„Dunărea de Jos" University of Galaţi

Doctoral School of Engineering and Fundamental Sciences

Formulation, chemical and functional characterization of some pharmaceutical products for external use with natural bioactive compounds and lactic acid bacteria (PhD thesis summary)

PhD student,

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Scientific coordinator,

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Seria I.1: BIOTEHNOLOGII No. 10

GALAŢI

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Content

INTRODUCTION

In the last decade, we have been facing a decline regarding the safety and the efficiency of the treatment for many skin disorders, doubled by a prevalence and premature manifestations increase, from the first years of life, diseases that are highly refractory to the usual drug treatments. Skin disorders have multiple causes which require advanced medical studies, lifestyle improvement, immune system boosting, as well as to study the individual characteristics of each human body.

The main objective of this research field is the identification of an alternative treatment for various skin disorders, which is non-invasive, safe and, most of all, efficient. In order to avoid long treatments with drug products that usually give allergic reactions or skin fragility, the pharmaceutical products with bioactive components from plants could prove to be a safe and viable choice. Furthermore, there are many scientific data that supports the many beneficial therapeutic effects that natural extracts exhibit.

Human body possesses a natural commensal flora that has protective and immunomodulatory properties. It has been scientifically proven that the appearance of multiple diseases that affect the global health is due to a lack of natural beneficial microbial flora. Moreover, the beneficial strains are capable to supply local and systemic protection by inhibiting pathogens, and also to provide post-biotic metabolites, that boost the immune system and for the general human health.

The skin microbial flora is almost similar for every human body, presenting the same probiotic microorganisms on the same body areas of different persons. Skin disorders were connected, in different studies, to the qualitative and quantitative imbalances of certain strains from the normal microbial flora, which are usually linked with individual characteristics or with lifestyle (food, hygiene etc.).

Probiotics have countless benefits supported by thousands of scientific articles. Most of them focus on their use as food or nutraceuticals for oral administration. In this context, in regards to the PhD thesis, the novelty of the undertaken research consists in trying to prove the probiotic strains efficacy by using them at a topical, external level on an affected area, on skin epidermis. The main benefit of this innovative pharmaceutical product is that it could offer an alternative treatment to those who developed intolerance to certain chemical substances or have allergic reactions or to those with cutaneous hypersensitivity.

Besides the multiple benefits of the presence of probiotics in this product, the bioactive compounds from *Aloe vera* add a nutraceutical and therapeutic value, that has been proven since antiquity and scientifically demonstrated over the years through randomized trials, on different batches of patients.

The PhD thesis entitled „***Formulation, chemical and functional characterization of some pharmaceutical products for external use with natural bioactive compounds and lactic acid bacteria"*** assessed the ability of some probiotic strains to grow in a minimal fermentative medium, enriched with *Aloe vera* (lyophilized powder or ethanolic extract), as active ingredients into a pharmaceutical formulation for topic use. Thus, modern techniques of analysis like FT-IR and HPLC were used for the qualitative and quantitative determination of the major bioactive compounds from *Aloe vera* leaves (*Aloe barbadensis* Miller). The vegetal samples from *Aloe vera* (lyophilized powder and 10% ethanolic extract of lyophilized powder) were used to supplement the cultivation media of the selected strains, *Lactobacillus plantarum* and *Lactobacillus casei.*

From the experimental samples, the fermentative product with the optimum functional properties (adhesive activity to epithelial HeLa-2 monolayer cells, antimicrobial activity) was

selected to be further incorporated as an active ingredient into a hydrophilic ointment, for topic use and for the prophylactic and medical purposes, in skin disorders.

The research studies followed the main scientific objectives that were formulated based on the experimental tests, as it follows:

1. The physico-chemical characterization of the vegetal samples from Aloe vera (cake, gel, whole leaf, lyophilized powder and ethanolic extracts).

2. The isolation, selection and testing of some lactic acid bacteria with fermentative potential and the optimum viability in a minimal culture media, in order to provide all the tri-biotic properties (pre-biotic, probiotic and post-biotic).

3. The formulation and characterization of a functional ointment with bioactive components and lactic acid bacteria, that are capable to adhere to epithelial cells and competitive dislocate the pathogens that usually colonize the skin surface (*Staphylococcus aureus* and *Candida albicans*).

4. *In vitro* tests to demonstrate the functionality of the proposed ingredients (fermentative medium with selected probiotic strains, derivatives from a minimal supplemented *Aloe vera* medium) and the efficacy of the innovative pharmaceutical ointment.

This PhD thesis is divided into two major parts, as it follows:

I. DOCUMENTARY STUDY has three chapters that present the most recent data from the scientific literature regarding the physiological and morphological characterization of lactic acid bacteria (*Lactobacillus* genra), the physico-chemical properties of the bioactive compounds from *Aloe vera,* which is a very well-known plant since antiquity for its extraordinary therapeutical, immuno-modulatory, antiinflamatory and antioxidant properties. More than that the data also acknowledges the skin health benefits, especially when it comes to natural products with lactic acid bacteria and bioactive compounds from vegetable sources.

Chapter 1 entitled **Probiotics - characterization and pharmaceutical applications** presents the main health benefits of probiotic administration and a detailed understanding of their action mechanisms, as well as their major therapeutical uses.
Although there are thousands of studies which revealed their benefits to the human health after oral administration, recent studies bring the attention over to their potential topical uses, by innovating new formulations, like vaginal suppositories, biofilms and scaffolds that contain different probiotic strains for external use. This research area could be enlarged by better understanding their action mechanisms and competitive inhibition of pathogens.

Chapter 2, entitled ***Aloe vera* – an important source of functional bioactive compounds** describes the phytochemical constituents and the main beneficial therapeutical effects, as well as the potential pharmaceutical uses of this plant. Countless studies sustain the various therapeutical effects that *Aloe vera* extract could manifest in skin disorders, thus it became a frequently used active ingredient in hundreds of dermato-cosmetical products.

Chapter 3, entitled **Modern trends in the formulation and characterization of functional pharmaceutical products for skin treatments** includes the characterization of pharmaceutical semisolid and bioadhesive forms, as well as the formulation regulations for a hydrophilic ointment preparation. The ointments defined as semisolid and bioadhesive products, are responsible for both for a local and also for a systemic action, in such a manner that by introducing in these types of products natural ingredients extracted from different vegetable materials may lead to a much more efficient, safe and compliant way of treatment.

II. EXPERIMENTAL PART includes all the original results of the experiments conducted during the doctoral stage, structured into 3 chapters, as follows:

Chapter 4, entitled **Extraction and characterization of bioactive compounds from *Aloe vera* (*barbadensis* Miller)** presents the original results from the experimental investigations after the separation of the gel and cortex, and after the lyophilization of the selected samples, respectively. The fresh gel and cortex and the lyophilized samples were analyzed by different methods in order to determine the moisture, dry weight, acidity, bioactive compounds content (total polyphenols, flavonoids, proteins, lipids etc.) and the radical scavenging capacity. In order to analyze the bioactive compounds, modern investigations were used like spectrophotometric method, high performance liquid chromatography and FT-IR analysis.

Chapter 5, entitled **The study of the fermentative and physiological behavior of some probiotic strains in an *Aloe vera* supplemented culture media** describes the kinetic parameters of the selected probiotic strains during fermentation, individual or mix culture, by inoculation in a MRS and minimal electrolytes medium enriched with *Aloe vera* (lyophilized powder or ethanolic extract). The growth curve for each strain was determined and the pH and the *Lactobacillus* viability in the fermented medium was also measured. Further, the fermented strains behavior was monitored for 21 days, under refrigerated conditions. *In vitro* tests demonstrated the adhesive capacity to HeLa-2 epithelial cells, as well as the competitive inhibition of pathogens adherence to monolayer cells (*Stahylococcus aureus* and *Candida albicans).* The fermented products presented antibacterial and antifungal activity against pathogenic strains that could accidentally invade the epidermis.

Chapter 6, entitled **The formulation of an innovative ointment with fermented probiotic products and *Aloe vera*** presents the investigation results that supported the formulation of this functional pharmaceutical product. This chapter includes the rheological characterization of hydrosoluble polyethylene glycol ointments, that are usually used for the formulation of an external use product, with viable *Lactobacillus* cells and bioactive compounds from *Aloe vera.* The experimental investigations were completed with the *in vitro* tests that proved the antibacterial and antifungal activity of the selected probiotic strains, as well as the functionality of the product over 21 days, stored at 0-4^{o}C.

Each chapter of the experimental part is organized into several subchapters: *Introduction*, where the opportunity of this research is presented under recent context; *Materials and methods*, that describes all the laboratory reagents, equipment and analytical methods, and also the means to interpret the experimental results; *Results and discussions,* where the original results are presented in comparison to similar data from the scientific literature; *Partial conclusions* and *References.*

Chapter 7, *General conclusions,* summarizes the main conclusions that were drawn from the scientific experiments, finally leading to the formulation and characterization of an innovative pharmaceutical product, for topic use, with two viable *Lactobacillus* strains and various natural bioactive compounds extracted from *Aloe vera*.

The fundamental and applicative approach described in the PhD thesis provides a novelty character not only for our country but also for the abroad scientific community, taking into account that very few investigations of this kind were reported. Most of the literature studies

reported data about either probiotics or bioactive compounds. By combining the active principles of probiotic lactic acid bacteria with the plant components, new research perspectives are opened up, so that a wide palette of pharmaceutical products for cosmetic or medical use could be developed, with plenty of health benefits and improvements of life standards.

Finally, the ***original contributions*** of the PhD thesis are presented. These contributions that may have a real impact on the knowledge development in the pharmaceutical technology, pharmacognosy and microbiology fields, by revealing the multidisciplinary and transdisciplinary character, that open up several research perspectives in terms of the optimization of the ingredients, product functionality increasement and therapeutical efficiency of this innovative pharmaceutical product.

The PhD thesis consists of 163 pages, of which 23 tables and 85 figures. The documentary study represents 25 % and the experimental part 75 %.

The research results were disseminated in three scientific articles, published or in press in ISI journals (*Romanian Biotechnology Letters, Journal of Biotechnology*) and one in a indexed international databases journal (*Brasov Medical Journal*). During the doctoral period, the results were presented nationally or internationally at several representative conferences (*Eurobiotech Conference, Bucharest, 2015*; *EuroAliment Symposium, 2017; Scientific Conferences of Doctoral School, „Dunărea de Jos", University of Galați, 2015, 2016 and 2017).*

The experimental research activities for the characterization of the selected probiotic strains and the bioactive compounds from *Aloe vera* were conducted in the *Integrated center for research, expertise and technological transfer in food industry: BioAliment – TehnIA*. (www.bioaliment.ugal.ro), Food Science and Engineering Faculty, and in the *Organic chemistry laboratory* ofSciences and Environment Faculty, „Dunărea de Jos" University of Galați. The *in vitro* tests for the cutaneous adherence and pathogen inhibition were conducted with the help of senior researches and by using the infrastructure of the *Microbiology laboratory* of the Biology Faculty of University ofBucharest whereas the antimicrobial research was done in the *Clinical Laboratory of Medical tests* of the Pediatric Hospital „St. Ioan", Galați.

The PhD thesis was carefully monitored and conducted under the coordination of the Scientific Committee, with the following members:

1. **Prof. dr. eng. – Gabriela-Elena BAHRIM** – PhD coordinator;
2. **Prof. dr. chem. – Rodica DINICĂ** – coordinator for the extraction and characterization studies of the bioactive compounds from *Aloe vera;*
3. **Prof. dr. chem. Ștefan DIMA** – coordinator for the rheological characterization studies and the formulation of hydrosoluble ointment;
4. **Assoc. prof. dr. biol. Vasilica BARBU** – coordinator for the strains identification, *in vitro* adherence and antimicrobial activity tests.

4. Extraction and characterization of bioactive compounds from *Aloe vera* (*Aloe barbadensis* Miller)

4.1. Introduction

This chapter presents the studies regarding the chemical analysis of the main bioactive compounds from *Aloe vera,* a plant grown under greenhouse climatic conditions in Romania, of 4 years old, compared to other varieties of *Aloe vera* grown in other areas. Thus, the ethanolic extract from the fresh material was analysed in order to determine the main classes of bioactive compounds, like polyphenols and flavonoids, and its radical scavenging activity. Also, for the solid, pressed and dehydrated (cake) residue resulting from the raw material that was processed in various manners (in the natural, dehydrated or freeze-dried state) specific parameters were determined.

The raw plant material characterization in terms of water content, dietary fiber content and physico-chemical properties was also required both to formulate the innovative pharmaceutical formulation and to preserve all the active principles with an increased therapeutical benefit of the final product.

4.2. Materials and methods

The *Aloe vera* plant, certified as origin, was purchased in 2016 from a greenhouse in Galați. From this 4 years old plant, the leaves with a 50-58 cm length and a 5-7 cm width were prelevated. The leaves that were cut from the base were washed several times with ultrapure water. The lateral spines and edges were removed before the dissection of the plant material.

The cortex was separated from the parenchyma with a scalpel. The extracted gel was washed twice with ultrapure water to remove the superficial exudate. Various quantities of the gel and of the epidermal plant residue were kept in the freezer at -40°C until the experiment was initiated. The extracted gel and pressed cortex were lyophilized separately using the Alpha 1-4 LD Plus freeze-dryer (Martin Christ, Germany). Before the freeze-drying, the gel was maintained for 1 hour at -20°C. The lyophilised products were stored at 4°C, in tightly sealed glass containers to prevent contamination and the absorption of moisture.
For each used technique, the plant extracts were diluted according to the protocols.

The physico-chemical characterization of the *Aloe vera* plant materials followed:

- the *Aloe vera* gel morphology determination by confocal microscopy;
- the determination of the fresh gel and cortex parameters: pH, acidity, protein content, lipids, fibers etc.;
- the determination of the hydrocolloid characteristics of the freeze-dried gel and cortex;
- antiradical activity (DPPH RSA) assessment;
- the spectrophotometric quantification of total polyphenols and flavonoids content from different natural or processed materials: oven-dried, freeze-dried.
- the separation, identification and quantification of the main polyphenolic compounds by HPLC;
- the identification of alloin by TLC and of the main functional groups by FT-IR.

4.3. Results and discussions

4.3.2. The physico-chemical characteristics of the fresh plant material

The gel extraction yield from the fresh product was reported to the full leaf mass (100%). The cortex extraction yield was 20.25% while for the gel it was 69.87%, with a loss of 9.88%. The yield obtained by pressing the cake was 96.3%, with a loss of 3.7% on the press filter. The gel yield was 0.9% whereas the lyophilized pressed cake registered a 0.95% yield hence confirming the large amount of free and bound water of this plant. To determine the water content of the plant or the water content that could be incorporated, the moisture content of the fresh material was determined (Table 4.2)

Table 4.2. *Physico-chemical characteristics of the products obtained from Aloe vera (Aloe barbadensis* Miller*)*

Characteristics	Fresh gel	Cake
Humidity (%)	**98.84%±0.46**	**89.26%±0.66**
pH	**4.53 ±0.12**	**4.69 ±0.06**
Acidity (% acid malic)	**0.065%±0.01**	**0.38%±0.02**

The pH of the samples was slightly acidic, ranging between 4.5 and 4.7. The pH value was closely correlated to the organic acids content, a content that increases during the night. Moreover, due to the fact that *Aloe vera* is a nocturnal CO_2 assimilation plant (crassulacean acid metabolism), the organic acids that were identified as part of its composition were citric, tartric and malic acid, although malic acid presented the highest concentration among the three. The acidity was rather similar in the gel and in the pressed cake, with a pH value of 4.53 and 4.69, and with an organic acids percentage of 0.065% and 0.38%, respectively.

The analysis of the nutrient content of the lyophilised material (gel and cortex) was performed according to the Weend's model. After a 7 days storage period at room temperature, the gel moisture was maintained at 4.23%, slightly higher than in the lyophilised cortex, which had a moisture value of 2.4% due to the fact that the gel was composed mostly of polysaccharides with the role of retaining water. The pressed cake exhibited a moisture content of 2.4% and because the yield was higher than expected, the residual gel of the cake was removed. The moisture of the gel depended on the freeze-dried gel storage. In this study, the lyophilised gel was kept at room temperature in tightly closed containers in the presence of a dehydrating agent, thus obtaining a moisture content of 4.23% (Table 4.3).

Table 4.3. *The chemical composition of the Aloe vera products*

Parameters	Lyophilised gel (g %)*	Cake (g %)*
Humidity	4.23 ± 1.05	2.4 ± 0.88
Proteins	8.07 ± 3.47	13.76 ± 1.64
Lipids	3.30 ± 0.61	2.20 ± 0.50
Fibers	1.25 ± 0.10	12.52 ± 2.18
Ash	18.33 ±0.99	11.15 ± 0.52
Non-nitrogen compounds	64.82 ± 3.78	57.97 ± 2.28

** Reported to dry matter content*

The crude fiber content of the pressed cake was 10 times higher than of the gel because the cortex and the chlorenchyma contain polyglucidic polymers such as cellulose,

various types of hemicellulose and lignin, as opposed to the gelthat contains only pectin and hemicellulose.

From these studies, it can be emphasized that the *Aloe vera* fibers can be used as dietary fibers, which are very useful in weight loss diets. The roughage fibers have a low nutritional value due to the fact that they contain a low amount of associated proteins, but they have benefits in treating constipation, lowering the blood sugar and cholesterol levels, and preventing colorectal cancer.

The amount of protein, determined by Bradford colorimetric method, revealed a 1.7-fold higher content in the pressed cake compared to the gel.

The mass lost by the incineration of the residue after a treatment with sulfuric acid and sodium hydroxide confirmed the high content of cortex fibers (12.52%) compared to the gel (1.25%) mainly because the cortex contained mostly parenchymal tissue as opposed to the gel which contained mostly polysaccharides.

4.3.3. Antioxidant properties

The antioxidant capacity of the fresh and lyophilized gel was assessed in order to determine the free radical scavenging activity. A slightly higher antioxidant capacity was observed in the case of the lyophilised gel compared to the fresh gel that quickly lost this property. After 2 days of refrigeration (2 -8°C) the gel passed through the process of enzymatic browning with its antioxidant capacity decreasing from 60.81% to 32.11% (Table 4.4).

Table 4.4. *Radical scavenging capacity of the fresh and lyophilised samples from Aloe vera*

Sample	Stock extract concentration	RSC %	RSC% (după 48 h)
Fresh gel	10%	60.81 ± 0.53	32,11 ± 0,2
Lyophylised gel	10%	66.67 ± 0.28	67,25 ± 0,34
Fresh cortex	10%	48.35 ± 0.1	-
Lyophylised cortex	10%	57.51 ±0.35	-

The anti-radical activity of the *Aloe vera* plant material was measured by subtracting the DPPH absorbance at 515 nm. The antioxidant compounds presence in the reaction medium interact with the DPPH radical, and thus resulting in a color intensity decrease.

The polyphenolic content was analyzed for the 96% ethanol extract. The results showed that the extract obtained from the lyophilised product had the most balanced proportion of the biologically active compounds required for the final pharmaceutical product. Although the results comprised within the range of values mentioned in the literature, however, a close correlation between the total polyphenol content, flavonoids content and the antioxidant activity of the three extracts could not be achieved. The concentrations of polyphenols and flavonoids were expressed as mg bioactive compounds/g dry weight (Table 4.5).

Table 4.5. *Bioactive compounds concentrations from Aloe vera samples*

Sample**	Flavonoids concentration (mg/g**)	Polyhenols concentration (mg/g**)	RSC (%)
NPP	12.812 ± 0.12	21.00 ± 1.1	5.00
LPP	38.26 ± 0.25	65.39 ± 2.32	43.60
DPP	11.85 ± 1.2	9.4 ± 0.17	43.38

** NPP = natural plant product; LPP = lyophylised plant product; DPP = dried plant product; ** Dry weight*

The total content of polyphenols varied depending on processing of the fresh plant material. From the data presented in Table 4.5, it can be observed that a higher concentration was obtained for the alcoholic extract of the lyophilised product compared to the other two extracts, values that were correlated to the antioxidant activity of this extract. The flavonoids concentration was clearly superior for the extract obtained from the lyophilised plant product, since the bioactive compounds with therapeutic action were retained by the plant mass compared to the drying treatment.

The lowest concentrations of flavonoids and polyphenols were obtained in the case of the dried plant material in the oven, which meant that with the loss of the constituent water, the important bioactive principles were lost and at the same time a higher temperature could influence the stability of the bioactive compounds.

In terms of the antioxidant activity, values between 5% (unprocessed plant) and 43.60% and 43.38% (lyophilised or dried plant) were obtained, values that were appreciably lower compared to ascorbic acid (80.5%) and rutin (86.7%) activities.

4.3.4. Hydrocolloidal properties

The hydrocolloidal properties of the plant material were closely related to the composition and structure of the polysaccharides and underwent various changes due to the molecular weight, ionic forms, pH, temperature etc. Of course, in terms of water retention capacity, the lyophilised gel exhibited a value superior to that of the lyophilised cortex, corroborated with the volume increase, according to the data presented in Table 4.6.

