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Evaluation Of the Possible Impact on Public Health of Microbiological Quality of Dried Herbs and Spices Used in Meat Industry*

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Introduction

Generally, spices, herbs and condiments are vegetable products or mixtures thereof, which are added to foods in small quantities to give them taste, flavour, aroma, smell, colour, and also to improve their conservation (PETER et al, 2006 [30]). For herbs the leaves are used, which contain flavour components, whereas spices store useful compounds in seeds, bark, and roots (OGBUNUGAFOR et al, 2017 [25]). However, terminology is less well defined, so herbs and spices may be used interchangeably, and it's accepted that they may be made from leaves, seeds, bark, roots, fruits, blossoms, stems, tubers, or bulbs (PEARSON et al, 1984 [29]; SOSPEDRA et al, 2010 [35]). These ingredients are used in the meat industry with different purposes: (i) to increase the variety of meat products, (ii) to potentiate the natural flavour of the meat, (iii) to give them a pleasant, appetizing or spicy taste, and (iv) to extend their shelf life (DUNCAN et al, 2017 [10]; GADEKAR et al, 2006 [12]). For some meat products, especially for traditional ones, certain ingredients confer the culinary specificity, a characteristic appreciated by consumers, on which, frequently, commercial selection is made (BENKERROUM et al, 2013 [4]; FRANCESCA et al, 2013 [11]; PETE et al, 2006 [30]). However, spices and herbs are potential source of microbial contamination for the foods in which they are added, as they are exposed to multiple sources of contamination during pre- and post-harvesting, transporting, processing, handling and storage conditions (BANERJEE et al, 2003 [3]; PAFUMI et al, 1986 [28]; VITULLO et al, 2011 [37]). Previous studies conducted at the level of different production units, of several countries, including Romania (ITTU et al, 2004 [19]; MAN et al, 2016 [24]; SALA et al, 2016 [32]), have demonstrated that spices can be an important vehicle for food-borne pathogens (BANERJEE et al, 2003 [3]; KŁĘBUKOWSKA et al, 2015 [23]; PETER et al, 2006 [30]; ZWEIFEL et al, 2012 [39]), and different bacterial and fungal origin microorganisms and/or mycotoxins. This fact can result in important economic losses derived from hospitalizations in case of foodborne outbreaks, as well as batch confiscations if the hazard is identified during the self-control process of the food producer. Overall, the results of previously conducted studies highlighted the necessity of the continuous monitoring of the safety of these products in order to evaluate the public health risk that is posed and to reduce the economic losses for the food producer. The study was undertaken (i) to analyse the microbiological quality of spices, herbs and condiments used in three industrial meat processing plant in Romania, in order to verify their compliance with the hygiene and safety criteria laid down in the national and European Legislation requirements; and (ii) to evaluate the public health risk that is posed to the consumer and to estimate the economic losses for the food producers in case of positive findings.

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Materials and Methods

A total of 68 batch samples, from three Romanian meat processing plants, noticed within the study with A, B, C, were randomly collected. The collected products included black pepper (*Piper nigrum*, n=6), green peppercorns (*Piper nigrum*, n=2), red pepper (*Piper nigrum*, n=2), ground black pepper (*Piper nigrum*, n=4), allspice grains (*Pimenta dioica*, n=4), allspice ground (*Pimenta dioica*, n=2), marjoram (*Origanum marjorana*, n=2), shredded basil (*Ocimum basilicum*, n=2), oregano grains (*Origanum vulgare*, n=2), oregano ground (*Origanum vulgare*, n=2), bay leaves (*Laurus nobilis*, n=4*)*, paprika (*Capsicum annuum*, n=10), mustard seeds (*Sinapis alba*, n=4), mustard powder (*Sinapis alba*, n=6), dried onion flakes (*Allium cepa*, n=4), curry powder (n=2), savory leaves (*Satureja hortensis*, n=2) , savory powder (*Satureja hortensis*, n=2), and garlic powder (*Allium sativum*, n=6).

