PhD THESIS

In vitro and *in vivo* evaluation of the probiotic properties of bacteria of the genus *Bacillus*

(SUMMARY OF Ph.D. THESIS)

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INTRODUCTION

Probiotics are considered living microorganisms that, when administered in appropriate amounts, are able to produce beneficial effects on the host. Currently, due to the growing resistance to antibiotics, attention has been directed to the administration of probiotics, as an alternative therapy in the treatment of gastrointestinal diseases. Intestinal health is directly related to the homeostasis of the resident microbial populations at this level, the populations that make up the intestinal microbiome. When an imbalance occurs at this level, there may be general symptoms that will influence the absorption of nutrients, digestion of food, and will cause local and systemic inflammatory syndromes that will affect the body as a whole. The imbalance of the intestinal microbiome is known as dysmicrobism. Moreover, this condition entails another pathology, namely systemic metabolic endotoxemia. In this context, probiotics can be used for therapeutic purposes, being able to modulate the intestinal microbial composition, resulting in a decrease in dysmicrobism and, consequently, the improvement of the general health of the host. For this reason, their widespread use is becoming increasingly popular. Another way to administer probiotics is as a prevention. One of the most common situations in which probiotics are given for this purpose is antibiotic treatment. The administration of antibiotics in various pathologies produces, in most cases, a secondary dysmicrobism. For this reason, the concomitant administration of probiotics with antibiotic therapy may reduce the degree of dysmicrobism, having a general beneficial effect on the host.

WORKING HYPOTHESIS AND RESEARCH OBJECTIVES

The aim of this research is fully found in its title: "***In vitro* and *in vivo* evaluation of the probiotic properties of bacteria of the genus *Bacillus***". Probiotic formulas based on sporulated bacteria have a higher resistance to gastrointestinal tract conditions (SCHMITZ and SUCHODOLSKI, 2016). At the present moment, unlike human medicine, probiotics commonly used in veterinary medicine do not include sporulated bacteria in their composition, such as those of the genus *Bacillus*. For this reason, there is not enough scientific evidence on the probiotic potential for animals of these bacteria.

The main objective of the thesis:

- ✓ Demonstration of the probiotic effect for animals of bacteria from the genus *Bacillus*

In order to achieve the general objective, the research was divided into 3 individual studies, each of which represents a **specific objective** of the research:

- ✓ *In vitro* evaluation of bacillus spores resistance by simulating canine gastrointestinal tract conditions:
  - Adaptation of an artificial digestion protocol to simulate the conditions of the canine gastrointestinal tract;
  - Determining the tolerability of probiotic formulas to simulated conditions of the gastrointestinal tract;
  - Assessing the antibacterial capacity of the various probiotic formulas tested;
- ✓ *In vivo* analysis of the probiotic capacity of bacillus spores in the context of intestinal dysbiosis, using the murine model:
  - Evaluation of the effects of bacillus-based probiotics in healthy rats and rats with dysmicrobism;
  - Assessing the impact on the feed conversion rate of probiotics and antibiotics;
  - Evaluation by histological and immunohistochemical techniques of the ability of bacillus-based probiotics to reduce the effects of antibiotic-induced dysmicrobism;
- ✓ Clinical evaluation of the probiotic potential of bacillus spores in the context of canine postprandial dysbiosis and endotoxemia:
  - Clinical and paraclinical evaluation of the subjects included in the study;
  - Determining the values and evolution pattern of postprandial endotoxemia in clinically healthy dogs;
  - Determining the values and pattern of evolution of postprandial endotoxemia in dogs with signs of apparent intestinal dysbiosis;
  - Determination of correlations between the presence of postprandial endotoxemia and markers of intestinal inflammation in dogs with signs of apparent intestinal dysbiosis.
  - Analysis of the fecal score during the 30 days of treatment;
  - Evaluation of the apparent digestibility coefficient before and after the 30 days of treatment.

STRUCTURE OF THE DOCTORAL THESIS

The PhD thesis entitled "*In vitro* and *in vivo* evaluation of probiotic properties of bacteria of the genus *Bacillus*" has 33 figures, 34 tables and is structured according

to the processing rules in two parts: **Part I - Current stage of knowledge** and **Part II - Personal contribution**.

