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*Research Article*

# *In Vitro* Proinflammatory and Cytotoxic Activity of Chicken-and Turkey-Based Würstels: A Preliminary Report

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Ultraprocessed foods represent a severe concern to human health due to their direct link with metabolic diseases. Among these foods, mechanically separated meat-based products are of particular interest due to the use of preservatives and the possible presence of antibiotic residues free or bound to animals' bone fragments. To demonstrate the potential harmfulness of these substances, 28 samples of commercially available würstels of different suppliers, price category, package size, and produced with mechanically separated chicken and turkey meat were randomly collected from the Italian market. The presence of antibiotics was assessed by LC/HRMS; bone fragments were identified using histological, histochemical, and microscopical analyses; the cytotoxic and proinflammatory activity of the würstels and their ingredients was assessed using ELISA. Bone fragments were detected in all samples, while only 9 out of 28 samples were positive for the presence of doxycycline, although at concentrations far from the maximum residue limits, ranging from 0.36 to 2.50 ug/kg. Most of the samples were cytotoxic at a dilution of 1 : 20 while all of the 3 tested exerted a proinflammatory effect, with significant cytokines' release (IL-1$\alpha$, IL-1$\beta$, IL-6, IL-8, INF-$\gamma$, TNF-$\alpha$, GM-CSF, and MCAF) at 24 and 36 h ($^{***}P < 0.001$). Part of the cytokine release was due to the presence of beech- and oak-based smoke flavoring, where a significant release of IL-1$\beta$ ($^{***}P < 0.001$), IL-8 ($^{***}P < 0.001$, $^{**}P < 0.01$), INF-$\gamma$ ($^{*}P < 0.05$ and $^{**}P < 0.01$), and MCAF ($^{***}P < 0.001$) was observed at 12 and/or 24 h. Although the results need further investigation to elucidate the cytotoxic and proinflammatory process, this can be considered one of the first reports shedding light on the possible toxic potential of some substances routinely used in food processing, even at allowed concentrations. Moreover, it provides new insights into the understanding of the link between high consumption of ultraprocessed meat, increased risk of inflammation, and progression of chronic diseases.

## 1. Introduction

Worldwide use of antibiotics, in particular in intensive farming, is a well-recognized concern [1, 2]. Most of these drugs, such as tetracyclines, sulphonamides, tylosin, aminoglycosides, $\beta$-lactams, macrolides, lincosamides, and quinolones, are used to prevent or treat overcrowding pathologies and improve animal growth and productivity in food-producing animals [3–8].

Despite the European Union banned the auxinic use of antibiotics in 2019 (Regulation (Eu) 2019/4 of [9]; Regulation (Eu) 2019/6 of [10]), literature studies reported an annual use of antibiotics higher than 100 mg per kilogram of production animal (i.e., cattle, chicken, and pig) [11], predicting a 67% global antibiotic consumption increase by 2030 [2].

Moreover, although the European Union [12] and Food and Drug Administration [13] set the maximum residue limits (MRLs) for antibiotics in foods of animal origin [14], the possible onset of drug resistance phenomena after prolonged intake of antibiotic-contaminated food, even below legal limits, has become more than a hypothesis [15–18].

In addition, the presence of such drugs, as residues or bound to animals' bones, in meat and meat-based products was shown to impact consumers' and pets' health [19–22]. In this regard, it is widely acknowledged that pet food production relies on meat meal (mainly poultry or turkey), with an important percentage of bone (20–30%) as an unavoidable consequence of mechanical boning [21]. The same phenomenon occurs in industrial food for human consumption, such as würstels, produced with mechanically separated meat. According to Regulation (EC) No 853/2004, "mechanically separated meat is obtained by removing meat from flesh-bearing bones after boning or from poultry carcasses, using mechanical means and resulting in the loss or modification of the muscle fiber structure" [23].

Besides antibiotics, food additives such as phosphates, nitrates, nitrites, and smoke flavorings used in ultraprocessed food have also been investigated in literature due to their possible toxic effects [24–29]. These additives are employed in food processing mostly to improve the bioavailability of functional compounds [30], increase the product quality [31, 32], exert antimicrobial and pH buffering activity [30, 33, 34], extend the product shelf-life [31, 32], and prevent discoloration [34].

Nitrates (sodium nitrate—E251, potassium nitrate—E252) and nitrites (sodium nitrite—E249, potassium nitrite—E250) are authorized as food additives by Regulation (EU) No 1129/2011 with a maximum limit of 150 mg/kg in processed meat [35], and an acceptable daily intake (ADI) of 3.7 and 0.07 mg/kg bw/day, respectively, was set in 2021 [36]. As far as concerns mono-, di-, tri-, and polyphosphates (E338—452), Regulation (EC) No 1333/2008 established a maximum limit of 5 g/kg in the processed meat [37], while the European Food Safety Authority panel on food additives and flavorings set their ADI to 40 mg/kg bw/day [38].

On the one hand, preservatives can improve product safety and stability, while on the other hand, they can potentially cause risks to human health once accumulated through food ingestion [25, 32, 39]. For instance, nitrates can overcome stomach acidity and enter the circulatory system, forming highly bioactive reactive nitrogen oxide species, which are involved in the generation of nitrosamines [40], while polyphosphate consumption should be limited, especially in patients with chronic kidney disease where increased morbidity and mortality were observed [27, 30].

Similarly, some liquid smoke flavorings (e.g., from beechwood) have been questioned for their potential toxicity on a daily intake, although no genotoxicity was reported [41]. Smoke flavorings are a specific category of flavorings subjected to Regulation (EC) No 1334/2008 on flavorings and certain food ingredients with flavoring properties for use in/on foods (1334/2008, 2008). They are produced by pyrolysis, subsequent condensation of the vapors, and fractionation of the resulting products (smoke condensates and tar fractions) [28]. Such products can be furtherly processed into smoke flavorings, which can be used in food processing [42]. Both smoke and derived products are complex mixtures of more than 400 compounds, including phenol derivatives, carbonyl compounds, alcohols, organic acids, and polycyclic aromatic hydrocarbons (PAH), which lend the typical flavor, color, and taste of a smoked product [43–45], and some are widely acknowledged for their toxicity [46–49].

Based on the abovementioned premises, this work aimed at demonstrating the presence of veterinary drugs in commercially available industrial würstels and correlating the potential toxicity of these ultraprocessed foods with antibiotics and food additives.

## 2. Materials and Methods

*2.1. Samples.* Twenty-eight commercially available würstels were purchased from the Italian market and analyzed for the presence of bone fragments and antibiotic residues. All samples were different in supplier, price category, and package size. According to the ingredients' list, all samples hold percentages of mechanically separated meat ranging from 0 to 94% (Table 1).

Part of each sample was homogenized with a blender (Model HGB2WT, Waring Commercial, Torrington, CT, USA), transferred into 50 mL Falcon tubes, and stored at −20°C until their use for the high-performance liquid chromatography (HPLC) analysis and for the enzyme-linked immunosorbent assay (ELISA) and cytotoxic and proinflammatory tests; the remaining part was used for histological, histochemical, and microscopical analyses.

*2.2. Histological and Histochemical Analysis.* Each würstel was trimmed to obtain three portions, subsequently fixed in 10% buffered formalin for 48 h, then processed to be embedded in paraffin wax, and sectioned at a thickness of 3 $\mu$m. Deparaffinized sections of each sample were stained with hematoxylin and eosin (HE) and Toluidine Blue (TB).

Table 1: List of samples and percentage of mechanically separated chicken and turkey meat.

| Sample | Mechanically separated chicken meat (%) | Mechanically separated turkey meat (%) |
|---|---|---|
| #1 | 94 | — |
| #2 | 91 | — |
| #3 | 91 | — |
| #4 | 83 | — |
| #5 | 73 | 21 |
| #6 | 49 | 42 |
| #7 | 45 | 27 |
| #8[1] | — | — |
| #9 | 46 | 38 |
| #10 | 91 | — |
| #11 | 78 | 78 |
| #12* | 94 | 94 |
| #13 | 91 | — |
| #14 | 49 | 42 |
| #15* | 84 | 84 |
| #16 | 11 | 54 |
| #17* | 65 | 65 |
| #18 | 45 | 35 |
| #19 | 49 | 38 |
| #20 | 68 | 22 |
| #21 | 51 | 34 |
| #22 | 38 | 41 |
| #23 | 81 | — |
| #24 | 11 | 54 |
| #25* | 78 | 78 |
| #26 | 43 | 31 |
| #27 | 78 | — |
| #28 | 91 | — |

[1]Control sample with 28% and 52% of chicken and turkey nonmechanically separated meat, respectively. *The percentage is referred to the mix of both species, as reported in the label.

Additional sections were histochemically stained with Von Kossa (VK) stain (Bio-Optica, Milan, Italy) to give more conclusive proof of the presence of calcium salts.

*2.3. Environmental Scanning Electron Microscopy (ESEM) Analysis.* An ESEM Quanta-200 (FEI Company, Thermo Fisher Scientific Inc., Hillsboro, Oregon, USA) equipped with energy-dispersive X-ray spectroscopy (X-EDS) microanalysis system Oxford INCA-350 was used to obtain micrographs and spectra of all würstels. Each sample was mounted on an aluminum stub (diameter 13 mm) via double-sided adhesive tape and observed with 10 nm Au sputtering at a high vacuum (≈10-5-10-6 Torr), with 20 kV accelerating voltage, 11 mm working distance, 4 μm spot size, and 1024 × 1024 pixels standard acquisition resolution. Images were acquired at 2000x and 5000x original magnifications, while sample areas of 140 $\mu$m × 140 $\mu$m were investigated. Back-scattered detector images were used to help the selection of particles for X-EDS analysis.

*2.4. ELISA for Oxytetracycline Detection.* All samples were analyzed in triplicate using oxytetracycline (OTC)-specific ELISA kit (Cat. # DEIA-XYZ35, Creative Diagnostics®, NY, USA), with cross-reactivity for chlortetracycline (CTC), tetracycline (TC), and doxycycline (DOXY) of 180, 180, and 110%, respectively. A microplate reader (Multiscan Ascent, Dasit S.p.a., Milan, Italy) was used to measure OTC concentration at 450 nm.

*2.5. Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) for Detection of Antimicrobial Residues in Muscle.* The analysis of würstels was performed by applying a multiclass method for detecting antimicrobial residues in muscle developed and validated by Istituto Zooprofilattico Sperimentale della Sardegna, according to Commission Decision 2002/657/EC [36]. Fifty-two antibiotics belonging to seven different drug families (beta-lactams, lincosamides, macrolides, pleuromutilins, quinolones, sulphonamides, and tetracyclines) were screened.

*2.5.1. Chemicals and Reagents.* Methanol for LC-HRMS and formic acid were purchased from VWR International s.r.l. (Milan, Italy). Acetonitrile (ACN) for LC-HRMS was supplied by Carlo Erba (Milan, Italy). Ultrapure water was produced using a Milli-Q purification apparatus (Millipore, Bedford, MA, USA). Solid phase extraction (SPE) Oasis PRIME HLB (60 mg, 3 mL) cartridges were obtained from Waters (Milford, MA, USA).

Penicillin G, amoxicillin, ampicillin, cloxacillin, dicloxacillin, oxacillin, nafcillin, penicillin V, ceftiofur, cefalexin, cefquinome, cefoperazone, penicillin G-d7,

ciprofloxacin, difloxacin, oxolinic acid, enrofloxacin, danofloxacin, marbofloxacin, flumequine, sarafloxacin, erythromycin A, spiramycin, tylosin A, tilmicosin, sulfanilamide, sulfamethazine, sulfapyridine, sulfadiazine, sulfadimethoxine, sulfaquinoxaline, sulfamerazine, sulfamethoxazole, sulfathiazole, sulfaguanidine, trimethoprim, chlortetracycline, oxytetracycline, doxycycline, tetracycline, lincomycin, clindamycin, tiamulin, and valnemulin standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ceftiofur and cefazolin were obtained from Dr. EhrenstorferTM (Augsburg, Germany); cephapirin from Aurogene (Roma, Italy); 4-epi-chlortetracycline, 4-epi-oxytetracycline, and 4-epi-tetracycline from Thermo Fisher Scientific Inc.; sulfanilamide-13C6 and enrofloxacin-d5 were obtained from WITEGA (Berlin, Germany) and Sigma Aldrich (St. Louis, MI, USA), respectively.

