SUMMARY OF THE PhD THESIS

Alternative phytotherapies in the conditioning of the carried microbiome and the immune response in pigs

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In Romania, swine farming in small farms with extensive systems is prevalent due to traditions and preference for self-supply. However, this housing system involves multiple risks, including animal welfare and biosecurity issues, which can affect the health of pigs and keepers.

External factors, such as the contact with the environment and overcrowding increase susceptibility to disease. Also, an important role is played by the carried microbiome, which, under appropriate conditions, can modify its pathogenicity and cause diseases. Identifying the species of the carried microbiome and the level of antibiotic resistance of the isolated strains has an important role in the therapy and prevention of diseases, as well as in establishing their involvement as a reservoir of resistance genes.

To protect the health of the pigs and reduce the excessive use of antibiotics, research is focused on the use of medicinal plants with immunostimulating and antimicrobial effects, offering natural alternatives in the prevention and treatment of diseases caused by opportunistic bacteria.

The doctoral thesis entitled "Alternative phytotherapies in the conditioning of the carried microbiome and the immune response in pigs", is structured in two parts, each comprising a series of chapters, designed according to the norms in force.

Part 1 entitled "The current state of knowledge" is divided into three chapters and describes the information from the specialized literature regarding the concept of the carried microbiome and its composition in pigs, the peculiarities of the immune system of pigs, the description of the influence of the environment on the health status of pigs, as well as the description of some plants with a medicinal role, regarding their composition and their antimicrobial and immunostimulating potential.

The first chapter provides information from the literature regarding the general description of the carried microbiome of pigs, its composition at the level of the skin, as well as at the level of the mucous membranes (oral and palatine mucosa, and intestinal mucosa).

The second chapter presents information from the literature regarding the immune system and its peculiarities in pigs, as well as the role of some environmental factors in the influence of the immune response of pigs.

In **the third chapter**, information is presented regarding the usefulness of medicinal plants in therapy and their immunostimulatory and antimicrobial potential.

The second part is represented by the personal contribution and is structured in 6 chapters that contain the results of own research according to the purpose and objectives presented.

In **Chapter 4**, data related to the purpose of using some medicinal plants are found; in the experiment carried out, envisaged are sea buckthorn, marigolds, thyme, garlic, coriander or pumpkin. Their active compounds are to be identified, to establish their usefulness in phytotherapy. The polyphenol concentration of a sea buckthorn syrup taken in the study was thus determined, as well as the composition of bioactive compounds and their concentration from alcoholic extracts of marigold, thyme, garlic, coriander and pumpkin. Due to the content of polyphenols, tocopherols, sterols, sulfoxides and methoxylated flavones (in concentrations specific to each plant) of the studied plants, which are bioactive compounds known for their antioxidant, immunostimulating and antimicrobial effects, it was decided to introduce them into the study, in order to establish the effects phytotherapy in pigs.

In **chapter 5**, data are presented about the origin of the samples, the phytotherapeutic protocol addressed, as well as the evaluation of the cell-mediated immunity in vitro in pigs, before and after the application of the phytotherapy schemes, in order to evaluate the immunostimulatory effect of the medicinal plants taken in the study. This chapter includes the steps of describing the experimental protocol, followed by results and discussion.

In a first stage, blood samples were collected from 9 clinically healthy pigs, of the same age, raised in an extensive system, in different households, which were simultaneously subjected to sea buckthorn syrup therapy (named group A). For better data analysis, they were divided into 2 groups, one treated (n = 5) and the other control (n = 4). In the second stage, 30 clinically healthy pigs from the same holding (called batch B) were included in the study, from which blood samples were also collected. At the same time, they were treated with marigold and thyme powder. For a better integration of the data, the pigs were divided into treated (n = 15) and control (n = 15) groups. Also, each batch was divided into age groups: piglets, fatteners and sows. All samples were individually identified by experimental variant and age group, followed by a running number to avoid confusion.

This protocol evaluated the *in vitro* proliferative response of mononuclear leukocytes to plant mitogens (plant extracts), which non-specifically stimulate these cells, by measuring glucose consumption as an indicator of cell growth.

