evaluate the effect of carbonylation of animal food proteins and allergenicity; Fenaille et al. [21] reported that resistance to digestion is associated with protein carbonylation may be related to the increase of food allergens. Likewise, Vojdani et al. (2020) and Corsini et al. (2013), in their works, establish that post-translational modification of proteins such as carbonylation can generate their recognition and stimulation of the immune system [45,46]. On the other hand, Nagai et al. (2008) demonstrated in their study the elevation in the content of carbonylated proteins in sputum of asthmatic patients compared to controls, finding that proteins such as albumin and α 1-antitrypsin were specifically carbonylated in allergic inflammation in humans and mice, demonstrating a relationship between these two phenomena [47].

This work demonstrated that antibiotics and pesticides that induced CASs carbonylation greater than 19 nmol of carbonyls/mg of proteins increased their IgE and IgG immunoreactivity. Moreover, the 1D5 α _{S1}-casein-specific mAb recognized the carbonylated epitope on caseins by dot-blot assay (Figure 2(a)).

According to the mapped epitope recognized by 1D5 on α _{S1}-casein [14], this fraction contains amino acids susceptible to oxidation, such as Arg, Glu, Pro, and Lys (Figure 5), whose carbonylation products (2-aminoadipic semialdehyde acid, glutamic semialdehyde, 2-pyrrolidone, and Michael adducts) are used as biomarkers of the oxidative stress of proteins.

To corroborate the findings of the *in vitro* assay, the IgE-mediated mouse model for milk allergy was used to assess *in vivo* allergenicity, which led us to conclude that carbonylation of CASs produced a more allergenic protein than the non-carbonylated proteins. Furthermore, we showed that this allergic animal model constitutes a biological tool to assess the power of an antibiotic or pesticide to increase the allergenicity of an allergen in food. We undoubtedly showed that IgE-sensitized mice developed more severe symptoms with the CASs fractions that exhibited the highest CI than non-oxidized CASs.

Although further studies should be done to more fully elucidate the mechanism by which carbonylation of CASs increased the IgE binding and allergenicity, we can suggest that the increased antibody recognition could be explained by the resistance to the digestion of carbonylated proteins [22,31,32,38] and by conformational changes promoted by carbonylation in the random coil casein structure in aqueous systems, this modification likely exposes cryptic epitopes or neoepitopes [48,49]. Carbonylation may enhance the casein resistance to gastric and duodenal degradation [31,32], thus increasing its accessibility to the intestinal cells to trigger inflammation or absorption through the epithelial compartment to gain access to blood and systemic distribution. The amino acid targets for the enzymes responsible for food protein digestion (pepsin, trypsin, chymotrypsin) (Figure 5) are those plausible of carbonylation in casein. According to Fenaille et al. [21], milk

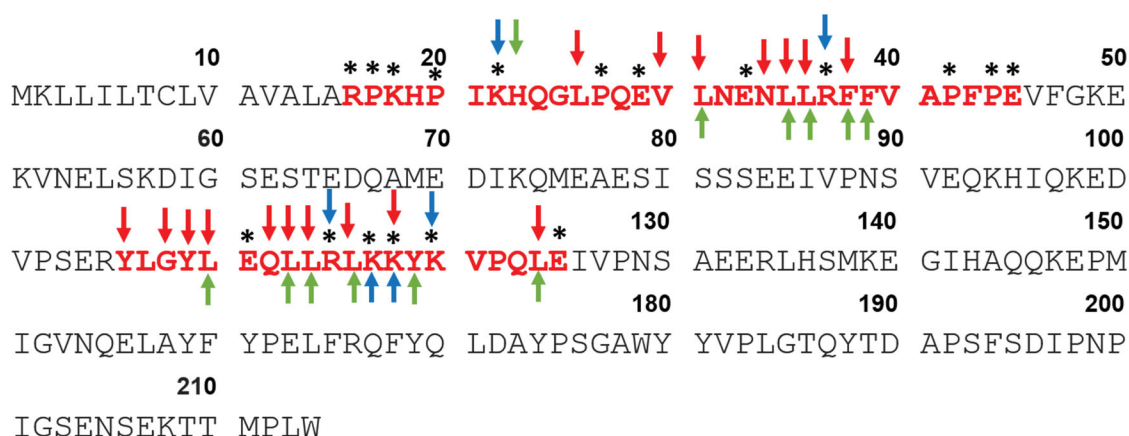


Figure 5. The aminoacidic sequence of α -S1 casein. The epitope mapped for the monoclonal antibody 1D5 mAb is shown with red letters. Arrows show the excision sites of pepsin (red), trypsin (blue) and chymotrypsin (green). The asterisks show the amino acids susceptible to carbonylation previously described in the scientific literature. The aminoacidic sequence was obtained from the Uniprot database, and the proteolytic sites were obtained from the PeptideCutter virtual tool of ExPASy.

protein species resistant to digestion could also constitute major food allergens.

The increased allergenicity found on caseins exposed to antibiotics and pesticides might be an additional factor to explain the rise in CMA observed worldwide [50]. Several studies have demonstrated a significant increase in CMA prevalence worldwide; Mehaudy et al. [51] estimated the prevalence of CMPA among children in a university community hospital in Argentina from 2004 to 2014, they found a CMPA prevalence of 1.2% in 2014, with an increase of 0.4% to 1.2% observed along the period studied; on the other hand, Rona et al. [52] through a meta-analysis of papers on food allergy published from January 1990 to December 2005 found a self-reported prevalence of CMA among 0.2% to 7%. Finally, Nwaru et al. (2014) found a self-report prevalence in CMA of 2.3% (95% CI 2.1%,2.5%) through a systematic review and meta-analysis of European studies published between 2000 and 2012 [53].

Pollutants in foods should be carefully considered when allergenicity is evaluated. They may represent an emerging public health problem if their use is not strictly controlled or monitored. The absence of sanitary policies, or noncompliance, can generate pollutants' residues in milk, which interfere with its biological and health properties, even in concentrations reported worldwide as harmless (MRLs) [25,31,32,54].

In conclusion, our findings showed that antibiotics (TCs, β -LTs, and FQs) and PTDs pesticide-derived residues, accepted worldwide as safe for frequent consumers, can promote the irreversible carbonylation of bovine CASs. In turn, the level of carbonylation induced by these contaminants on CASs significantly increased their recognition of IgE *in vitro* with carbonyl indices greater than 19 nmol of carbonyls/mg of proteins.

Furthermore, the 1D5 mAb, specific for α _{S1}-casein, recognized a carbonylated epitope on this milk fraction. Finally, we employed an IgE-mediated milk allergy model to demonstrate the significantly increased allergenicity of the carbonylated caseins. We found that the modified proteins induced an increased mast cell degranulation, thus indicating that their IgE-binding capacity was enhanced.

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Disclosure statement

The authors confirm that do not are no conflicts of interest.

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