*2.5.2. Standard Solutions.* Individual stock standard solutions (1000 $\mu$g/mL) were prepared in MeOH (lincosamides, macrolides, pleuromutilins, sulphonamides, and tetracyclines) or $H_2O$/ACN 75/25 (v/v) for beta-lactams. Quinolones were dissolved in MeOH, except for ciprofloxacin and oxolinic acid, dissolved in 2 M NaOH in MeOH. These stock solutions were stored in a freezer from 1 month (cefquinome) to 12 months (sulphonamides). Intermediate (10 $\mu g \cdot mL^{-1}$) and working (1 and 0.1 $\mu g \cdot mL^{-1}$) solutions were prepared in $H_2O$/ACN 75/25 (v/v) for beta-lactams and MeOH for all other antibiotics. The internal standards solutions were prepared by using the same solvent or a mixture of isotopically labeled native compounds.

*2.5.3. LC/HRMS Conditions.* Chromatography was performed on a Thermo Ultimate 3000 High-Performance Liquid Chromatography system (Thermo Fisher Scientific, San Jose, CA, USA). Analytes were separated on a Poroshell 120 EC-C18 column (100 × 3.0 mm, 2.7 $\mu$m; Agilent Technologies, Santa Clara, CA, USA) equipped with the guard column Poroshell (2.1 × 5 mm). Formic acid 0.1% in water (A) and MeOH (B) were used as mobile phases. The gradient was initiated with 5% eluent B for 1 min and continued with a linear increase to 95% B in 19 min. This condition was maintained for 5 min. The system returned to 5% B in 1 min and was re-equilibrated for 4 min (total run time: 30 min). The column compartment was kept at 30°C while the autosampler at 10°C. The flow rate was 0.25 $mL \cdot min^{-1}$, and the injection volume was 5 $\mu$L. The mass spectrometer Q-Orbitrap (Thermo Fisher Scientific) was equipped with a heated electrospray ionization (HESI-II) source with an optimized temperature of 320°C, a capillary temperature of 300°C, and an electrospray voltage of 3.00 kV working on positive ion mode. Sheath and auxiliary gas were 35 and 15 arbitrary units.

The mass spectrometer was controlled by the Xcalibur 3.0 software (Thermo Fisher Scientific). The exact mass of the compounds was calculated using the Qual browser in Xcalibur 3.0. Instrument calibration in positive mode was done in every analytical batch with direct infusion of an LTQ Velos ESI Positive Ion Calibration Solution (Pierce Biotechnology Inc., Rockford, IL, USA). The acquisition was achieved in full scan/dd-MS2.

All quantitative data were calculated using the full scan data. The mass range in the full scan was within m/z 150-1000. The data were acquired at a resolution of 70000 Full Width at Half-Maximum (FWHM) (m/z 200). The Automatic Gain Control (AGC) representing the maximum capacity in C-trap was set at $3 \times 10^6$ ions for a maximum injection time of 100 ms. As for the Data-Dependent Scan Mode (ddMS2) mode, an inclusion list was used with the precursor ion masses, their expected retention time (with a minute acquisition time window centered on each retention time), and their Normalized Collision Energy (NCE). The precursor ions were filtered by the quadrupole, which operated at an isolation window of m/z 2.4. A resolution of 35000 FWHM (m/z 200) was used. The AGC target was set at $1 \times 10^6$ ions for a maximum injection time of 100 ms. The main MS acquisition parameters are listed in Table 2. All extracted mass traces were based on a 5-ppm mass window (accuracy).

*2.6. Conditioned Culture Medium and Cell Cultures Preparation.* The conditioned culture medium (CCM) was prepared according to Di Cerbo et al. [3]. Briefly, 1 g of each sample was weighted on a precision balance (Explorer E12140, OHAUS Europe GmbH, Nänikon, Switzerland), added to 10 mL of PBS (pH 7.4) in a 20 mL beaker, and left to shake for 48 h at room temperature. Then, each sample was placed into a 50 mL plastic tube and centrifuged at 6000 rpm for 10 minutes using a Sorvall RC5C + centrifuge (Kendro Laboratory, Products, Asheville, NC, USA). After centrifugation, each supernatant was collected and transferred into a 15 mL Falcon tube.

CCM was obtained by lyophilization (Lio 5p Digital, Vetrotecnica, Padua, Italy) of 2 mL of each supernatant, followed by resuspension in 2 mL of RPMI-1640 medium and filtration through a 0.22 $\mu$m filter. Serial dilutions of each CCM (1 : 2, 1 : 4, 1 : 10, 1 : 20) were prepared for cell viability assay.

K562 myelogenous leukemia cell line was chosen as the validated *in vitro* model used in other research studies of some of the authors [8, 50–52], purchased from American Type Culture Collection (ATCC) (LGC Standards S.r.l., Milan, Italy), grown in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 g/mL streptomycin, 100 U/mL penicillin, 2 mM glutamine (Euroclone S.p.a., Milan, Italy), incubated at 37°C with 95% oxygen and 5% $CO_2$, and used for cytotoxicity assay against würstel-derived CCM.

*2.7. Food Additive Solutions.* Sodium pyrophosphate, sodium tripolyphosphate, and sodium metaphosphate were purchased from Merk Life Science S.r.l. (Milan, Italy); sodium nitrite was purchased from Fisher Scientific Italia (Milan, Italy), while beech- and oak-based smoke flavoring was purchased from Droghe Palma Commerciale S.r.l. (Treviso, Italy). All additives were tested at different concentrations. More in detail, pyrophosphate,

Table 2: Molecular formulas, adducts, exact masses, and fragments of the fifty-seven analytes.

| Chemical family | Analyte | Molecular formula | Adduct | Precursor exact mass (m/z) | Fragment 1 | Fragment 2 | *N* (CE) |
|---|---|---|---|---|---|---|---|
| Beta-lactams Penicillins (8) | Amoxicillin | $C_{16}H_{19}N_3O_5S$ | $[M+H]+$ | 366.1118 | 208.0 | 349.1 | 10 |
| | Ampicillin | $C_{16}H_{19}N_3O_4S$ | $[M+H]+$ | 350.1169 | 106.1 | 192.0 | 20 |
| | Cloxacillin | $C_{19}H_{18}ClN_3O_5S$ | $[M+H]+$ | 436.0728 | 277.0 | 160.0 | 10 |
| | Dicloxacillin | $C_{19}H_{17}Cl_2N_3O_5S$ | $[M+H]+$ | 470.0339 | 160.0 | 311.0 | 15 |
| | Nafcillin | $C_{21}H_{22}N_2O_5S$ | $[M+H]+$ | 415.1322 | 199.1 | 256.1 | 20 |
| | Oxacillin | $C_{19}H_{19}N_3O_5S$ | $[M+H]+$ | 402.1118 | 160.0 | 243.1 | 15 |
| | Penicillin G | $C_{16}H_{18}N_2O_4S$ | $[M+Na]+$ | 357.0882 | 160.0 | 176.1 | 10 |
| | Penicillin V | $C_{16}H_{18}N_2O_5S$ | $[M+Na]+$ | 373.0829 | 160.0 | 192.1 | 15 |
| Beta-lactams Cephalosporins (6) | Cefalexin | $C_{16}H_{17}N_3O_4S$ | $[M+H]+$ | 348.1013 | 158.0 | 174.1 | 40 |
| | Cefazolin | $C_{14}H_{14}N_8O_4S_3$ | $[M+H]+$ | 455.0373 | 156.0 | 153.0 | 15 |
| | Cefapirin | $C_{17}H_{17}N_3O_6S_2$ | $[M+H]+$ | 424.0632 | 152.0 | 292.1 | 25 |
| | Cefquinome | $C_{23}H_{24}N_6O_5S_2$ | $[M+H]+$ | 529.1322 | 134.1 | 324.1 | 16 |
| | Cefoperazone | $C_{25}H_{27}N_9O_8S_2$ | $[M+H]+$ | 646.1497 | 143.1 | 290.1 | 16 |
| | Ceftiofur | $C_{19}H_{17}N_5O_7S_3$ | $[M+H]+$ | 524.0363 | 241.0 | 210.0 | 25 |
| Tetracyclines (8) | Chlortetracycline | $C_{22}H_{23}ClN_2O_8$ | $[M+H]+$ | 479.1216 | 444.1 | 154.0 | 26 |
| | Doxycycline | $C_{22}H_{24}N_2O_8$ | $[M+H]+$ | 445.1621 | 428.1 | 410.1 | 30 |
| | Oxytetracycline | $C_{22}H_{24}N_2O_9$ | $[M+H]+$ | 461.1555 | 426.1 | 337.1 | 30 |
| | Tetracycline | $C_{22}H_{24}N_2O_8$ | $[M+H]+$ | 445.1605 | 154.0 | 410.1 | 30 |
| | Epi-chlortetracycline | $C_{22}H_{23}ClN_2O_8$ | $[M+H]+$ | 479.1216 | 444.1 | 154.0 | 26 |
| | Epi-doxycicline | $C_{22}H_{24}N_2O_8$ | $[M+H]+$ | 445.1605 | 428.1 | 410.1 | 30 |
| | Epi-oxytetracycline | $C_{22}H_{24}N_2O_9$ | $[M+H]+$ | 461.1555 | 426.1 | 201.1 | 30 |
| | Epi-tetracycline | $C_{22}H_{24}N_2O_8$ | $[M+H]+$ | 445.1605 | 410.1 | 392.1 | 30 |
| Pleuromutilins (2) | Tiamulin | $C_{28}H_{47}NO_4S$ | $[M+H]+$ | 494.3299 | 192.1 | 119.0 | 30 |
| | Valnemulin | $C_{31}H_{52}N_2O_5S$ | $[M+H]+$ | 565.3670 | 263.1 | 164.1 | 30 |
| Macrolides (5) | Tilmicosin | $C_{46}H_{80}N_2O_{13}$ | $[M+2H]+$ | 435.2903 | 174.1 | 696.5 | 32 |
| | Tilosin | $C_{46}H_{77}NO_{17}$ | $[M+H]+$ | 916.5264 | 174.1 | 101.1 | 25 |
| | Azithromycin | $C_{38}H_{72}N_2O_{12}$ | $[M+H]+$ | 749.5171 | 158.1 | 83.0 | 28 |
| | Erythromycin | $C_{37}H_{67}NO_{13}$ | $[M+H]+$ | 734.4685 | 158.1 | 83.0 | 20 |
| | Spiramycin | $C_{43}H_{74}N_2O_{14}$ | $[M+2H]++$ | 422.2643 | 540.3 | 699.4 | 30 |
| Lincosamides (2) | Clindamycin | $C_{18}H_{33}ClN_2O_5S$ | $[M+H]+$ | 425.1872 | 126.1 | 377.2 | 30 |
| | Lincomycin | $C_{18}H_{34}N_2O_6S$ | $[M+H]+$ | 407.2210 | 126.1 | 359.2 | 30 |
| Quinolones (11) | Nalidixic acid | $C_{12}H_{12}N_2O_3$ | $[M+H]+$ | 233.0921 | 205.1 | 159.1 | 70 |
| | Oxolinic acid | $C_{13}H_{11}NO_5$ | $[M+H]+$ | 262.0710 | 160.0 | 234.0 | 80 |
| | Ciprofloxacin | $C_{17}H_{18}FN_3O_3$ | $[M+H]+$ | 332.1405 | 231.1 | 203.1 | 65 |
| | Danofloxacin | $C_{19}H_{20}FN_3O_3$ | $[M+H]+$ | 358.1562 | 82.1 | 255.1 | 70 |
| | Difloxacin | $C_{21}H_{19}F_2N_3O_3$ | $[M+H]+$ | 400.1467 | 299.1 | 58.1 | 65 |
| | Enrofloxacin | $C_{19}H_{22}FN_3O_3$ | $[M+H]+$ | 360.1718 | 203.1 | 245.1 | 60 |
| | Flumequine | $C_{14}H_{12}FNO_3$ | $[M+H]+$ | 262.0874 | 238.1 | 220.0 | 80 |
| | Marbofloxacin | $C_{17}H_{19}FN_4O_4$ | $[M+H]+$ | 363.1463 | 72.1 | 320.1 | 25 |
| | Norfloxacin | $C_{16}H_{18}FN_3O_3$ | $[M+H]+$ | 320.1405 | 231.1 | 203.1 | 80 |
| | Ofloxacin | $C_{18}H_{20}FN_3O_4$ | $[M+H]+$ | 362.1511 | 261.1 | 221.1 | 50 |
| | Sarafloxacin | $C_{20}H_{17}F_2N_3O_3$ | $[M+H]+$ | 386.1311 | 299.1 | 338.1 | 60 |
| Sulfonamides (15) | Sulfaquinoxaline | $C_{14}H_{12}N_4O_2S$ | $[M+H]+$ | 301.0754 | 156.0 | 108.0 | 38 |
| | Sulfachloropyridazine | $C_{10}H_9ClN_4O_2S$ | $[M+H]+$ | 285.0208 | 156.0 | 108.0 | 35 |
| | Sulfadiazine | $C_{10}H_{10}N_4O_2S$ | $[M+H]+$ | 251.0597 | 156.0 | 108.0 | 35 |
| | Sulfadimethoxine | $C_{12}H_{14}N_4O_4S$ | $[M+H]+$ | 311.0809 | 156.1 | 108.0 | 42 |
| | Sulfaguanidine | $C_7H_{10}N_4O_2S$ | $[M+H]+$ | 215.0597 | 156.0 | 108.0 | 40 |
| | Sulfamerazine | $C_{11}H_{12}N_4O_2S$ | $[M+Na]+$ | 287.0573 | 156.0 | 190.0 | 42 |
| | Sulfamethazine | $C_{12}H_{14}N_4O_2S$ | $[M+H]+$ | 279.0910 | 124.1 | 156.0 | 42 |
| | Sulfamethizole | $C_9H_{10}N_4O_2S_2$ | $[M+H]+$ | 271.0318 | 156.0 | 108.0 | 40 |
| | Sulfamethoxazole | $C_{10}H_{11}N_3O_3S$ | $[M+H]+$ | 254.0594 | 156.0 | 108.0 | 40 |
| | Sulfamethoxypyridazine | $C_{11}H_{12}N_4O_3S$ | $[M+H]+$ | 281.0703 | 126.1 | 108.0 | 50 |
| | Sulfamonomethoxine | $C_{11}H_{12}N_4O_3S$ | $[M+H]+$ | 281.0710 | 156.0 | 108.0 | 41 |
| | Sulfanilamide | $C_6H_8N_2O_2S$ | $[M+H]$ $[NH_3]+$ | 156.0114 | 65.0 | 92.0 | 70 |
| | Sulfapyridine | $C_{11}H_{11}N_3O_2S$ | $[M+H]+$ | 250.0645 | 156.0 | 184.1 | 43 |
| | Sulfathiazole | $C_9H_9N_3O_2S_2$ | $[M+H]+$ | 256.0209 | 156.0 | 108.0 | 38 |
| | Trimethoprim | $C_{14}H_{18}N_4O_3$ | $[M+H]+$ | 291.1452 | 123.1 | 261.1 | 60 |