The batch sample comprised a pool of five individual samples, according to the sampling strategy established by the Romanian Sanitary Veterinary and Food Safety Authority within the national Order No. 27/2011 [26]. The detailed sampling methodology, together with the types of microbiological analysis carried out on herbs and spices are summary presented in the Table 1.

Detection of yeast and mould was performed according to the official reference method EN/ ISO 21527-2:2008 [16]. A total of 10 g portions of spices and herbs were aseptically weighed, homogenized, and diluted in 90 ml buffered peptone water (BPW, Oxoid, Thermo Fisher Scientific co., Massachusetts, USA). For each sample six serial decimal dilution were made. Subsequently, for quantitative microbiological analysis, 1 ml of the dilution was inoculated in duplicate plates, using the appropriate agar media, as is described below. Consecutive counts were performed on dichloran 18% glycerol (DG18, Oxoid, Thermo Fisher Scientific co., Massachusetts, USA) agar, using the dilution plating methods. Plates were incubated at 25°C, for 5 days. The total number of identified colonies was expressed in colony-forming unit (cfu)/g. The development of the fungi was daily monitored. The grown colonies were identified, at species level, based on their cultural and morphological characteristics, and using Dichotomous keys (ISO 21527-2:2008 [16]).

Detection of *Enterobacteriaceae* was performed in agreement with the official reference method EN/ ISO 21528-2:2004 [17]. After the dilutions, done by the same technique described for yeasts and molds, the *Enterobacteriaceae* counts were determined using violet red bile glucose agar (VRBG, Oxoid, Thermo Fisher Scientific co., Massachusetts, USA), duplicate pour plates with 1 ml of each dilution. The plates were incubated at 37 °C, for 24 h. The resulted counts were expressed in cfu/g (ISO 21528-2:2004 [17]).

The obtained results for yeast and molds and for *Enterobacteriaceae* were classified according to standard guidelines in satisfactory, acceptable and unsatisfactory level.

Salmonella spp. detection was performed according to the official method EN/ ISO 6579:2002 - Cor. 1:2004 [18]. In the first step, 25 g of each sample were diluted, mixed with 225 ml buffered peptone water (BPW) and incubated at 37°C, for 24 h. Following the incubation, 0.1 ml sample from the culture was inoculated in 10 ml of Rappaport-Vassiliadis enrichment broth (Oxoid, Thermo Fisher Scientific co., Massachusetts, USA) and incubated at 41,5 °C, for 24 h. Next, 1 ml from the culture was inoculated in 10 ml of Muller-Kauffmann Tetrathionate Novobiocin broth (MKTTn- Oxoid, Thermo Fisher Scientific co., Massachusetts, USA) and incubated at 37 °C temperatures. In the next step, one loop cultures were streaked on xylose lysine deoxycholate agar (XLD, Oxoid, Thermo Fisher Scientific co., Massachusetts, USA), and other loop cultures - onto Rambach agar (CHROMagar, Paris, France). The prepared plates were incubated at 37 °C for 24 h (ISO 6579:2002 [18]).

L. monocytogenes identification was performed by streaking on ALOA (Oxoid, Basingstoke, UK) and PALCAM (Oxoid, Basingstoke, UK) selective agars. Typical colonies were selected and identified by morphological and biochemical tests, namely, the Gram staining, ß haemolysis in blood agar, rhamnose and xylose fermentation, along with catalase and CAMP tests (ISO 11290-1:1996 [15]).

Determination of mycotoxin levels in spices was performed using the microtiter plate enzyme-linked immunosorbent assay (ELISA) methodology using RIDASCREEN® Aflatoxin Total kit and RIDASCREEN® Ochratoxin A kit (R-Biopharm AG, Germany) according to the manufacturer recommendations [40, 41]. The determination was caried out only for the products in which yeast and mold were detected at unsatisfactory level.

For the interpretation of the results Regulation (EC) No 1881/2006 [7] was used as a reference document, which establishes the level of mycotoxins admitted in food.

