SUMMARY

of the doctoral thesis entitled:

RESEARCH ON THE USE OF MODERN METHODS OF DETECTION AND ANALYSIS OF PATHOGENS IN DIFFERENT FOOD MATRICES

PhD-student: **ALZAIDI Quthama Jasmin**

Scientific coordinator: **Prof. Univ. Dr. MATEI Florentina**

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During the last decade the promotion of a healthier and more sustainable lifestyle has led to an increasing demand for fresh vegetables and other ready-to-eat products. With this wave of pre-packaged products that have a long shelf life, outbreaks of food-borne infection have also appeared more frequently. Detection of pathogens and spoilage microorganisms is a crucial aspect of maintaining public health, preventing outbreaks, and ensuring the safety of food, soil, water, and other biological samples.

The real-time PCR (qPCR) technique has improved pathogen detection and quantification through increased assay sensitivity and specificity of tests. Also, the speed of analyzes offered by the multiplexing and automation of devices is an important plus for various industries, such as food or pharmaceutical. However, this technique presents a number of limitations related to the accuracy of the results, especially in the case of samples containing mixtures of viable and non-viable cells, generating false negative results. A promising strategy to avoid this problem is "viability-qPCR" (v-qPCR), which is based on a series of permeabilizers that can help the process of penetration of the outer membrane in the case of Gram-negative bacteria by photoactive dyes (propidium monoazide PMA), thus increasing the accuracy of the results.

The general purpose of the research aimed at the development of rapid molecular methods for the detection and quantification of spoilage microorganisms (*Botrytis cinerea*) or pathogens (*E. coli, Salmonella sp*.) from different food matrices, respectively perishable fruits and vegetables (raspberries, mixes of packaged salads) and milk. Specifically, it was aimed to optimize the v-qPCR technique by identifying an accessible, safe and efficient substance that allows photoactive dyes to efficiently penetrate any type of matrix, namely lactic acid.

Therefore, the specific objectives were the following:

1. The development of a qPCR method to quantify the contamination of fresh raspberries with *B. cinerea*;
2. The development of a method for detection and quantification of *E. coli* and *Salmonella* by v-qPCR;
3. The use of the v-qPCR method for the detection of *E. coli* in food matrices.

In the theoretical part of the work, an extensive documentation structured in several chapters was made regarding the main pathogens encountered in the food industry and the development and improvement of detection methods. Thus, the molecular methods (PCR, qPCR, v-qPCR) and how they evolved, as well as their disadvantages and limitations, were described. Also, the use of permeabilizers for the detection and quantification of Gram-negative pathogens was described in order to reach the most accurate results.

The second part of the study, structured in three experimental chapters and a chapter of conclusions, addresses the development of a molecular method to quantify the contamination of raspberry

fruits with *Botrytis cinerea*, the improvements made to the viability qPCR technique and the testing of the new analysis on food matrices such as milk and spinach leaves.

The first experimental chapter aimed at the comparative study of the detection results of the spoilage microorganism *B. cinerea* on packaged raspberry samples, by using the traditional plate cultivation method and a qPCR method developed in the laboratory.

By plating raspberry samples in specific medium, no mycelium indicating the presence of *B. cinerea* species was detected on any of the three commercial samples packaged and on the shelf. Considering the uncertain results obtained by the classical microbiological technique, the qPCR technique was used, and the detection method with the pair of Bc3F/Bc3R primers was developed. The linearity of the standard curve obtained was observed throughout the range used, having a correlation coefficient $(R_2=0,9929)$ indicating a very low inter-assay variability.

An amplification efficiency of 85.18% was obtained. Under the conditions described, the maximum C_t value that could be used was 32 and corresponds to a DNA concentration of 9.8 fg. Through the developed technique, the presence of *B. cinerea* was detected in two of the three commercial samples, with values around 3.8-4.2 fg.

Thus, the developed qPCR method can detect even the smallest amount of *B. cinerea* present in the sample, by using the primers Bc3F and Bc3R, obtaining accurate results. The proposed simple technique can be effective for the routine examination of several samples per day.

The following two experimental chapters, related to pathogenic bacteria, specifically aimed at optimizing the v-qPCR technique by identifying an accessible, safe and efficient substance that allows photoactive dyes to efficiently penetrate any type of matrix, namely lactic acid. A v-q-PCR method was developed that used lactic acid as a permeabilizer for the detection and quantification of *E. coli* and *Salmonella sp*.

In a first phase, it was found that lactic acid in low concentration (5-20 mM) does not affect the viability of the strain of *E. coli*, while the strain of *Salmonella* sp. it is not inhibited at concentrations lower than 10 mM. In both strains lactic acid (LA) acts synergistically with the antibiotic ciprofloxacin (CIP 1) and can be used to increase the antibacterial action. Pretreatment with 10 mM LA has the maximum effect of cell membrane permeabilization in *E. coli*, and in this case the dCt-nonviable index that quantifies the reduction of the qPCR signal has an average value of 14.5. In the S. Typhimurium strain, PMA efficiently penetrates the outer membrane, and the signal reduction values (non-viable dCt) for PMA-treated samples and 10 mM PMA + LA samples were not statistically different. The detection limit and standard curve for the *E. coli* strain were also calculated to evaluate the performance of the implemented detection technique.

Subsequently, lactic acid permeabilization assays and qPCR analysis with PMA dye were applied to milk samples and commercially purchased spinach leaves. The use of lactic acid together with v-qPCR has been shown to be a new breakthrough for the specific detection and accurate quantification of viable Gram-negative pathogens, both under culture conditions in the food matrix. This approach can help advance pathogen detection methodologies and support efforts to protect public health and food safety.

The studies carried out provide useful information on the use of lactic acid, which proves to be an efficient, cheap and low-toxicity permeabilizer, which has the potential to be used in the detection and quantification of Gram-negative pathogens by the PMA-qPCR method because it improves the efficiency of the reaction.