tripolyphosphate, and metaphosphate were tested at 1.25, 2.5, 5, and 10 $\mu$g/kg; sodium nitrite at 50, 100, 150, and 300 $\mu$g/kg; beech- and oak-based smoke flavoring at 0.05, 0.1, and 0.2%.

*2.8. Cell Viability Assay.* Cell viability was assessed after 48 h of continuous exposure to different dilutions of the CCM of each würstel sample and after 12 and 24 h to different concentrations of food additives. Cell Counting Kit-8 (CCK-8) assays (Dojindo Laboratories, Kumamoto, Japan) were used to measure CCM and food additive cytotoxicity based on detecting the content of the produced formazan by living cells. Briefly, the K562 cells were plated on 96-well plates (Euroclone S.p.a.) at a concentration of 7000 cells/cm$^2$. After exposure to different CCM dilutions for 48 h and food additive concentration for 12 and 24 h, 10 $\mu$L of CCK-solution was added to each well and incubated for 2 h at 37°C. Finally, absorption was measured at 450 nm using a multiplate reader Multiscan FC (Thermo Scientific). Dimethyl sulfoxide (DMSO) 3% was used as a toxicity reference drug. Cell viability data correspond to the mean ± SD of three different experiments done in quadruplicate and expressed as a percentage of live cells.

*2.9. ELISA Multiplex Human Cytokine Assay.* To test the potentially toxic role of würstels-derived CCM and single food additives, a simultaneous quantitative determination of proinflammatory cytokines measurements was performed on K562 supernatant medium using the Multiplex cytokine ELISA assay (Anogen, Mississauga, Ontario, Canada). The kit contains precoated well with specific monoclonal antibodies for interleukin (IL)-1$\alpha$, IL-1$\beta$, IL-6, IL-8, interferon-gamma (INF-$\gamma$), tumor necrosis factor-alpha (TNF-$\alpha$), granulocyte-macrophage colony-stimulating factor (GM-CSF), and monocyte chemotactic and activating factor (MCAF/MCP-1). Standard curves and calculation of cytokine production were obtained using Curve Expert Professional 2.6.0 software.

*2.10. Statistical Analysis.* All the ELISA experiments were carried out in duplicate. Data were analyzed using GraphPad Prism 9 software (GraphPad Software, Inc., La Jolla, CA, USA). All data are presented as the means ± standard deviation (SD) and were first checked for normality using the D'Agostino–Pearson normality test. Differences in cytokines concentration were analyzed using a Two-Way Analysis of Variance (ANOVA) followed by Tukey multiple comparison test, while differences in cell viability were analyzed using a Kruskal–Wallis test followed by Dunn's post-test A $^*P < 0.05$ was considered significant.

## 3. Results

*3.1. Histological and Histochemical Analysis.* The histological examination of all commercially available würstels containing mainly mechanically separated meat (chicken and turkey) revealed the presence of scattered foci of chondroid matrix and mineralized spicules (Figure 1). The mineralized spicules were basophilic, granular, and fragmented (Figure 1(a)) and stained deeply black with VK, revealing calcium salts consistent with bone matrix (Figure 1(b)). The foci of the chondroid matrix were composed of chondrocytes arranged in islands and surrounded by an amorphous basophilic matrix (hyaline cartilage) that stained metachromatically with TB (Figure 1(c)) and appeared black with VK (Figure 1(d)). Interestingly, the only specimen prepared without mechanically separated chicken meat showed mineralized material stained with VK and chondroid matrix.

*3.2. ESEM Analysis.* To further confirm the presence of bone fragments at the ultrastructural level and their chemical composition (mainly phosphorous, calcium, and carbon) [53], ESEM images were acquired on all samples (Figure 2).

According to the analysis and the relative chemical spectra, microsized bone fragments or aggregates as a consequence of the high-pressure mechanical separation of meat were clearly observed.

*3.3. ELISA and LC-HRMS Analyses.* According to the standard procedures to determine the presence of antibiotics, the ELISA screening was performed before carrying out the Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS). According to our previous research studies where antibiotics were found to be chemically bound to antibiotics, despite all samples resulted below the limit of detection but the presence of bones was confirmed, we decided to evaluate the possible presence of antibiotics at lower concentration than that of the ELISA. The results of the ELISA screening and LC-HRMS analysis concerning the possible presence of antibiotics in the würstels are presented in Table 3.

According to the ELISA screening, tetracyclines were below the detection limit in all samples, while the LC-HRMS analysis revealed the presence of DOXY in 9 out of 28 samples, in the range of 0.4–2.5 $\mu$g/kg, therefore lower than the MRL (Figure 3) [12].

DOXY belongs to the tetracycline antibiotic group, with a broad spectrum of activity, mainly bacteriostatic [4, 5], and its residues are generally found in the muscle and bone of treated animals. Nevertheless, the levels of DOXY found in the würstels samples were far below the MRL (100 $\mu$g/kg for all TCs in food of animal origin) [12]. However, it should be noted that 9 out of 28 samples showed antibiotic residues. Although not alarming, this observation confirms the frequent presence of veterinary drugs in highly processed foods.

*3.4. Cytotoxicity Assay.* Results concerning the K562 cell viability challenged with different CCM dilutions (1 : 2, 1 : 4, 1 : 10, and 1 : 20) after 48 h of incubation are summarized in Figure 4.

The results clearly showed that all samples of the würstels-derived conditioned medium, at the lowest dilutions, significantly decreased the cell viability of K562 cell

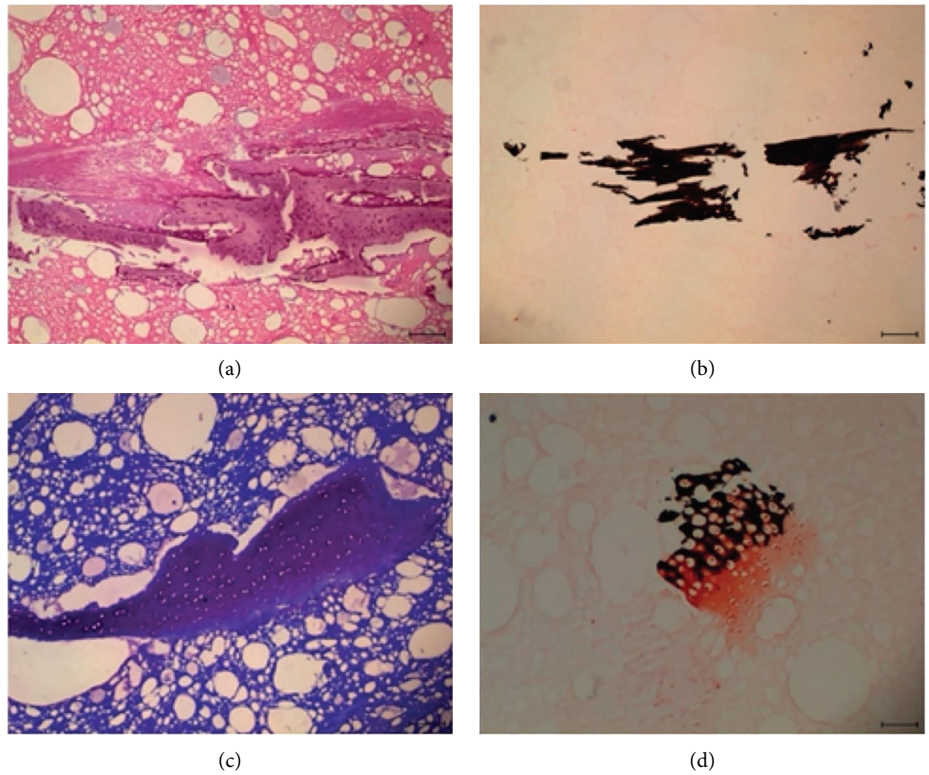


FIGURE 1: Section of würstels (5 microscopical fields per section) containing mineralized matrix ((a) HE, bar: 100 $\mu$m) composed of calcium salts ((b) VK, bar: 100 $\mu$m) and containing cartilage matrix metachromatic ((c) BT, bar: 100 $\mu$m) and also calcified ((d) VK, bar 50 $\mu$m).

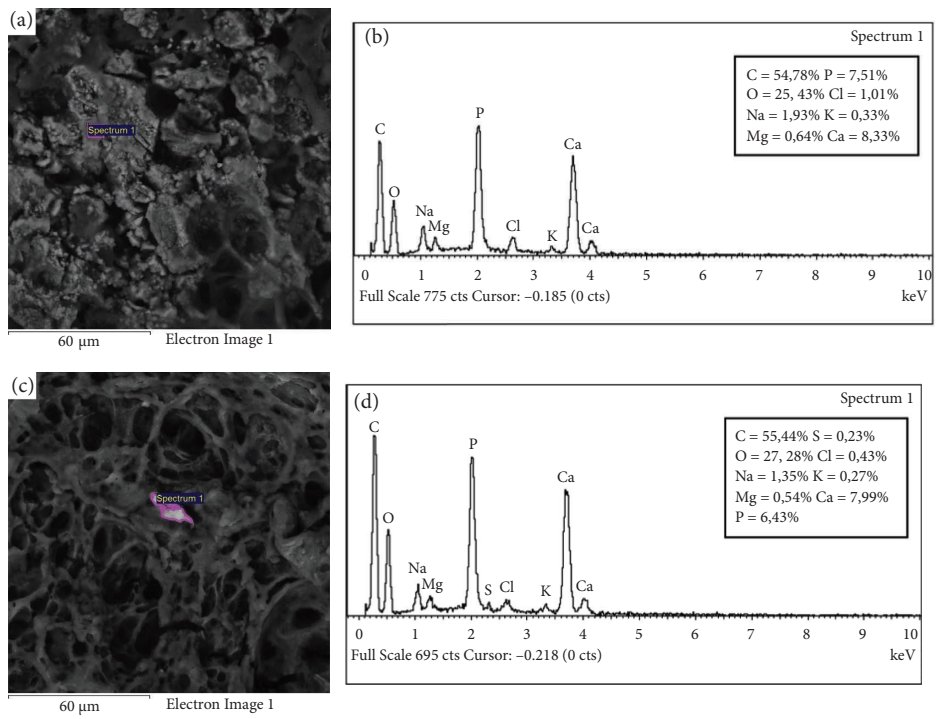


FIGURE 2: Environmental scanning microscopy morphological analysis on two different würstels observed at 60 $\mu$m (5 microscopical fields per section) showing (a) bone aggregates or (c) bone fragments along with their spectra (b, d).

