SUMMARY

RESEARCHES ON THE DYNAMICS OF MICROBIAL POPULATIONS IN THE FERMENTATION PROCESSES

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The research is the subject of the doctoral thesis entitled "RESEARCH ON THE DYNAMICS OF SOME MICROBIAL POPULATIONS THROUGH THE FERMENTAL PROCESSES" within the Doctoral School of USAMV in Bucharest.

Fermentation is a metabolic process by which an organism converts a carbohydrate, in different compounds like an acid or an alcohol. For example, yeast transforms sugar into alcohol through the fermentation process. Lactic bacteria transform carbohydrates into lactic acid through fermentation. This complex process is used to produce different food products like beer, wine, yogurt or other farmaco-comestics products.

Generally, fermentation is konw as a natural process. Humans used the fermentation process to produce products such as beer, wine or cheese long before the biochemical process was understood. Louis Pasteur, in 1850, showed that fermentation is caused by living cells and became the first scientist to study fermentation. The most important fermentation processes are: lactic fermentation, alcoholic fermentation and acetic fermentation. During the alcoholic fermentation, yeasts and certain bacteria carry out glucides' transformation, a process by which pyruvic acid is transformed into ethanol and carbon dioxide; such process is to be used for the production of bread, wine or beer. Along the lactic fermentation, the lactose is methabolozed via the pyruvic acid mainly into an acid, respectively the lactic acid. Such process is specific for the production of different dairy products, as cheese and other fermented mile derivates. The acetic bacteria are the main microorganism involved in the acetic fermentation and as an intermediate product result the acetic acid while the final food products is the vinegarş this fermentation is also used to preserve vegetables, like pickles. As a specificity, despide the fact that it is considered fermentation, it is carried out in the presence of oxygen.

These fermentations run on natural sugar sources like grapemust, milk, grains, while the biodiversity and microbial levels are in permanently changing. In order to obtain the best final product, the optimisiation of the fermentation process is a must, and, for that the involved microorganisms are an important factor.

A complex approach is requested to characterize the microbial biodiversity during the fermentative processes, and both phenotypic and genotypic methods should be taken into account, while a correlation between the results of these methods should be established. In the context where fermentation processes are basic processes for obtaining food, including functional ones, the use of modern techniques for monitoring microbial populations during these processes has become a necessity, if not an obligation.

The experiments were carried out in two main locations, namely in the Applied Microbiology laboratory of the Faculty of Biotechnology in USAMV Bucharest and in the molecular diagnostic laboratory of Vetwork Diagnostics in Tulcea, which provides laboratory services in the field of animal health and food safety.

The specific objectives were the following:

1. Dezvoltarea metodologiei qPCR de cuantificare a populațiilor de bacterii lactice şi bacterii acetice în produse fermentate de tip Kombucha

2. Dezvoltarea unei metode qPCR pentru cuantificarea populaţiilor totale de *Hanseniospora* versus *Saccaharomyces* în cadrul procesului de vinificaţie
3. Detectarea şi cuantificarea populaţiilor viabile ale unor specii *Saccharomyces şi non-Saccharomyces* din procesele de vinificaţie prin tehnica qPCR

The thesis is structured in two main parts: the documentation part and the part of own research, which were presented in 6 chapters, to which were added the introduction, summary, general conclusions and recommendations and the bibliography.

The first part of the work contains 2 chapters.

In the first chapter, a synthesis of specialized literature was made regarding the main fermentations of food interest, with an emphasis on microbial dynamics in the process of alcoholic fermentation in winemaking and microbial dynamics in Kombucha-type drinks, products that were taken over in the experimental part. .
In the second chapter, the main methods of monitoring microbial populations during microbial processes were reviewed, with emphasis on molecular methods, especially the qPCR technique.

The second part of the doctoral thesis includes 4 chapters, which describe the own experimental research.