Sample preparation. Samples were milled until a fine powder was obtained. Five g from each sample was extracted with 25 ml of 70% methanol. This mixture was stirred for three minutes. Subsequently, the supernatant was centrifuged at 3000 rpm, for 10 minutes. Following centrifugation, 100 µl of extract was diluted with 1.3 ml of distilled water.

For the analysis 50 µl of the extract was added to the microwells along with 50 µl conjugate enzyme and 50 µl of the antibody solution and were incubated at room temperature for 30 minutes. The wells were washed three times with 250 µl distilled water/well. Subsequently, 100 µl of substrate/chromogen was added to each well and the mixture was slightly homogenized by manual stirring, followed by an incubation for 15 minutes at room temperature in the dark. After the incubation 100 µl of stop solution was added to each well and homogenized slightly by manual stirring. Finally, the absorbance at 450 nm was read with a 630 nm differential filter [40, 41].

Results and Discussions

***Yeast and mold* counts.** From a total of 68 analysed samples, 56 (82.3%; 95% C.I. 71-89), showed satisfactory results, which means that the values obtained for the individual samples were less than 10^5 cfu/g. Twelve (17.7%; 95% C.I. 10-28) individual samples were contaminated with yeasts and molds (Table 2).

A number of four (5.9%; 95% C.I. 2-14%) samples, including ground black pepper and curry powder, showed unsatisfactory microbiological quality (Table 2). According to requirements of National Order No. 27/2011 [26] for yeast and molds, unsatisfactory microbiological quality means that at least one sample had greater level than 10^6 cfu/g or 4/10 individual samples showed values higher than 10^5 cfu/g, but less than 10^6 cfu/g.

Eight (11.8%; 95% C.I. 6-21%) samples, including black pepper**,** dried flakes, paprika and mustard powder, showed acceptable microbiological quality, which means that 4/10 individual sample showed values ranging from 10^5 cfu/g to 10^6 cfu/g. Most samples with unsatisfactory and acceptable level of yeasts and molds were obtained on ground spices, except black berry pepper. These positive findings can be related by the fact that the milled spices are considered less microbiologically stable products comparing with the grain's ones, because they are structural elements devoid of defence. This fact can favour the direct contact of the pollutant microorganisms with nutrients necessary for their development, resulting in a higher degree of susceptibility of these substances to fungal contamination. The higher moisture content of milled spices, as a result of increasing the contact surface with humidity from the atmosphere, can be considered another explanation. Presently, the survival of molds on dehydrated products is well documented (AHENE et al, 2011 [2]; DE BOER et al 1985 [9]; HASHEM et al, 2010 [13]; WAN AINIZA et al, 2015 [38]).

Mycological examinations. The analysed spices and herbs showed the involvement of several fungal species, as presented in Table 3.

***Mycotoxin* levels.** The detection of mycotoxins was caried out from the two non-compliant samples with unsatisfactory level of contamination of yeasts and molds, namely the black ground pepper from which *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus clavatus*, *Penicillium expansum* and *Penicillium urtice* were isolated, and curry contaminated with *Aspergillus flavus* and *Aspergillus ochraceus.* No aflatoxins or ochratoxins were detected in the analysed spices. Studies caried out by other researchers indicate that *Aspergillus niger* and *Aspergillus flavus* were the most common species isolated from spices with an emergence between 46.7% and 93% (SOSPEDRA et al, 2010 [13]; SIMION et al, 2018 [34]), in our study the two species were identified in four out of 19 (21%; 95% C.I. 8-43%) analysed types of spices and herbs.

As shown in the present study, although the occurrence of aflatoxins was not detected, using extensive investigations based on enzyme-linked immunosorbent assays, reveals that mycotoxins, particularly aflatoxins, are contaminants of spices and implicitly of foods in which they are added (DARWISH et al, 2014 [8]; KHAZAELI et al, 2017 [21]; OZBEY et al, 2012 [27]; SIMION et al, 2018 [34]; WAN AINIZA et al, 2015 [38]), generating possible confiscations of final products. It has been demonstrated that organic spices are significantly more contaminated with mycotoxins than conventional condiments (TOSUN et al, 2013 [36]).