Table 3: Antibiotic concentrations in würstels detected by ELISA and LC-HRMS.

| Sample | OTC ($\mu$g/Kg)[a] | DOXY ($\mu$g/Kg)[b] |
|---|---|---|
| #1 | <LOD | — |
| #2 | <LOD | 1.46 |
| #3 | <LOD | 0.83 |
| #4 | <LOD | — |
| #5 | <LOD | — |
| #6 | <LOD | — |
| #7 | <LOD | — |
| #8 | <LOD | — |
| #9 | <LOD | — |
| #10 | <LOD | 0.37 |
| #11 | <LOD | — |
| #12 | <LOD | — |
| #13 | <LOD | — |
| #14 | <LOD | — |
| #15 | <LOD | — |
| #16 | <LOD | 0.86 |
| #17 | <LOD | — |
| #18 | <LOD | 0.36 |
| #19 | <LOD | — |
| #20 | <LOD | 0.50 |
| #21 | <LOD | — |
| #22 | <LOD | — |
| #23 | <LOD | 2.50 |
| #24 | <LOD | 1.75 |
| #25 | <LOD | — |
| #26 | <LOD | — |
| #27 | <LOD | 0.61 |
| #28 | <LOD | — |

(a) ELISA screening, (b) LC-HRMS confirmation analysis; LOD = limit of detection.

line ($^{***}P < 0.001$ vs Ctrl) (Figure 4(a)). Indeed except for 8 samples (1, 4, 8, 9, 17, 25, 26, 27), the extent of the reduction elicited by the other samples is similar to that one obtained by DMSO used as a reference toxic compound. As dilution increases, cytotoxicity drops and barely a few samples retain the ability to decrease significantly K562 cell viability. Indeed, as shown in Figure 4(d), only samples 10, 11, 12, 17, 21, 22, and 23 retain their cytotoxicity (Figure 4(d)).

*3.5. Multiplex Human Cytokines Assay.* Based on the results achieved by the cytotoxicity assay, 2 out of 7 cytotoxic samples (10 and 23) at the lowest dilution (1 : 20) were selected and evaluated for their proinflammatory activity at 24 and 36 h. Moreover, 1 out of 20 "noncytotoxic" samples (26) was randomly selected as a further control at the same dilution (Figure 5).

Interestingly, both cytotoxic and "noncytotoxic" samples exerted a significant proinflammatory effect at 24 and 36 h ($^{***}P < 0.001$) compared to control cells. IL-1$\alpha$ significantly increased for all samples compared to the control (from 0 to $130.7 \pm 2.75$, $758.5 \pm 7.77$, and $195.3 \pm 0.45$ pg/100 $\mu$l, $^{***}P < 0.001$, respectively for samples 10, 23, and 26) at 24 h. A significant increase, although at a lower extent, was also observed at 36 h, from 0 to $43.27 \pm 1.32$, $100.08 \pm 1.17$, and $69.65 \pm 0.84$ pg/100 $\mu$l, $^{***}P < 0.001$, respectively (Figure 5(a)). Moreover, IL-1$\alpha$ resulted significantly increased at 24 h compared to 36 h for all samples ($^{***}P < 0.001$). As far as concerns IL-1$\beta$, it showed a significant increase in its release for all three samples compared to the control (from 0 to $262.6 \pm 1.75$, $272 \pm 2.48$, and $1486.0 \pm 8.14$ pg/100 $\mu$l, $^{***}P < 0.001$, respectively) after 24 h and 36 h (from 0 to $177.3 \pm 0.39$, $488.29 \pm 2.05$, and $1332 \pm 4.98$ pg/100 $\mu$l, respectively, $^{***}P < 0.001$) (Figure 5(b)). Moreover, it significantly decreased for samples 10 and 26 after 36 h compared to 24 h ($^{***}P < 0.001$), while it significantly increased for sample 23 after 36 h compared to 24 h ($^{***}P < 0.001$). IL-8 showed a significant increase for all samples compared to the control (from 0 to $1613.0 \pm 1.98$, 1614.0 1.31, and $1673 \pm 10.88$ pg/100 $\mu$l, $^{***}P < 0.001$, respectively) after 24 h and 36 h (from 0 to $1686.0 \pm 5.98$, $1528.0 \pm 4.99$, and $1697.0 \pm 3.63$ pg/100 $\mu$l, $^{***}P < 0.001$, respectively) (Figure 5(d)). Differently from IL-1$\beta$, IL-8 significantly increased for samples 10 and 26 after 36 h compared to 24 h ($^{***}P < 0.001$) while significantly decreasing in sample 23 after 36 h compared to 24 h ($^{***}P < 0.001$). Similarly, IL-6 showed a significant increase in all three samples compared to the control (from 0 to $701.5 \pm 1.32$, $706.3 \pm 1.49$, and $705.8 \pm 3.47$ pg/100 $\mu$l, $^{***}P < 0.001$, respectively) after 24 h and 36 h (from 0 to $701.6 \pm 0.0$, $704.8 \pm 1.53$, and $707.1 \pm 0.87$ pg/100 $\mu$l, $^{***}P < 0.001$, respectively) (Figure 5(c)). However, no significant differences among samples were observed by comparing the release after 24 and 36 h. Even GM-CSF, MCAF, and TNF-$\alpha$ showed significant increases in their release for all samples compared to the control after 24 and 36 h. In particular, GM-CSF increased from 0 to $1822.0 \pm 4.10$, $1783 \pm 4.12$, and $1701 \pm 2.39$ pg/100 $\mu$l ($^{***}P < 0.001$), respectively, after 24 h, and from 0 to $1723.0 \pm 10.95$, $1715.0 \pm 4.08$, and $1613.0 \pm 1.70$ pg/100 $\mu$l ($^{***}P < 0.001$), respectively, after 36 h (Figure 5(e)). Moreover, it significantly decreased for all samples at 36 h compared to 24 h ($^{***}P < 0.001$). INF-$\gamma$ showed a significant increase in all three samples compared to the control (from 0 to $511.2 \pm 2.44$, $589.3 \pm 1.19$, and $464.9 \pm 1.23$ pg/100 $\mu$l, $^{***}P < 0.001$, respectively) at 24 h and 36 h (from 0 to $11.1 \pm 2.44$, $35.32 \pm 0.84$, and $42.7 \pm 1.51$ pg/100 $\mu$l, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{***}P < 0.001$, respectively). In addition, INF-$\gamma$ significantly increased after 24 h compared to 36 h in all samples ($^{***}P < 0.001$). MCAF increased for all three samples after 24 h from 0 to $155.20 \pm 1.01$, $200.50 \pm 1.28$, and $82.37 \pm 3.69$ pg/100 $\mu$l ($^{***}P < 0.001$), respectively, and at a higher extent at 36 h from 0 to $666.7 \pm 2.30$, $261.9 \pm 2.24$, and $243.1 \pm 2.01$ pg/100 $\mu$l ($^{***}P < 0.001$), respectively (Figure 5(g)). It also significantly increased after 36 h compared to 24 h for all samples ($^{***}P < 0.001$). TNF-$\alpha$ increased after 24 h in all samples compared to the control, from 0 to $949.3 \pm 2.41$, $1030.0 \pm 1.52$, and $1847.0 \pm 3.40$ pg/100 $\mu$l, respectively, $^{***}P < 0.001$, and at a lower extent at 36 h from 0 to $588.30 \pm 1.64$, $486.90 \pm 0.64$, and $1770.0 \pm 1.52$ pg/100 $\mu$l, $^{***}P < 0.001$, respectively (Figure 5(h)). TNF-$\alpha$ also significantly decreased for all samples after 36 h compared to 24 h ($^{***}P < 0.001$).

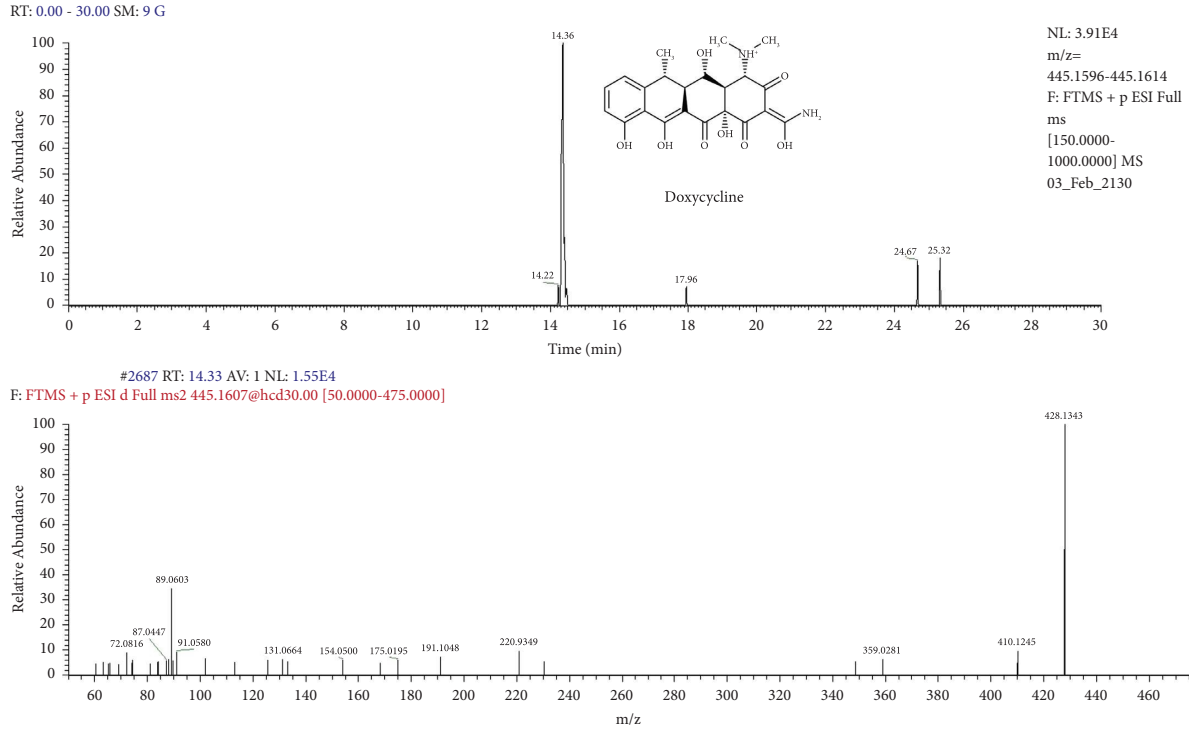


Figure 3: Chromatogram and full-MS spectrum of doxycycline in sample 23.

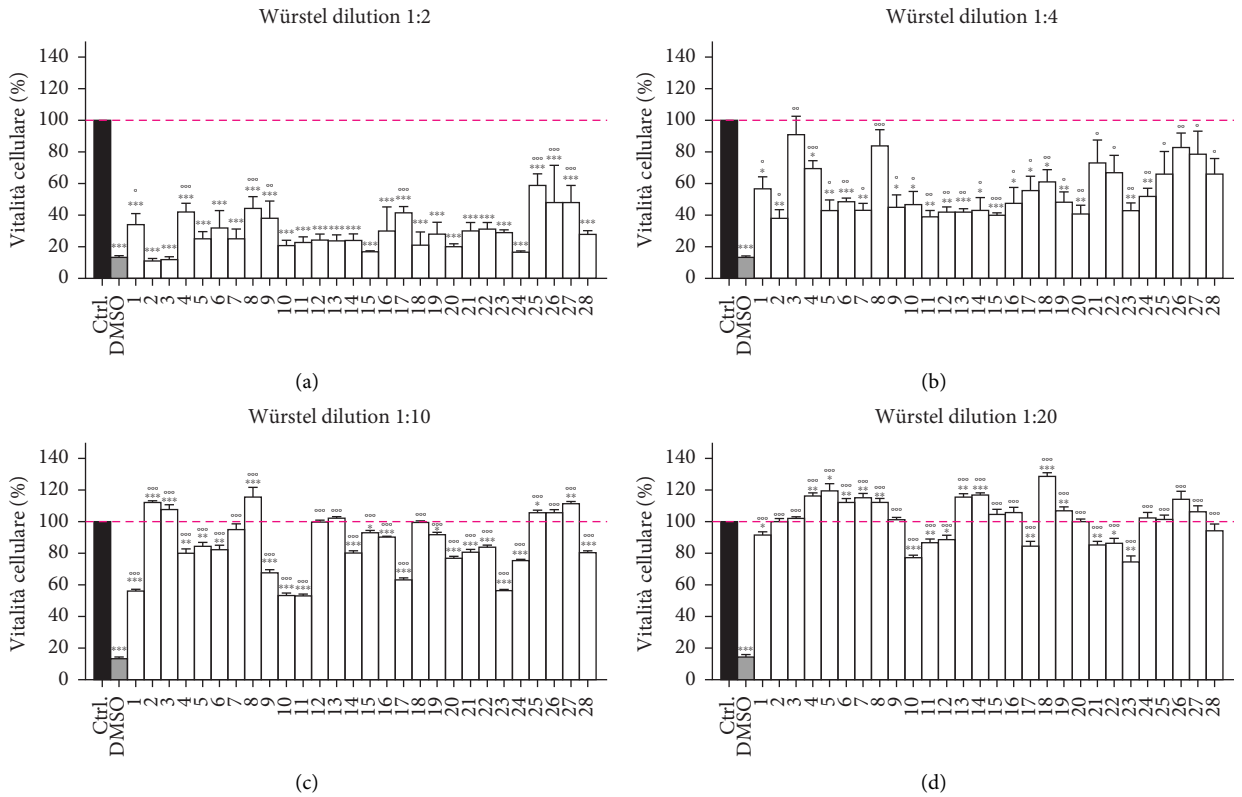


Figure 4: Graphical representation of K562 cell viability after 48 h challenge with würstels-derived conditioned culture medium at different dilutions. Panels (a–d): $^{***}P < 0.001$ vs. Ctrl; $^{**}P < 0.01$ vs. Ctrl; $^{*}P < 0.05$ vs. Ctrl; $^{\circ}P < 0.05$ vs. DMSO; $^{\circ\circ}P < 0.01$ vs. DMSO; $^{\circ\circ\circ}P < 0.001$ vs. DMSO.

