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DOCTORAL THESIS

RESEARCH REGARDING THE SIMULTANEOUS QUANTITATIVE DETECTION OF PATHOGENIC BACTERIA IN SOME FOOD PRODUCTS BY MULTIPLEX REAL-TIME PCR

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SUMMARY

The thesis has 224 pages and it is structured according to usage into two parts, and in order to complete it 372 bibliographical titles were used as sources of information and documentation.

The first part consists of 52 pages (31,5%) and reflects the current stage of knowledge regarding the discussed theme, being structured into 3 chapters, 10 sub-chapters. Also, it is illustrated with 7 figures and 2 tables.

Chapter I entitled **Meat quality and hygiene** is made of 2 sub-chapters and contains notions that regard the nutritive and sensory quality of meat and also its hygienic quality. Due to the fact that meat in its composition favors the growth of indigenous and foodborne microorganisms, it is necessary to know all prevention measures, in order to avoid meat contamination. Besides hygiene measures, enforced by law, many countries apply a series of measures regarding the Biocontrol of meat contamination with pathogenic microorganisms.

Chapter II entitled **Foodborne pathogens implicated in meat contamination** is structured into 4 sub-chapters, each chapter containing the brief description of a bacterial species with pathogenic potential to humans' health. 4 foodborne pathogens were described: *Escherichia coli O157:H7, Salmonella spp*., *Listeria monocytogenes* and *Campylobacter spp*., with special reference to the *Campylobacter jejuni* species. For each species it was synthetically described the history of the disease with animal origin, the morphological and cultural aspects, the virulence factors, the sources of human contamination, as well as the methods and techniques of their isolation from aliments. Both morphological and virulence factors aspects are important, because by getting to know them well nowadays it is possible to synthesize a series of nucleotide fragments which afterwards are used in molecular biology tests.

Chapter III entitled **PCR and PCR real-time technique and their applications in food microbiology** is structured into 4 sub-chapters. The first sub-chapter contains notions concerning classic PCR reactions. Several aspects were described: the DNA structure and replication, the DNA extraction techniques, the principles of PCR reaction and amplification steps, the necessary instruments for PCR reaction. Also, the elements of PCR reaction were detailed, together with the importance of the reaction conditions. The second sub-chapter presents the principle of real-

time PCR, the graphical analysis of experimental data, the detection systems, the necessary instruments for reaction, and also the quantification systems implied by this technique. The third sub-chapter presents notions regarding multiplex PCR reactions and the last sub-chapter describes the first studies in which these techniques were applied for detecting foodborne pathogenic microorganisms.

The second part has 117 pages (68,5%) and contains the results of own researches regarding the detection and the quantification simulated by pathogenic bacteria in food, by real-time PCR technique. It is structured into 5 chapters and it is illustrated with 70 figures and 47 tables. Each research chapter contains: the material and the work method, the obtained results and the discussion upon them and also the partial conclusions.

In chapter IX the 19 final conclusions are synthesized, that is the main aspects concluded after undertaking the researches.

In the IVth chapter of part II the goal and the researchers' objectives are described.

The objectives of the research are the following:

- The isolation and identification of 4 pathogenic bacteria from different type of samples, often involved in foodborne: *Salmonella spp*., *Escherichia coli O157:H7*, *Listeria monocytogenes* and *Campylobacter jejuni*, by classical microbiological techniques, by using the national accepted standards;
- The qualitative and quantitative detection of isolated strains, by TaqMan real-time PCR technique, by using TaqMan Pathogen Detection kits (Applied Biosystems) and the comparison between the obtained results after the amplification of DNA extracted from pure cultures and from artificially inoculated samples;
- The qualitative and quantitative detection of isolated bacterial strains, by TaqMan real-time PCR methods, with primers pairs and approved probes and the comparison between the obtained results after the amplification of DNA extracted from pure cultures and from artificially inoculated samples;
- The study of the specificity, the sensitivity (detection limit) and the repeatability of approved TaqMan real-time PCR methods;
- The qualitative and quantitative detection of some strains of *Salmonella spp., E. coli O157:H7, Listeria monocytogenes* and *Campylobacter jejuni* by TaqMan real-time PCR techniques using primers and unapproved probes obtained with PrimerExpress application;

In chapter V entitled **Research regarding the isolation and identification of *Salmonella Spp., Listeria Monocytogenes, Escherichia coli O157:H7* and *Campylobacter Jejuni* from aliments by classical microbiological methods** the investigations followed the

isolation and identification of the 4 bacterial species by classical tests, according to legal standards. In order to isolate the *Salmonella* strains 135 meat samples were analyzed (poultry, pork, mutton and beef), for *Listeria monocytogenes* 129 samples were tested (mutton, pork, snail and beef), for *E. coli O157:H7* 40 samples were tested from beef and minced meat, and to isolate the *Campylobacter jejuni* species there were analyzed 69 meat samples from poultry and pork. The researches were undergone at the Microbiology Laboratory at the Veterinary Medicine Faculty in Iasi, by using the SREN ISO 6579/AC/2006 standards to identify *Salmonella* strains, SREN ISO. 11290-1-A_1-2004 to identify *Listeria monocytogenes,* EN ISO 16654/2001 for *E. coli O157:H7* and SREN ISO 10272/2007 for *Campylobacter jejuni*. The work techniques included one pre-enrichment or enrichment step, one selective isolation step and a confirmation step.

