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ABSTRACT

„MICROBIOLOGICAL RESEARCH ON BACTERIAL AND FUNGAL FLORA ISOLATED FROM FOOD ADDITIVES AND SPICES"

Key words: *food additives, spices, contamination, bacterial and fungal flora, mycotoxins.*

Lately, in the entire political or geographical diversity, with more and more precarious natural resources and with time that lost its patience, for enslaving without recourse our existence, it becomes vital the usage of preservatives, adjuvants, ameliorators, favours - natural or of synthesis of herbs, spices or of combinations of spices. The study of the potential biological dangers from food additives and spices represents a quick reaction adapted to the problems and to the problematic of food safety due on the one hand and poor legislation for these ingredients.

The doctoral thesis, *„**Microbiological research on bacterial and fungal flora isolated from food additives and spices**"* is structured in two distinct parts: the first part, theoretically substantiated on 233 bibliographic references consulted, comprises 55 pages and represents 27,50 % and the second part, personal contributions, is extended on 145 pages, representing 72,50 % from the paper.

Chapter I *„Food additives. Definitions. Classification. Codification. Regulations, standards and methodological norms"*, presents general data on food additives, their usage and classification according to the effect had in view and to the standards which they must comply with.

In chapter II *„Spices. Definitions. Classification. Description. Regulations, standards and methodological norms"*, there are presented data regarding the most often used spices in our country and studies regarding microbial contamination and the specific legislation.

Chapter III *„Objectives of research. Scope of the investigations. Work materials and methods"*, concisely presents the scientific arguments that support the strategy of the investigations performed regarding the bacterial and fungal flora from spices and food additives.

The researches had the following objectives:

1. perform bacteriologic and mycological investigations to food additives used usually on local/regional plan in the technology of food products of animal and non-animal origin; microbiological exam comparatively on the additives from different technological and operational groups;
2. determine the main quantitative and qualitative microbiologic indicators associated to the spices and aromatic herbs with "tradition" in the local food technology; the evaluation of quantitative and qualitative microbiologic particularities of aromatic herbs and spices' species which are present in the consumption and economic preoccupation;
3. comply with the specific regulations regarding ensuring the innocuity and security of using food additives and spices, as ingredients, technological adjuvants or even raw material of some food products, mainly complying with the norms regarding microbiological risks;
4. define the effects probable from microbiological point of view on the "target" final product and correlation of the results inadequate to the mycological parameters with supplementary determinations regarding the probability to increase the mycotoxins level to alarming values.

At the same time, there are presented the essential data of the organizational and institutional framework where the researches were performed.

The stages monitored according to the experimental protocol designed in 2009 had developed as follows: in the first stage there were performed quantitative, bacteriological and mycological determinations for food additives, in the second stage the same determinations were done for spices, naturally monitoring the isolation, identification and taxonomic classification of the micro flora isolated both from food additives and from spices.

The following was taken into consideration: identifying the yeasts and moulds; detection, counting and identifying the total number of germs, enterobacteriaceae, sulphite-reducers; mycotoxicological exam for the samples with the fungal contamination over the maximum limit allowed by the legislation in force.

Hereinafter, there were described the methods, equipments used and the samples that made the object of the research. The following were sampled:

- 56 samples of mixture of additives from meat industry, belonging to 16 assortiments from 4 different batches;
- 74 samples of additives from pastry – bread manufacture, belonging to 27 assortments from 3 different batches;
- 67 samples of usual spices from 23 different assortments from 3 units of distribution and commercialization: garlic (*Allium sativum*), tarragon (*Artemsia dracunculus*), paprika (*Capsicum annuum*), caraway (*Carum carvi*), cinnamon (*Cinnamomum verum),* coriander (*Coriandrum sativum*), ginger (*Zingiber officinale*), laurel (*Laurus nobilis*), lovage (*Levisticum officinale*), basil (*Ocinum basilicum*), oregano (*Origanum vulgare),* poppy (*Papaver somniferum*), pepper (*Piper nigrum*), sage (*Salvia officinalis*), sesame (*Sesam indicum*), mustard (*Sinapis alba),* cloves (*Syzygium aromaticum*), thyme *(Satureja hortensis),* allspice *(Pimenta dioica*), parsley (*Petroselinum crispum*), nutmeg *(Myristica fragrans),* saffron (*Crocus sativus*), rosemary (*Rosmarinus officinalis*).