Table 4.6. The hydrocolloidal properties of *Aloe vera* lyophilised gel and cake

Sample	Water retention capacity (g water/g lyophilised)	Swelling capacity (mL water/ g lyophilised)	Fat adsorbtion capacity (g oil/ g lyophilised)
Lyophilised gel	32.21 ± 2.35	33.67 ± 4.0	4.5 ± 0.03
Lyophilised cortex	23.15 ± 0.26	8.27 ± 0.15	6.9 ± 0.05

In the lyophilised gel, the water retention capacity (WRC) was 32.21% g water/g lyophilised gel. The behavior differences were probably due to the different water removal processes, which influenced the cortex and parenchyma dehydration.

The WRC of the cortex was 23.15 g water/g sample, which demonstrated once again that *Aloe vera* is a CAM plant with a high content of polysaccharides, acemannan, an acetylated galactoglucomannan, (that has an excellent water binding capacity by employing hydrogen bonds), present in the gel, and the pectins and glycosylated proteins present in the parenchymal cell walls, which are also capable of retaining a large amount of water.

The swelling capacity (SC) showed a value of 33.67% for the lyophilised gel after 16h of hydration, which revealed a good ability to retain water or other polar solvents. The cortex had a weak swelling capacity being 4 times lower compared to the gel because the fibers in its content were a barrier to water loss, and had no retention capacity.

4.3.5. The characterization of the *Aloe vera* ethanol extract composition by chromatographic analysis

- HPLC analysis revealed the presence of various polyphenolic compounds with antioxidant activity (Figure 4.13), as follows:

- Peak 1 corresponded to galic acid, at a concentration of 7.97 µg/mL.

- Peak 2 was specific to vanilic and cafeic acid; the elution elapsed due to close retention times, at a concentration of 7.34 µg/mL.
- Peak 3 and peak 4 corresponded to epicatechin and respectively catechin, at a concentration of 20.82 µg/mL, respectively 12.25 µg/mL.
- Peak 5 corresponded to ferulic acid at a concentration of 9.86 µg/mL.
- Peak 6 corresponded to quercetin at a concentration of 204.75 µg/mL.

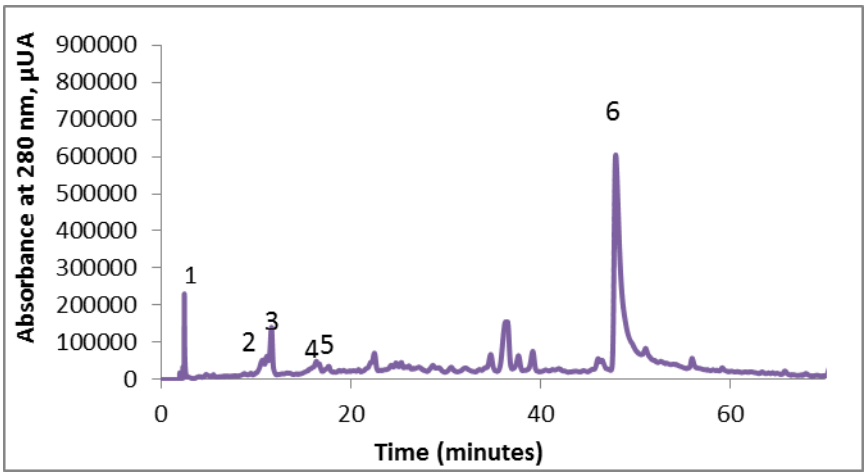

Figure 4.13. *HPLC chromatogram of the ethanol extract from Aloe vera lyophilised powder*

The comparative composition of the bioactive compounds identified by HPLC was also shown in Figure 4.14 in which one can clearly see that the quercetin content was the highest, and thus the superior antioxidant activity was explained.

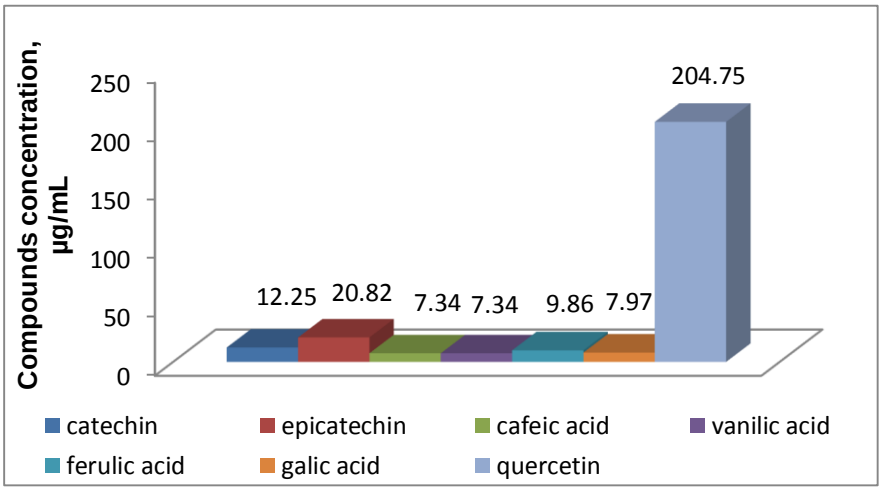

Figure 4.14. The bioactive compounds concentration in the ethanol extract from *Aloe vera* lyophilised powder

4.3.6. FT-IR analysis

The FT-IR analysis was performed to highlight the predominant functional groups present in the ethanol extracts of the natural, lyophilised and dried plant product. The FT-IR analysis of the *Aloe vera* cortex, gel and juice samples revealed the presence of specific functional groups: -OH- specific to the absorption band of 3426 cm^{-1}; C=O- with characteristic absorption bands between 1750-1655 cm^{-1} and amino acids - with specific absorption bands between 3300-3250 cm^{-1}, and the absorption band of 1550 cm^{-1} specific for the carboxyl COO- groups.

The comparative analysis of the FT-IR spectra from the ethanol extracts obtained by processing the *Aloe vera* leaf (natural, dehydrated, lyophilised) in different ways revealed close absorption bands for all three samples. The highest intensity peaks were recorded for the ethanol extract obtained from the lyophilised powder, thus indicating an optimal degree of maintenance of the biologically active compounds following this type of processing of the plant material.

The phenolic hydroxyls presence indicated an increased concentration of specific phenolic compounds in the ethanolic extracts obtained from *Aloe vera* - flavonoids, phenolic acids, anthraquinones, and the highlighted intensity band in the phenolic region indicated the accumulation of phenolic compounds in a high percentage.

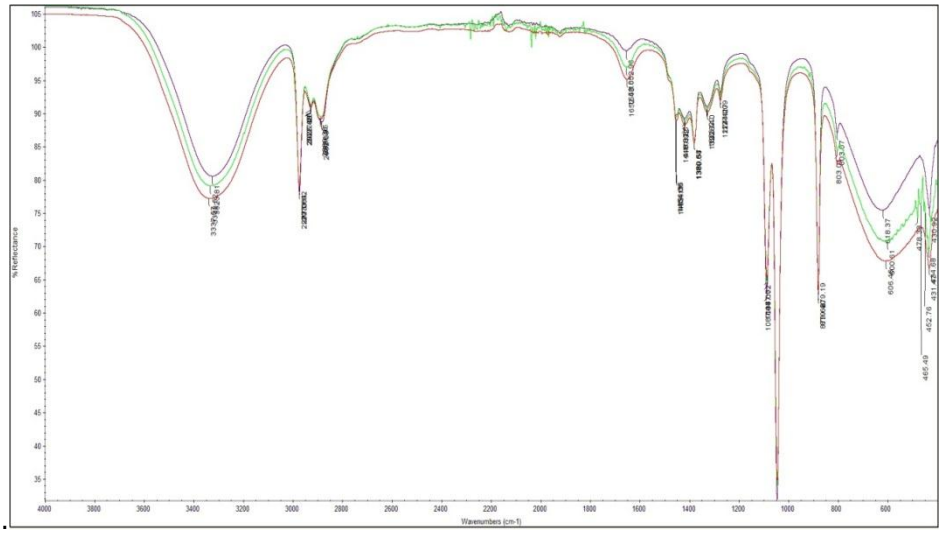

Figure 4.22. *FT-IR spectra of the ethanol extract from Aloe vera: lyophylised powder –red; dehydrated powder – green; fresh product – purple.*

These functional groups were responsible for the polar nature of the polyphenolic compounds from the *Aloe vera* ethanol extract. The average intensity specific band for the C-O groups was about 1653 cm^{-1}, thus indicating the presence of anthraquinone-type carbonyl compounds. The low intensity bands at the frequency of about 800 cm^{-1} were attributed to the C-H bonds present in the polymeric compounds from the studied extracts. The absorption bands in the 1332 cm^{-1} region corresponded to the symmetrical NO_2 bonds characteristic to the nitrogenous aromatic compounds.

The absorption around the 1620-1610 cm^{-1} values corresponded to the C=C bonds, and thus indicating the presence of specific vinyl ether and aloin groups in the sample. In the extracts analysed by FT-IR method, the chlorophyll fraction was removed, which also resulted in the separation of aloin, although this compound was present in the *Aloe vera* cortex sample.

The peaks at 2926 cm^{-1} corresponded to the C-H bonds that resulted from the formation of numerous hydrogen bonds at high concentrations of bioactive compounds. The average intensity bands were observed at 1720 and 1255 cm^{-1} and indicated the presence of C=O and C-O-C groups, which were specific to the acetyl and carboxyl groups in the samples. The identification of enhanced bands in the 1050 cm^{-1} area confirmed the presence of monosaccharide units in nearby regions, such as galactose, glucose etc..

4.4. Partial conclusions

1. The chemical and phenolic composition of various *Aloe vera* (*Aloe barbadensis* miller) samples such as leaf gel, gel, cake, ethanol extracts obtained from the unprocessed, oven-dried or freeze-dried leaves were studied.

2. It has been shown that the plant exhibited a rich chemical composition that provided a high nutritional value and also an important functionally, such as polyphenolic compounds which induced an antioxidant activity that was comparable to the standard compounds, in the case of the ethanol extracts obtained from the lyophilised or dried leaves.

3. In terms of the chemical composition and functional value, the processed products derived from *Aloe vera* (gel, cakes, powders, ethanol extracts) can become beneficial substrates for the development and metabolic activity of microorganisms, especially of lactic acid bacteria.

4. By considering the ability of water retention and fat adsorption, the *Aloe vera* plant has many health beneficial properties making it suitable for cosmetics and pharmaceuticals products.

5. For the following studies, the ethanol freeze-dried powder extract had been chosen for the growth of lactic acid bacteria, the metabolic activity stimulation, the antimicrobial activity, the potential benefits on skin disorders and for the formulation of a hydrophilic ointment with a potential therapeutic use in atopic dermatitis, psoriasis, various types of eczema and dermatitis, and uncomplicated first and second degree burns.

5. The fermentative and physiological behavior of some probiotic strains in an *Aloe vera* supplemented culture media

5.1. Introduction

The studies presented in this chapter aimed to evaluate the fermentative and functional behavior of three strains of lactic probiotic bacteria of the *Lactobacillus* genus, *L. rhamnosus, L. plantarum* and *L. casei*, by cultivation in a specific MRS environment and in an unconventional environment based of electrolytes enriched with 0.5% freeze-dried *Aloe vera* powder.

The analysed parameters, both during the fermentation and also during the preservation period of the fermented products (at 4°C for 21 days) were:

- the lactic fermentation dynamics;
- the probiotic bacteria multiplication dynamics and kinetic parameters;
- the probiotic bacteria viability in the fermented environments, during storage;
- the adherence capacity of the probiotic bacteria multiplied in different fermentative media (*in vitro* tests on epithelial cell cultures);
- the antimicrobial activity of the fermented products.

The obtained results are innovative because the probiotic bacteria strains isolated from commercial products were first grown in an electrolyte solution supplemented with 0.5% freeze-dried powder obtained from *Aloe barbadensis* leaves. The studies carried out at this stage are biotechnologically important as they allowed the selection of some probiotic strains that were capable of multiplying and surviving in an *Aloe vera* fermented media in order to formulate new functional ointments for the prevention and treatment of skin disorders.

5.2. Materials and methods

The *Lactobacillus rhamnosus* GG (Gyniel plus®, Hyllan Pharma, Romania), *Lactobacillus plantarum* (from the BioAliment Platform, Faculty of Food Science and Engineering, Galati), *Lactobacillus casei* (GynOphylus®, Beaufour Ipsen Industries, UK) species were isolated by successive cultivations on a MRS agar medium. For each strain the inoculum was measured spectrophotometrically at the λ = 600 nm (with the O.D. of 0.500) , in a liquid MRS medium, liquid MRS supplemented with *Aloe vera* lyophilised powder and in an electrolyte solution supplemented with *Aloe vera* lyophilised powder. The following parameters were:

- ✓ pH;
- ✓ the number of colony-forming units (cfu/mL) by the indirect counting method Koch;
- ✓ lactic bacteria viability during storage.

Subsequently, the *L. casei* and *L. plantarum* strains were selected to be further tested in a combined inoculum on an experimental medium of electrolyte solution supplemented with *Aloe vera* lyophilised powder.

For the *L. casei* and *L. plantarum* strains, the ability to adhere to the HeLa-2 epithelial cell monolayer was determined as well as the antimicrobial and antifungal activity on different pathogenic microorganisms: *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* (clinically isolated from a skin wound), *Candida albicans* ATCC 10231, *Candida albicans* (clinically isolated from a vaginal exudate) by using the radial diffusion method (Kirby - Bauer) and by competitive inhibition of the adhesion to HeLa - 2 cell monolayer.

The used reagents were: methanol 96%, ethanol 96%, acetone 96% (purchased from Chemical Co.) and saline phosphate buffer (Sigma-Aldrich, Germany). The used equipment was: UV-VIS 6505 Spectrophotometer (Jenwey, Germany), Separate Multiparametric Seven Easy F20K Analyzer (Mettler Toledo, Switzerland), Thermostatic Incubator ICT-18 (Falc Instruments, Italy), Immersion and Epifluorescence Microscope BX 41 (Olympus, USA), High-precision analytical balance XS403SM (Mettler-Toledo, Switzerland).

5.3. Results and discussion

5.3.1. Lactic fermentation dynamics

The pH evolution in all the fermentative media for the three probiotic strains, *Lactobacillus plantarum, Lactobacillus rhamnosus* and *Lactobacillus casei*, was analyzed during both the fermentation period (48 hours) and after 7, 14 and 21 days (during the storage period).

For the studied strains, the growth dynamics, viability and adaptability to different substrates on which they were grown were followed, in two stages, one in regards to the fermentation and the other for the storage period.

Following the performed experiments, it was established that the optimal fermentation period was 72 hours while the optimal storage period was 21 days. For this reason, the growth dynamics was assayed over 72 hours, and the viability within 21 days. In order to obtain a the most beneficial environment for the development of probiotic bacteria, the main objective of this study was to assess the behavior of the three strains by inoculating them on three different compositions: a control medium (MRS broth selective for lactobacilli) (MRS broth and *Aloe vera* lyophilisate at a 0.5% concentration) and on an electrolyte-based medium supplemented with 0.5% *Aloe vera* lyophilisate with a pH of 6.8, a medium that has not been tested up until this study.

The acidification capacity was closely correlated to the degree of lactic bacteria multiplication, and the almost constant pH over the storage period was directly corroborated with the maintenance of the viability of the studied strains.

In the case of the *L. rhamnosus* strain, a sudden reduction of the pH value was observed in the first 24 hours of fermentation for all three samples, from 5.4 to 3.9. Nonetheless, no difference was observed between the blank sample and the other two samples supplemented with *A. vera* (Figure 5.1).

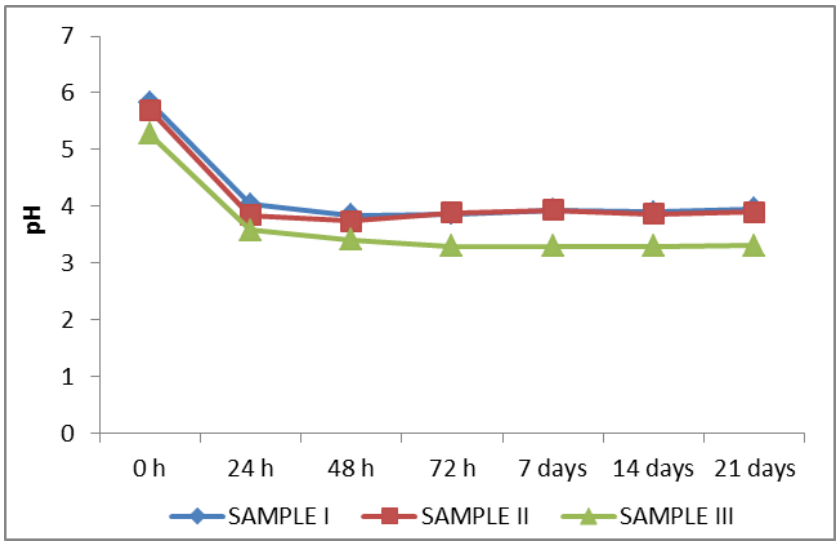

Figure 5.1. *The pH variation during the cultivation and the storage period of the L. rhamnosus strain*

Also, during the storage period, from 7 to 21 days, at 4°C, the pH was maintained for all the three samples, with a slight increase towards the end of the storage period, in the case of the cultivation on the liquid MRS supplemented with 0.5% lyophilised *A. vera* powder.

The most constant pH was recorded for the *L. plantarum* culture, from 24 h to 21 days (Figure 5.2). The most significant decrease of the pH was recorded between 0 and 24 h, afterwards it was maintained constant, although the multiplication was progressive. The innovative electrolyte environment showed a constant pH of 3.77 throughout the whole fermentation and preservation period.

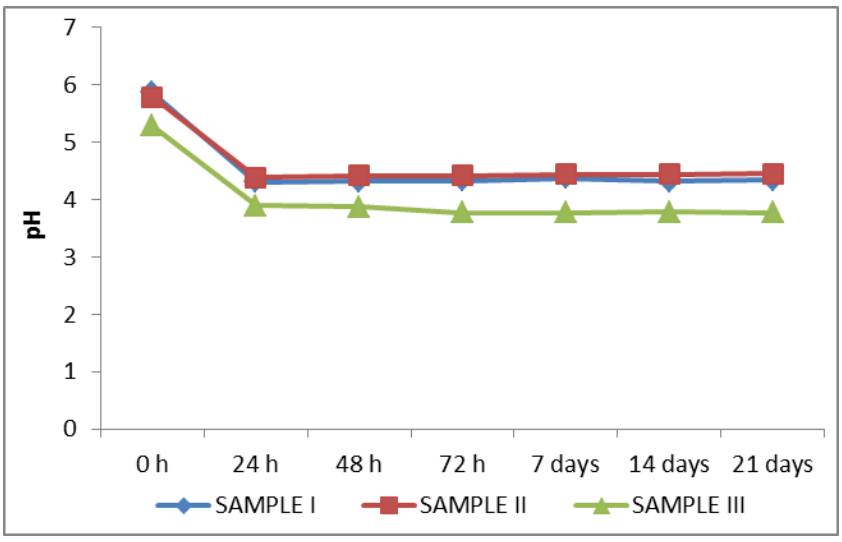

Figure 5.2. *The pH variation during the cultivation and the storage period of the L. plantarum strain*

In the case of *L. casei* culture (Figure 5.3), the pH evolution showed a similar dynamics to the cultures previously studied, showing also an increase of the pH values at 4.23 after 72 hours of fermentation at 37°C, during the storage of the fermented MRS supplemented with *Aloe vera*. The lowest pH value was recorded for the electrolyte fermentation medium, 3.19, after 72 hours of incubation at 37°C.

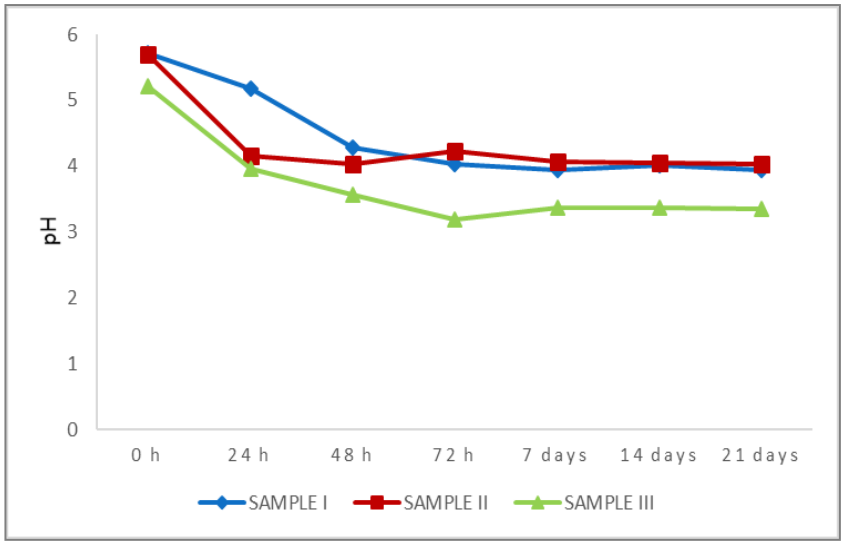

Figure 5.3. The pH variation during the cultivation and the storage period of the L. casei strain

5.3.2. Multiplication dynamics and the viability of the probiotic bacteria during fermentation and preservation of the fermented media

The multiplication dynamics during the fermentation period

For the *L. rhamnosus* strain, the growth dynamics of the probiotic strain cultured on the liquid MRS supplemented with 0.5% *Aloe vera* powder, the fastest multiplication rate was recorded in the first 24 hours, with a sharp decrease over the next 24 hours, followed by a CFU/ mL maintenance for the next 24 hours (Figure 5.4.).