Thus, the blood samples collected from all the pigs included in the study were subjected to the leukocyte blast transformation test, by which the stimulation percentage of some plant extracts was measured. In the case of batch A, 7 alcoholic plant extracts (*Echinacea angustifolia, Echinacea purpurea, Hippophae rhamnoides, Thymus vulgaris, Vaccinium myrtillus, Sylibum marianum, Arnica montana*) and 2 hydro-alcoholic extracts (*Hippophae rhamnoides, Betula* spp.) were used, and in in the case of batch B, 5 plant extracts were used (*Calendula officinalis, Satureja hortensis, Allium sativum, Coriandrum sativum* and *Cucurbita pepo*) whose composition was previously studied. Stimulation/inhibition indices (BTI%) were determined by measuring the concentration of glucose remaining in the culture medium.

In the case of group A, a stimulation response to the plant extracts was observed when testing before the administration of sea buckthorn syrup, the highest values being in the case of Echinacea purpurea (71.42%). The solvent also influenced the response to mitogens, the alcoholic extract being more active than the hydro-alcoholic one (in the case of sea buckthorn used in vitro – 61.76% alcoholic extract and 60.88% hydro-alcoholic extract). The weakest stimulating effect was presented by the alcoholic extract of Arnica montana, with a stimulation percentage of 54.06%. The test was also repeated after the application of the therapeutic protocol, in order to observe whether sea buckthorn has an impact on the immune response.

In the second test, an increase in the spontaneous blast index can be observed, the response to classical mitogens increased, and the plant extracts used in vitro stimulated an increased response especially to *Echinacea purpurea* (84.85%, compared to 70.42% in the first test). The weakest response could be observed for *Thymus vulgaris* extract, with a stimulation percentage of 68.73%.

Statistical analysis of the obtained data indicates that sea buckthorn treatment led to a significant increase in the stimulation percentage, while the results obtained in the control group did not show statistical relevance.

Table 1. Statistical significance of BTI increase in treated group after sea buckthorn syrup therapy compared to controls

		M	**PHA**	**Alc**	**Ea**	**Ep**	**Cat1**	**Thy**	**Vm**	**Sy**	**Ar**	**Cat2**	**Mest**
T	**t**	0.02	0.03	0.13	0.03	0.02	0.01	0.02	0.01	0.15	0.04	0.01	0.16
	Semn.	sign	sign	non-sign	sign	sign	sign	sign	sign	non-sign	sign	sign	non-sign
C	**t**	0.28	0.2	0.1	0.32	0.01	0.42	0.19	0.31	0.48	0.27	0.25	0.15
	Semn.	non-sign	non-sign	non-sign	non-sign	sign	non-sign	non-sign	non-sign	non-sign	non-sign	non-sign	non-sign

Legend: T=treated, C=control group

The BTI corresponding to batch B were tested by age group, the pigs being divided into 3 categories: piglets, fatteners and sows. The testing was performed before the *in vivo* administration of medicinal plant products, to observe whether the plant extracts under study have a positive effect on the BTI.

In the case of samples collected from piglets, a positive response to the classic mitogen was observed, but all plant extracts, except *Calendula officinalis*, showed an inhibitory effect. In pigs, the BTI results against the mitogens of the plant extracts are higher than in the case of piglets, but lower than those of the classical mitogen. In this category, garlic extract stimulated cell growth compared to the untreated control, but insignificantly compared to the solvent control. BTI values in sows were below those of the untreated control for all plant extracts tested. Due to the weak stimulation activity of the mitogens obtained from the plant extracts studied, their post-treatment testing was no longer carried out.

Results indicated statistically significant differences between age groups, at very low p-values for most plant extracts, indicating a clear dependence on age category.

Table 2. P-values for statistical significance of differences between experimental categories

	M	PHA	Alco	*Calendula*	*Satureja*	*Allium*	*Coriandrum*	*Cucurbita*
Suckling piglets - weaned pigs	0.00016	0.017	0.00002	0.18 NS	0.00002	0.00001	0.0002	0.0022
Weaned pigs-sows	0.1982 NS	0.8285 NS	0.0066	0.0359	0.0071	0.0013	0.0003	0.0001
Suckling piglets - sows	0.065725 NS	0.0254	0.0238	0.14094	0.00347	0.04609	0.28475 NS	0.106031 NS

The *in vitro* stimulation behavior of plant extracts following the application of the therapeutic protocol to group A, proves the immunostimulating effect of sea buckthorn and is encouraging for the eventual association of some extracts. Regarding group B, it was observed that in fattening pigs and sows, garlic and thyme extracts showed the greatest improvement in the cellular response in vitro. Comparing the results obtained in fatteners and sows with those of less responsive piglets, it was observed that all plants acted in an age-dependent manner.