After the tests, 7 *Salmonella spp.* strains were isolated from pork and poultry, 11 *Listeria monocytogenes* strains from mutton and snail meat and 9 *Campylobacter jejuni* strains from poultry. The samples tested to isolate the *E. coli O157:H7* species turned out negative, and the biochemical characters and the molecularly tests were made on 3 standard strains.

In chapter VI entitled **Research regarding qualitative and quantitative detection of *Salmonella spp., Listeria Monocytogenes, Escherichia coli O157:H7* and *Campylobacter jejuni* from cultures and aliments by TaqMan real-time PCR (TaqMan Pathogen Detection Kits)** it was intended to detect and quantify bacterial DNA in pure cultures and artificially inoculated samples, by using 4 kits and respecting a common amplification program. The inoculation of meat was realized with a dilution of 1 ml, and by using 5 dilutions (10^{-1}-10^{-5}), while the quantification of DNA was done by using a standard scale made up of 5 calibration points of one ATCC strain, with the known stock concentration. 2 experiments were realized on every 2 strains (on DNA extracted from pure culture and DNA extracted from artificially inoculated meat), for each species. The obtained results varied for each species: for the *Salmonella* strains, from pure cultures, the DNA quantity varied from 36, 13 and 57,7 ng/ µl at highest dilutions to 0,09-0,06 ng/µl at lowest dilutions. According to the conversion from ng/µl to UFC/reaction, the following values were obtained: $3,6x10^6$ and $5,5x10^6$ UFC/reaction at highest dilutions and $9x10^3$ and $1x10^2$ UFC/reaction at lowest dilutions. For the artificially inoculated meat, the DNA quantity varied from 170 and 31,6 ng/µl at highest dilutions to 0,01-0,001 ng/µl at lowest dilutions. The bacterial load was established at values between $1,7x10^7$ and $3,1x10^6$ UFC/reaction at highest dilutions and $1x10^3$ and $1x10^2$ UFC/reaction at low dilutions.

For the *Listeria monocytogenes* strains form pure cultures, the DNA quantity varied form 28,6 and 709,2 ng/µl at highest dilutions up to 0,02 and 0,1 ng/µl at 10^{-3} dilution. According to the conversion from ng/µl to UFC/reaction, the bacterial load varied from $2,8x10^6$ and $7x10^7$ UFC/reaction at highest dilutions to $2x10^3$ and $1x10^4$ UFC/reaction at 10^{-3} dilution. For the DNA

extracted form artificially inoculated meat, the values varied from 66,2 and 14,5 ng/µl at high dilutions to 0,6 and 0,1 ng/µl at 10^{-3} dilution, and the bacterial load was set between $6,6x10^6$ and $1,4x10^6$ UFC/reaction at high dilutions and $6x10^4$ and $1x10^4$ UFC/reaction at 10^{-3} dilution. The last 2 dilutions could not be quantified for both types of DNA.

For the *E. coli O157:H7* strains the DNA quantity from pure cultures had the following values: 100 and 400 ng/µl at highest dilutions and 1,2 and 0,4 ng/µl at 10^{-3} dilution. The bacterial load varied from $1x10^7$ and $4x10^7$ UFC/reaction at highest dilutions and $1,2x10^5$ and $4x10^4$ at 10^{-3} dilution. Similar to the *Listeria monocytogenes* case, the 2 last dilutions could not be quantified. The DNA extracted from artificially inoculated meat presented values of la 0,1 and 0,2 ng/µl at highest dilutions and 0,00001 şi 0,0002 ng/µl at lowest dilutions. After conversion bacterial load varied between $1x10^4$şi $2x10^4$ UFC/reaction at highest dilutions to 10 şi $2x10^1$ UFC/reaction at lowest dilutions.

For the *Campylobacter jejuni* species 3 strains were tested form pure cultures, and the DNA quantity varied from 49,4, 73,9 and 21,06 ng/µl at first dilution, to 0,004, 0,007 and 0,02 ng/µl at last dilutions. The bacterial quantity varied from $4,9x10^6$, $7,3x10^6$ and $2,1x10^6$ UFC/reaction at first dilutions, to $4x10^2$, $7x10^2$ and $2x10^3$ UFC/reaction at lowest dilutions. The values obtained from the artificially inoculated meat varied from 531 and 786 ng/µl at first dilutions to 0,0005 and 0,0007 ng/µl at last dilutions. The quantitative conversion showed bacterial quantities set between $5,3x10^7$ and $7,8x10^7$ UFC/reaction at first dilutions and $5x10^1$ and $7x10^1$ UFC/reaction at last dilutions.