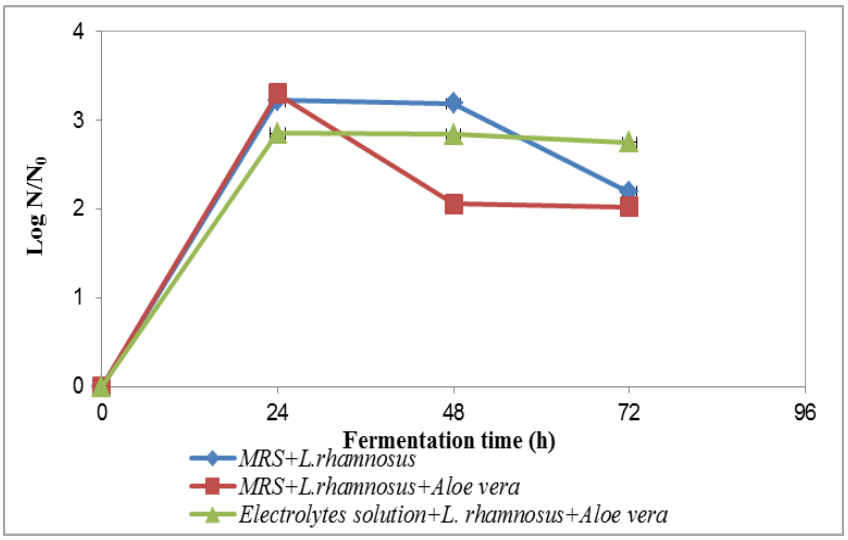

Figure 5.4. *The multiplication dynamics of L. rhamnosus during the fermentation period*

The blank sample showed a predictive growth curve, with a maximum increase, a standstill period, followed by a decline phase. The most interesting behavior was observed for

the sample III (electrolyte-based medium, pH = 6.8, supplemented with 0.5% lyophilised *Aloe vera* powder). On this medium, a slower multiplication rate was recorded over the first 24 hours compared to the blank but the culture showed the best stability in regards to cell viability (CFU / mL after 48 and 72 hours of cultivation).

For the *L. plantarum* culture, a similar behavior was observed in the *Aloe vera* supplemented medium (Figure 5.5). In this case, the culture multiplied slower and had a lower viability after 48 and 72 hours in the liquid MRS with 0.5% lyophilised *Aloe vera* powder.

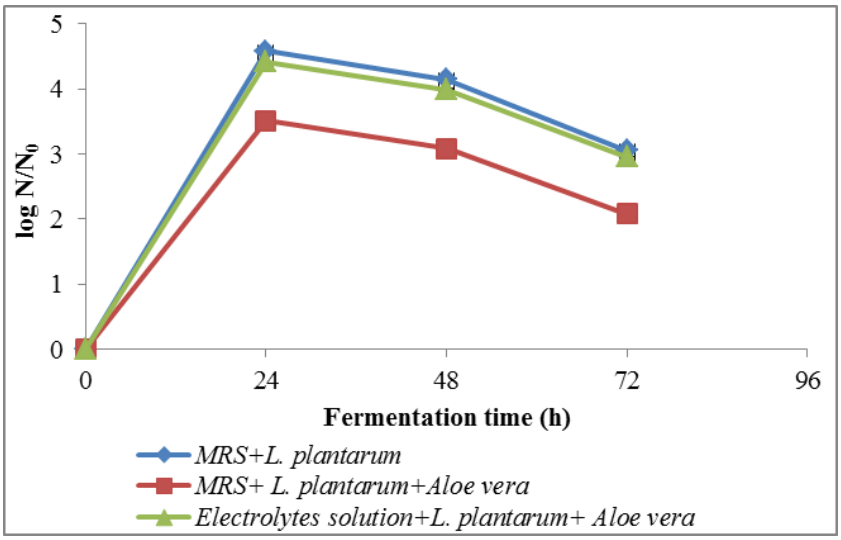

Figure 5.5. *The multiplication dynamics of L. plantarum during the fermentation period*

The maximum cell counts were at the same level after 24 hours of fermentation for the control sample, respectively, with the innovative electrolyte fermentative broth, $1.4 \cdot 10^9$ CFU/mL, and after 72 hours the viability slightly decreased to $1.2 \cdot 10^8$ CFU/mL, and $1.28 \cdot 10^8$ CFU/mL for the same two samples, respectively. The sample in which the probiotic lactic bacteria were grown on the MRS with the addition of *Aloe vera* lyophilised powder showed a different dynamic with lower values of 10^9 CFU/mL at 24 h and $9.95 \cdot 10^7$/mL at 72 hours, respectively. Figure 5.5. revealed a viability regression rate proportional to the other two samples.

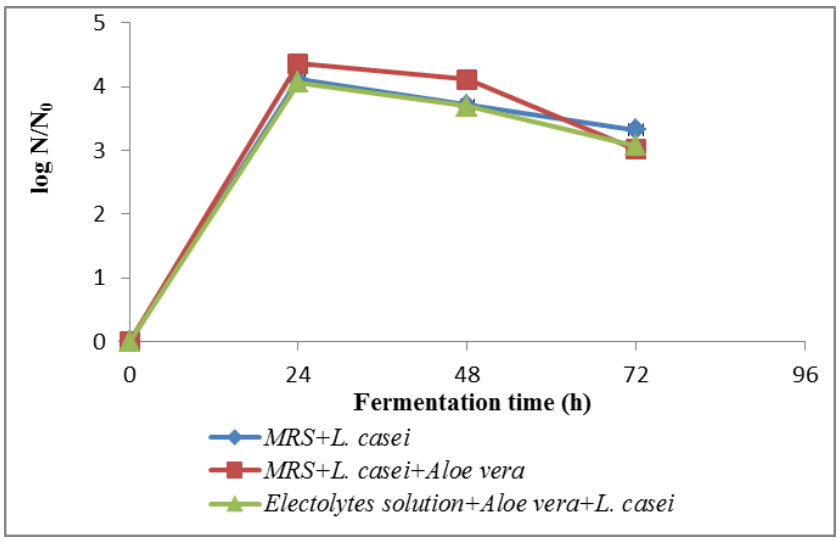

Figure 5.6. *The multiplication dynamics of L. casei during the fermentation period*

The *L. casei* strain had a maximum increase over the first 24 hours, the highest multiplication rate being recorded by cultivation in the liquid MRS medium supplemented with *Aloe vera* (Figure 5.6), 2.8 • 10^9 CFU/mL, compared to 4 • 10^8 CFU/mL for the control sample.

Following the *L. casei* dynamics, in sample III, in the medium based on the electrolytes solution and *Aloe vera*, a higher multiplication rate was observed compared to the control sample over the first 24 hours, 1.14 • 10^9 CFU/mL and after 72 hours of fermentation slightly lower values were obtained compared to the control sample, 8.35 • 10^8 UFC/mL. After 72 hours, samples II and III displayed the same CFU/mL values, 2.18 • 10^8 and 4.64 • 10^8, which were slightly lower compared to the control sample.

For each studied strain, the kinetic parameters describing the multiplication, number of generations, multiplication rate, and generation time were calculated. The obtained data were presented in Table 5.4.

Table 5.4. *Multiplication kinetic parameters of probiotic bacteria (after 72 hours of cultivation, at 37°C)*

Strain	Number of generations			Multiplication rate (h^{-1})			Generation time (h)			Viability (%)		
	S I	S II	S III	S I	S II	S III	S I	S II	S III	S I	S II	S III
L. plantarum	13.82	16.77	13.32	0.57	0.69	0.55	1.75	1.43	1.8	71.10	61.56	63.81
L. rhamnosus	10.66	10.96	9.47	0.44	0.45	0.39	2.27	2.19	2.53	73.98	69.23	61.55
L.casei	10.59	13.71	12.19	0.44	0.57	0.50	2.27	1.75	1.97	61.93	61.49	69.84

The results demonstrated the multiplication capacity of *L. plantarum* and *L. casei* cultures in all the studied media, especially in the *Aloe vera* supplemented media. In terms of viability, it was reduced by 29-30% after 72 hours of cultivation, depending on the strain and the used fermentation medium. The kinetic parameters of *L. casei* deployed the most favorable evolution in the electrolyte experimental medium supplemented with 0.5% lyophilised *A. vera* powder, exhibiting a multiplication rate of ~0.5±0.7 h^{-1}.

The probiotic bacteria viability during the preservation of fermented media

The fermented media were maintained under refrigeration (2-8°C) for 21 days and analysed after 7, 14 and 21 days. For the *L. rhamnosus* strain, the three samples maintained at 4°C showed a similar evolution in terms of viability.

The control sample displayed a decrease of its viability after 7 days, then a slight decrease to 21 days (Figure 5.7). Compared to this, the samples supplemented with *Aloe vera* exhibiter a lower decrease of the viability and a higher CFU/mL than the control sample.

The highest viability was manifested by the cells grown and maintained in the electrolyte-based medium supplemented with 0.5% lyophilised *Aloe vera* powder.

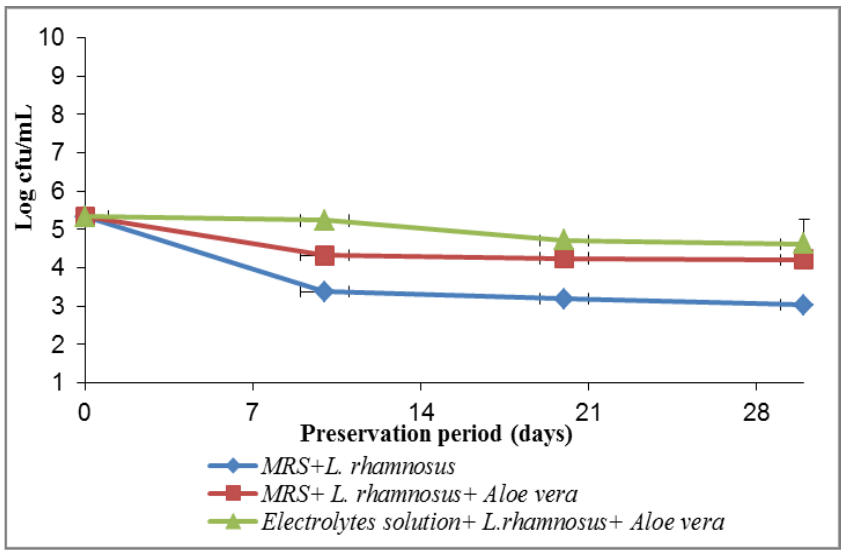

Figure 5.7. *The viability of L. rhamnosus during the preservation of fermented media*

During the preservation period, *L. plantarum* had the highest viability in the experimental samples II and III, compared to the control sample. Thus, as it can be seen from Figure 5.8, the highest viability after 21 days was observed for the sample where the bacteria was cultured in the electrolyte solution (ME) medium supplemented with 0.5% *Aloe vera* powder. This fact demonstrated that the strain could be used in the experimental study and that the addition of *Aloe vera* lyophilised powder was a good growth substrate for the lactic bacteria. At the same time, a future study should also follow the behavior of *L. planarum* in the proposed experimental environment supplemented with *Aloe vera* by co-cultivation with *L. rhamnosus* and / or *L. casei.*

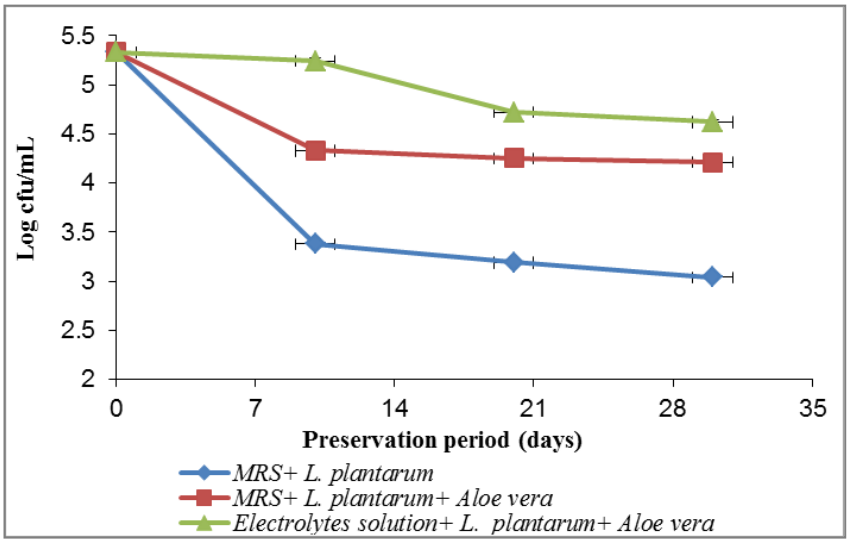

Figure 5.8. *The viability of L. plantarum during the preservation of fermented media*

In the case of *L. casei* cultures (Figure 5.9), the highest degree of viability was also obtained after 21 days in the case of the culture cultured in the the electrolytes with *Aloe vera* enriched based medium. After 7 days, the most drastic cell viability decrease was observed in the culture grown on the MRS medium and on the MRS supplemented with *Aloe vera*, afterwards the cell death rate slowed down, which certified the protective effect of the plant on the cells. Thus, after 21 days, in the experimental sample, based on MRS broth supplemented with *Aloe vera* lyophilised powder, a higher CFU/mL value, $5.63 \cdot 10^7$ CFU/mL, was determined compared to that of the control sample, $2.52 \cdot 10^5$ CFU/mL.

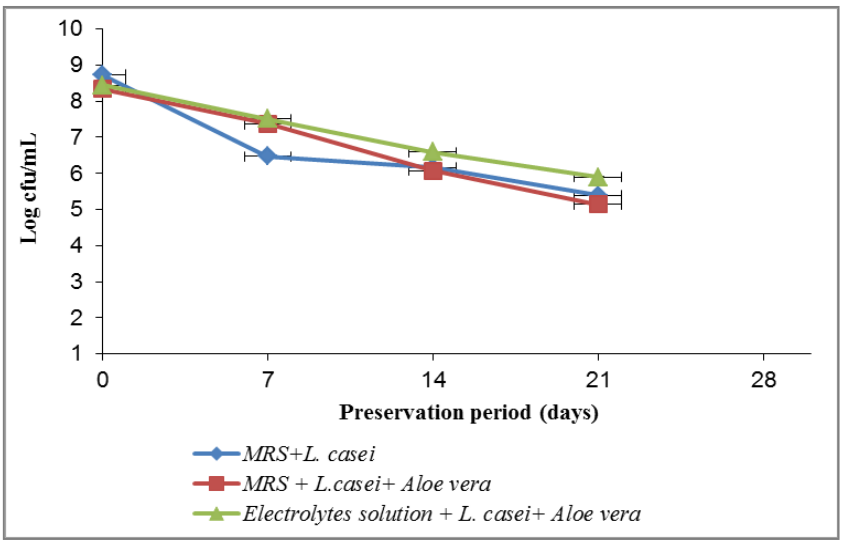

Figure 5.9. *The viability of L. casei during the preservation of fermented media*

L. plantarum and *L. casei* strains demonstrated a good adaptability by cultivation on the electrolyte solution (ME) supplemented with 0.5% *Aloe vera* powder and the ability to maintain their cell viability for 21 days through the preservation of the fermented media under refrigeration conditions (2-8°C).

The possible association of 2 probiotic strains will increase the functionality of the innovative pharmaceutical product, thus benefiting from both the antioxidant, anti-inflammatory, healing properties of *Aloe vera* as well as the antimicrobial and immunomodulatory effect of the probiotic strains.

Between 72 hours and 21 days, the samples were maintained at 4°C, and the studied strains showed different degrees of viability.

In the case of the *L. rhamnosus* strain, a marked decrease of its viability was observed from 72 hours to 7 days, at the temperature transition from 37°C to 4°C (Figure 5.10).

The highest decrease of the viability was observed for sample III, on the experimental electrolyte-based solution (EM), where the nutrients were probably consumed more quickly. Sample II registered a significant decrease of viability at the temperature change, but afterwards a stationary phase was recorded, so after 21 days, the experimental samples had close viability values compared to the control sample, 61.56% and 63.81%, compared to 71.1%.

To improve the viability, future studies were proposed to optimize the composition of the electrolyte-based medium (EM) by nutritionally balancing or increasing the concentration of *Aloe vera* lyophilised powder.

At the same time, it will also be possible to perform tests on their use in a micro encapsulated form, matrix yielding, substrate adaptation and colonization of the targeted surface.

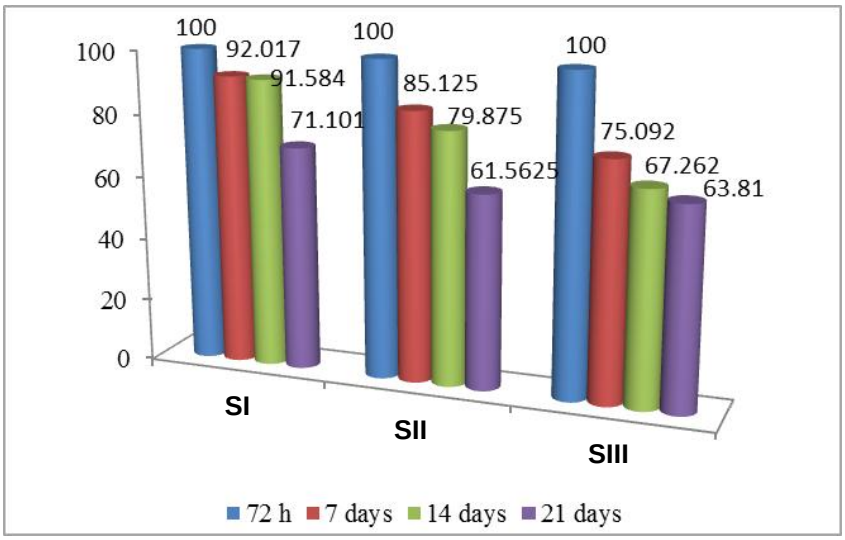

Figure 5.10. *The viability maintenance degree of L. rhamnosus in the fermented media (kept at 0-4°C)*

L. plantarum showed a superior viability degree after 21 days in the case of sample III (cultivation on EM medium, supplemented with *Aloe vera*) compared to the control sample (Figure 5.11). Although, compared to the control sample, sample III displayed a viability decrease after 7 and 14 days, while sample II exhibited approximately the same viability curve compared to the control sample.

L. plantarum exhibited a good viability in regards to sample III at the end of the 21 days of the fermented media storage under refrigeration conditions, which meant that *L. plantarum* was a strain that was suitable for the formulation of the targeted pharmaceutical product (external use).

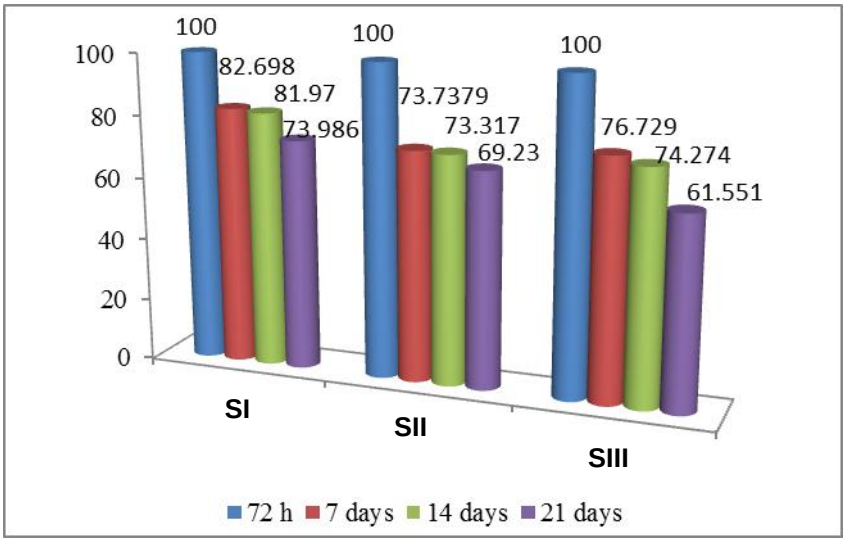

Figure 5.11. *The viability maintenance degree of L. plantarum in the fermented media (kept at 0-4°C)*

Undoubtedly, the best viability maintenance degree and stability of *L. casei* was observed for the EM medium supplemented with *Aloe vera*. Thus, in all the analysed samples this culture manifested the best behavior compared to *L. rhamnosus* and *L. plantarum*, having the highest values of viability, compared to the control, after 7 ,14 and respectively 21 days (Figure 5.12). Sample II showed the same survival rate after 7 days, but the cell viability began to drop after 14 days and after 21 days it had the same viability as the control.