Chapter 6 describes the methods of isolation and identification of the carried microbiome from skin and oral mucosa of pigs. At the same time, the influence of the plants used on the microbiome was observed. The study was carried out in 2 stages, before and after phytotherapeutic treatment. For the isolation of the strains, nutrient broth and nutrient agar were used as culture media. After isolation, Gram staining was performed to determine the morphological characteristics and catalase and oxidase tests were performed to determine the biochemical characteristics of the isolated colonies.

Special microbiological tests such as the API test, the Remel RapID ONE test and the Remel RapID Staph Plus test were used to determine the bacterial species.

In the case of batch A, based on morphological characters and special tests, the following bacterial strains were identified, when tested before the administration of sea buckthorn syrup: *Shighella spp., Salmonella spp., Citrobacter freundii, Staphylococcus sciuri, Staphylococcus warneri* and *Kytococcus sedentarius*. Testing after the administration of sea buckthorn syrup resulted in the isolation of bacterial species with the same morpho-functional characters, inferring that sea buckthorn syrup had no impact on the microbiome at the skin level.

In the case of batch B, based on the morpho-functional characters and special tests, bacterial strains from the Bacillota phylum were isolated on day 0 of the test, in a proportion of 57.89% of the total isolated strains, the Pseudomonadota phylum, in a proportion of 40.78 % and the Actinomycetota group, in proportion of 1.31%. The most commonly isolated bacterial strains fall into the genera Enterococcus, Enterobacter, Streptococcus and Staphylococcus.

Samples were also collected and isolated on day 14 from the start of the experiment, and changes in the oral microbial flora were observed. In the case of pigs that received marigold and thyme powder, a decrease in the proportion of strains isolated from the Bacillota phylum was noted. It was also noted the increase in the share of bacterial isolates from the Pseudomonadota phylum, a consequence of the decrease in those from the Bacillota phylum.

To observe whether this inhibitory trend of the medicinal plants is maintained in the treated pigs, sampling and bacterial isolation was also performed on day 28 of the experiment. On day 28, a return to the initial percentage of the bacterial strains from the 3 mentioned tusks was observed. This results in the temporary inhibitory character of the administration of marigolds and thyme in pig feed.

Statistically comparing the values of the share of the strains isolated on day 0 compared to the other harvesting and isolation stages, a result of 0.274 between day 0 and 14, and 0.362 between day 0 and 28 emerges. The values obtained have no statistical relevance, due to their temporary and selective nature on the isolated strains of the medicinal plants used.

Chapter 7 presents the antibiotic susceptibility assessment technique of the isolated strains, the antibiotics used in this study, the results of the initial susceptibility tests to the antibiotics used, the definition of the antibiotic resistance profile of the isolated bacteria and the monitoring of its re-profiling in the case of pigs that were part of treated lots.

In this study, the antibiotic susceptibility of all bacterial strains isolated from pig skin and oral cavity mucosa was initially tested by the Kirby-Bauer diffusimetric method. Subsequently, the antibiotic susceptibility testing of the strains isolated from the oral mucosa of the pigs in group B, the treated group, was performed on day 14 of the experiment, when changes in the composition of the carried microbiome from this level were observed.

In the case of group A, 9 types of antibiotics were applied: gentamicin-GN, tulathromycin-TUL, cefotaxime-CTX, doxycycline-DO, streptomycin-S, amoxacillin with clavulanic acid-AMC, marbofloxacin-MAR, oxytetracycline-T and tylosin- TY. In the case of group B, 7 types of antibiotics were applied: amoxacillin-AMX25, ampicillin-AMP30, chloramphenicol-C30, oxytetracycline-T30, tulathromycin-TUL30, erythromycin-E30 and enrofloxacin-E5. These were selected based on their use in the test area.

In the case of batch A, the presence of resistant bacterial colonies was observed, but if the MAR index, equal to 33.33%, is analyzed, the presence of an increased number of resistant strains cannot be concluded. However, when analyzed by antibiotic class, a low efficiency of oxytetracycline is noted, where 75% of the strains are resistant. Of all the isolated strains, Shigella spp. proved to be the most resistant, being classified as a multi-drug resistant (MDR) strain, posing a risk to animals and caretakers. At the same time, all the isolated strains proved to be resistant to different classes of antibiotics, with MAR indices varying between 0.22 and 0.33.