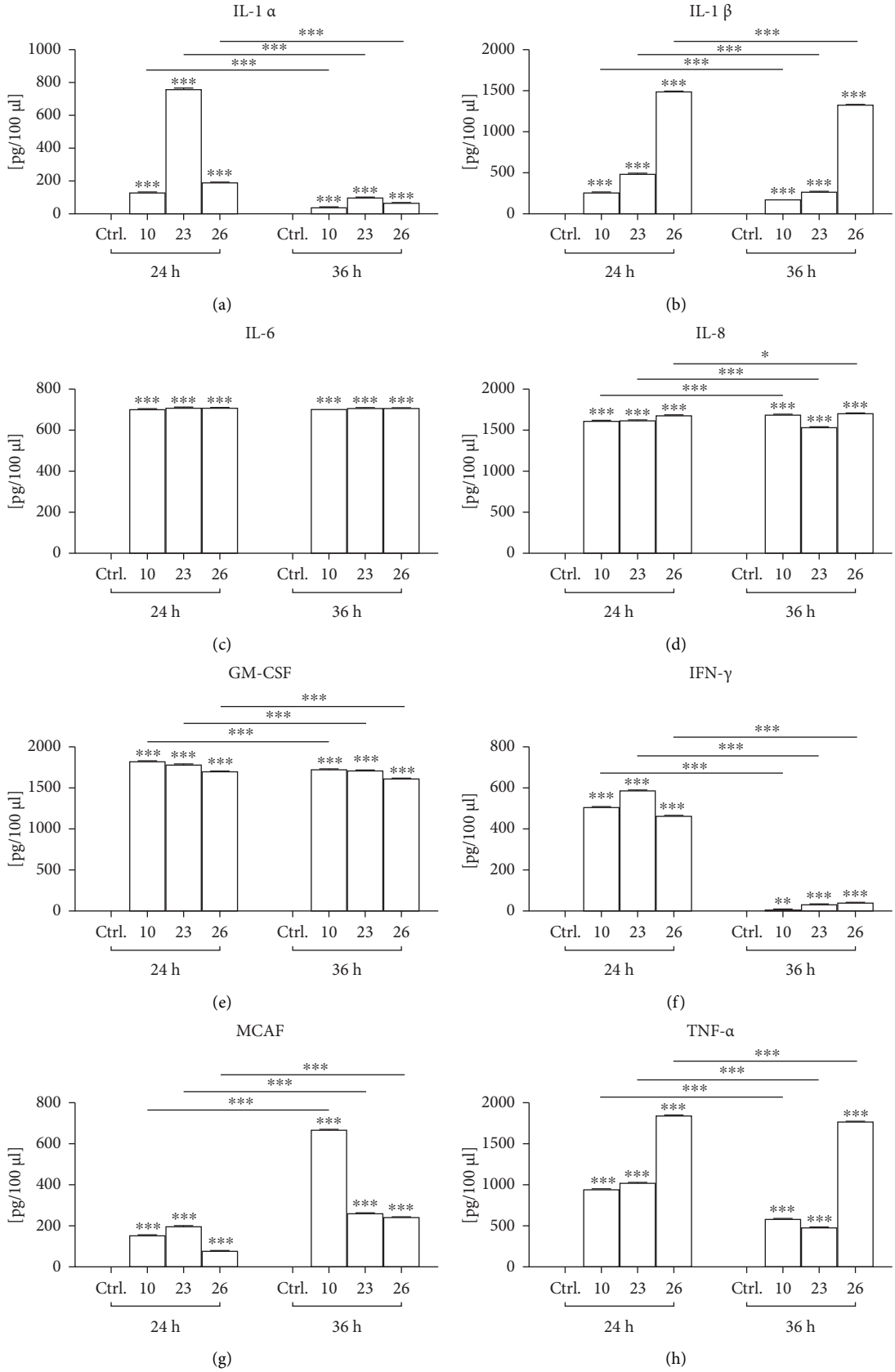


Figure 5: Graphical representation of the cytokines' release by K562 following 24 and 36 h challenge with three different würstel-derived conditioned culture media at 1 : 20 dilution ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

*3.6. Food Additive Cytotoxicity Assay.* As a first step in assessing the compounds responsible for the cytotoxicity observed in the whole samples, some potentially cytotoxic food additives (pyrophosphate, tripolyphosphate, metaphosphate, sodium nitrite, and one beech- and oak-based smoke flavoring) used in industrial würstel preparation were tested according to their maximum allowed amount [54, 55]) (Figure 6).

Pyrophosphate showed an overall significant reduction in cell viability compared to the control, which varied depending on the concentration used (Figure 6(a)); a significant 20% reduction after 24 and 48 h ($^{***}P < 0.001$) was observed at 1.25 $\mu$g/ml; a similar reduction in cell viability was also observed at 2.5 $\mu$g/ml after 24 h, furtherly decreasing to 30% after 48 h ($^{***}P < 0.001$); an inverted trend was observed at 10 $\mu$g/ml, reaching a 25% reduction after 24 h and 17% after 48 h ($^{***}P < 0.001$). Interestingly, a 10% reduction was observed at 5 $\mu$g/ml (the maximum permitted amount) after 24 and 48 h ($^{**}P < 0.01$). Cell viability was significantly reduced at 2.5 $\mu$g/ml and significantly increased at 10 $\mu$g/ml after 48 h compared to 24 h ($^{***}P < 0.001$ and $^{**}P < 0.01$, respectively).

As for tripolyphosphate, no significant cell viability decrease was observed after 24 h compared to the control, while a significant 10% decrease was observed at 1.25, 2.5, and 10 $\mu$g/ml after 48 h ($^{**}P < 0.01$, $^{***}P < 0.001$, and $^{**}P < 0.01$, respectively) (Figure 6(b)). Furthermore, cell viability was significantly reduced at 1.25 and 5 $\mu$g/ml after 48 h compared to 24 h ($^{**}P < 0.01$). The metaphosphate exerted a significant cytotoxic activity after 24 h only at 2.5 and 5 $\mu$g/ml compared to the control, with an 8 and 9% cell viability decrease ($^{**}P < 0.01$ and $^{***}P < 0.001$, respectively) (Figure 6(c)). After 48 h, 1.25, 2.5, and 10 $\mu$g/ml induced a significant 9, 17, and 9% cell viability decrease, respectively, compared to the control ($^{***}P < 0.001$). Moreover, cell viability was significantly reduced at all concentrations after 48 h compared to 24 h ($^{**}P < 0.01$ and $^{*}P < 0.05$). No significant cell viability decrease was observed for sodium nitrite compared to the control after 24 and 48 h (Figure 6(d)). Interestingly, the beech- and oak-based smoke flavoring induced a significant 23, 80, and 81% cell viability decrease at 50 mg/kg, 100 mg/kg, and 200 mg/kg (between the maximum suggested amount and above), respectively, after 24 h ($^{***}P < 0.001$) (Figure 6(e)). All the values significantly decreased to 65, 88, and 86%, respectively, after 48 h ($^{***}P < 0.001$). Cell viability was significantly reduced at all concentrations after 48 h compared to 24 h ($^{***}P < 0.01$).

*3.7. Food Additive Multiplex Human Cytokines Assay.* No cytokine release was observed for pyrophosphate, tripolyphosphate, metaphosphate, or sodium nitrite (data not shown). Since the results achieved with the beech- and oak-based smoke flavoring at 0.10 and 0.20% were similar, only the minimum (0.05%) and maximum (0.20%) suggested concentrations were evaluated. Considering the observed cytotoxic effect of the oak-based smoke flavoring at 24 h (Figure 6(e)), the presence of a proinflammatory effect was evaluated at 12 and 24 h to assess a possible role of the tested cytokines in the cytotoxic effect (Figure 7).

Interestingly, only four cytokines (IL-1$\beta$, IL-8, INF-$\gamma$, and MCAF) were stimulated after challenging with the beech- and oak-based smoke flavoring compared to the control. In particular, the beech- and oak-based smoke flavoring induced a significant increase in IL-1$\beta$ and MCAF at 200 mg/kg, from 0 to $11.33 \pm 0.57$ pg/100 $\mu$l ($^{***}P < 0.001$) and from 0 to $7.16 \pm 0.11$ pg/100 $\mu$l, ($^{***}P < 0.001$), respectively, after 24 h (Figures 7(a) and 7(d)).

Conversely, IL-8 showed a significant increase at 50 mg/kg of the beech- and oak-based smoke flavoring after 12 h, from 0 to $53.00 \pm 21.73$ pg/100 $\mu$l ($^{***}P < 0.001$), and after 24 h, from 0 to $57.33 \pm 0.57$ pg/100 $\mu$l ($^{***}P < 0.001$) (Figure 7(b)). Moreover, IL-8 significantly increased at 24 h compared to 12 h ($^{**}P < 0.01$).

INF-$\gamma$ resulted significantly increased after 12 h at 200 mg/kg of the beech- and oak-based smoke flavoring, from 0 to $1.96 \pm 0.23$ pg/100 $\mu$l ($^{*}P < 0.05$), and after 24 h, from 0 to $4.50 \pm 0.17$ pg/100 $\mu$l ($^{**}P < 0.01$) (Figure 7(c)). However, it significantly increased after 24 h at 50 mg/kg, from 0 to $9.13 \pm 1.15$ pg/100 $\mu$l ($^{**}P < 0.01$). Moreover, INF-$\gamma$ significantly increased at 24 h compared to 12 h at 50 and 200 mg/kg ($^{*}P < 0.05$), respectively.

## 4. Discussion

The present research builds upon previous studies carried out between 2014 and 2020 on intensive farmed animals' bone, one of the ingredients used in pet food, which revealed the presence of antibiotics, particularly OTC, even respecting the withdrawal times [3, 8, 52].

We demonstrated that such an antibiotic, as well as any other tetracycline, could remain fixed to the bone tissue of treated animals, mainly chickens, due to its covalent binding to $Ca^{2+}$ forming a protein complex responsible for the cytotoxic and proinflammatory activity *in vitro* [3–5, 8, 50]. We also reported the presence of such an antibiotic in pets and humans that had never been treated with it, inducing several clinical manifestations ranging from adverse food reactions to intense itch, neck eczema, otitis, dermatitis, diarrhea, and generalized anxiety [19, 21, 50, 56, 57]. Concerning the pets, we hypothesized that the bone in the kibbles, derived by a mechanical separation from the meat, could act as a possible antibiotic dragger able to be first accumulated in the animal's body (as a consequence of continuous ingestion) and then gradually released over time [21, 50]. On the contrary, we hypothesized that the onset of the food intolerances observed in the gym-trained human subjects could be the consequence of a long and continuous sensitization process fostered by the presence of low amounts of OTC but also DOXY, present in the chicken meat-based diet that they assumed daily (300 to 600 g/day) [19].