Part I – Current stage of knowledge

Part I contains 2 chapters and represents the synthesis and summary of knowledge about the intestinal microbiome, probiotics, their use for curative and preventive purposes, but also the general characteristics of bacteria of the genus *Bacillus* and their probiotic potential.

Chapter I is entitled "Microbiome" and includes 3 subchapters: "General and importance", "Microbiome in canids" and "Changes in the microbiome in the context of gastrointestinal disorders in dogs (dysmicrobism-antibiotics, endotoxemia)".

Chapter II is entitled "Probiotics" and contains 5 subchapters entitled: "Definition and classification", "Mechanisms of action", "Importance of probiotics", "Alternative therapy with probiotics in dogs" and "Use of *Bacillus* spp. as a probiotic".

Part II - Personal contribution

Part II contains 9 chapters and represents the personal contribution. These chapters include the working hypothesis and research objectives, the material and methods used, the results obtained, the general conclusions, the recommendations extracted from the research and elements of originality of the thesis.

Chapter III is entitled "Working hypothesis and research objectives", which describes the working hypothesis, but also the general objective and specific objectives of the thesis.

Chapter IV is named "Materials and methods - general aspects" and is structured and systematized in 5 subchapters in which are described in a general way all the materials and methods used, from the organization of investigations to statistical analysis of data obtained.

Chapter V is entitled "*In vitro* evaluation of the resistance of various probiotic formulas to simulated conditions of the gastrointestinal tract" and is structured and systematized into 4 subchapters in which it is presented, in a logical manner, during this first study. Thus, the purpose and objectives of the study, the materials and methods specific to this study, the results obtained and the partial conclusions are presented. This chapter provides data on tolerance to artificial gastric juice, as well as bile salts of various probiotic formulas tested, as well as information on the antimicrobial capacity of a probiotic bacterial combination that includes bacillus spores.

Chapter VI, named "*In vivo* determination of the probiotic capacity of bacillus spores in the context of intestinal dysbiosis, using the murine model" is structured in 4 subchapters to illustrate the logical thread of the second study in this thesis. This chapter provides information on the impact of antibiotics and probiotics in experimentally induced intestinal dysmicrobism, by analyzing the evolution of conventional growth indices for the murine population used in the study, and by evaluating the histological and immunohistochemical aspects obtained.

Chapter VII is entitled "Clinical evaluation of the probiotic potential of

bacillus spores in the context of canine dysbiosis and postprandial endotoxemia" contains 4 subchapters. This chapter presents clinical and paraclinical data of particular importance (the assessment of postprandial endotoxemia and inflammatory markers) that illustrate the probiotic potential of bacillus spores. Data on the impact of the probiotic formula on digestion are also available.

Chapter VIII is entitled "General conclusions and recommendations" and summarizes the conclusions of our studies and the recommendations derived from them, which are intended to improve basic research.

Chapter IX is entitled "Originality and innovative contributions of the thesis" and summarizes the unique and original elements of the thesis and the innovative contribution of newly implemented methods for assessing the probiotic potential.

RESEARCH RESULTS

In vitro evaluation of the resistance of various probiotic formulas to simulated conditions of the gastrointestinal tract

Resistance of probiotic bacteria to simulated conditions of the gastrointestinal tract

Given that the results of applied *in vitro* biological tests are qualitative results, we used the principle of mathematical functions to demonstrate the validity of the applied method for testing the inhibitory effect of gastric juice. The logical mathematical function on which this demonstration is based assigns to each fundamental element (gastric juice, probiotic, food) a constant, as follows: f (x) = a + b + c, where a represents gastric juice, b represents probiotic, and c represents food. Thus, depending on the experimental protocol, two basic functions were created, taking into account the expected effect of the experiment (the probiotics tested retain their viability after artificial digestion).

Using this method of mathematical interpretation we obtained the following results:

For the artificial digestion protocol of samples P1, P2 and P3, in the absence of food, the results of the mathematical functions were divided into two situations:

- f(x)=a for samples P1 şi P2
- f(x)=a+b for P3

Thus, for P1 and P2, where f (x) = a, there is a total inhibition of the viability of probiotic bacteria following artificial digestion. For P3, where f (x) = a + b, it can be seen that probiotic bacteria have maintained a certain level of viability.