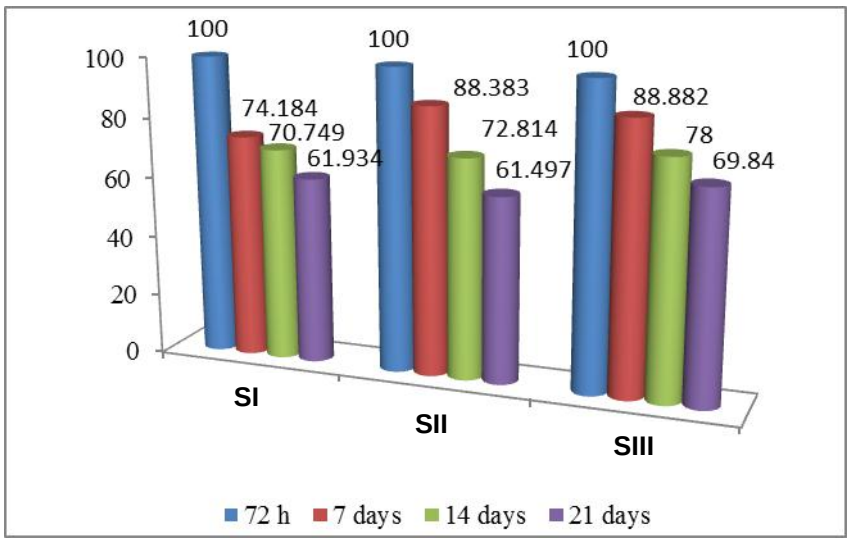

Figure 5.12. *The viability maintenance degree of L. casei in the fermented media (kept at 0-4°C)*

The majority of the studied research also recommended this strain to be used for the pharmaceutical product formulation. Nevertheless, the co-culture of *L. plantarum* and *L. casei* could be an optimum variant both technologically and functionally.

5.3.3. pH variation during fermentation and preservation of the fermented media

For all the tested variants, during the cultivation period, a pH decrease correlated with the lactic bacteria multiplication was observed (Figure 5.13 and 5.14). These developments confirmed the good functionality of the bacteria in the used fermentative media. The most pronounced pH reduction was recorded in the first 48 hours of cultivation. During the storage of the fermented product, the pH registered relatively constant values.

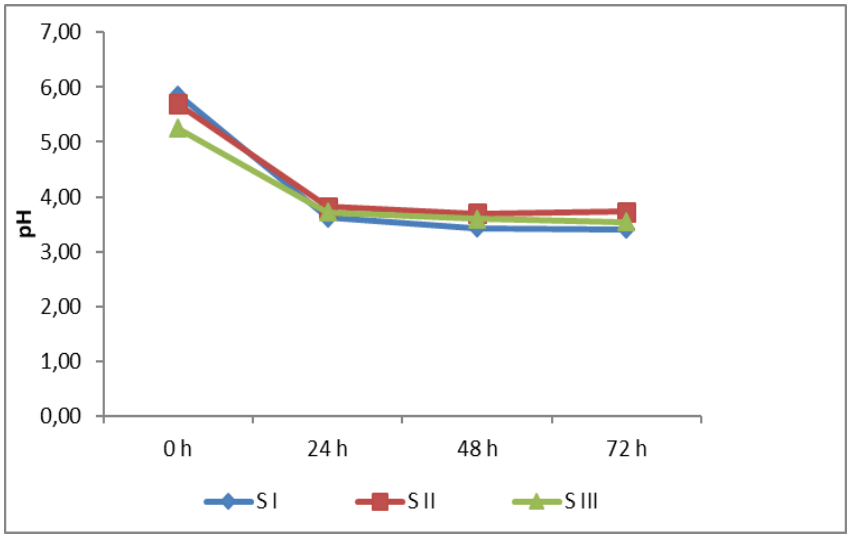

Figure 5.13. *The pH variation during the fermentation period*

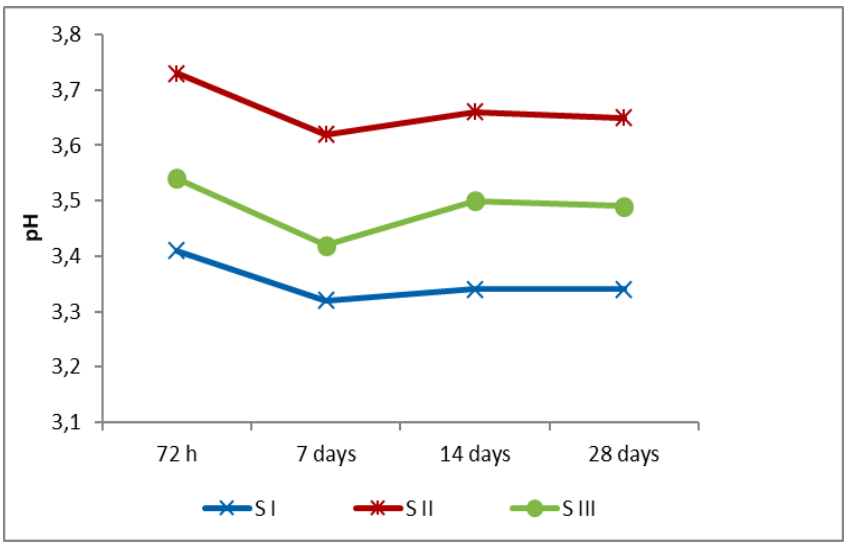

Figure 5.14. *The pH variation of the fermented medium during the preservation period*

5.3.4. Multiplication dynamics and the stability of the probiotic bacteria in the fermented media

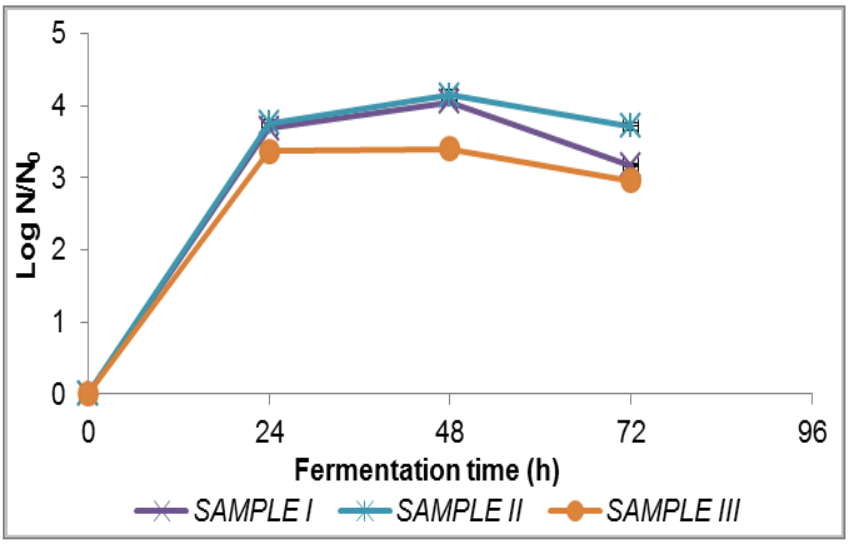

Figure 5.15. *Multiplication dynamics of probiotic bacteria during the fermentation period*

Concerning the lactic acid bacteria multiplication dynamics in the electrolyte-based medium supplemented with 0.5% *Aloe vera* lyophilised powder (Figure 5.15), a significant multiplication was observed for all the samples in the first 24 hours, followed by a slight increase up to 48 hours, and by a stationary stage up to 72 hours of cultivation. Although the cultures recorded a lower rate of multiplication, in the sample containing the two species cultured together, the bacteria were more stable from the viability point of view.

The lactic acid bacteria multiplication kinetic parameters

In Table 5.5., the kinetic parameters are shown during the multiplication and the degree of viability maintenance of lactic acid bacteria, parameters that were used to assess the lactic acid bacteria stability and their ability to maintain their viability of $\geq 10^6$ CFU/mL, required to produce beneficial health effects.

Table 5.5. *The lactic acid bacteria multiplication kinetic parameters in the the electrolyte-based medium supplemented with 0.5% Aloe vera lyophilised powder*

Samples	Number of generations	Multiplication speed (h-1)	Time of generations (h)	Viability maintenance percent(%)
Sample I	12.19	0.43	1.972	91.73
Sample II	13.32	0.38	1.801	95.36
Sample III	11.25	0.32	2.136	95.21

A slow multiplication of the lactic bacteria in the minimal environment was observed, the *L.casei* species being the most adapted. In a 1:1 ratio mixture of the two species, the multiplication speed and the generation time demonstrated a slightly slow propagation, but the degree of viability was comparable to that of the single *L.casei* culture.

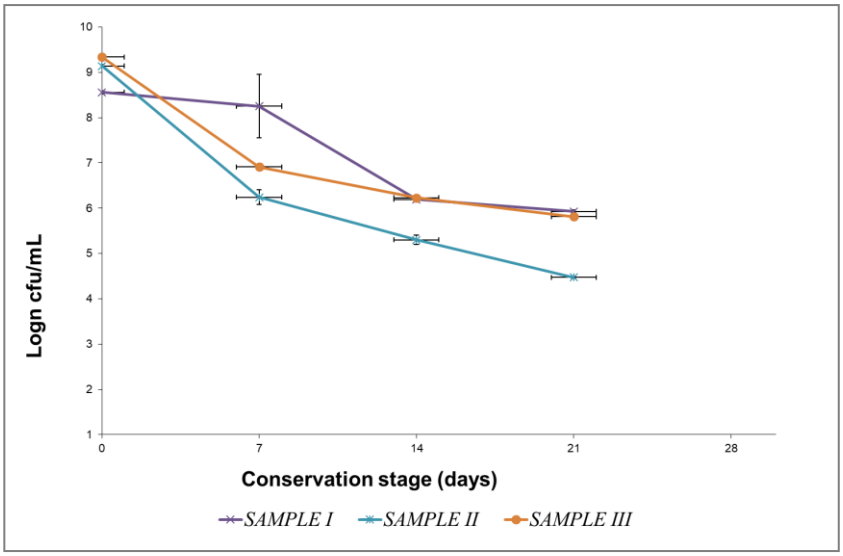

Figure 5.16. *The viability maintenance of probiotic bacteria during the preservation period of the fermented media*

From Figure 5.16. it could be observed the fact that a constant maintenance of the lactic acid bacteria viability from Sample III was assessed, of 10^6 CFU/mL, compared to samples I and II which either had a discontinuous dynamics with sudden decreases or were continuously decreasing over the preservation period, reaching values of 10^4 CFU/mL.

5.3.5. Antimicrobial activity of the fermented media

Using the variants of cultures and environments encoded in the materials and methods chapter, the antimicrobial activity of the fermented products was tested after 48 hours of cultivation at 37^{o}C. Following the assay technique described above, using as indicator the strains of *Staphylococcus aureus* and *Candida albicans*, the best results being recorded against the *S. aureus* ATTC bacteria, results obtained with the fermented environments encoded as 12, 8′ and 5′ (Figure 5.17.).

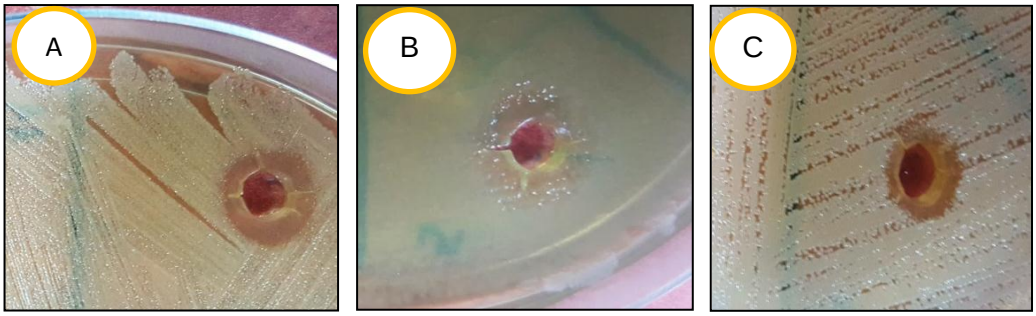

Figure 5.17. *The antimicrobial activity against S.aureus ATCC of some fermented media A) sample 12; B) sample 8'; C) sample 5'*

The inhibition diameters measured after 24 hours of incubation at 37°C ranged from 7 to 12 mm.

The inhibition diameters obtained for the *S. aureus* ATCC and clinical trials showed moderate values of 8-15 mm, with a more obvious antimicrobial activity for the samples that benefited from the supplementation of the fermentation medium with 2 mL of ethanol lyophilised *A vera*. It must be noted the fact that most of the experimental samples used in this study, which showed a relevant antimicrobial capacity, samples 12, 8' and 5' (Figure 5.17) were those optimized by the addition of lyophilised *Aloe vera* alcohol extract. Hence, the fermentation medium of the innovative pharmaceutical product for topical use will be further supplemented with ethanolic lyophilised *Aloe vera* extract, which had shown its beneficial activity in both the growth of the viable cell numbers and the antimicrobial activity.

Testing the antifungal activity on one of the most prevalent and adaptable pathogenic yeast strains of *Candida albicans* revealed a rather low inhibitory growth capacity by cultivation on the specific Sabouraud medium. Better results were obtained with respect to the inhibition of growth of the *C. albicans* strain from a vaginal exudate, where the inhibition diameters ranged from 10 to 12 mm were recorded for 5 samples: 5', 6', 7', 9, 10, compared to the inhibition diameters recorded on *C. albicans* ATCC, between 8-10 mm, only for three samples: 5, 5 'and 7. The samples obtained by *L. casei* and *L. plantarum* fermentation in the mediums with added 10% alcoholic *Aloe vera* extract showed an average antifungal activity (Figure 5.18).

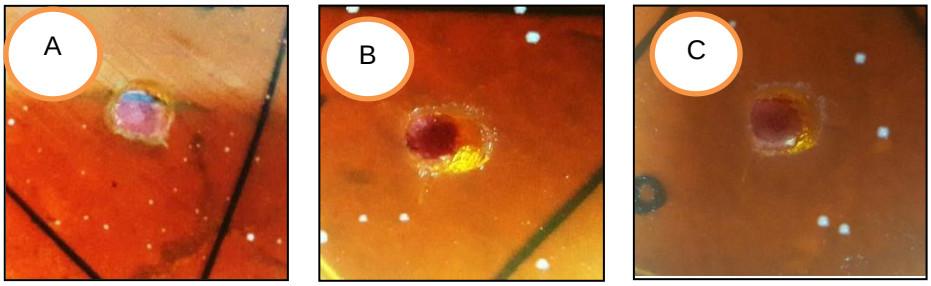

Figure 5.18. *Antimicrobial activity against C. albicans of some fermented media A) sample 6'; B) sample 10'; C) sample 9*

5.3.6. Probiotic lactic bacteria adhesion to the HeLa-2 cell line

The study aimed to test the adherence capacity of *L. casei* and *L. plantarum* probiotic lactic strains to adhere and colonize the HeLa-2 cellular monolayer. The adherence capacity varied depending on the probiotic strain tested, the used fermentation medium, MRS or the electrolyte environment (EM) and the addition of 2 mL of *Aloe vera* alcoholic extract (AAE) in different fermentative media variants (Figure 5.19).

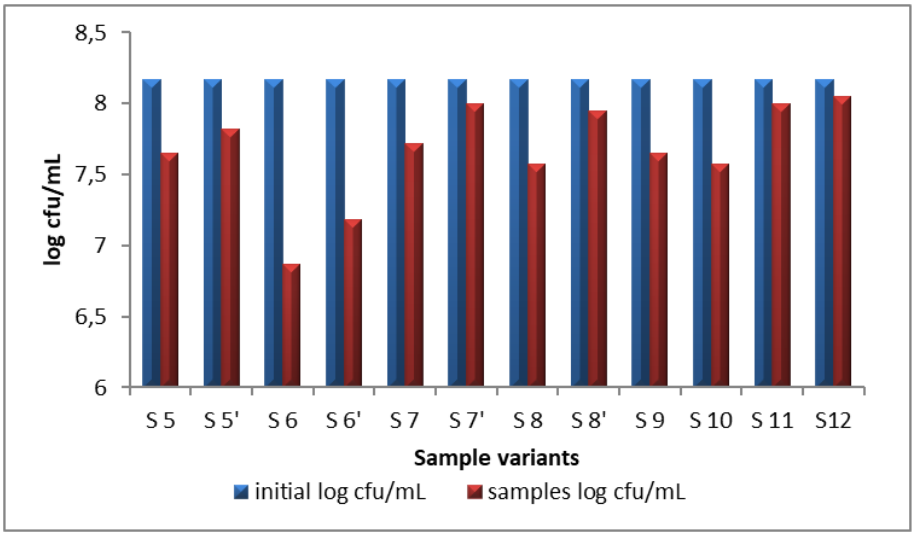

Figure 5.19. *Adhesion degree of L. casei and L. plantarum to the HeLa-2 cell line*

Thus, when the *L. casei* probiotic strain was individually cultivated in the MRS it exhibited a 30% adhesion degree corresponding to 4.5 • 10^7 CFU/mL, and respectively a 45% adhesion capability optimization corresponding to 6.75 • 10^7 CFU/mL, for the fermentative medium supplemented with 2 mL of AAE. At the same time, a similar behavior was also observed for the *L. plantarum* probiotic strain that was cultivated individually on the MRS supplemented with AAE, showing an adhesion degree of 70% and 1 • 10^8 CFU/mL, compared to only 35% in the case of the MRS cultivation variant (Figure 5.19).

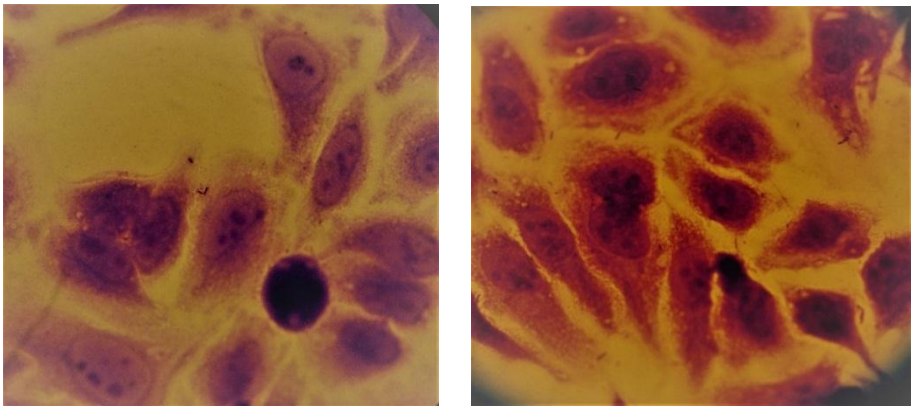

Figure 5.20*. Adhesion ability of L. casei (sample 5') –on the left and L.plantarum (sample 7')-on the right*

The variants that had the probiotic lactic bacteria strains individually cultivated in the experimental electrolytes media (EM) showed a moderate adhesion to the HeLa-2 cell monolayer, except for *L. plantarum* in the EM supplemented with AAE medium, which achieved an adhesion degree of 60%, corresponding to the value of 9 • 10^7 CFU/mL (Figure 5.20).

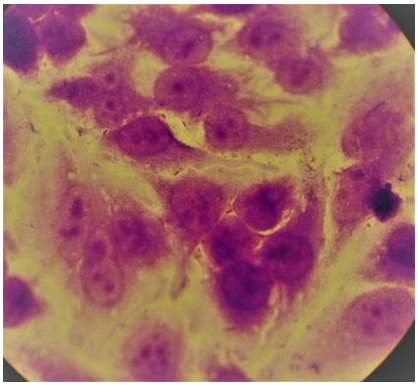

Figure 5.21. *Adhesion ability of L. plantarum (sample 8')*

The adhesion stimulation in this case was solely due to the addition of AAE, since the AAE-free electrolytes environment reached a 25% adhesion degree.

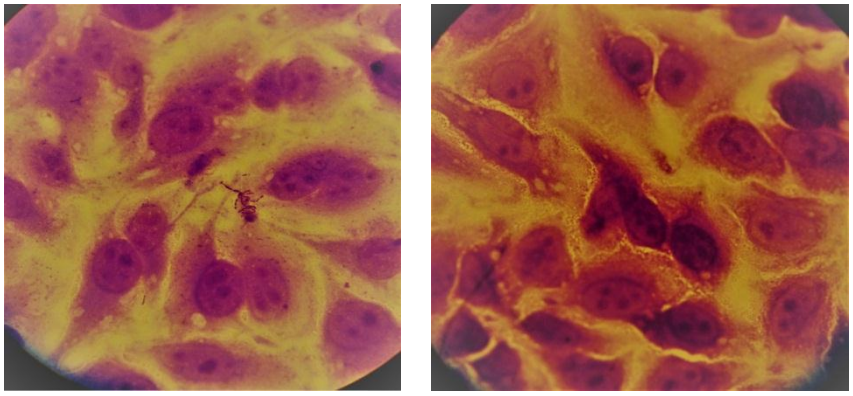

Figure 5.22. *Adhesion ability of the co-cultivated strains L. casei : L.plantarum in a 1:1 ratio (sample 11) – on the left – (sample 12) – on the right*

The optimal adhesion degree values were recorded for the fermented media where the probiotic strains were co-cultivated 1:1 when the cultures also benefited from the presence of bioactive compounds from the *A. vera* alcoholic extract. Samples 11 and 12 exhibited a 70% and 75% adhesion degree, respectively, which represented a CFU/mL of the lactic bacteria adhered to the cell monolayer of $1.0 \cdot 10^8$ CFU/mL and $1.13 \cdot 10^8$ CFU/mL, the adhesion being stimulated by the introduction of the *A. vera* extract into the fermentation substrate (Figure 5.22).