Being aware of the new regulations set in 2019 concerning the phase-out of the routine use of antibiotics for disease prevention in farming animals and reserving only the prophylactic use for exceptional circumstances [10], we decided to investigate the presence of bone and antibiotic residues in 28 commercially available chicken-based würstels obtained by mechanical separation to have an

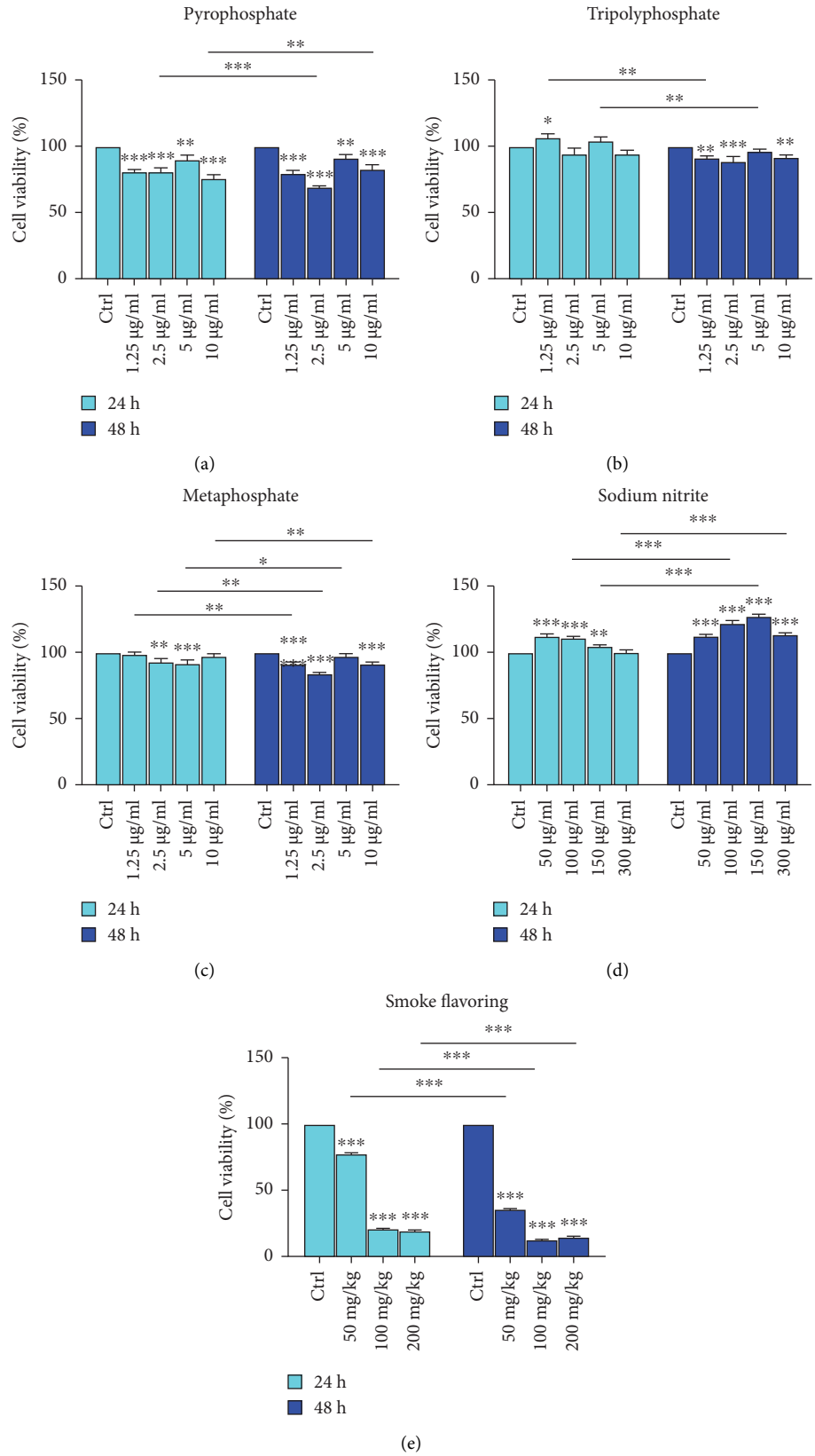


Figure 6: Graphical representation of K562 cell viability after 24 and 48 h challenge with different würstel-derived potentially cytotoxic preservatives at different concentrations ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

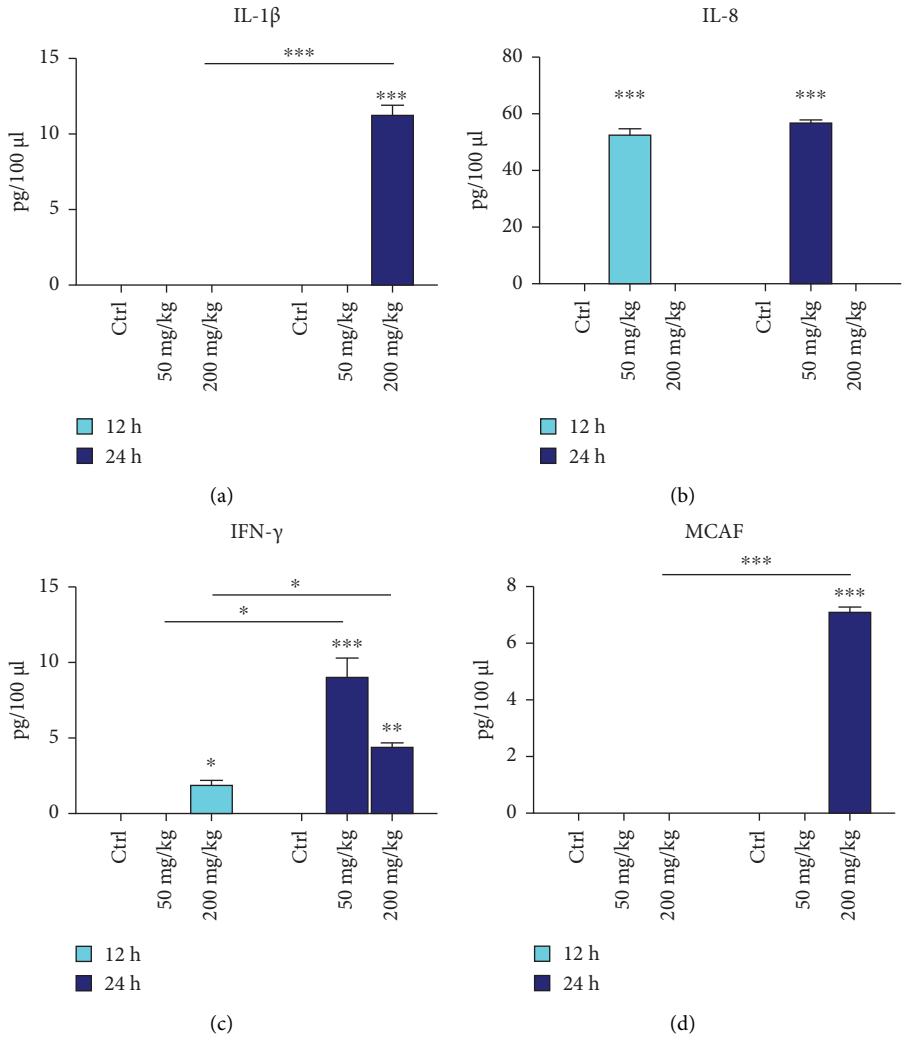


Figure 7: Graphical representation of the cytokines' release by K562 following the beech- and oak-based smoke flavoring challenge at different concentrations ($^{*}P < 0.05$, $^{***}P < 0.001$).

updated picture of the direct effects of such regulations on foods of animal origin.

The histochemical and microscopy analyses revealed the presence of calcium salts within the foci of the bone matrix in all samples. However, the ELISA did not show the presence of OTC, while the LC-HRMS method for antibiotic identification revealed the presence of DOXY in 9 out of 28 samples in the range of 0.4–2.5 $\mu$g/kg, although significantly lower than the maximum residual limits for all considered matrices.

Interestingly, the CCK-8 cytotoxicity assay carried out on all samples at different dilutions (1 : 2, 1 : 4, 1 : 10, 1 : 20) revealed a significant cytotoxic effect for all samples at 1 : 2 and 1 : 4, while at 1 : 10, such effect was detected in 19 samples and at 1 : 20 only in 9 out of 28 samples. According to Ermak et al., who demonstrated the negative effect of DOXY on cell viability at concentrations as low as 200 $\mu$g/kg [58], we ruled out that the cytotoxic effect observed could be related to the presence of the antibiotic since the concentration reported by LC-HRMS was too low, but, at the same time, we did not exclude the involvement of other substances. Therefore, we hypothesized that the cytotoxicity could be mediated by other ingredients, including food additives such as pyrophosphate, tripolyphosphate, metaphosphate, and sodium nitrite, which have been widely acknowledged as potentially harmful substances [27, 28, 59–61]. However, none of the aforementioned compounds was proven to be highly cytotoxic (with a maximum cell viability decrease of 10–30%), neither at the allowed amounts nor above them, probably in light of their high acceptable daily intake ascertained by EFSA [62–64].

Conversely, the beech- and oak-based smoke flavoring, another food preservative present in all the samples, exerted a significantly high cytotoxic effect (with a cell viability decrease of 80–86%) even below the allowed amount, probably due to a high concentration of polycyclic aromatic hydrocarbons (PAHs), such as benz[a]anthracene, chrysene, benzo[b]fluoranthene, and benzo[a]pyrene, formed through incomplete pyrolysis of the organic fuel (typically wood) used to generate smoke and able to raise serious health concerns [65–70].

Smoke flavorings are subjected to the general Regulations (EC) No 2065/2003 and No 1334/2008 on flavorings and certain food ingredients with flavoring properties for their use in foods, which lay down the general requirements for safe use of flavorings, define the different types of flavorings, set out flavoring substances for which an evaluation and approval is required, and establish a community procedure for the safety assessment and the authorization of smoke flavorings intended for use in or on foods based on a high level of protection of human health and consumers' interests [55, 71].

In a recent paper from Racovita et al. [67], the authors quantified the PAHs concentration from seven types of hardwoods (plum, Alder, birch, beech, oak, apple, and walnut) and observed that their sum increased continuously with higher temperatures (55–95°C) and longer smoking periods (2–9 h) [67]. Although plum, Alder, and birch yielded the highest concentrations of PAHs compared to beech- and oak ones, it is reasonable to hypothesize that the high cytotoxic and proinflammatory effect observed in our assays could be due to the sum of their concentrations.

Based on our previous *in vitro* observations [3, 8, 50, 72], we also investigated the potential proinflammatory effect of 3 würstel extracts (2 cytotoxic and 1 noncytotoxic) at the highest dilution (1 : 20). The rationale for choosing the highest dilution was based on the assumption that the possible effect on cytokine production could be mediated at concentrations lower than those that interfere with cell viability. Pyrophosphate, tripolyphosphate, metaphosphate, sodium nitrite (data not shown), and beech- and oak smoke flavoring solutions were also tested at different concentrations for their potential proinflammatory effect.

Regarding the 3 würstel extracts, all samples induced a significant release of the considered cytokines with proinflammatory activity after 24 and 36 h, partially similar to beech- and oak-based smoke flavoring. This led us to hypothesize a prominent involvement of such smoke flavoring in cytokine production and, therefore, in the possible onset and progression of inflammation-based metabolic disorders such as diabetes and obesity. In fact, prolonged exposure to IL-1$\beta$ has been shown to be involved in obesity and insulin resistance by reducing insulin-induced glucose uptake following a decreased expression of Glut 4 and a marked inhibition of its translocation to the plasma membrane [73]. Therefore, the IL-1$\beta$ increased release even at 48 h observed in our study corroborates the possible link between processed meat intake and aforementioned metabolic disorders. IL-1$\beta$, MCAF, IL-6, and TNF-$\alpha$ are also known to be released by macrophages infiltrating adipose tissue in obesity, thus contributing to the pathogenesis of obesity-induced insulin resistance [74]. Moreover, inflammation (e.g., MCAF and C-reactive protein production) and insulin resistance were observed in obese cohorts, as a consequence of red and processed meat consumption, and related to an excess of adipose tissue [75]. Among contributing factors to oxidative stress and inflammation status observed in the progression of metabolic disorders related to red and processed meat consumption iron, trimethylamine-N-oxide (TMAO), preservation methods (smoking, salting, and curing), and preservatives (sodium, nitrates, and advanced glycation end products) have been questioned [75–78]. In fact, iron, highly present in red and processed meat can result from the high-temperature cooking, leading to the formation of carcinogenic chemicals such as N-nitroso-compounds and PAHs [76, 79]. Conversely, TMAO was shown to activate TXNIP-NLRP3 inflammasome, which in turn releases inflammatory cytokines such as IL-6, IL-1$\beta$, and TNF-$\alpha$ and increases oxidative stress [80]. Regarding advanced glycation end products, these were shown to increase oxidation of low-density lipoproteins, resulting in obesity and insulin resistance [81], while dietary nitrites can be converted back to biologically active NO, by means of several nitrite reductase enzymes (e.g., hemoglobin, methemoglobin, and neuroglobin), a molecule known to play a pivotal role in the inflammation pathogenesis [82–84]. All these evidences thus support the hypothesis of a link between massive consumption of processed meat, such as würstels, and the onset and progression of the obesity-related inflammation [85–87]. As far as concerns the INF-$\gamma$, the release trend observed for the beech- and oak-based smoke flavoring was quite similar to that observed in a CCM incubated with a ground bone derived from chicken treated with OTC, showing a rapid onset at 12 h and a peak at 24 h [3], indicating an acute inflammation occurring in the early stages following food consumption.