Chapter IV, *„Results obtained regarding bacterial and fungal flora at food additives"* presents in detail the results regarding the bacterial and fungal load at food additives and namely, the total number of aerobe mesophilic germs, total number of yeasts and moulds and the results obtained regarding the isolation, identification and taxonomic classification of the bacterial and fungal flora from food additives.

From percentage point of view, 21,43% from the additives from meat industry and 31,4% from the additives for bread manufacture- pastry were not adequate to the criteria provided in the product's technical standards for the total number of germs (ufc/g).

The percentage of the ones that exceeded the maximum limit of total number of yeasts and moulds (NTD+M/g) provided in the technical document was situated at the level of 14,28% for the additives from meat industry and 33,78% for the ones from bread manufacture - pastry sector; out of the bread manufacture-pastry additives, the most intense contamination was recorded at the assortments dedicated to the bread specialties: Graham, multi cereals, multi vitamins. This is explained by the increased percentage of vegetable ingredients (flour, starch, gluten, millet, sesame, caraway) that constitutes a nourishing substratum favorable to microbial development.

85% from the bacterial stems isolated from meat industry additives and 70% from the bacterial species isolated from bread manufacture-pastry products' additives belong to *Enterobacteriaceae* family. 18,51 % from the assortments of additives for bread manufacture – pastry were not adequate in what concerns *Bacillus cereus* parameter and 14,81% not adequate due to the presence of the species *Escherichia coli; Aspergillus spp.* was isolated in 62,76% from the

assortments of additives; 23,25% developed *Aspergillus niger* and 9,30% *Aspergillus flavus; Penicillium spp.* was isolated in 34,88% from the examined additives assortments.

In **chapter V *„Results obtained regarding bacterial and fungal flora from spices"*** there are exposed the results regarding the bacterial and fungal load at spices: total number of aerobe mesophilic germs, total number of anaerobe germs, enumerating the enterobacteriaceae and total number of yeasts and moulds.

According to ANSVSA Order no. 27/2011 regarding approval of microbiological and hygiene criteria which apply to food products, other than the ones mentioned in Regulation (CE) no. 2073/2005 regarding the microbiological criteria for food products, the total number of *Enterobacteriaceae* exceeded the maximum allowed limits for a number of 18 samples representing a percentage of 26,18 % from the total number of samples of spices examined: coriander (*Coriandrum sativum*), poppy (*Papaver somniferum*), pepper (*Piper nigrum*), allspice *(Pimenta dioica*), nutmeg *(Myristica fragrans).* The largest load in what concerns the total number of germs that develop at 30ºC was registered at: lovage (*Levisticum officinale*), poppy (*Papaver somniferum*), pepper (*Piper nigrum*), mustard (*Sinapis alba),* parsley (*Petroselinum crispum*), paprika (*Capsicum annuum*). The largest load in what concerns the total number of anaerobe germs appeared at the following assortments: paprika (*Capsicum annuum*), pepper (*Piper nigrum*), allspice *(Pimenta dioica*), parsley (*Petroselinum crispum*).

The maximum allowed limits for the total number of yeasts and moulds expressed in ufc/g, provided by ANSVSA Order no. 27/2011, were exceeded for 19 samples of spices, representing 28,36% from the total of the samples examined; the largest fungal load was registered at the following assortments: parsley (*Petroselinum crispum*), allspice *(Pimenta dioica*), thyme *(Satureja hortensis),* mustard (*Sinapis alba),* pepper (*Piper nigrum*), poppy (*Papaver somniferum*) and lovage (*Levisticum officinale*).