5.3.7. Pathogenic strainc adhesion capacity în the presence of the fermented media with probiotic lactic bacteria

The starting point of this study was to demonstrate the antagonistic ability of probiotics against some intestinal pathogens and uro-pathogens and gonococci.

To investigate this innovative, safe and potentially effective therapy, this research study was conducted to investigate the possible interactions of two lactic bacteria (*L. plantarum* and *L. casei*) with *Staphylococcus aureus* and *Candida albicans* pathogenic strains, clinically isolated from contaminated environments.

Considering the adaptability of the pathogenic species, both the bacterial species and especially of *C. albicans* strains, which are resistant to most antibiotics or antimycotics, the ability to competitively dislodge or exclude these pathogenic strains by the intake of fermented products with probiotic lactic bacteria, is an important breakthrough. The dramatic spread of the

antibiotics or antimycotics resistance may also have as an alternative treatment the use of probiotic strains in the prevention or the cure of certain skin conditions. Furthermore, the bioactive compounds of *Aloe vera* plant played an important prebiotic role by stimulating the multiplication, viability and adherence of probiotic bacteria.

The study followed, primarly, the anti-adhesive property that the *L. casei* and *L. plantarum* strains exhibited against the bacterial strains *Staphylococcus aureus* ATCC 25922 and *Staphylococcus aureus* isolated from a skin disorder and against *Candida albicans* ATCC10231 and *Candida albicans* strain isolated from a vaginal exudates. For all the pathogenic strains, the competitive method was applied and the pattern of adhesion as well as the adhesion degree to HeLa-2 epithelial cells variation were followed by the co-cultivation of the indicator microorganisms, and thus several variants of fermented media (lactic bacteria and lyophilised powder *Aloe* alcoholic extract) were obtained.

In some cases, the indicator microorganism maintained its adhesion pattern, although the cell line adhesion capacity decreased by 90%. Moreover, the adhesion degree failed to record values above 50%, but the adhesion pattern change could have beneficial consequences on reducing the pathogenicity manifestation (Figure 5.23).

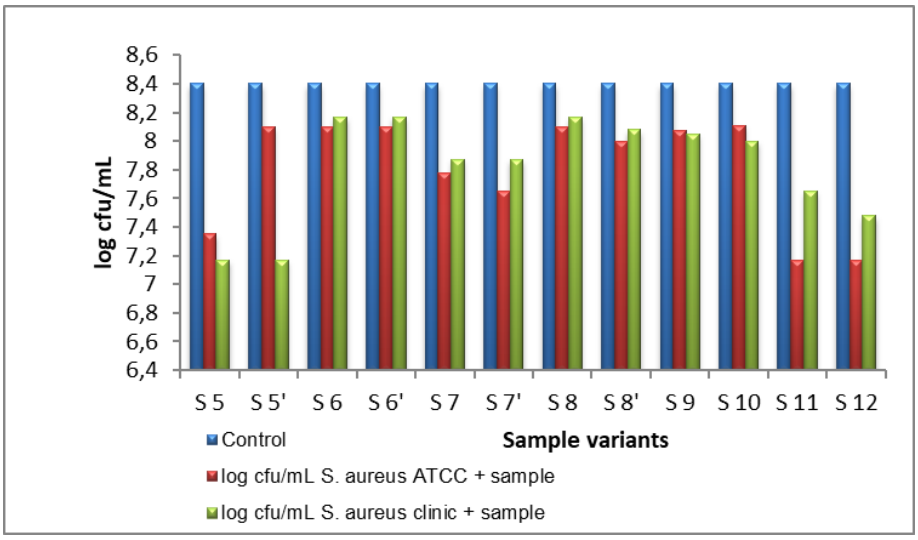

Figure 5.23. *The adhesion degree modification for Staphylococcus aureus ATCC 25922 and Staphylococcus aureus strains in the presence of the fermented media with Lactobacillus spp. and Aloe vera alcoholic extract*

The individually cultivated probiotic *L. casei* strain (S5) presented a competitive inhibition of *S. aureus* ATCC adhesion of 85%, producing a 1:10 log/CFU/mL decrease when the MRS broth medium was used compared to an inhibition of only 15% for the cultivation on the MRS medium supplemented with *Aloe vera* alcoholic extract. If in the case of the *L. casei* strain, the presence of *Aloe vera* compounds did not have a stimulating effect, in the case of the fermented media containing *L. plantarum* in the MRS (S7') and electrolytes medium (P8'), the addition of alcoholic extract of *Aloe* (AAE) produced a stimulating effect by reducing competitively the adhesion degree of *S. aureus* by 10% and 30%, respectively. Regarding the cultivation of *L. plantarum* in the MRS and electrolytes environment without the AAE, the pathogenic microorganism reached the following values: $1.5 \cdot 10^8$, $2.25 \cdot 10^7$ in the presence of *L. casei* supernatants (S5) and $4.5 \cdot 10^7$ in the presence of *L. plantarum* supernatants (S7'). The optimal anti-adhesion action was recorded for the S11 and S12 variants, where the co-cultivation of *L. casei* and *L. plantarum* probiotic strains, in both of the fermentation media supplemented with 2 mL of AAE, resulted in a maximum adhesion inhibition of *S. aureus.*

The adhesion inhibition of the clinically isolated *S. aureus* to the HeLa-2 cells monolayer

showed approximately the same evolution after applying the above-described competitive method. *L. casei* presented the maximum ability to inhibit the adhesion of the clinically isolated pathogenic strain both in its individual cultivation and in the 1:1 co-cultivation variant with *L. plantarum*, where the addition of the *A. vera* alcohol extract had a clear stimulating effect. Thus, the 1:1 co-cultured lactic bacteria supernatants in the MRS medium and electrolytes medium (ME), both supplemented with AAE (S11 and S12), reduced the adhesion of the clinically isolated *S. aureus* by 70% and 80%, the pathogen reaching a cell count of $4.5 \cdot 10^7$ CFU/ mL, and $3 \cdot 10^7$ CFU/mL, respectively, compared to the control sample, $1.5 \cdot 10^8$ CFU/mL.

As observed on the Olympus BX 41 Microscope (with a 100x immersion objective), the competitive method of the co-cultivation of *S. aureus* with several variants of the fermented samples containing lactic acid supernatants (*L. casei, L. plantarum*) cultured in the medium supplemented with the extract of *Aloe vera* revealed the adhesion pattern alteration, which either determines the inability to use ligands for the cell binding sites or to reduce the pathogenity.

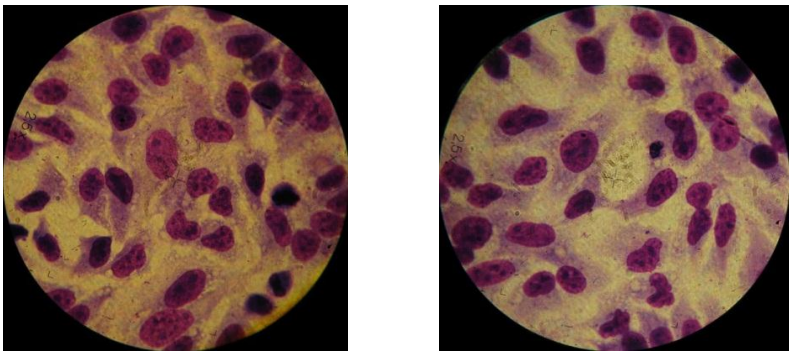

Figure 5.24. *The adhesion inhibition of S. aureus ATCC 25922 to the HeLa-2 cell line, in co-cultures with sample 5' – on the left and 7' – on the right*

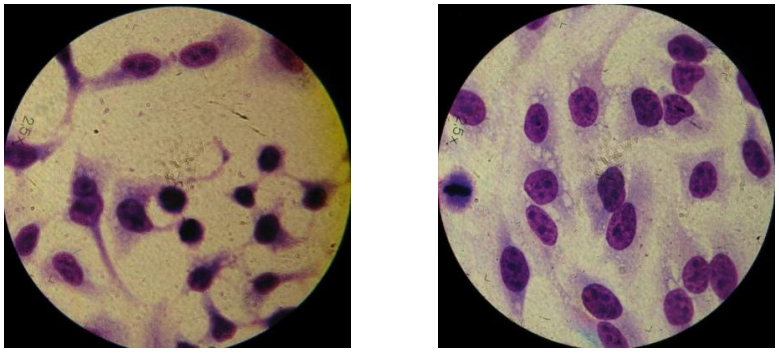

Figure 5.25. *The adhesion inhibition of S. aureus ATCC 25922 to the HeLa-2 cell line, in co-cultures with sample 11 – on the left and 12 – on the right*

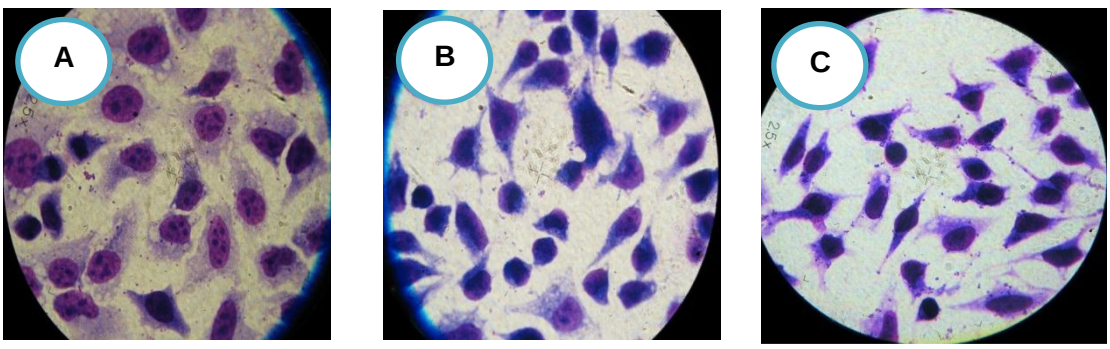

Figure 5.27. *The adhesion inhibition of S. aureus ATCC 25922 to the HeLa-2 cell line, in the presence of the L. casei and L. plantarum supernatants A - S_5'; B – $S_{11;}$ C – S_{12}*

The study also assessed the behavior of the fungal strains of *Candida albicans* ATCC 10231 and *Candida albicans* from a vaginal exudate in the presence of lactic acid supernatants cultivated for 48 hours in different fermentative media, MRS broth or in a nutrient poor electrolytes medium with or without the addition of *Aloe vera* alcohol extract from lyophilised powder (Figure 5.28).

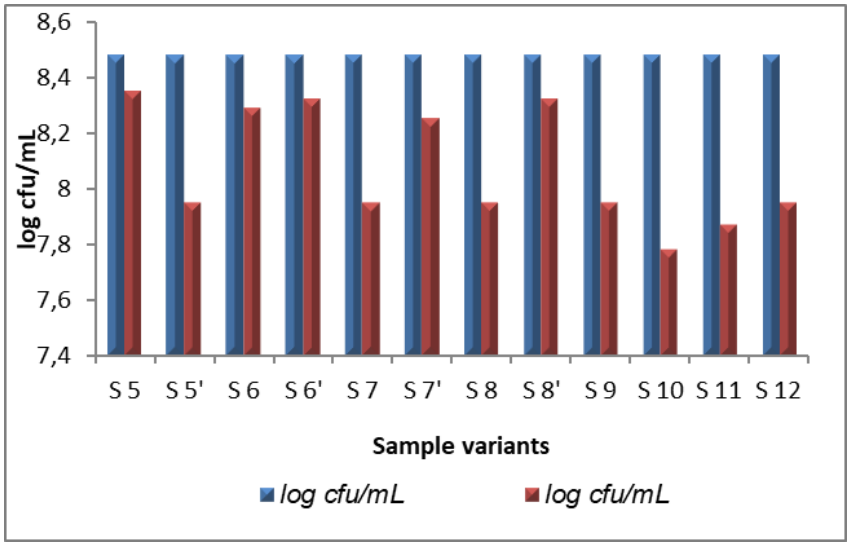

Figure 5.28. *The adhesion degree modification for Candida albicans ATCC 10231 strain in the presence of the fermented media with Lactobacillus spp. and Aloe vera alcoholic extract*

The *L. casei* probiotic strain showed an anti-adhesive property towards the *C. albicans* ATCC pathogenic yeast strain both in the individual cultivation variant (S5, S5') and in the 1:1 co-cultivation variant with *L. plantarum* (P9, P12). Thus, *L. casei* had a 70% adhesion reduction to the cellular monolayer of *C. albicans*, reaching a log of 7.95 CFU/mL, compared to the control, log 8.48 CFU/mL. Supplementing the fermentative medium with 4% from the *Aloe vera* alcoholic extract 10% (AAE) has proven to be extremely beneficial, thus increasing the capacity of *L. casei* to inhibit the pathogen from 25% (P5) to 70% (S5'). In the electrolytic fermentation medium, *L. casei* showed an anti-adhesive capacity of ~ 30%. However, the lowest adhesion degree was reached in the co-cultivation of the *C. albicans* ATCC pathogen with the supernatants obtained by culturing the two strains of lactic acid bacteria, *L. casei* and *L. plantarum,* in MRS broth supplemented with AAE, the pathogenic yeast achieving a CFU/mL of $6 \bullet 10^7$ compared to the control, $3 \bullet 10^8$ CFU/mL.

Unlike *L. casei*, *L. plantarum* showed anti-adhesive properties towards *C. albicans* ATCC of 70% only in the variants of its cultivation in MRS and EM without the supplementation with AAE (S7, S8). This fact led to the assumption that the adhesion inhibition of *C. albicans* in the co-cultivation with the supernatants obtained from the complex variants S10 and S11 was mainly due to the *L. casei* probiotic strain.

As observed on the Olympus BX 41 Optical Microscope (with a 100x immersion objective), the competitive method of *C. albicans* co-cultivation with the fermented variants *containing L. casei* and *L. plantarum* supernatants in a culture medium supplemented with lyophilised *Aloe vera*, revealed a pattern change of the adhesion which had consequences on their adhesive capacity.

The *L. casei* probiotic strain showed the most important antifungal action, both by inhibiting the *C. albicans* adhesion to the HeLa-2 monolayer substrate, by modifying the adhesion pattern of the control, hence achieving a competitive dislocation of up to 70-80% and by preventing the fungal filamentation development at the surface of the substrate. The presence of the 10% *Aloe vera* alcoholic extract from the lyophilised powder had a stimulating

effect on the the antiadhesive capacity of *L. casei* against *C. albicans*.

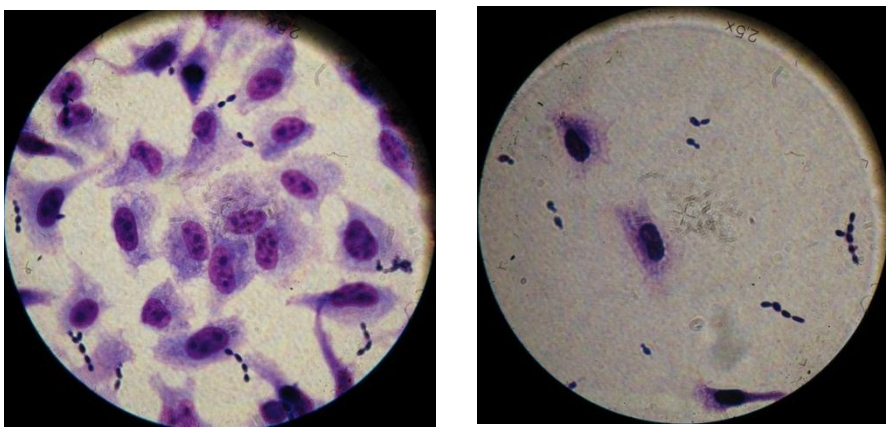

Figure 5.30. *The adhesion inhibition of C.albicans ATCC 10231, in the presence of the L. casei and L. plantarum supernatants S9 (on the left) and S10 (on the right)*

The *C. albicans* fungal strain, especially the clinically isolated one, had an extraordinary adaptability, which increased not only the high infection prevalence but also the inefficiency of the most conventional drug treatments. The results of the studies had shown satisfactory, but not optimal data. Therefore, the study also followed the behavior of *C. albicans* isolated from a vaginal exudate in the presence of *L. casei* and *L. plantarum* lactic acid bacteria supernatants, cultivated on different fermentative media with the addition of *Aloe vera* alcoholic extract.

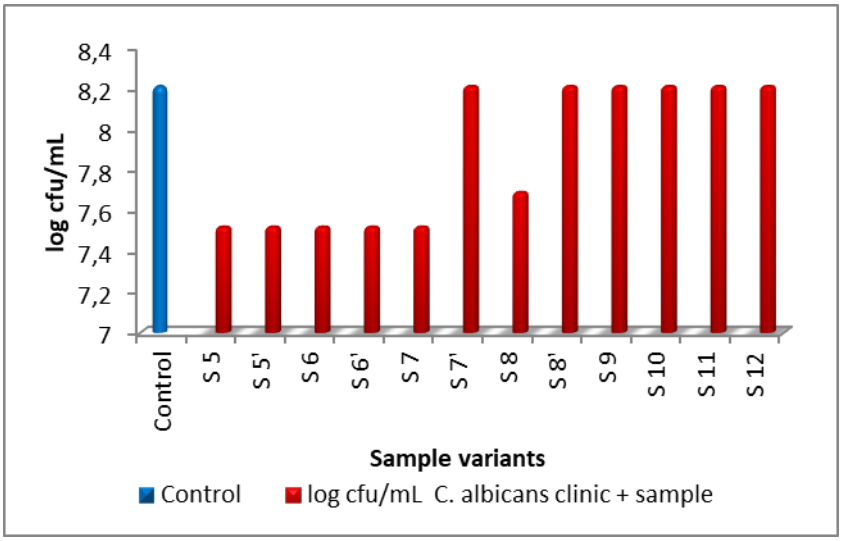

Figure 5.31. *The adhesion degree modification for the clinically isolated Candida albicans strain in the presence of the fermented media with Lactobacillus spp. and Aloe vera alcoholic extract*

The individually cultivated probiotic strain, *L. casei,* in the MRS broth medium and in the experimental electrolytes medium, with/without the *Aloe vera* alcohol extract (AAE), showed the highest anti-adhesive capacity towards the *C. albicans* pathogenic yeast, by achieving a 35% adhesion inhibition. The adhesion of *C. albicans* to the cell monolayer was reduced from 1.65 • 10^8 CFU/mL to 3.3 • 10^7 CFU/mL by the co-cultivation of the fungal strain with the lactic bacteria supernatants. The presence of the *Aloe vera* alcoholic extract did not result in any major changes of the ability to inhibit the *C. albicans* adhesion to the HeLa-2 cell monolayer, and it also did not represent an impediment to the beneficial properties of *L. casei*.

In contrast, the *L. plantarum* probiotic strain showed the ability to inhibit the adherence of the clinically isolated *C. albicans* only in two situations. Only the supernatants of the individually cultivated *L. plantarum* in different fermentative media but without AAE were able to induce the

C. albicans adhesion reduction by 35% (P7) and by 25% (P8), respectively. Thus, in the presence of the lactic bacteria supernatants, the clinically isolated *C. albicans* showed an adhesion reduction and a decrease of the number of adherent cells to the substrate of about 0.69 log CFU/mL (*L. casei*), respectively 0, 59 log CFU/mL (*L. plantarum*).

As observed with the Olympus BX 41 microscope (with a 100x immersion objective), the competitive method of the clinically isolated *C. albicans* co-cultivation (Figure 5.33), with the fermented samples containing lactic acid supernatants of *L. casei and L. plantarum,* in the culture media supplemented with lyophilised *Aloe vera* extract, revealed a modification of the adherence pattern which resulted in several conformational changes of the cell surface (with consequences in their adhesive capacity).

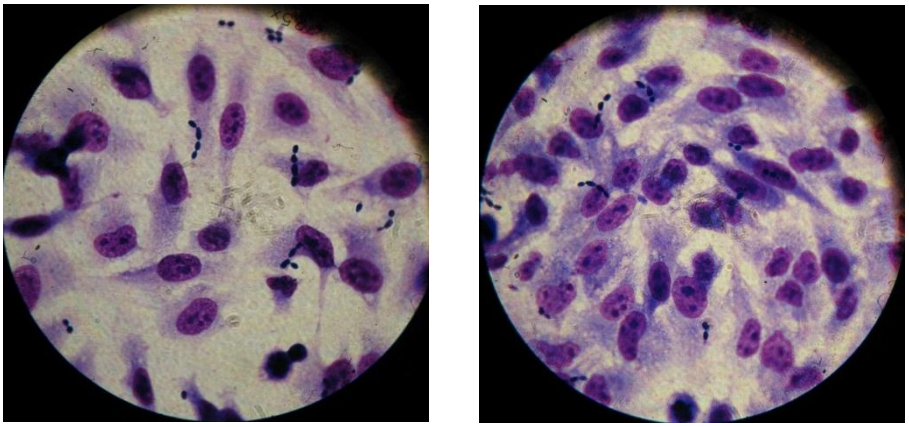

Figure 5.33. *The adhesion inhibition of the clinically isolated C.albicans in the presence of the co-culture of L. casei (S6' on the left) and L. plantarum (S10 on the right)*

Of the total samples of the lactic acid bacteria fermented media with a different medium composition (MRS or EM) and supplemented with 2 mL of 10% ethanolic lyophilised Aloe vera powder, 85% exhibited an adhesion to the monolayer epithelial cells, an essential requirement for a probiotic strain.