To further support the direct involvement of considered cytokines in metabolic disorders, a study on 224 Iranian women in 2023 showed a relation between higher adherence to processed meat consumption, TGF-1$\beta$, IL-1$\beta$, IL-6, and MCAF production, and increasing odds of metabolically unhealthy obesity phenotype [88].

Based on the aforementioned considerations, we hypothesized that the proinflammatory activity of the beech- and oak-based smoke flavoring could be correlated to PAHs' cytotoxic potential. In fact, PAHs are largely known for their toxic, mutagenic/genotoxic, and carcinogenic effects in humans and laboratory animals [89].

Hence, our study investigated the potential cytotoxic and proinflammatory effect of processed meat, i.e., würstels and their ingredients, particularly beech- and oak-based smoke flavoring, widening the panel of potentially dangerous compounds besides antibiotics. We can speculate these compounds' possible dualistic and synergic activity in the würstel extract, particularly beech- and oak-based smoke flavoring and antibiotics: one observed at low concentrations on proinflammatory cytokines and one observed at high concentrations directed to the metabolic activity of cells as shown by the inhibition assay. It must be underlined that this last effect could also be a direct consequence of the possible massive release of cytokines mediated by the highest concentrations tested.

At the same time, we cannot rule out the involvement of a possible release of an antibiotic-protein complex from the bone fragments present in all würstel samples, which was already proven to be cytotoxic and proinflammatory once released in the medium after 24–48 h of incubation [50]. In addition, such a hypothesis might explain the gap in the cytokines' release observed for the whole wurstels and the beech- and oak-based smoke flavouring.

Although we are aware that this can be considered only a preliminary study and that all of the ingredients should be thoroughly investigated even at a molecular level, it provides new insights into the understanding of the link among high consumption of ultraprocessed meat, increased risk of inflammation, and progression of chronic diseases.

## Data Availability

The data presented in this study are available from the corresponding authors upon request.

## Disclosure

No persons or third-party services were involved in the research and manuscript preparation. Moreover, no AI software packages have been used to prepare the manuscript.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

## Authors' Contributions

A.D.C., L.C., and C.S. conceptualized the study. S.S., N.R., G.E.M., C.M., S.R., A.R.L., and R.S. proposed the methodology. A.D.C., L.C., J.C., and A.T. performed formal analysis. S.S., N.R., R.C., C.V., and M.A. investigated the study. R.S., S.R., A.D.C., and C.M. provided resources. A.T., C.V., C.S., M.A., S.S., and N.R. contributed to data analysis and interpretation. A.D.C., R.C., L.C., S.S., N.R., C.V., A.T., and G.E.M. wrote the original draft. A.R.L., J.C., M.A., C.S., S.R., A.D.C., C.M., and R.S. reviewed and edited the article. L.C., A.D.C., and C.S. supervised the study. A.D.C. performed project administration. All authors agree to be accountable for the content and conclusions of the article. Carla Sabia and Alessandro Di Cerbo shared senior co-authorship. The authors Lorenzo Corsi, Nicola Rubattu, and Severyn Salis contributed equally.

## References


[1] R. Mulchandani, Y. Wang, M. Gilbert, and T. P. Van Boeckel, "Global trends in antimicrobial use in food-producing animals: 2020 to 2030," *PLOS Global Public Health*, vol. 3, no. 2, Article ID e0001305, 2023.

[2] T. P. Van Boeckel, C. Brower, M. Gilbert et al., "Global trends in antimicrobial use in food animals," *Proceedings of the National Academy of Sciences of the U S A*, vol. 112, no. 18, pp. 5649–5654, 2015.

[3] A. Di Cerbo, A. T. Palatucci, V. Rubino et al., "Toxicological implications and inflammatory response in human lymphocytes challenged with oxytetracycline," *Journal of Biochemical and Molecular Toxicology*, vol. 30, no. 4, pp. 170–177, 2016.

[4] A. Di Cerbo, F. Pezzuto, G. Guidetti, S. Canello, and L. Corsi, "Tetracyclines: insights and updates of their use in human and animal pathology and their potential toxicity," *The Open Biochemistry Journal*, vol. 13, pp. 1–12, 2019.

[5] A. Di Cerbo, F. Pezzuto, A. Scarano, G. Guidetti, and S. Canello, "The contradictory world of tetracyclines," *Panminerva Medica*, vol. 62, no. 2, pp. 116-117, 2020.

[6] A. B. Falowo and O. F. Akimoladun, "Veterinary drug residues in meat and meat products: occurrence, detection and implications," in *Veterinary Medicine and Pharmaceuticals*, S. Oppong Bekoe, M. Saravanan, R. Kwame Adosraku, and P. K. Ramkumar, Eds., pp. 1–18, IntechOpen, Croatia, 2019.

[7] M. F. Landoni and G. Albarellos, "The use of antimicrobial agents in broiler chickens," *The Veterinary Journal*, vol. 205, no. 1, pp. 21–27, 2015.

[8] R. Odore, M. De Marco, L. Gasco, L. Rotolo, V. Meucci, and A. T. Palatucci, "Cytotoxic effects of oxytetracycline residues in the bones of broiler chickens following therapeutic oral administration of a water formulation," *Poultry Science*, vol. 94, no. 8, pp. 1979–1985, 2015.

[9] European Parliament and of the Council, "Regulation (Eu) 2019/4 on the manufacture, placing on the market and use of medicated feed, amending regulation (EC) No 183/2005 of the European parliament and of the council and repealing council directive 90/167/EEC," *Official Journal of the European Union*, 2018, https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32019R0004.

[10] European Parliament and of the Council, "Regulation (Eu) 2019/6 on veterinary medicinal products and repealing Directive 2001/82/EC," *Official Journal of the European Union*, 2018, https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32019R0006&qid=1689946961644.

[11] M. R. Vishnuraj, G. Kandeepan, K. H. Rao, S. Chand, and V. Kumbhar, "Occurrence, public health hazards and detection methods of antibiotic residues in foods of animal origin: a comprehensive review," *Cogent Food and Agriculture*, vol. 2, no. 1, Article ID 1235458, 2016.

[12] European Commission, "Commission regulation (EU) no 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin," 2010, https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32010R0037&from=EN.

[13] FDA, "Tolerances for residues of new animal drugs in food," 2021, https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=556.1.

[14] Alimentarius, "Codex alimentarius," 2022, https://www.fao.org/fao-who-codexalimentarius/codex-texts/dbs/vetdrugs/en/.

[15] L. L. Founou, R. C. Founou, and S. Y. Essack, "Antibiotic resistance in the food chain: a developing country-perspective," *Frontiers in Microbiology*, vol. 7, p. 1881, 2016.

[16] R. Iseppi, A. Di Cerbo, P. Aloisi, M. Manelli, V. Pellesi, and C. Provenzano, "In vitro activity of essential oils against planktonic and biofilm cells of extended-spectrum beta-lactamase (esbl)/carbapenamase-producing gram-negative bacteria involved in human nosocomial infections," *Antibiotics*, vol. 9, no. 5, 2020.

[17] R. Iseppi, A. Di Cerbo, P. Messi, and C. Sabia, "Antibiotic resistance and virulence traits in vancomycin-resistant enterococci (VRE) and extended-spectrum beta-Lactamase/AmpC-producing (ESBL/AmpC) enterobacteriaceae from humans and pets," *Antibiotics*, vol. 9, no. 4, 2020.

[18] N. Vragovic, D. Bazulic, and B. Njari, "Risk assessment of streptomycin and tetracycline residues in meat and milk on Croatian market," *Food and Chemical Toxicology*, vol. 49, no. 2, pp. 352–355, 2011.

[19] A. Di Cerbo, S. Canello, G. Guidetti, C. Laurino, and B. Palmieri, "Unusual antibiotic presence in gym trained

subjects with food intolerance; a case report," *Nutricion Hospitalaria*, vol. 30, no. 2, pp. 395–398, 2014.

[20] A. Di Cerbo, V. Rubino, F. Morelli et al., "Mechanical phenotyping of K562 cells by the Micropipette Aspiration Technique allows identifying mechanical changes induced by drugs," *Scientific Reports*, vol. 8, no. 1, p. 1219, 2018.

[21] F. Mazzeranghi, C. Zanotti, A. Di Cerbo et al., "Clinical efficacy of nutraceutical diet for cats with clinical signs of cutaneus adverse food reaction (CAFR)," *Polish Journal of Veterinary Sciences*, vol. 20, no. 2, pp. 269–276, 2017.

[22] D. Sanz, P. Razquin, S. Condón, T. Juan, B. Herraiz, and L. Mata, "Incidence of antimicrobial residues in meat using a broad spectrum screening strategy," *European Journal of Nutrition and Food Safety*, vol. 5, no. 3, pp. 156–165, 2015.

[23] European Parliament and of the Council, "Regulation (Ec) No 853/2004 of the European Parliament and of the Council laying down specific hygiene rules for on the hygiene of foodstuffs," *Official Journal of the European Communities*, 2004, https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32004R0853&qid=1687612394870.

[24] S. Erem and M. S. Razzaque, "Dietary phosphate toxicity: an emerging global health concern," *Histochemistry and Cell Biology*, vol. 150, no. 6, pp. 711–719, 2018.

[25] M. Karwowska and A. Kononiuk, "Nitrates/nitrites in food-risk for nitrosative stress and benefits," *Antioxidants*, vol. 9, no. 3, 2020.

[26] C. A. Monteiro, G. Cannon, R. B. Levy, J. C. Moubarac, M. L. Louzada, and F. Rauber, "Ultra-processed foods: what they are and how to identify them," *Public Health Nutrition*, vol. 22, no. 5, pp. 936–941, 2019.

[27] E. Ritz, K. Hahn, M. Ketteler, M. K. Kuhlmann, and J. Mann, "Phosphate additives in food--a health risk," *Deutsches Ärzteblatt International*, vol. 109, no. 4, pp. 49–55, 2012.

[28] A. Theobald, D. Arcella, A. Carere et al., "Safety assessment of smoke flavouring primary products by the European Food Safety Authority," *Trends in Food Science and Technology*, vol. 27, no. 2, pp. 97–108, 2012.

[29] S. Yuan, J. Chen, T. Fu et al., "Ultra-processed food intake and incident venous thromboembolism risk: prospective cohort study," *Clinical Nutrition*, vol. 42, no. 8, pp. 1268–1275, 2023.

[30] O. Goemaere, S. Glorieux, M. Govaert, L. Steen, and I. Fraeye, "Phosphate elimination in emulsified meat products: impact of protein-based ingredients on quality characteristics," *Foods*, vol. 10, no. 4, 2021.

[31] N. Bach, S. Long, R. Gál, and F. Buňka, "Use of phosphates in meat products," *African Journal of Biotechnology*, vol. 10, 2011.

[32] R. Pavlovic, F. Di Cesare, F. Longo, F. Abballe, S. Panseri, and R. C. Bonanni, "Undeclared (Poly)phosphates detection in food of animal origin as a potential tool toward fraud prevention," *Foods*, vol. 10, no. 7, p. 1547, 2021.

[33] T. V. Duncan, "Applications of nanotechnology in food packaging and food safety: barrier materials, antimicrobials and sensors," *Journal of Colloid and Interface Science*, vol. 363, no. 1, pp. 1–24, 2011.

[34] F. C. O. L. Martins, M. A. Sentanin, and D. De Souza, "Analytical methods in food additives determination: compounds with functional applications," *Food Chemistry*, vol. 272, pp. 732–750, 2019.

[35] FAO/WHO Expert Committee, "General standard for food additives," 2021, https://www.fao.org/fao-who-codexalimentarius/shproxy/en/?lnk=1&url=%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FMeetings%252FCX-711-52%252F2021SepWD%252Ffa52_10e.pdf.

[36] Commission of the European Communities, "Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (Text with EEA relevance) (notified under document number C(2002) 3044)," 2002, https://eur-lex.europa.eu/eli/dec/2002/657/oj.

[37] European Parliament and Council, "Regulation 1333/2008 on food additives," 2008, https://eurlex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32008R1333&from=IT.

[38] EFSA Panel on Food Additives and Flavourings, "Re-evaluation of phosphoric acid–phosphates – di-, tri- and polyphosphates (E 338–341, E 343, E 450–452) as food additives and the safety of proposed extension of use," *EFSA Journal*, vol. 17, p. 5674, 2019.