In what concerns the bacterial flora isolated from spices, the following results were registered: 60,86% from the assortments of spices analyzed were contaminated with germs from *Bacillus spp.* genus, being situated on the top of the classification, out of the total of those 18 genera isolated. Out of this genus, most samples of spices were contaminated with *Bacillus cereus*: garlic (*Allium sativum*), tarragon (*Artemsia dracunculus*), paprika (*Capsicum annuum*), oregano (*Origanum vulgare)* and cloves (*Syzygium aromaticum*); 34,78% from the spices assortments were contaminated with different species of *Enterobacter spp.*: *Enterobacter cloacae:* garlic (*Allium sativum*), lovage (*Levisticum officinale*) and parsley (*Petroselinum crispum*); *Enterobacter aerogenes:* cloves (*Syzygium aromaticum*) and mustard (*Sinapis alba); Enterobacter amnigenus:* saffron (*Crocus sativus*) and nutmeg *(Myristica fragrans).*

From 30, 43% of the spices samples examined, different species were isolated from *Clostridium spp.* genus: *Clostridium subterminale* at pepper (*Piper nigrum*), *Clostridium aerotolerans* at garlic (*Allium sativum*), *Clostridium bifermentas* at poppy (*Papaver somniferum*), *Clostridium difficile* at paprika (*Capsicum annuum*), *Clostridium novyi* at caraway (*Carum carvi*), *Clostridium sporogenes* at thyme *(Satureja hortensis), Clostridium baratii* at lovage (*Levisticum officinale*).

Regarding the fungal flora isolated from spices, 90,47% from the spices assortments examined were contaminated with different species from *Aspergillus spp.* genus: 21,73% with *Aspergillus niger*, 11,11% with *Aspergillus flavus* and 39,13% with other species of *Aspergillus spp.* At 50,87% from the spices assortments analyzed, there were isolated micelle from *Penicillium spp*. genus; at 34,78% *Mucor spp.*, 26,08%, *Rhisopus spp.* and 17,39% *Fusarium spp.;* 66,07% from the genera of fungi isolated are potentially mycotoxigenous.

In **chapter VI** there are exposed mycotoxicological exam results. The large number of samples that were inadequate from mycologic point of view, has naturally lead to suspecting those respective batches as being potentially contaminated with mycotoxins belonging to the isolated genera. According to CE Regula tion no. 1881/2006/2010, there are imposed limits for aflatoxin B1, for total aflatoxins and for ochratoxin A to few species of spices and to the mixtures from which they are part of. The following assortments of spices are in scope: pepper *(Capsicum spp.)* under derived forms (dry, whole or grinded fruits, including hot pepper, hot pepper powder, Cayenne pepper and paprika); pepper *(Piper spp.*) all species, including white and black pepper; nutmeg (*Myristica fragans);* ginger *(Zingiber officinale);* saffron *(Curcuma longa),* Indian saffron and mixtures of spices that comprise one of the above mentioned spices.
There were performed mycotoxicological exams for aflatoxin B1, for total aflatoxins and for ochratoxin A to the additives and spices samples that had presented mycological contamination over the maximum allowed limits: nitrites additive, salami and sausages ameliorator, Graham additives, toast bread additives, rye bread additives, multi cereals bread additives, multi vitamins bread additives, lovage (*Levisticum officinale*), poppy (*Papaver somniferum*), Black pepper (*Piper nigrum*), mustard (*Sinapis alba),* thyme *(Satureja hortensis),* Allspice *(Pimenta dioica*), parsley (*Petroselinum crispum*).From the analysis of the results for aflatoxin B1, it is observed that out of the total of 14 samples examined, a number of 8 additives and spices assortments had a concentration in alfatoxin B1 over 5 µg/ kg. These are: Graham additives, toast bread additives, multi cereals bread additives, multi vitamins bread additives, lovage (*Levisticum officinale*), mustard (*Sinapis alba),* allspices *(Pimenta dioica*) and parsley (*Petroselinum crispum*).

In **Chapter VII** there are presented the ***„Final conclusions and recommendations***" regarding the objectives had in view and their results. It was pointed the fact that: the additives meant for usage in food products for human consumption are authorized if they comply with certain physical, chemical, microbiological or of purity parameters, as well as the maximum percentage (dosage) from the constituency of the final product; for the additives whose support favors microbial development, the specific legislation (like for example ANSVSA Order no. 132/ 2011 regarding food additives for usage in food products for human consumption) provides also certain microbiological parameters (total number of germs, number of yeasts and moulds, *Salmonella spp.*, or *Bacillus cereus*, as case may be);

Extremely few additives assortments can be used under the form of pure substance, as it was authorized; most of them are distributed under the form of additives premixes belonging to some very different categories in what concerns the action and the effect had in view (for example, a premix of additive for pastry that comprises: acidifiers E330, E331; preservative E 202; gelifier E440; antifoam E900; thickening agent E509; colouring material E120; colouring material E150; aroma;).