The study focused on the inhibition ability, through a competitive manner or by exclusion, of some pathogenic strains to the epithelial cells substrate in the presence of the probiotic strains supernatants obtained after 48 hours of fermentation at 37°C. From the 12 samples, 9 had the ability to inhibit the adhesion of both *S. aureus* bacteria, the ATCC and the clinically isolated strain, to the cellular substrate (HeLa-2 epithelial cells), with a percentage between 15-90%. With regard to the *C. albicans* pathogenic yeast strains, the lactobacilli fermented samples had a more inhibitory effect on the clinically isolated strain when the maximum inhibition percentage of the adhesion to the cellular substrate was 80% compared to the ATCC strain, at which the maximum inhibitory percentage was 70%. Noteworthy is that, in the case of the assays performed on the clinically isolated *C. albicans* strain, all the probiotic lactic bacteria supernatants, individually cultivated on the fermentative media supplemented with 10% alcoholic *Aloe vera* extract, inhibited its adhesion to the substrate in a proportion of 80%.

The direct interference of the pathogen growth inhibition on the cutaneous substrate had been studied on the same strains, the clinically isolated *Staphylococcus aureus* and the *Staphylococcus aureus* ATCC as well as on *Candida albicans* ATCC and the clinically isolated *Candida albicans*.

5.4. Partial conclusions

1. The studies from this chapter tested the possibility of obtaining fermented functional media from the simple culture media (MRS and EM) supplemented with (0.5% (w/v) lyophilised powder and 4% (v / v) lyophilised powder ethanolic extract 10%) by the cultivation of some probiotic bacteria (*L. rhamnosus, L. plantarum* and *L. casei*).

2. The beneficial effect of the *Aloe vera* bioactive compounds on the multiplication and the viability maintenance of the probiotic lactic bacteria grown in different culture media (MRS and in a minimal electrolytes solution) has been demonstrated.

3. Based on their fermentative behavior and resistance during the storage of the fermented media, two strains were further selected, in the presence of *L. plantarum* and *L. casei* strains, which were grown in a monoculture and multiculture, and then tested for their antimicrobial and antifungal activity, for the ability to adhere to epithelial cells and for the competitive potential to reduce the adherence of pathogenic microorganisms to epithelial cells.

4. It has also been shown the ability of the probiotic strains to maintain a superior degree of viability, especially for the *L. casei* culture, during the storage period of the fermented medium obtained by cultivation on the minimal EM medium supplemented with *Aloe vera* for 21 days under refrigeration conditions.

5. The products fermented with *L. plantarum* and *L. casei* in single cultures or as a combined inoculum (1:1) exhibited antimicrobial activity against some pathogenic microorganisms (*Staphylococcus aureus* and *Candida albicans*) with different potential depending on the culture medium. The supplementation of the media with *Aloe vera* potentiated the inhibitory capacity.

6. The *L. casei* and *L. plantarum* lactic acid strains exhibited a certain adhesion degree to the HeLa-2 cell line, the optimal variant being the 1:1 combined inoculum grown in the electrolyte media supplemented with 10% *Aloe vera* alcohol extract.

7. The antimicrobial and antifungal activity was also evaluated by *in vitro* cultivation on epithelial cell cultures (HeLa-2) in co-cultures with the pathogenic microorganisms and in the fermented medium with lactic acid bacteria. The last option produced a beneficial effect in inhibiting the adherence of the pathogenic microorganisms (*Staphylococcus aureus* and *Candida albicans*) to the cellular substrate, which affected the pathogenicity of the indicator microorganisms.

6. The formulation of an innovative ointment with fermented probiotic products and *Aloe vera*

6.1. Introduction

The overall objective of the study at this stage was the formulation of a functional ointment, an innovative, semi-solid bioadhesive system supplemented with a lactic acid-fermented product in a minimal *Aloe vera* environment.

The innovative obtained pharmaceutical product had as a physiological function the optimal adhesion to the biological tissue of some beneficial probiotic organisms, with the role of restoring the physiological functions of the skin, as well as the competitive dislocation of some pathogenic microorganisms, in order to regain the health of the epidermis.

The innovative pharmaceutical product was a hydrophilic ointment based on

polyethylene glycol, a water-miscible anhydrous ointment containing 4000 polyethylene glycol (macrogol), glycerol, the result being an organogel with gelling properties for the topical action at the epidermal and dermal level. The route of administration was the skin and its annexes.

The ointments are semisolid pharmaceutical forms intended for skin or mucosa administration for therapeutic or protective purposes. They consist of excipients (ointment bases) in which the active substance is dispersed. Depending on the degree of dispersion of the active substances, the ointments may be: ointments-solutions, ointments-emulsions, ointments-suspensions or polyphasic ointments (FR X).

Topical ointments are a viable, non-invasive and compliant alternative to treating skin conditions such as skin allergies, atopic dermatitis, eczema or varicose ulcer. For each affection, there are various classes of medicine that have health benefits and are capable of restoring the normal skin functions.

The ability of some probiotic lactic bacteria species to compete against some species of pathogenic microorganisms commonly known for skin disorders and to properly colonize the skin surface was demonstrated in order to promote the skin barrier function restoration and to display a healthy appearance.

6.2. Materials and methods

The experimental ointments were prepared according to the formulation conditions and preparation required by the Romanian Pharmacopoeia X, having the following ingredients:

Rp: PEG 4000 …….. ……..….20 g
Glycerol 40%.....5 mL
Fermented medium .....…….....7 mL.

The fermented media were prepared according to previous protocols and were added after the cooling of the ointment base. The used reagents: PEG 4000, 40% glycerol, liquid MRS, MRS agar (Merck, Germany).

The experiments followed:

- the rheological characteristics of the experimental ointments;
- the viability determination of the inoculated lactic bacteria strains;
- the antimicrobial capacity of the ointment samples.

In order to observe the homogeneity of the samples, a LSM 710 confocal microscope (Carl Zeiss, Germany) was used, while for the rheology experiments, an AR2000ex rotary rheometer (TA Instruments, USA) was used.

The formulated bioactive ointment had as a novel feature as the use of two *Lactobacillus* strains *casei* and *plantarum* in a ratio of 1:1, obtained after a fermentation of 48 hours at 37°C, maintained at a BF 4000 Forced Binder incubator, in different culture media: a classic, selective lactobacilli Man Rogosa and Sharp (MRS) and another experimental, innovative electrolyte mix (EM), both of which had a near pH of 6.5±0.2 for the liquid MRS, respectively 6.7±0.2, for the electrolyte solution.

The ointment samples were prepared under the same conditions, in a sterile area, with sterile utensils by using both the lactic bacteria strains (after 48 hours of fermentation at 37°C) in the liquid MRS medium and in the electrolytes medium supplemented with 10% *Aloe vera* alcoholic extract.

The co-cultured *Lactobacillus* suspensions, fermented for 48 hours at 37°C, in the two different culture media, had the initial titer of CFU/mL of 6.9×10^7 ($\log_{CFU/mL}$ of 7.84) for the MRS-cultivated strains and a CFU/mL of 1.5×10^7 and a $\log_{CFU/mL}$ of 7.18, respectively, for the strains grown in the electrolyte solution.

The fermented media used to formulate the ointments were as follows:

- Sample I - 0.5 mL of the *L. casei* inoculum + 0.5 mL of the *L. plantarum* inoculum + 2 mL of 10% ethanolic extract of *Aloe vera* powder in 50 mL of liquid MRS.
- Sample II - 0.5 mL of the *L. casei* inoculum + 0.5 mL of the *L. plantarum* inoculum + 2 mL of 10% ethanolic extract of *Aloe vera* powder in 50 mL of electrolyte solutions (EM).

To demonstrate the functionality of the formulated ointments, the viability of the lactic acid bacteria in the ointments over a 28 days storage (2-8°C) period was analysed.

The use of a hydrophilic base (polyethylene glycol 4000 and glycerol) in the ointment formulation presented the advantage of a homogeneous dispersion of 1g of sample of the experimental ointment in the saline solution to achieve successive decimal dilutions (Koch method).

6.3. Results and discussion

6.3.1. Organoleptic characterization of the ointment samples

The ointment samples were prepared under aseptic conditions to avoid the contamination of the formulas with microorganisms. The topical formulations in accordance to the **Rp** had the following characteristics:

✓ **Appearance**:

Organoleptically, the ointment samples were yellowish in the case of the fermented medium derived from the liquid MRS and pearlescent white with respect to the fermented medium derived from the electrolyte solution. The consistency was semi-solid, more viscous after refrigeration, although it returned to a softer consistency after being brought to room temperature. The spreadability on the skin was easy, it was also noticed that in a thin layer, a plastic film was formed. The film was easily removed, by washing with water, it was not greasy, left no traces on the skin and did not irritate. It also presented a pleasant and sweetish smell.

Kept for a long period of 2-3 months, the studied samples did not show signs of oxidation or any possible contamination with microorganisms (bacteria or fungi).

✓ **pH**:

The pH of the ointment samples ranged from 4.8 - 5.7, values that respect the physiological pH of the skin – 4.0 - 6.5.

6.3.2. Rheological characteristics of the bioactive ointments

Generally, the gels are viscoelastic or plastic pharmaceutical forms. These properties can be characterized by varying the mechanical modules: the storage modulus G' and the loss modulus G". The storage module G' measures the elasticity of the material and represents its ability to retain the received energy while the loss modulus G" characterizes the viscosity of the material and expresses the ability of the material to dissipate the energy.

In this study, the dynamic viscosity variance correlated with the shear velocity was analysed, observing in all the cases a decrease of it with the frequency, which denoted that the analysed ointments had a non-Newtonian rheological behavior.

6.3.2.1. Variation of the modules in terms of frequency

By increasing the frequency, one can see an increase in the storage module, similar to the blank test module and a maintenance loss module, to a value three times lower. This aspect demonstrated the ability of the polyethylene glycols from the ointment base to exhibit a good stability and to maintain a homogeneity on the skin (Figure 6.1, Figure 6.2, Figure 6.3, Figure 6.4).

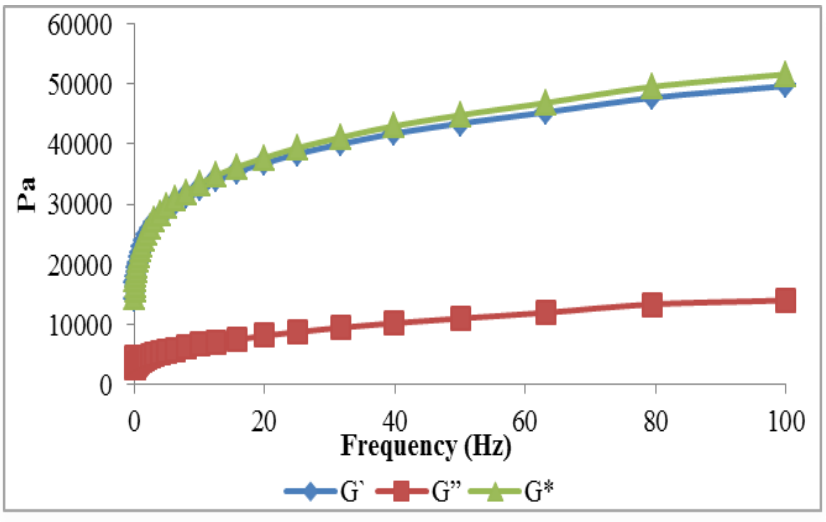

Figure 6.1. *The variation of the modules with frequency in the case of the ointment obtained with the fermented medium (sample 5')*

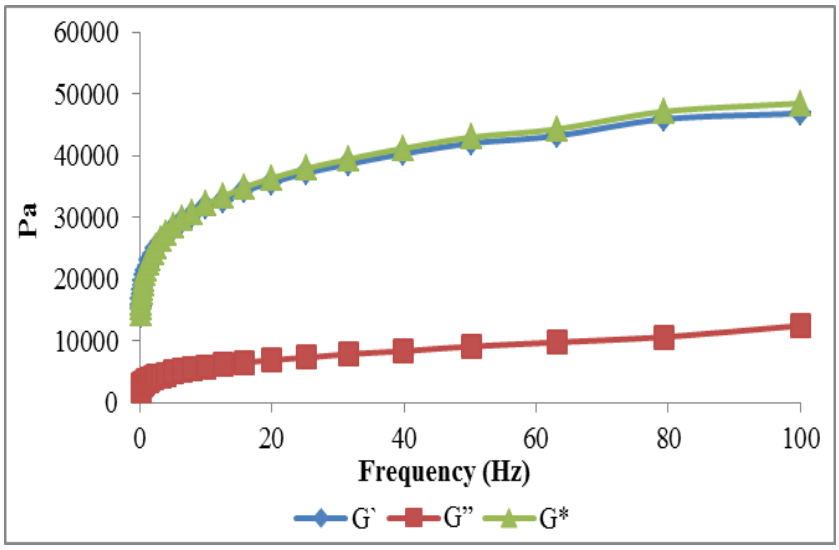

Figure 6.2. *The variation of the modules with frequency in the case of the ointment obtained with the fermented medium (sample 7')*

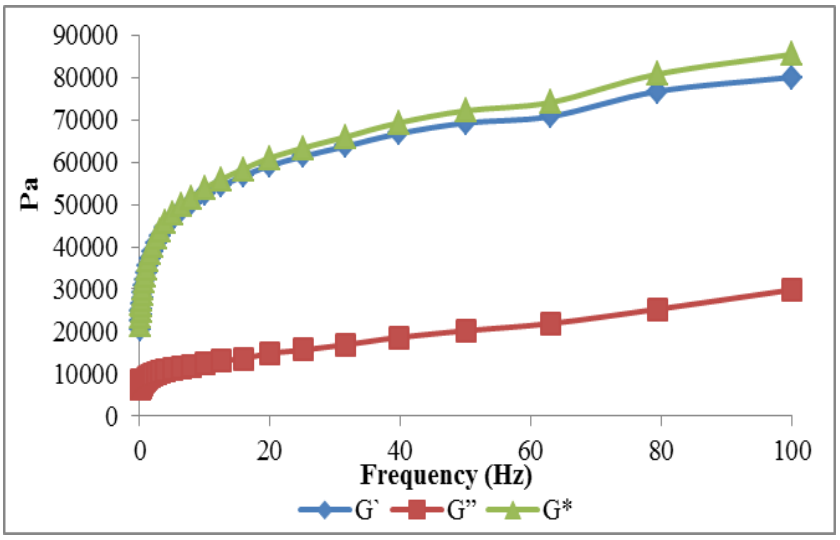

Figure 6.3. *The variation of the modules with frequency in the case of the ointment obtained with the fermented medium (sample 11)*

Figure 6.3 showed an increased value of the loss modulus, twice as much compared to the ointments obtained with the fermented medium (sample 5' and 7'), being corroborated with a value of up to 80000 Pa of the storage module. This fact reinforced the belief that the samples containing both of the lactic bacteria species were stable over the preservation period thus being effective on the skin.

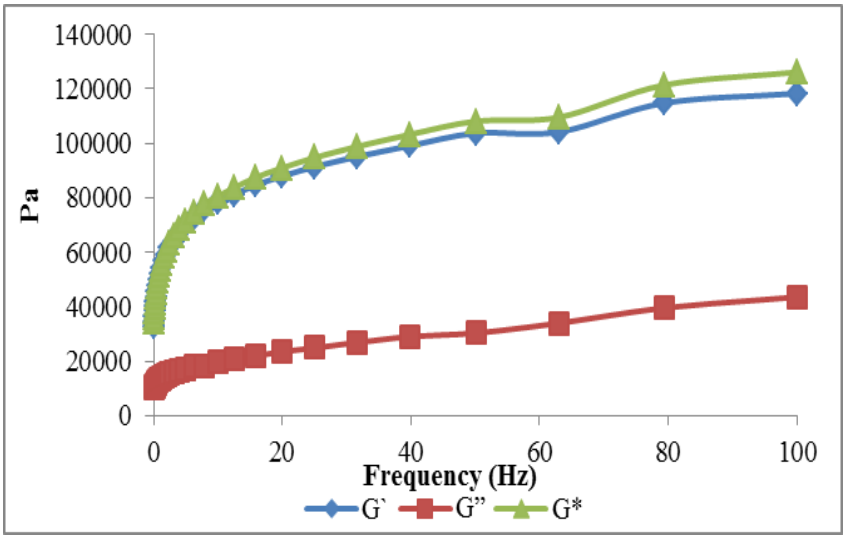

Figure 6.4. *The variation of the modules with frequency in the case of the ointment obtained with the fermented medium (sample 12)*

The variation of the analysed sample modules was similar. The highest values of the modules were obtained with the fermented medium ointments samples 11 and 12, which varied between 80000-120000 Pa.

These data confirmed the organoleptic observations made on the studied samples so that they exhibited the same spreadability properties, approximately the same viscosity, and the film formed on the skin after the oinments were spread had a 3-second installation time (the film could easily be removed by washing with water).

6.3.2.2. Variation of the shear stress in terms of shear velocity

The ointments obtained with the fermented medium (samples 5' and 7') exhibited a plastic flow, for example the modification rate of the gel structure varied proportionally with the shear stress applied to the ointment (Figure 6.5, Figure 6.6, Figure 6.7 and Figure 6.8.).

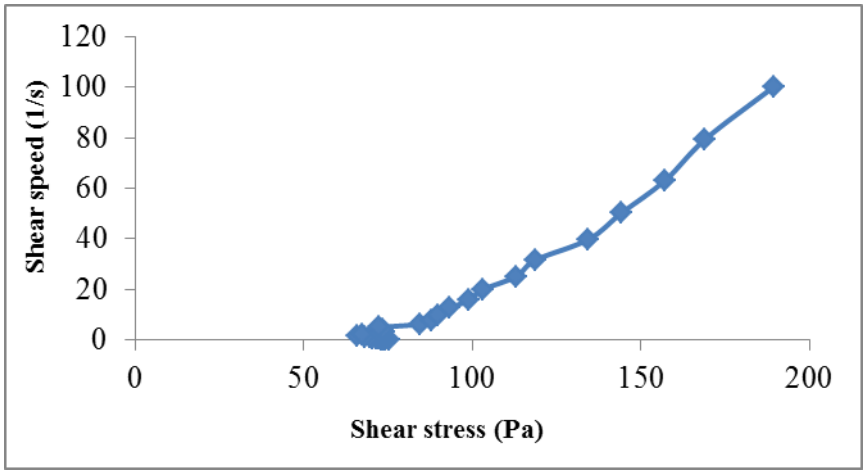

Figure 6.5. *Reogram of the ointment obtained with the fermented medium (sample 5')*

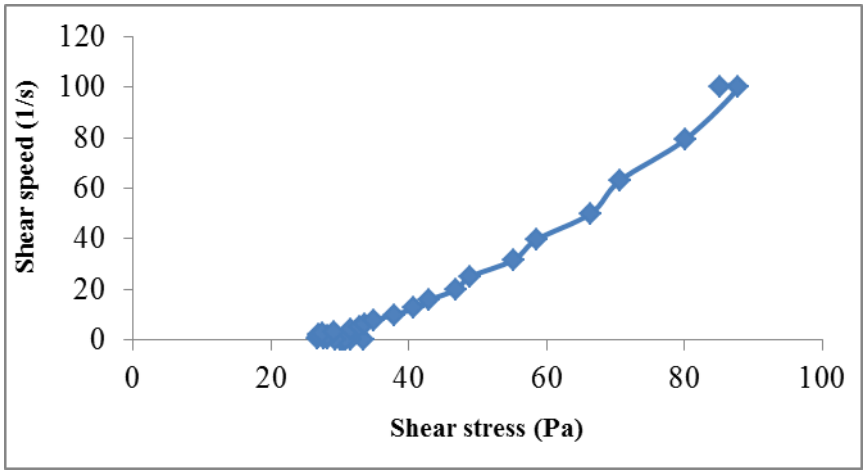

Figure 6.6. *Reogram of the ointment obtained with the fermented medium (sample 7')*

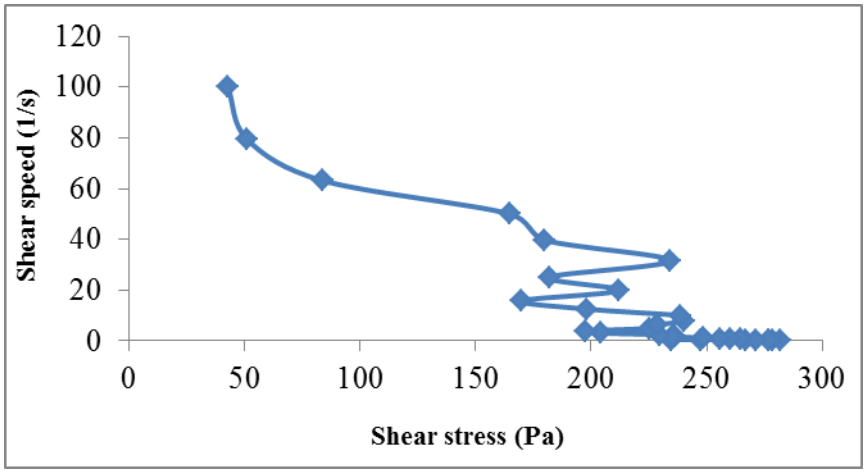

Figure 6.7. *Reogram of the ointment obtained with the fermented medium (sample 11)*

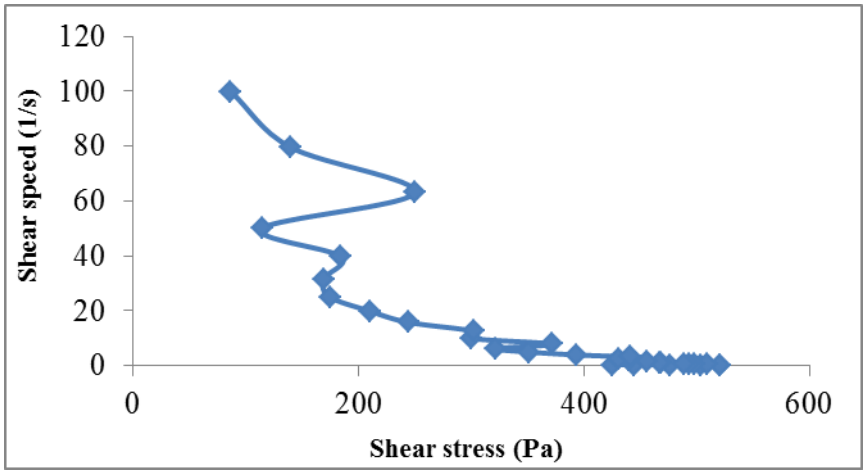

Figure 6.8. *Reogram of the ointment obtained with the fermented medium (sample 12)*

The rheological curves of the ointments obtained with the fermented medium (samples 5' and 7') demonstrated that these fluids had a flow corresponding to the plastics with different flow thresholds ranging between 30-80 Pa.s. In the case of the samples obtained with the fermented medium (samples 11 and 12), which contained both of the probiotic lactic bacteria species, a non-uniform flow was observed, although the organoleptic and skin-displaying characteristics of these ointments were similar to the others.