For the protocol of artificial digestion of samples P1, P2, P3, in the presence of food, the results of the mathematical function were summarized in one case:

- f(x)=a+b+c.

Thus, for all three samples included in the study, it is noted that they maintained a certain percentage of viability following the process of artificial digestion in the presence of food.

Regarding the **tolerance to bile secretion**, for P1 and P3 it was observed the presence of a weak bactericidal activity on the bacterial strains in the composition of probiotics (lysis area of 1.5-2 mm around the ball washer). Thus, probiotic bacteria

show a low tolerance to fresh pork bile, their viability being directly influenced by the presence of this digestive secretion. However, the bactericidal activity of bile is weak.

Antimicrobial capacity

It is known that one of the mechanisms of action of probiotics is the replacement of pathogenic bacteria in the gut (LEE et al., 2003). Also, the production of antimicrobials, the stimulation of the immune response (JONES and VERSALOVIC, 2009) or the regulation of the concentration of metabolites (SOO and et al., 2008) are mentioned.

In the present study, the inhibition of competition manifested by the probiotic bacteria tested was noted. Thus, in the case of cultures of *Salmonella enteritidis*, *Staphilococcus aureus* and *Escherichia coli* this inhibition of competition is obvious. In the case of the pathogenic culture of *Bacillus cereus* we can observe the character of the pathogenic colonies, of much smaller dimensions in the central area, around the probiotic colony, compared to the dimensions of the colonies in the rest of the plaque.

In vivo determination of the probiotic capacity of bacillus spores in the context of intestinal dysbiosis, using the murine model

Analysis of conventional growth indices

The highest value of the feed conversion factor was recorded in group 1-control group (Table 1). For group 2 (14-day amoxicillin administration) and group 3 (14-day probiotic administration), the value of the feed conversion rate was negative. Amoxicillin has been used due to its increased intestinal bioavailability (CHESA-JIMEJNEZ et al., 1994). The negative values can be explained due to the ability of the 2 administered products to modify the intestinal microbiome and implicitly to influence the intestinal absorption rate. Thus, although the animals consumed a normal amount of food, similar to the control group (Table 1), they lost weight, an effect that is directly attributable to the experimental intervention. Regarding group 4 (antibiotic and probiotic concomitant for 14 days), the food conversion factor recorded positive values. The amount of food consumed was also for this group within physiological parameters, being comparable to other groups. Thus, the positive value recorded for FCR in this group is the result of the concomitant administration of the 2 treatments. Regarding group 5 (antibiotic 7 days, probiotic 7 days), the FCR value was negative. In the context of a quantity of food consumed approximately equal to the other groups, it can be considered that the probiotic administered after the cessation of the antibiotic treatment does not have the expected effect.

Table 1

Mean values for all the growth conventional indices

Group	Feed consumed	Lot Gain	FCR/Group	SGR
L1	885.8	4.50	196.8444	0.021967
L2	896	-16.50	-54.303	-0.08514
L3	1003	-15.50	-64.7097	-0.07613
L4	1079	10.00	107.9	0.047923
L5	984.5	-26.00	-37.8654	-0.12782

FCR- feed conversion rate; SGR- specific growth rate

Histological and immunohistochemical evaluation

On **histological** examination of the Goldner's Tricrome stained samples, the morphological appearance of the three intestinal segments revealed significant changes in groups 2, 3 and 5. In the control group, as well as in group 4 - concomitantly treated with antibiotic and probiotic, the changes occurred were absent or reduced. The most important changes observed were at the duodenal level, these being represented by the presence of subepithelial spaces, maintaining the integrity and cohesion of the epithelium of the intestinal villi, the lesion being found mainly at the apical pole of the duodenal villi (Fig. 1 A, B).