In default of the legislative provisions, for the microbiological exams performed, there were taken into consideration only the product technical standards; reported to these technical data, the results were inadequate regarding the total number of germs (NTG/g) at a percentage of 28%, and the total number of yeasts and moulds (NTD+M/g) at a percentage of 17,6% out of the total number of samples of additives examined; in what concerns the identification of contaminated bacterial and fungal genera/species, the following predominated: *Bacillus cereus, Proteus mirabilis , Escherichia coli* and *Aspergillus niger*.

There is no real justification for omitting the food additives from the category of the objectives monitored from microbiological point of view, due to the fact that the majority of the additives is commercialised under the form of "additive – electuary" mixture – usually, the electuary being from the category of nutrient substances that favours the development of bacteria, yeasts and moulds, so implicitly mycotoxins elaboration.

The legislation regarding spices underwent significant changes in the last four years. Health Ministry's Order 975/1998, abrogated in 2009, provided for spices the following microbiological criteria: total number of germs, *Enterobacteriaceae, Salmonella, Escherichia coli*, coagulase-positive *Staphylococcus*, sulphite-reducing clostridia, *Bacillus cereus* and the total number of yeasts and moulds.

After the abrogation of Order no. 975 in 2009 and until the approval of ANSVSA Order no. 27/2011, the microbiological criteria for spices were not provided either in Regulation no.

2073/2005 or in the legislation of our country, or in the commercialisation standards; the ANSVSA Order no. 27/2011 provides as microbiological criteria for spices only the total number of *Enterobacteriaceae* and the total number of yeasts and moulds; for these reasons, there were chosen for study only the parameters for which there were terms of comparison similar to the actual legislation: total number of germs, *Enterobacteriaceae,* total number of anaerobe germs (sulphite-reducing clostridia), total number of yeasts and moulds. 67% of the types of fungus isolated are potential mycotoxins producers. For the total number of germs from spices, there are not legally provided the allowable limits; taking into consideration the provisions of Health Ministry's Order 975/1998, abrogated in 2009, we consider that the allowed limits were exceeded for 56,71% from the samples examined. In what concerns the anaerobe bacterial flora, due to the fact that for this parameter there are not legislatively provided the microbiological criteria, there were taken into consideration again the precedent norms, according to which 17,91% from samples are not adequate. By analysing the total number of *Enterobacteriaceae* for which ANSVSA Order no. 27/2011 provides maximum = 100 ufc/g or maximum 1000 ufc/g at 2 samples out of 5, the results certify that: 26,18 % from the samples of spices examined were not adequate; the maximum allowable limits for the total number of yeasts and moulds were exceeded for 19 samples, representing 28,36% from the total of the samples examined; the largest fungal load was registered at the following assortments: parsley(*Petroselinum crispum*), allspice*(Pimenta dioica*), thyme *(Satureja hortensis),* mustard(*Sinapis alba),* pepper (*Piper nigrum*), poppy (*Papaver somniferum*) and lovage (*Levisticum officinale*).

Without legislative provisions to this extent, it cannot be decided on the innocuity of some assortments, batches or samples of spices from the point of view of the isolated bacterial or fungal genera and species; statistically, the data were as follows: 60,86% from the assortments of spices analysed were contaminated with the germs from the genus *Bacillus spp.: B. Cereus, B. Subtilis, B. Licheniformis*; 34,78% from the assortments of spices were contaminated with miscellaneous species of *Enterobacter spp.*; from 30,43% of the samples of spices examined, there were isolated miscellaneous species from the genus *Clostridium spp.* Genus *Aspergillus spp*. was isolated in 90,47% from the samples of spices analysed.