6.3.2.3. Antimicrobial activity of the bioactive ointments

Thixotropy is the property of the visco-elastic bodies to return to their initial semi-solid state after the removal of the shear force or of an used temperature over them. The switching from one state to the other can be done indefinitely.

The tested samples were subjected to a variable shear stress, thus observing the rheological behavior, the destructuring and restructuring of the formulas after the force removal.

Depending on the shear velocity values obtained for the analysed samples, at the application of a variable shear stress, the hysteresis curves were drawn, which allowed the characterization of the flow type (Newtonian or non-Newtonian) of the ointment (Figure 6.9. 6.10, Figure 6.11, Figure 6.12).

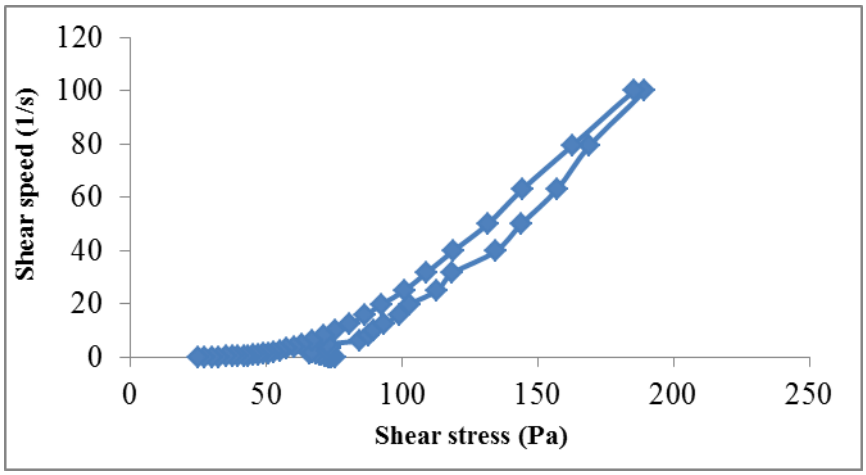

Figure 6.9. *The flow curve for the ointment obtained with the fermented medium (sample 5')*

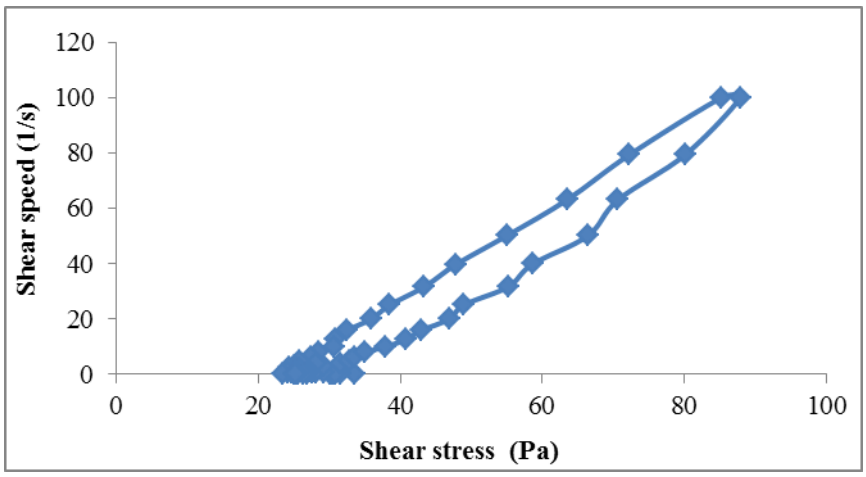

Figure 6.10. *The flow curve for the ointment obtained with the fermented medium (sample 7')*

It was noted that the two rheological curves (ascending and descending) did not overlap, which meant that the experimental hydrophilic ointment did not restructure immediately after the shear stress has been removed. It is well-known that the gels based on high molecular weight polyethylene glycols fit into the category of reopex fluids with a marked thixotropy, especially when a combined polyol - glycerol ointment base is used.

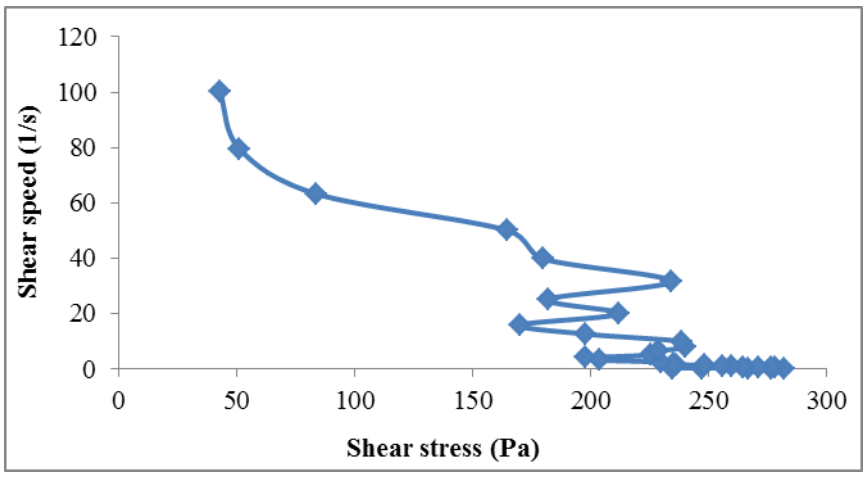

Figure 6.11. *The flow curve for the ointment obtained with the fermented medium (sample 11)*

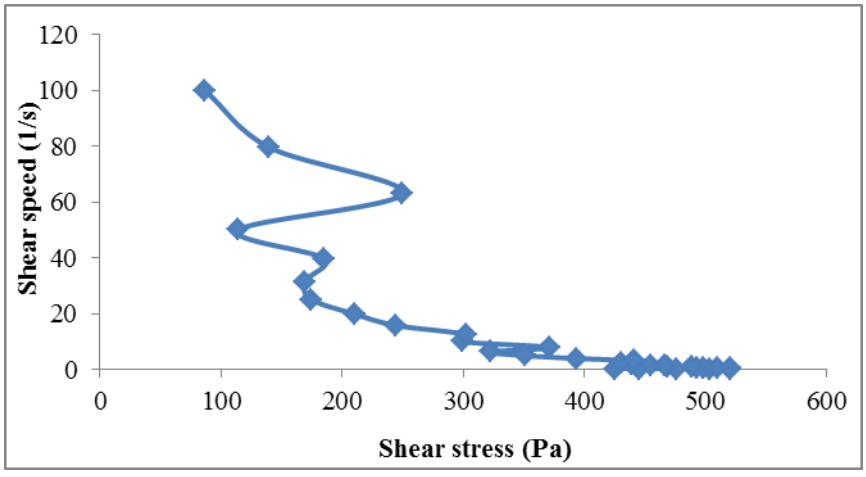

Figure 6.12. *The flow curve for the ointment obtained with the fermented medium (sample 12)*

The ointments obtained with fermented media (samples 5' and 7') exhibited similar thixotropic properties, specific to gels and visco-elastic forms. The thixotropic properties of the

studied gels were highlighted by the appearance of a hysteresis curve in the graphic representation of the shear velocity according to the applied shear stress (Figure 6.9, Figure 6.10).

Due to the presence of the aqueous inoculum in the ointment formulation and of the high molecular weight polyethylene glycol 4000, the ointment exhibited a pseudoplastic behavior. When a high shear stress was applied to the ointments formulated with this anhydrous base, a reopex phenomenon was observed, followed by a slight thixotropy associated with the polymeric molecular structure as a result of the temporary crosslinks.

The ointments obtained with the fermented media (samples 11 and 12) had an uncontrolled flow, probably due to the temporary breakage of the polymeric bonds that degraded the coherence of the gel (Figure 6.11, Figure 6.12.). After resting, the return of the initial viscosity was observed.

Organoleptically, there were no notable differences of the ointments flow, and the behavior of the ointments to the skin was similar.

6.3.2.5. Hysteresis rheogrammes

Depending on the flow shown for each test sample and by observing the viscosity variation, the hysteresis curves were drawn (Figure 6.15, Figure 6.16, Figure 6.17, Figure 6.18).

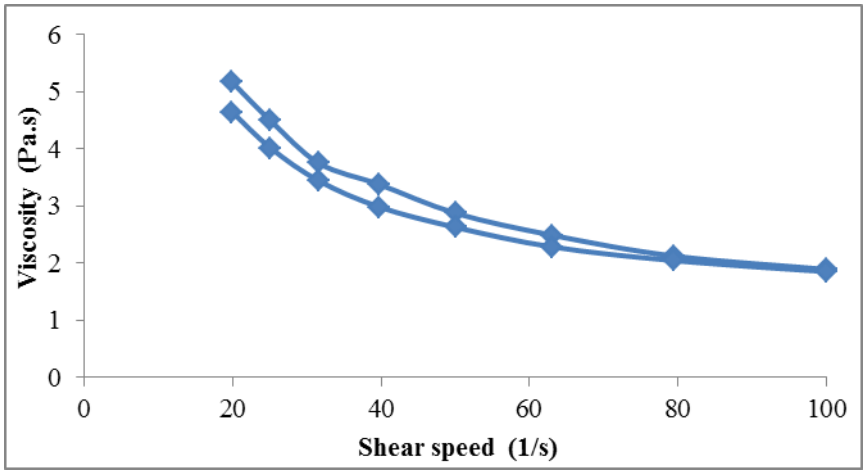

Figure 6.15. *The hysteresis curve of the bioactive oinment (formula 5')*

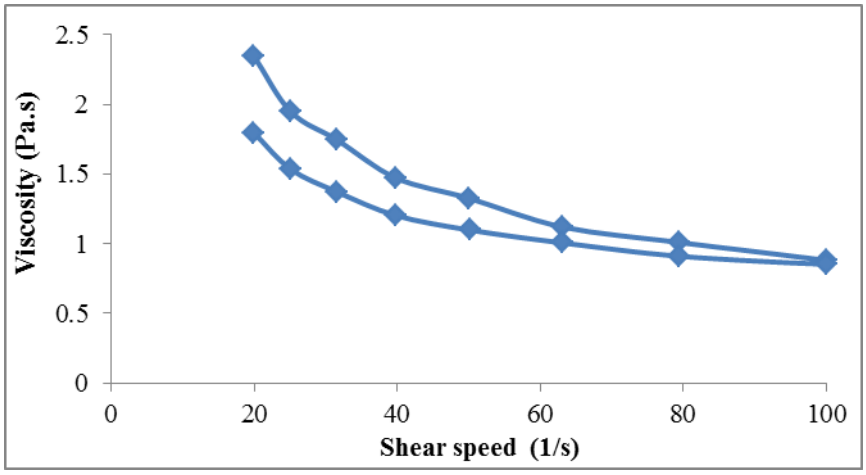

Figure 6.16. *The hysteresis curve of the bioactive oinment (formula 7')*

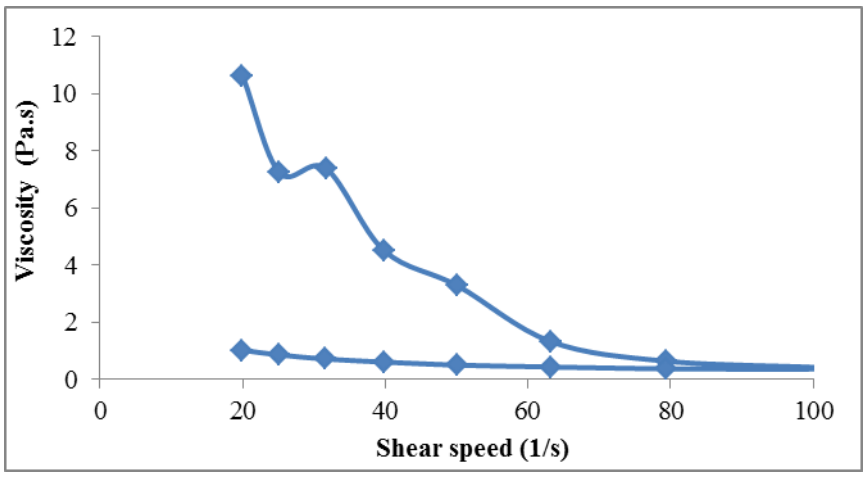

Figure 6.17. *The hysteresis curve of the bioactive oinment (formula 11)*

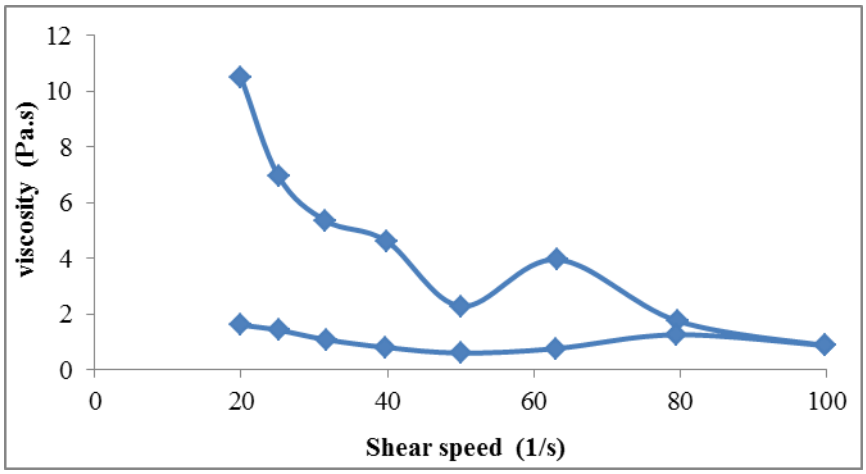

Figure 6.18. *The hysteresis curve of the bioactive oinment (formula 12)*

Depending on the shear rate increase or decrease, the viscosity variation was assessed. It was observed that S5' and S7' exhibited a hysteresis phenomenon due to the variation in time of the viscosity. In samples 11 and 12, the hysteresis was very large and irregular, due to the uneven gel structure and to the pseudo-plastic flow of the ointment base. The gels that have high molecular weight polyethylene glycols have a specific reopex character.

Usually, the presence of the electrolytes quantities caused a decrease in the absolute viscosity of the substances dispersion in PEG, but the presence of polyols, e.g. glycerol, gave it a continuous increase of the viscosity to a point where it had a similar configuration to the PEG molecules.

In conclusion, the ointments formulations (11 and 12) based on the fermented media derived from the liquid MRS media or the electrolyte solution medium (EM) with equal proportions of inoculum (1:1) of *Lactobacillus plantarum* and *Lactobacillus casei* were recommended in the dermatological treatment of several conditions.

6.3.3. Functional properties of the formulated bioactive ointments

6.3.3.1. Viability of the lactic bacteria in the ointments

The novel bioactive ointment was formulated by using two strains of lactic acid bacteria *Lactobacillus casei* and *Lactobacillus plantarum* in a ratio of 1:1, strains that were kept under fermentation for 48 hours at 37°C in a Binder Forced air circulation BF 4000 incubator, in different culture media: one selective for the *Lactobacillus* species growth, Man Rogosa and

Sharp (MRS), and in other experimental, innovative mixture of electrolytes (EM), both having a pH near 6.5 ± 0.2 for the MRS liquid, respectively 6.7 ± 0.2, for the electrolyte solution.

The ointment samples were prepared under the sterile same conditions, using two strains of lactic acid bacteria after 48 hours of fermentation at 37°C, cultured in a MRS liquid medium or in an electrolytic solution.

The novelty and originality of the experiments consisted not only in the co-culture of two strains of lactic acid bacteria, but also in the enrichment of the culture with an alcohol extract (10%) of a freeze-dried powder obtained from the leaves of *Aloe vera*, a beneficial additive that was used both for the nutritive capacity increase of the substrate and for the beneficial therapeutic properties harnessing of this plant, known from antiquity.

The co-cultured *Lactobacillus* suspensions, fermented for 48 hours at 37°C, in the two different culture media, had the initial CFU/ mL value of 6.9×10^7 and the log CFU/mL of 7.84 for the liquid MRS, and a CFU/mL of 1.5×10^7 with a log CFU/mL of 7.18, respectively, for the strains grown in the electrolyte solution.

The bioactive ointments were formulated based on the following composition:

Macrogol(PEG 4000)	**20 g**
Glycerol	**3 mL**
Fermented medium with lactic bacteria and *Aloe vera*	**7 mL**

The fermented media used for the ointment formulation were as follows:

- Sample I - 0.5 mL of *L. casei* inoculum + 0.5 mL of *L. plantarum* inoculum + 2 mL of 10% ethanolic extract of the *Aloe vera* powder in 50 mL of liquid MRS.
- Sample II - 0.5 mL *L. casei* inoculum + 0.5 mL *L. plantarum* inoculum + 2 mL 10% ethanolic extract of the *Aloe vera* powder in 50 mL electrolyte solutions (EM)

To demonstrate the functionality of the formulated ointments, the lactic acid bacteria viability in the ointments was analysed over a 28 days refrigerated storage period (2-8°C).

The use of a hydrophilic base with polyethylene glycol 4000 and glycerol in the formulation of the ointments presented the advantage of a homogeneously dispersing in the physiological saline solution of 1g of sample from the experimental ointment in order to achieve successive decimal dilutions.

The number of colony forming units was determined immediately after preparation, then at 24 hour intervals, up to 96 hours (Figure 6.20). The following samples were checked at 7, 14, 21 and 28 days to assess the viability loss percentage of the probiotic lactic bacteria in the ointment (Figure 6.21). Based on this parameter, it was possible to determine the shelf life of the ointments.

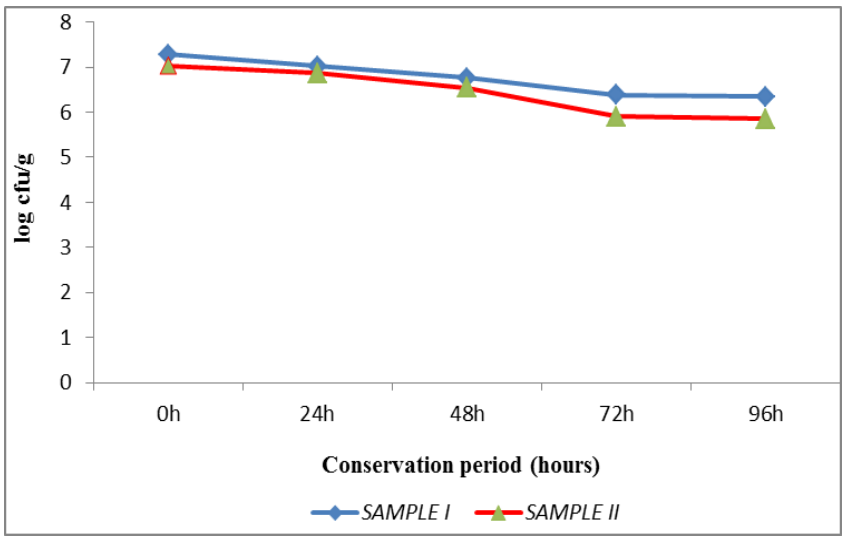

Figure 6.20. *The viability of L. casei and L. plantarum in the oinment*

The lactic bacteria viability in the hydrophilic ointment was influenced by the used cultivation medium, liquid MRS (SI) or the electrolyte solution (S II) medium. After 72 hours of refrigeration, both of the ointment samples had a viable lactobacilli content of 6 log CFU/g, with a reduction of 0.89 log CFU/g of the viability compared to the T_0 moment of the ointment preparation, which determined in term their capacity to positively influence the therapeutic effect.