In chapter III, the development of the qPCR methodology for quantifying the population of lactic and acetic bacteria in Kombucha-type fermented products was pursued. The purpose of the experiments was to obtain a complex product, which includes various health compounds, by fermenting the pollen collected from bees with a Kombucha consortium. During the process of maceration-fermentation of pollen with Kombucha, the levels of lactic acid bacteria (LAB) and acetic bacteria populations were determined by classical and molecular techniques, compared to the levels of these groups of bacteria in the Kombucha fermented product. By adding pollen at the beginning of fermentation, the initial level of LAB increases by almost two logarithmic units, up to 10^7 CFU / ml. This important increase in the LAB population is due to the content of LAB in the pollen most likely from *Lactobacillus* sp. species in the bee stomach. Thus, it has been proven that the addition of pollen increases the probiotic properties of the final product. At the laboratory level a lower level was observed (10^7 CFU/ml) than that resulting from the 100L pilot studies (10^9 CFU/ml). Thus, high throughput fermentation plants improve the pollen effect of LAB microbial population growth. Regarding acetic bacteria, their initial level differs by one logarithmic unit in the case of pollen addition (10^3 cells/mL in Kombucha without pollen and 10^4 cells/mL in Kombucha with pollen), but at the end of maceration-fermentation the level stabilizes in both fall to 10^6 cells/mL. Technically, qPCR has been shown to be a feasible technique for quantifying the total load of lactic and acetic bacteria in Kombucha. Also, during the experiments, the qPCR technique was used successfully and can be used to evaluate the pollen content in lactic acid bacteria.

In chapter IV the study proposed the development of a molecular method for the quantification of *Saccharomyces* and *Hanseniospora* populations during the winemaking process, while initially was employed the addition of selected starter cultures. For the real-time qPCR method were employed specific primers and SYBR Green probe as well as dilutions from reference strains of the genus *Saccharomyces* and *Hanseniospora* were used to desing the calibration curve. The goal was to use the developed method to study different samples of grape must and wines in different feremntation steps, during natural or controlled fermentation with slected yeast strains.

When desigining the standard curve, it was obtained a very good correlation coefficient, respectively R^2 = 0.9927 and a value of RSDr = 0.25%. The detection limit showed the need for a Ct maximum equal to 32 to obtain a positive SYBR-Green reaction. The amplification efficiency value was 90% for this qPCR reaction, meeting the validation criteria with an R^2 higher than 0.98 and a slope ranging from -3.1 to -3.6.

In the case of *Hanseniospora* population, in the big majority of the analyzed wine samples, almost similar values were obtained for the yeast levels; for the grape must samples a higher initial level was obsrved compared with the wines, while the must samples with sulfites recorded the highest populational levels. In the case of must samples, the concentration of *Hanseniospora* populations was higher than that of *Saccharomyces* populations in the initial and final phases of fermentation, being exceeded during the fermentation period.

These results are in accordance with other reported data and support the ideea that qPCR is a sensitive, rapid, direct (non-cultivable) and reliable technique to quantify the total cell numbers of different yeast species.

Chapter V sought to develop a method for quantifying viable populations of some Saccharomyces and non-Saccharomyces species using propidium monoazide (PMA), a chemical compound that is able to penetrate membrane-compromised or dead cells and bind covalently to the nucleic acid after photoactivation. It was proposed to develop a quantification method for the species *Candida stellata*, *Torulaspora delbrueckii* and *Wickerhamomyces anomalus*, maintaining as a central element the yeast *S.cerevisiae*. Considering the fact that, from a metabolic point of view, viable cells are important, because they lead to different organoleptic characteristics of the wine, this time it was proposed to quantify the viable cells of these species. During testing, a slight decrease in viability was observed following PMA treatment of viable cells.

The slopes of the standard curves were almost similar, which corresponded to amplification efficiencies between 91.17% and 95.98%, highlighting a linear relationship (R^2) between 0.9908-0.9959.

The detection limit ranged from 38 fg/µL to 49 fg/µL, which corresponds to quantification limits of 70 CFU/mL to $1.03x10^2$ CFU/mL. In the case of *W. anomalus*, the detection and quantification limits are too high requiring further optimization.

Following the optimization process, we found that applying PMA-qPCR to different wine samples may lead to results within one working day, which can be cobsidered as a great advantage compared to the 5-7 days required to obtain results by the conventional culture methods .