In chapter VII entitled **Research regarding detection and quantification of *Salmonella spp., Listeria Monocytogenes, Escherichia coli O157:H7* and *Campylobacter jejuni* from aliments by TaqMan real-time PCR approved methods** the following aspects were studied: the specificity, the sensitivity (detection limit) and the repeatability of some TaqMan real-time PCR approved techniques, for the 4 bacterial species isolated from meat. The 4 techniques had as targets the following genes: *ttr* (*Salmonella spp.*), *hlyA* (*L. monocytogenes*), *STX1*, *STX2* and *rfbE* (*E. coli O157:H7*) and *VS1* (*C. jejuni*), and the amplification programs were developed in 2 or 3 steps. The specificity of the 4 techniques was 100% for the 4 bacterial species, and there were not registered any false negative reactions for the non-spp. strains that were used, neither false negative nor false negative reactions for the confirmed strains or the standardized ones.

The detection limit was tested comparatively, for the DNA taken from pure cultures and the one extracted form artificially inoculated meat samples. 5 known bacterial loads were analyzed (10^5-10^1UFC/ml), on every 3 or 2 bacterial strains. The detection limit (the smallest amount detectable by the method) varied for the 4 bacterial species as follows: for the *Salmonella* strains between 10-100 UFC/ml for cultural DNA and 100-1000 UFC/ml for the DNA extracted from meat; for the *Listeria monocytogenes* strains between 10-1000 UFC/ml for cultural DNA and

100-1000 UFC/ml for the one extracted from meat; for the *E. coli O157:H7* strains the minimum detectable quantity was 10 UFC/ml for cultural DNA and 100 UFC/ml for the DNA extracted from meat and the *Campylobacter jejuni* values varied from 10-100 UFC/ml for cultural DNA and 100-1000 UFC/ml for the one extracted from meat. The obtained results show that the detection limit is better for the pure cultures than for the artificially inoculated meat. The repeatability was realized in 3 consecutive tests, at 10^{-3} dilution, on one ATCC strain for each bacterial species, and the values of the standard relative deviation varied from 0,33-0,93% for *Salmonella spp*., 0,64-1,46% for *L. monocytogenes*, 1,11-2,38% for *E. coli O157:H7* and 0,24-0,73% for *C. jejuni*, which indicated the high precision of the methods.

Chapter VIII entitled **Research regarding detection and quantification of *Salmonella spp., Listeria Monocytogenes, Escherichia coli O157:H7* and *Campylobacter jejuni* from aliments by TaqMan real-time PCR unapproved methods** followed the specificity of some primers pairs and TaqMan probes obtained "in silico", by PrimerExpress application for 4 gene fragments: *ttr (Salmonella spp.), hlyA (L. monocytogenes), rfbE (E. coli O157:H7)* and *VS1 (C. jejuni)*. Both the pairs of primers and the nucleotide probes have Tm (melting temperatures) at close values, in order to realize a common amplification program: initial denaturation of 10 minutes at 95ºC, followed by 45 cycles 15 sec, at 95ºC and 1 min. at 60ºC. Out of the 4 primers pairs and TaqMan probes, only 3 furnished relevant results, the specificity being 100%. The primers pair and the TaqMan probe for the *hlyA* gene fragment did not give relevant results, the lack of the amplification signal revealing this aspect. The quantification by these methods was made on each 2 strains for every species, in triplicates, on DNA from pure cultures. The bacterial load varied as follows: 10^3 UFC/reaction for the *Salmonella* strain isolated from goose meat and 10^4-10^5UFC/reaction for the strain isolated form egg; between 10^6 and10^7 UFC/reaction for the first *E. coli O157:H7* strain and 10^7 UFC/reaction for the second strain; higher values were registered for the *C. jejuni* strains: between 10^7 and 10^8 UFC/reaction for the first strain isolated from poultry meat and 10^6 and 10^7 CFU/reaction for the second strain.

From the obtained data, we can affirm that the aliments represent an important source of pathogenic bacteria for humans, and rapid detection tests at species level or even serotype are absolutely necessary.

The real-time PCR reaction can be easily used in the qualitative and quantitative detection of these bacteria after an anterior enrichment or even directly from the alimentary matrix, as long as the bacterial quantity is detectable by the used instrument, however the costs of the reagents and of the necessary tools are pretty high. The molecular methods are specific, sensitive and reproducible, which makes it easier for the specialists who work in laboratories.