By following the lactic acid bacteria viability from the two ointment samples, it was observed that after 72 hours, a higher viability decrease (9,63%) was registered in the ointment that contained the lactic acid bacteria cultured in the electrolyte solution medium compared to the bacteria cultured in the MRS (5,47%).

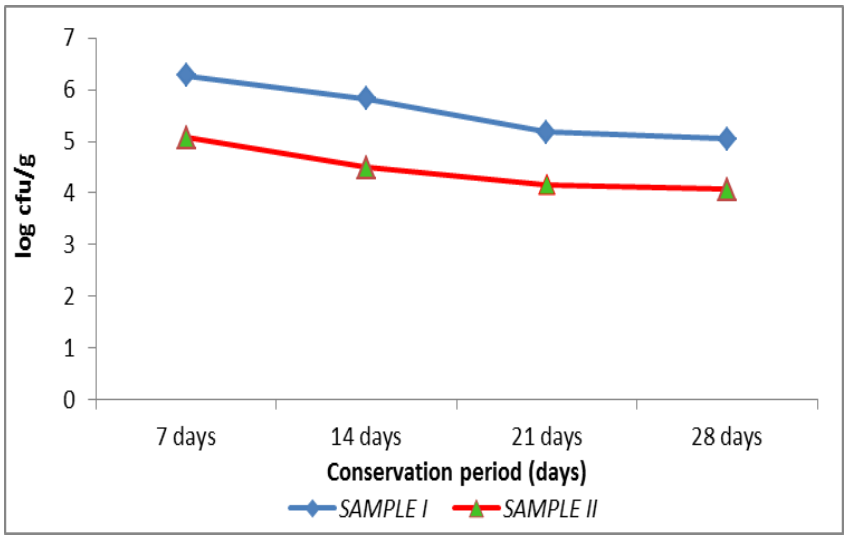

Figure 6.21. *The viability of L. casei and L. plantarum in the oinment*

From the data presented in Figure 6.21, it was observed that the culture medium influenced the viability of the lactic acid bacteria for a longer period. It should also be mentioned the fact that lactic acid bacteria inoculum that was used for the preparation of the two ointment samples presented at T_0 a log CFU/mL of 7.84, for the bacteria cultivated in MRS and a respectiv log CFU/mL of 7.83, for the bacteria cultivated in the EM medium.

In the ointment at T_0, the two samples had a log CFU/g of 7.28, for the lactic acid bacteria cultured in MRS, and a log CFU/g of 7.04, for the lactic acid bacteria cultured in the EM solution.

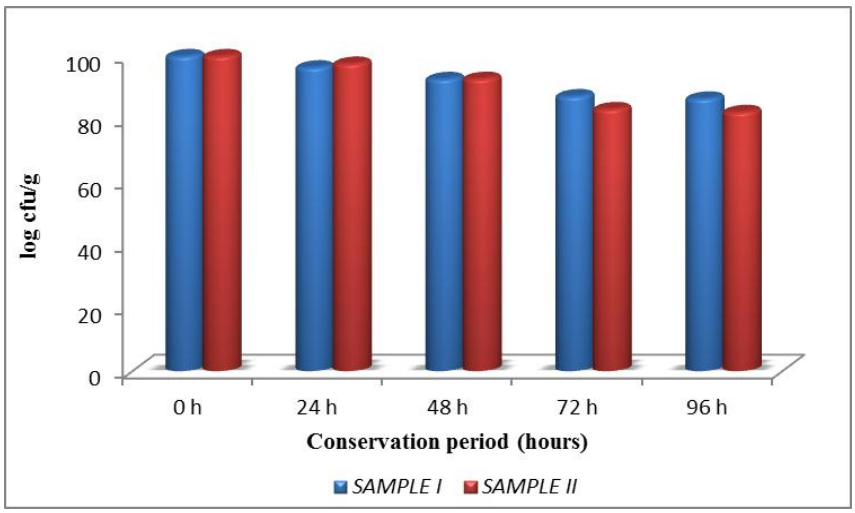

Figure 6.22. *The viability degree maintenance of the lactic bacteria in the ointments*

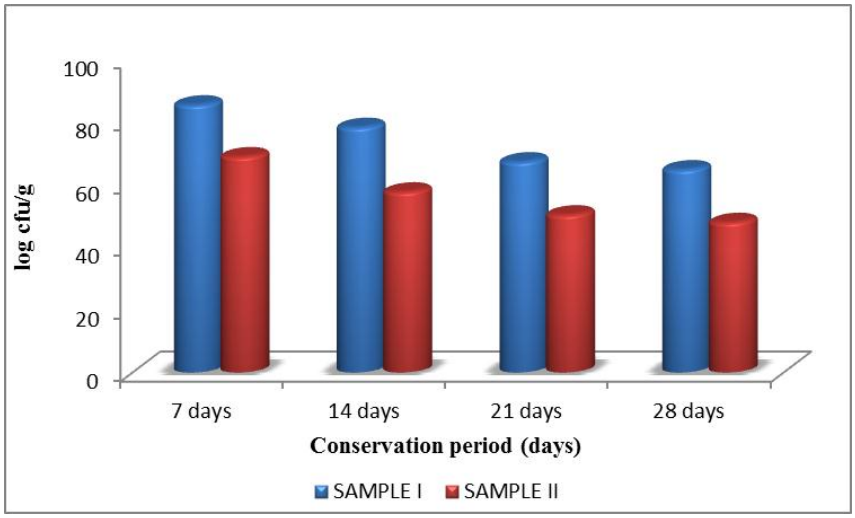

Figure 6.23. *The viability degree maintenance of the lactic bacteria in the ointments*

The lactic acid bacteria showed a rather stable viability degree for 28 days with a marked decrease of the colony-forming units number at 14 and 21 days respectively, that was recorded for sample II (Figure 6.22), due to the depletion of the nutrient substrate composed of electrolytes.

During the storage period of the two ointment samples in the refrigerator, at 2-8^{o}C, for 28 days, the number of colony forming units recorded a decrease of 30.63% - sample I and 42.18% - sample II, compared to the initial sample.

The concentration of the probiotic lactic acid bacteria strains decreased in the ointment, after 28 days, and recorded values between 2.23 $\log_{CFU/g}$ in the ointment sample where the lactic bacteria were grown in MRS and 2.97 log $\log_{CFU/g}$ in the sample where the electrolytes solution was used.

The experimental bioactive ointments were formulated to contain both *L. casei* and *L. plantarum*, which led to a prolonged viability of 21 days.

6.3.3.2. Antimicrobial activity of the bioactive ointments

The formulated ointments did not show a relevant antimicrobial activity. In the case of the ointment formulated with the electrolyte solution fermented medium (sample II), a 10 mm inhibition area was observed only against the clinically isolated *Staphylococcus aureus* strain.

None of the two bioactive ointment formulas showed an inhibitory activity against the *Candida albicans* yeast strains.

The fungal strains used in this experiment were the most adaptable to the common antimycotic treatments as they were the most difficult to treat.

For these reasons, it is imperative in future studies to optimize the composition of the ointments by increasing the addition of the fermented medium and also the fermentation conditions for the antimicrobial compounds biosynthesis.

6.4. Partial conclusions

1. The bioactive ointments formulated with lactic acid bacteria fermented in the presence of the *Aloe vera* extract exhibited rheological properties specific to pseudo-plastic bodies with a viscosity and a non-Newtonian flow, which imparted a thixotropic, reopex behavior that could reversibly pass from the semi-solid phase to the liquid phase.

2. From a rheological point of view, the proposed ointments had a pleasant color, from white to a yellowish-white, pearlescent, had no specific smell, didn't oxidize, were lightly spread on the skin, forming a film that was removed easily by washing with water.

3. The ointments had as excipients polyethylene glycol 4000, an anhydrous and water-soluble ointment base with a low capacity to incorporate water, which resulted in a good sample preservation at cold (2-8°C) or at room temperature (20 ± 5°C), the ointment being stable as no contamination with microorganisms took place. Due to the addition of the L. casei and L. plantarum probiotic strains as active ingredients, it is advisable to keep these samples under cold refrigeration conditions to increase the storage period.

4. The addition of glycerol in the ointment formulation was beneficial as the polyethylene glycol 400 was removed from the official formulation, as a liquid excipient, the glycerol had a double action, both as plasticizer and preservative, so that the viability of the probiotic lactic bacteria strains was prolonged.

5. The probiotic lactic bacteria strains, *Lactobacillus casei* and *Lactobacillus plantarum*, grown into two different culture media supplemented with 10% alcoholic extract of the lyophilised *Aloe vera* leaves, retained a superior degree of viability after a period of 28 days of refrigeration. The loss of viability was 10^2 CFU/g for the ointment with the fermented medium derived from the liquid MRS and 10^3 CFU/g in the case of the ointment with the fermented medium derived from the electrolyte-based solution.

6. The formulated ointment with the fermented medium derived from the electrolyte-based solution, containing both the Lactobacillus plantarum and Lactobacillus casei probiotic bacteria and the Aloe vera extract, deployed an inhibition of the clinically isolated Staphylococcus aureus strains. Nevertheless, none of the two tested formula inhibited the Candida albicans strains.

7. General conclusions

The Ph.D. thesis entitled "***Formulation, chemical and functional characterization of some pharmaceutical products for external use with natural bioactive compounds and***

lactic acid bacteria" aimed at the obtainment of a functional ointment with an innovative formula based on the incorporation of live lactic bacteria together with a fermented product containing an extract or a freeze-dried *Aloe vera* powder into a proper base in order to maintain the lactic acid bacteria viability during shelf-life. The functional ingredients, the fermented lactic acid bacteria medium and the *Aloe vera* leaf extracts (*Barbadensis Miller* variety) were characterized from a physico-chemical lly and microbiological point of view in order to demonstrate their functional role. Based on the obtained experimental results and the partial conclusions presented at the end of each chapter of the experimental part, the following general conclusions are summarized as follows:

1. *Aloe vera* is an excellent raw material for the formulation of some pharmaceutical or cosmetic functional products due to the increased concentration of polyphenolic compounds with a predominantly antioxidant activity as well as to the moisture and fat adsorption properties.
2. The processing of the *Aloe vera* plant material by lyophilisation has proven to be the most effective processing technique with respect to the biologically active compounds concentration.
3. The biologically active compounds extracted from the *Aloe vera* plant (*barbadensis* Miller variety) may constitute the functional substrates for the development and metabolic activity of the lactic bacteria strains, *Lactobacillus casei* and *Lactobacillus plantarum*.
4. The *Lactobacillus casei* and *Lactobacillus plantarum* species demonstrated an increased adaptability and stability in the minimal electrolyte-based medium, and the supplementation with the *Aloe vera* lyophilised powder had also a beneficial effect on the lactic bacteria metabolism and viability.
5. The co-cultivation of the *Lactobacillus casei* and *Lactobacillus plantarum* species proved to be beneficial for the obtainment of the fermented product in terms of bacterial multiplication and stability of the viable cells during the preservation period compared to the single cultures.
6. The tested *Lactobacillus casei* and *Lactobacillus plantarum* cultures demonstrated an increased adhesion to HeLa-2 cell monolayer, hence demonstrating the adhesion to the epidermal cells.
7. The products fermented with the *Lactobacillus casei* and *Lactobacillus plantarum* combined cultures for 48 hours at 37°C in the minimal electrolyte medium supplemented with the ethanolic *Aloe vera* extract demonstrated the ability to competitively inhibit the adherence to the HeLa-2 cellular monolayer (epithelial cells) of the *Staphylococcus aureus* and *Candida albicans* pathogenic strains, isolated from the skin or vaginal exudate, by occupying the binding sites of the cell membrane.
8. The fermented media obtained in the tested variants with combined cultures of *Lactobacillus casei* and *Lactobacillus plantarum* exhibited antimicrobial, antibacterial and moderate antifungal activity. In order to enhance the antimicrobial activity, additional studies are needed to optimize the fermentative conditions.
9. The hydrophilic ointments formulated with natural ingredients that contained fermented probiotic lactic bacteria and ethanolic *Aloe vera* extract are innovative pharmaceutical products that have a beneficial effect in preventing, treating and curing skin problems.
10. The use of the ointment base with polyethylene glycol 4000 and glycerol had the advantage of imprinting a thixotropic and reopex flow, characteristic to the viscoelastic

gels and viscoelastic forms as well as a hydrophilic character which allowed the film to be removed from the skin by washing with water.

11. The selection of the PEG 4000 compound as an ointment anhydrous base rose the microbiological stability of the finished product and the glycerol served both as a plasticizer and a protector for the lactic bacteria by maintaining the water activity index of the product at optimal values.

12. The hydrophilic ointment containing the product fermented with *Lactobacillus casei* and *Lactobacillus plantarum* (1:1) by cultivation on the electrolyte medium supplemented with 6% ethanolic extract (10%) of *Aloe vera* for 24 hours at 30°C, presented a high antimicrobial activity against the *Staphylococcus aureus* strain isolated from a skin wound, and a low antifungal activity against *Candida albicans* strains.

13. The proposed functional pharmaceutical product was found to be suitable for atopic dermatitis, minor injuries, uncomplicated first or second degree burns, psoriasis or eczema, after completing the experiments with *in vivo* trials.

14. These studies offer the prospect of a new research concerning the use of lactic bacteria in pharmaceutical products for the prevention and treatment of skin diseases, given their metabolic and functional diversity and their ability to adapt and resist in minimal environments in the presence of biologically active compounds from plant sources, especially polyphenolic compounds.

8. Contributions to field knowledge development and future perspectives

The pharmaceutical field opens up new perspectives regarding the field knowledge and scientific research, as pharmaceutical technology is in a continuous dynamic by responding to patients' needs constantly, with the goal to increase the efficiency and the compliance of the treatment. On the other hand, consumers are more and more interested in using bioactive natural products in order to replace those obtained by chemical synthesis.

Nowadays, finding the most effective solutions by exploiting our natural resources is a continuing concern of mankind, some of these remedies being used long before the characterization of the source. It is also the case of lactic acid bacteria, which are now considered the solution for many health, metabolic functionality and conservability problems. The natural microbiome responsible for the majority of the health benefits associated to living beings is a multi-conglomerate of useful microorganisms that are linked between them based on symbiosis and synergism criterias. The development that mankind is currently going through creates serious imbalances upon this microbiome with disastrous consequences on the health and quality of life. Hence, the research, technological development and innovation have as a main target the formulation of products that sustain the useful microbiota, whether they are food, cosmetics or pharmaceuticals.

In this respect, the PhD thesis, through the proposed scientific objectives and results, offers an innovative product alternative for the epidermal health, whose effectiveness has been demonstrated through *in vitro* studies. Thus, the originality of the PhD thesis is based on the following research innovation aspects:

- The lactic bacteria strains capable of multiplying and surviving in a minimal, unconventional, electrolyte environment supplemented with pre-biotic *Aloe vera*

bioactive compounds have been studied and selected for further experiments. This type of fermentation medium has not been studied so far, so that the obtained results offer multiple perspectives both from the fundamental and applicative research point of view.

- The functionality of the *Lactobacillus spp.* bacteria fermented medium in the presence of *Aloe vera* was assessed through the epithelial cell adhesion and the antimicrobial inhibitory activity against pathogens that could contaminate the epidermis *(Staphylococcus aureus* and *Candida albicans).*
- The formula of a functional, simple, tribiotic (prebiotic, probiotic and postbiotic) product has been proposed and tested, a formula for which it has been demonstrated the capacity to maintain the viability of lactic bacteria for 21 days at refrigeration temperatures. The obtained ointment was effective against strains of *Staphylococcus aureus* and *Candida albicans* (strains isolated through clinical trials).
- The studies carried out in their entirety are original and the results obtained are unique in Romania and even in the world, fact certified by the scientific literature that offers very little data in this sense.

In perspective, the optimization of the fermentative conditions and of the ointment composition will be followed in order to both increase the efficiency and the functionality of the product and also to patent the obtained formula.

9. Dissemination of research results

The PhD thesis results dissemination during the doctoral studies was done through the publication or communication of scientific papers as follows:

A. Published articles in ISI journals

1. NICOLETA MAFTEI ARON, **MONICA (GĂUREANU) BOEV**, GABRIELA BAHRIM, ***Probiotics and therapeutic effect in clinical practice – review***, Romanian Biotechnological Letters, vol 20, nr1/2015, pg. 10162-10175, IF 0,412.
2. **MONICA (GĂUREANU) BOEV,** NICOLETA MAFTEI, GABRIELA BAHRIM, ***The biotechnological behaviour evaluation of some lactic bacteria in Aloe vera enriched medium,*** Journal of Biotechnology, 2015, 208:102, IF 2,75.

B. Published articles in international database journals

1. **GĂUREANU (BOEV) MONICA,** MAFTEI NICOLETA-MARICICA, BAHRIM GABRIELA-ELENA**, *Formularea unui unguent bioactiv cu Aloe vera și bacterii lactice - Topical formulation with bioactive components from Aloe vera and lactic acid bacteria,*** Revista Jurnal Medical Brașovean, nr. 2/2018.

C. Books/Book chapters published by international publishing houses

1. MAFTEI, NM; COTARLET, M; **BOEV (GAUREANU) M**; BAHRIM GE, 2017, ***Probiotics in health promotion and their therapeutic effect,*** LAP LAMBERT Academic Publishing, International Book Market Service Ltd,member of Omniscriptum Publishing Group, ISBN 978-613-4-90751.

D. Papers communicated at international scientific conferences

1. **MONICA (GĂUREANU) BOEV,** NICOLETA MAFTEI, GABRIELA BAHRIM, ***The biotechnological behaviour evaluation of some lactic bacteria in Aloe vera enriched medium,*** European Biotechnology Congress, 7th-9th of May, 2015, Bucharest, Romania.

2. **BOEV GAUREANU MONICA**, NICOLETA M. MAFTEI, GABRIELA BAHRIM, 2017, ***Sustain of the Metabolic Activity and Stability of Lactic Acid Bacteria by Bioactive Compounds from Aloe***, *8th International Euroaliment Symposium Mutatis Mutandis in Foods*, 7th – 8th of September, Galați, Romania.

3. MAFTEI NICOLETA M., RAMOS-VILLARROEL ANA Y., **GĂUREANU (BOEV) MONICA**, Chesaru Bianca I., Iancu Alina V, Paltenea Elpida, Nicolau Anca I., 2017, ***The Inactivation Effect on Pulsed Light on Aspergillus spores*** , 8th International Euroaliment Symposium Mutatis Mutandis in Foods, 7th – 8th of September 2017, Galați, Romania.

E. Papers communicated at national scientific conferences

1. **MONICA (BOEV) GĂUREANU**, NICOLETA MAFTEI-ARON, GABRIELA BAHRIM, ***Microbiological quality evaluation of some commercial nutraceuticals containing probiotic bacteria,*** Scientific Conference of Doctoral Schools from UDJ – Galati CSSD-UDJG, 15th-16th of May 2014, Galați, Romania.

2. **MONICA (BOEV) GĂUREANU**, NICOLETA MAFTEI-ARON, GABRIELA BAHRIM, ***Cultivation and Preservation of Lactic Bacteria in Aloe Vera Enriched Medium, for Topic Use in Pharmaceuticals Formulation*****,** Scientific Conference of Doctoral Schools from UDJ – Galati CSSD-UDJG, 4th-5th of June 2015, Galați, Romania.

3. **MONICA (BOEV) GĂUREANU**, GABRIELA BAHRIM, LIA MARA DIȚU, ***The evaluation of antimicrobial activity of fermented products contains lactic acid bacteria and Aloe vera extract based of cellular adherence capacity***, Scientific Conference of Doctoral Schools from UDJ – Galati CSSD-UDJG, 2-3 June 2016, Galați, Romania.

F. Research Projects

„Development of a versatile fingerprinting system with applications in bitterness analysis of pharmaceuticals", PN-II-RU-TE-2014-4-1093, Contract: 40/01.10.2015 Period: 1.10.2015 - 30.09.2017 – researcher

Published articles in ISI journals

IRINA MIRELA APETREI, ADRIANA AURORA BEJINARU, **MONICA BOEV**, CONSTANTIN APETREI, OLIMPIA DUMITRIU BUZIA, ***Determination of ibuprofen based on screen-printed electrodes modified with carbon nanofibers***, Revista Farmacia, 65, nr. 5/2017, 790-795, IF 0,918.

Papers communicated at international scientific conferences

C. APETREI, **M. BOEV**, A. DUMITRACHE, I. M. APETREI, ***Novel biosensor based on L-amino-acid oxidase and polypyrrole for detection of L-Tyrosine in pharmaceuticals***, International Conference of Physical Chemistry – ROMPHYSCHEM 2016, Galati, September 21-23, poster. Abstract published in: Book of abstracts, ISSN 2286-1327, ISSN-L 2286-1327, page 60.
http://gw-chimie.math.unibuc.ro/romphyschem16/ROMPHYSCHEM16-AbstractBook.pdf