Bacteriological examinations.

***Enterobacteriaceae* levels.** The results of the present study showed that 58 (85,3%; 95% C.I. 75-91%) samples out of 68 have satisfactory microbiological quality (Table 2). Four (5.9%; 95% C.I. 2-14%) samples showed acceptable quality. As it is shown in Table 2, a number of 6 samples (8.8%; 95% C.I. 4-17%) exhibited unsatisfactory microbiological quality. According to the limits establish by national legislation (Ord. 27/2011 [26]), for *Enterobacteriaceae*, unsatisfactory microbiological quality means that at least one individual sample had values greater than 10^3 cfu/g or 4/10 individual samples had values higher than 10^2 cfu/g, but less than 10^3 cfu/g.

Enterobacteriaceae acceptable microbiological quality was found on black berry pepper and paprika, whereas unacceptable microbiological quality was obtained on ground black pepper, ground oregano, and curry. Compared to the results obtained for molds and yeasts contamination, milled spices had the most samples with acceptable and unsatisfactory microbiological quality in both determinations.

The microbiological contamination of spices and herbs, including *Enterobacteriaceae*, as has been previously demonstrated in several studies, can occur during their harvesting, handling or packing (AHENE et al, 2011 [2]; KIMIRAN-ERDEM et al, 2013 [22]; SALARI et al, 2012 [31]). Generally, *Enterobacteriaceae* counts are used as indicator of hygienic quality of the spices and dried herbs (ABHANI et al, 2016 [1]; BANERJEE et al, 2003 [3]; PETER et al, 2006 [30]).

***Listeria monocytogenes* detection.** The presence of *Listeria monocytogenes*, as one of the most feared food-borne pathogens, was identified in two (0.9%) samples of black berry pepper out of 22 investigated. The presence of *Listeria monocytogenes* has been previously confirmed in spices including red chili pepper and cumin in another study (KARA et al, 2015 [20]), but its presence in the black pepper was not detected. *L. monocytogenes* has been reported being highly resistant in several products, meaning up to 180 days at a level of 3.37±0.43 log cfu/g in black pepper, chickpeas and sesame seeds at a 75% relative humidity level (SALAZAR et al, 2019 [33]). The presence of *Listeria* spp. showed a substantial concern

***Salmonella* detection.** None of the samples were found to be positive for *Salmonella* spp. Interpretation of the results was performed using as a reference Regulation (EC) No. 1441/2007 [6]. However, the presence of *Salmonella* spp. has been reported in a wide variety of spices, demonstrating, at the same time, its implications in several foodborne outbreaks of salmonellosis (IMRE et al, 2020 [14]; PETER et al, 2006 [30]). For example, in a period of three years (2017-2020) Food and Feed Safety Alerts of the European Commission reported eight alerts for herbs and spice, from which *Salmonella* spp. was reported in 5 cases [5].

Conclusions

The study results demonstrated the occurrence of several fungal species, without the presence of mycotoxins, the exceeding of the maximum limit for *Enterobacteriaceae* count in certain ground spices (black pepper, oregano, curry), and the presence of *Listeria monocytogenes* (black berry pepper), showing potential risk for consumers. These microbiological nonconformities can generate economic losses for the food producer resulting in confiscation and seizure of batches of finished products, which largely depends by the quantity of the produced contaminated food from a certain batch. Also, the obtained results indicate a microbiological instability of ground spices compared to those with structural protection elements.

The absence of *Salmonella* spp. revealed a good microbial quality of the analysed spices. However, further surveillance studies regarding the microbiological quality of the condiments are appropriate in order to evaluate the real risk for public health.

Conflict of Interest

The authors have no conflict of interest to declare.

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