[39] N. O'Brien and E. Cummins, "Ranking initial environmental and human health risk resulting from environmentally relevant nanomaterials," *Journal of Environmental Science and Health*, vol. 45, no. 8, pp. 992–1007, 2010.

[40] Z. Ding, S. Johanningsmeier, R. Price et al., "Evaluation of nitrate and nitrite contents in pickled fruit and vegetable products," *Food Control*, vol. 90, 2018.

[41] EFSA Panel on Food Contact Materials, "Scientific opinion on the safety of smoke flavouring Primary Product AM 01–2012 Update," *EFSA Journal*, vol. 10, no. 2, p. 2580, 2012.

[42] D. Meier, "Liquid smoke-an analytical challenge," *Fleischwirtschaft International: Journal for Meat Production and Meat Processing*, pp. 37–40, 2005.

[43] M. a. D. Guillén and M. a. J. Manzanos, "Study of the volatile composition of an aqueous oak smoke preparation," *Food Chemistry*, vol. 79, no. 3, pp. 283–292, 2002.

[44] M. D. Guillen and M. L. Ibargoitia, "Influence of the moisture content on the composition of the liquid smoke produced in the pyrolysis process of Fagus sylvatica L. wood," *Journal of Agricultural and Food Chemistry*, vol. 47, no. 10, pp. 4126–4136, 1999.

[45] J. A. Maga and Z. Chen, "Pyrazine composition of wood smoke as influenced by wood source and smoke generation variables," *Flavour and Fragrance Journal*, vol. 1, no. 1, pp. 37–42, 1985.

[46] B. Bukowska and S. Adamczyk, "The presence and toxicity of phenol derivatives – their effect on human erythrocytes," *Current Topics in Biophysics*, vol. 27, pp. 43–51, 2003.

[47] Y. K. Koleva, J. C. Madden, and M. T. Cronin, "Formation of categories from structure-activity relationships to allow read-across for risk assessment: toxicity of alpha,beta-unsaturated carbonyl compounds," *Chemical Research in Toxicology*, vol. 21, no. 12, pp. 2300–2312, 2008.

[48] A. B. Patel, S. Shaikh, K. R. Jain, C. Desai, and D. Madamwar, "Polycyclic aromatic hydrocarbons: sources, toxicity, and remediation approaches," *Frontiers in Microbiology*, vol. 11, 2020.

[49] X. Xu, A. Liu, S. Hu et al., "Synthetic phenolic antioxidants: metabolism, hazards and mechanism of action," *Food Chemistry*, vol. 353, Article ID 129488, 2021.

[50] A. Di Cerbo, S. Canello, G. Guidetti et al., "Adverse food reactions in dogs due to antibiotic residues in pet food: a preliminary study," *Veterinaria Italiana*, vol. 54, no. 2, pp. 137–146, 2018.

[51] A. Di Cerbo, A. Scarano, F. Pezzuto et al., "Oxytetracycline-protein complex: the dark side of pet food," *The Open Public Health Journal*, vol. 11, pp. 162–169, 2018.

[52] C. Pacelli, A. Di Cerbo, L. Lecce et al., "Effect of chicken bone extracts on metabolic and mitochondrial functions of K562 cell line," *Pharmaceuticals*, vol. 13, no. 6, 2020.

[53] C. Rey, C. Combes, C. Drouet, and M. J. Glimcher, "Bone mineral: update on chemical composition and structure," *Osteoporosis International*, vol. 20, no. 6, pp. 1013–1021, 2009.

[54] European Commission, "Commission regulation 1129/2011, C. R. E. N," *List of food additives Text with EEA relevance*, 2011, https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32011R1129.

[55] European Parliament and of the Council, "Regulation (Ec) No 1334/2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC," *Official Journal of the European Union*, 2008, https://eur-lex.europa.eu/legalcontent/EN/TXT/HTML/?uri=CELEX:32008R1334.

[56] A. Di Cerbo, S. Sechi, S. Canello, G. Guidetti, F. Fiore, and R. Cocco, "Behavioral disturbances: an innovative approach to monitor the modulatory effects of a nutraceutical diet," *Journal of Visualized Experiments: JoVE*, vol. 119, 2017.

[57] S. Sechi, A. Di Cerbo, S. Canello et al., "Effects in dogs with behavioural disorders of a commercial nutraceutical diet on stress and neuroendocrine parameters," *The Veterinary Record*, vol. 180, no. 1, p. 18, 2017.

[58] G. Ermak, V. J. Cancasci, and K. J. Davies, "Cytotoxic effect of doxycycline and its implications for tet-on gene expression systems," *Analytical Biochemistry*, vol. 318, no. 1, pp. 152–154, 2003.

[59] Y. M. Abd-Elhakim, M. M. Hashem, A. Anwar et al., "Effects of the food additives sodium acid pyrophosphate, sodium acetate, and citric acid on hemato-immunological pathological biomarkers in rats: relation to PPAR-alpha, PPAR-gamma and tnfalpha signaling pathway," *Environmental Toxicology and Pharmacology*, vol. 62, pp. 98–106, 2018.

[60] D. Cvetković, V. Živković, V. Lukić, and S. Nikolić, "Sodium nitrite food poisoning in one family," *Forensic Science, Medicine and Pathology*, vol. 15, no. 1, pp. 102–105, 2019.

[61] P. K. Datta, A. C. Frazer, M. Sharratt, and H. G. Sammons, "Biological effects of food additives. II.—sodium pyrophosphate," *Journal of the Science of Food and Agriculture*, vol. 13, no. 11, pp. 556–566, 1962.

[62] Efsa Panel on Food Additives and Flavourings, "Re-evaluation of phosphoric acid–phosphates – di-, tri- and polyphosphates (E 338–341, E 343, E 450–452) as food additives and the safety of proposed extension of use," *EFSA Journal*, vol. 17, p. 5674, 2019.

[63] Efsa Panel on Food Additives and Nutrient Sources added to Food, "Re-evaluation of sodium nitrate (E 251) and potassium nitrate (E 252) as food additives," *EFSA Journal*, vol. 15, no. 6, Article ID e04787, 2017.

[64] Efsa Panel on Food Additives and Flavourings, "Re-evaluation of potassium nitrite (E 249) and sodium nitrite (E 250) as food additives," *EFSA Journal*, vol. 15, no. 6, Article ID e04786, 2017.

[65] B. Bukowska, K. Mokra, and J. Michalowicz, "Benzo[a] pyrene-Environmental occurrence, human exposure, and Mechanisms of toxicity," *International Journal of Molecular Sciences*, vol. 23, no. 11, 2022.

[66] J. L. Domingo, "Concentrations of environmental organic contaminants in meat and meat products and human dietary exposure: a review," *Food and Chemical Toxicology*, vol. 107, pp. 20–26, 2017.

[67] R. C. Racovita, C. Secuianu, M. D. Ciuca, and F. Israel-Roming, "Effects of smoking temperature, smoking time, and type of wood sawdust on polycyclic aromatic hydrocarbon accumulation levels in directly smoked pork sausages," *Journal of Agricultural and Food Chemistry*, vol. 68, no. 35, pp. 9530–9536, 2020.

[68] G. R. Sampaio, G. M. Guizellini, S. A. da Silva et al., "Polycyclic aromatic hydrocarbons in foods: biological effects, legislation, occurrence, analytical methods, and strategies to reduce their formation," *International Journal of Molecular Sciences*, vol. 22, no. 11, 2021.

[69] M. G. Soni, "Benz[a]anthracene," in *Encyclopedia of Toxicology*, P. Wexler, Ed., pp. 250-251, Elsevier, New York, NY, USA, 2nd edition, 2005.

[70] L.-P. Tao, X. Li, M.-Z. Zhao et al., "Chrysene, a four-ring polycyclic aromatic hydrocarbon, induces hepatotoxicity in mice by activation of the aryl hydrocarbon receptor (AhR)," *Chemosphere*, vol. 276, Article ID 130108, 2021.

[71] European Parliament and of the Council, "Regulation (Ec) No 2065/2003 on smoke flavourings used or intended for use in or on foods," *Official Journal of the European Union*, 2003, https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:02003R2065-20210327.

[72] G. Guidetti, A. Di Cerbo, A. Giovazzino et al., "In vitro effects of some botanicals with anti-inflammatory and antitoxic activity," *Journal of Immunology Research*, vol. 2016, Article ID 5457010, 2016.

[73] J. Jager, T. Gremeaux, M. Cormont, Y. Le Marchand-Brustel, and J. F. Tanti, "Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression," *Endocrinology*, vol. 148, no. 1, pp. 241–251, 2007.

[74] D. B. Ballak, R. Stienstra, C. J. Tack, C. A. Dinarello, and J. A. van Diepen, "IL-1 family members in the pathogenesis and treatment of metabolic disease: Focus on adipose tissue inflammation and insulin resistance," *Cytokine*, vol. 75, no. 2, pp. 280–290, 2015.

[75] F. Shiraseb, D. Hosseininasab, A. Mirzababaei et al., "Red, white, and processed meat consumption related to inflammatory and metabolic biomarkers among overweight and obese women," *Frontiers in Nutrition*, vol. 9, Article ID 1015566, 2022.

[76] V. Bouvard, D. Loomis, K. Z. Guyton, Y. Grosse, F. E. Ghissassi, and L. Benbrahim-Tallaa, "Carcinogenicity of consumption of red and processed meat," *The Lancet Oncology*, vol. 16, no. 16, pp. 1599-1600, 2015.

[77] Z. Wang, N. Bergeron, B. S. Levison et al., "Impact of chronic dietary red meat, white meat, or non-meat protein on trimethylamine N-oxide metabolism and renal excretion in healthy men and women," *European Heart Journal*, vol. 40, no. 7, pp. 583–594, 2019.

[78] A. Wolk, "Potential health hazards of eating red meat," *Journal of Internal Medicine*, vol. 281, no. 2, pp. 106–122, 2017.

[79] N. D. Turner and S. K. Lloyd, "Association between red meat consumption and colon cancer: a systematic review of experimental results," *Experimental Biology and Medicine*, vol. 242, no. 8, pp. 813–839, 2017.

[80] X. Sun, X. Jiao, Y. Ma et al., "Trimethylamine N-oxide induces inflammation and endothelial dysfunction in human umbilical vein endothelial cells via activating ROS-TXNIP-

NLRP3 inflammasome," *Biochemical and Biophysical Research Communications*, vol. 481, no. 1-2, pp. 63–70, 2016.

[81] W. Rungratanawanich, Y. Qu, X. Wang, M. M. Essa, and B. J. Song, "Advanced glycation end products (AGEs) and other adducts in aging-related diseases and alcohol-mediated tissue injury," *Experimental and Molecular Medicine*, vol. 53, no. 2, pp. 168–188, 2021.

[82] C. Sharma, R. Dhiman, N. Rokana, and H. Panwar, "Nanotechnology: an untapped resource for food packaging," *Frontiers in Microbiology*, vol. 8, 2017.

[83] S. Shiva, "Nitrite: a physiological store of nitric oxide and modulator of mitochondrial function," *Redox Biology*, vol. 1, no. 1, pp. 40–44, 2013.

[84] P. Waltz, D. Escobar, A. M. Botero, and B. S. Zuckerbraun, "Nitrate/nitrite as critical mediators to limit oxidative injury and inflammation," *Antioxidants and Redox Signaling*, vol. 23, no. 4, pp. 328–339, 2015.

[85] G. Cildir, S. C. Akincilar, and V. Tergaonkar, "Chronic adipose tissue inflammation: all immune cells on the stage," *Trends in Molecular Medicine*, vol. 19, no. 8, pp. 487–500, 2013.

[86] S. P. Weisberg, D. McCann, M. Desai, M. Rosenbaum, R. L. Leibel, and A. W. Ferrante Jr., "Obesity is associated with macrophage accumulation in adipose tissue," *Journal of Clinical Investigation*, vol. 112, no. 12, pp. 1796–1808, 2003.

[87] H. Xu, G. T. Barnes, Q. Yang et al., "Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance," *Journal of Clinical Investigation*, vol. 112, no. 12, pp. 1821–1830, 2003.

[88] A. Mohamadi, F. Shiraseb, A. Mirzababaei et al., "Inflammatory markers may mediate the relationship between processed meat consumption and metabolic unhealthy obesity in women: a cross sectional study," *Scientific Reports*, vol. 13, no. 1, p. 9261, 2023.

[89] P. Šimko, "Determination of polycyclic aromatic hydrocarbons in smoked meat products and smoke flavouring food additives," *Journal of Chromatography B*, vol. 770, no. 1, pp. 3–18, 2002.