Global results can not be generalized. Only parameter ranges NTG are exponentially and statistically acceptable mits. In Spain, the differences were in the regions studied and outcomes were the same of different. Studies in India give the same advice to adapt legislation at regional and even assortment. In Brazil the results on the number of anaerobes intrigue authorities while

other studies recommended especially for their antimicrobial properties. All these differences could be due to their climatic parculiarities of production and post-harvest processing.

The intense fungal contamination with mycotoxigen genera of micelles determined extending the objectives of the study towards the mycotoxicological analysis of the assortments of additives and spices that exceeded the maximum limits allowed to this extent; the legislation in force provides regulations in what concerns the contamination with aflatoxin B1, total aflatoxins and ochratoxin A for: paprika, pepper, cardamom and saffron; there are no provisions to this extent for the rest of assortments of spices, and in what concerns the range of additives that are commercialised under the form and in real conditions of usage, there are neither legislative provisions, nor standards of producer, nor active monitoring regarding food safety; the determinations performed show contaminations with total aflatoxin and aflatoxin B1 over the limits allowed to certain assortments of spices, for the other assortments examined there are no value benchmarks from this point of view; many of the assortments of additives examined can be included in categories what make object to the Regulation (CE) 1881/2006 reviewed in 2012. Here, there can be had in view the products that are used as such, or as ingredients of the final product, without further transformations for which the limits allowed for mycotoxins are 5 times smaller than the ones provided for spices (for example, there can be included certain assortments of additives for bread making – pastry that have as support proteins, carbohydrates, fats of vegetal origin);

Approximately 60% of the bacterial flora isolated from food additives, 83% of the isolated flora from spices and 28% fall within the fungal flora are from microbial spoilage flora, special in food impairment. Worldwide rearch is directed towards finding natural solutions to renew bioactive packaging food using antimicrobial and antioxidant role. Biodegradable packaging LDPE, Chitosan, chitosan nanocomposites and nanoclays, grape seed extract, Irganox and vitamin E show antimicrobial and antioxidative properties. They certainly are a natural solution for the protection of flora and extension of confiscatory valid providing wholesome food.

Having in view the lack of norms regarding these additives, additives mixtures, premixes, mixes of spices and additives that represent over 80% out of their commercialisation form, a warning signal should be drawn regarding their inclusion in the program of mandatory monitoring, including in what concerns the contamination with mycotoxins. More so, the researches from previous years had discovered new substances, germs, secondary metabolisms or unsuspected harmful effects. For example, if until 2009 in the nourishment for animals it was monitored only aflatoxin B1, there were introduced step by step all other mycotoxins that are quantified in food. The proof is CE Recommendation as of 27th March 2013 regarding the

presence of toxins T-2 and HT-2 in cereals and in the products based on cereals, through which there are provided the limits of quantification and detection mandatory for the methods of analysis, both at food and at the nourishment for animals, as well as the necessity that this entire group of mycotoxins: fumonisine, zearalenone, deoxynivalenol, toxin T-2 and HT-2 to be simultaneously monitored.

Taking into account the diversity of microorganisms that develop within these premixes, it is necessary to enlarge the range of monitored microbiological parameters (Health Ministry's Order 975/1998 regarding hygiene-sanitary norms for food was much more clearly formulated, and microbiological criteria had in view parameters that covered the entire range of microbial flora). This recommendation is directed also towards spices because the ANSVSA Order 27/2011 has also summarily provisions to this extent. It is true that this contamination over the limits provided can be interpreted as supportable by an immunity stable organism, but it is not raised the question of accumulation of contaminated agents from all assortments of food daily consumed.

It is necessary the monitoring within the annual strategic programs regarding the safety of food and of the units that produce, store, transport and commercialise these assortments of additives and their premixes. There were many cases intensively broadcasted on the improper hygiene conditions in which they are produced, stored or transported (fact found in this study on the occasion of sampling for laboratory exams).

Legislation should be adapted to the specific climatic and traditional area in the spices, as the use of additives can it be right to risk short shelf life of food than to affect consciously or not, health (concept in constant reformulation) of generations or perhaps would be more appropriate to find natural solutions for environmental protection and food for us all to follow.