In the case of group B, on day 0 of testing, the presence of resistant strains, but also strains sensitive to the tested antibiotics, was noted. Analyzing by antibiotic classes, it could be seen that the most potent antimicrobials were chloramphenicol and enrofloxacin, with a resistance percentage of 0%. Instead, in the case of erythromycin, a resistance percentage of 63.63% could be observed. Among the isolated strains, the most resistant were Enterobacter spp., Aeromonas hydrophila, Rahnella aquatilis and Vibrio parahaemolyticus. The calculated MAR index for antibiotic susceptibility tests on day 0 was 0.225 (percentage of 22.72%).

On day 14, tests were carried out to determine the level of antibiotic resistance of the strains isolated from pigs whose feed was supplemented with marigolds and thyme. As on day 0, the most potent antibiotics were chloramphenicol and enrofloxacin, with a resistance percentage of 0%. In contrast to day 0, the increase in the percentage of resistance in the case of ampicillin was noted, with a percentage of resistance of 58.82%. In the case of erythromycin, the results were similar to day 0. The most resistant strain isolated was Citrobacter freundii. The MAR index calculated on day 14 was 0.23, 0.005 higher than on day 0, statistically insignificant difference. These results are due to the selective effect of medicinal plants, inhibiting Gram-positive strains, which favored the development of Gram-negative strains.

The general conclusions that emerge from the work entitled: "Alternative phytotherapies in the conditioning of the carried microbiome and the immune response in pigs", can be found in **chapter 8**.

The wealth of active principles of the medicinal plants studied advocated their use in different forms, to establish their usefulness in therapy, either as antimicrobials or as immunomodulators.

Differences were observed in leukocyte reactivity to in vitro treatment with alcoholic extracts of the studied plants. While sea buckthorn extracts had a potent stimulatory effect, the results obtained with the other plant extracts did not indicate a strong stimulatory effect.

Differences in the carried microbiome were observed, depending on the location of these strains in the body (skin or oral mucosa), but also on the location of the farms studied. The composition of the bacterial microflora depended on the age of the animals, the time of sampling and the therapy applied to the pigs.

Although the MAR index was not increased (MAR index lot A equal to 0.275, MAR index lot B on day 0 equal to 0.225 and MAR index lot B on day 14 equal to 0.23), an increased resistance of some species can be observed bacterial resistance to certain antimicrobials, suggesting an increased risk to pigs and keepers. Similarly, they could represent a source for further contamination and spread of resistance around pig farms.

Increased resistance observed to oxytetracycline with 75% resistance and tylosin with 50% resistance in isolates from group A; erythromycin with a resistance of 58.258%, ampicillin with a resistance of 58.82% in the case of strains isolated from group B require their exclusion from current therapy.

While sea buckthorn did not produce changes in the microbial flora of the skin and the level of antibiotic resistance, the administration of Calendula officinalis and Satureja hortensis powder caused post-administration changes in the flora of the mouth and antibiotic resistance.

The positive results, emphasizing the response to mitogens of plant origin in the case of pigs that were administered sea buckthorn syrup, indicate the need to implement an immunostimulation protocol through the combined administration of medicinal plants.

Chapter 9 highlights the originality and innovative contributions of the thesis:

It is the first study that investigated and characterized the composition of the carried microbiome from the skin and the oral mucosa of clinically healthy pigs, from the study area, evaluating the response and the changes that occurred in the case of the introduction of some medicinal plants into the pigs' diet (sea buckthorn, calendula, thyme).

This research provides crucial data on the resistance profile of bacteria that are part of the normal flora of the skin and oral mucosa of pigs reared in an extensive system, necessary for the effective management of infections occurring on farms and in the prevention of the transmission of pathogens, carriers of resistance genes, but also the changes caused by them in the case of the administration of plants with a medicinal role.

The study is innovative by highlighting the variability of the antimicrobial and immunostimulating effect of these plants, considering that a strong immunostimulating effect of sea buckthorn and a selective and temporary antimicrobial effect of calendula and thyme were pronounced.