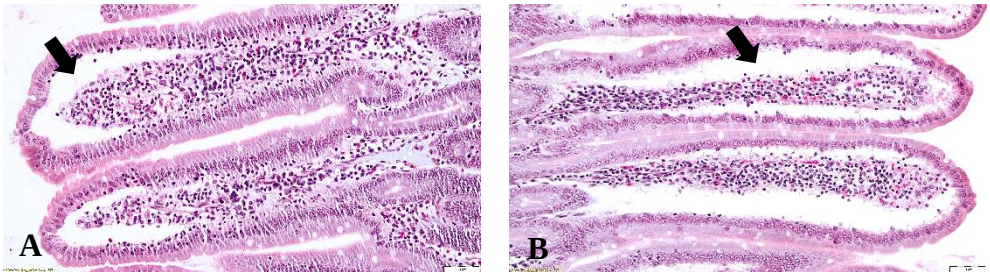

Fig. 1 Appearance of duodenal mucosa in group 2 (A) and group 5 (B), TG staining: presence of subepithelial spaces, maintaining the integrity and cohesion of the intestinal villi epithelium

At the level of the jejunal mucosa, the observed changes were moderate, and at the level of the colon, the changes were classified as poorly represented in all 5 groups, with differences in intensity between groups.

Immunohistochemically, the intestinal segment with the highest intensity of the reaction, for both LBP and TLR4, was in the duodenal mucosa followed by the jejunum (in which the mucosal lymphoid infiltrate is lower in quantity compared to the duodenum), in while in the colon the expression of the two markers is significantly lower compared to the previous segments (Table 2). Regarding the details of the immunohistochemical reaction, both in the case of LBP and in the case of TLR4, the immunolabeling is highlighted by shades of brown (of different intensities) strictly at the membrane of macrophages related to GALT (gut associated lymphoid tissue). Also, the color intensity of the immunohistochemical reaction is comparable to the two markers analyzed, finding a direct correlation of LBP expression with the TLR4

marker in each segment studied. Regarding the place where the two markers are expressed, they were identified in the lymphoid tissue associated with the intestinal mucosa (GALT), mainly in the macrophages of the lamina propria.

Table 2

Immunopositivity score for LBP and TLR 4 markers in the 3 intestinal segments

Group	Intestinal segment	LBP	TLR4
Lot 1 Group 1	Duoden	±	±
	Jejun	±	±
	Colon	±	±
Lot 2 Group 2	Duoden	+++	+++
	Jejun	+++	+++
	Colon	+	+
Lot 3 Group 3	Duoden	+++	+++
	Jejun	++	++
	Colon	+	+
Lot 4 Group 4	Duoden	+++	++
	Jejun	++	++
	Colon	+	+
Lot 5 Group 5	Duoden	+++	++
	Jejun	++	++
	Colon	+	+

± weakly positive reaction, + moderately positive reaction, ++ clearly positive reaction, +++ intense positive reaction

Clinical evaluation of the probiotic potential of bacillus spores in the context of canine dysbiosis and postprandial endotoxemia

Clinical exam

At the clinical examination on day 0, no dog showed significant changes in physiological parameters. At the clinical examination on day 30, this aspect remained constant, and the remission of gastrointestinal signs in dogs attributed to group 2 with apparent intestinal dysbiosis was also observed.

Paraclinical investigations

The pre-treatment probiotic coproparasitological examination was negative for the subjects of both groups. At the post-treatment examination all samples were negative, except for one subject from group 2 in which eggs of *Uncinaria* spp./*Ancylostoma* spp. were present.

Hematological parameters showed a remarkable heterogeneity in all dogs included in the study. However, the differences remained within the physiological reference range for each parameter.

Like hematological parameters, heterogeneity was also present in the case of biochemical ones with some exceptions. However, both pre-treatment and post-treatment results were within the reference range.

The evolution of oxidative stress parameters does not suggest a direct influence of probiotic treatment on the assessed indicators. Although their evolution changed, their dynamics were not linear.

Evolution of endotoxemia: in the present study, the values of endotoxemia were analyzed in dynamics, both anteprandial and after food administration at 6 and 12 hours, respectively. The results obtained before the probiotic treatment show higher values of endotoxemia, compared to those obtained at the end of the treatment. There are also differences between the values obtained in the group of healthy dogs, compared to the group of patients with apparent dysbiosis (Fig. 2).

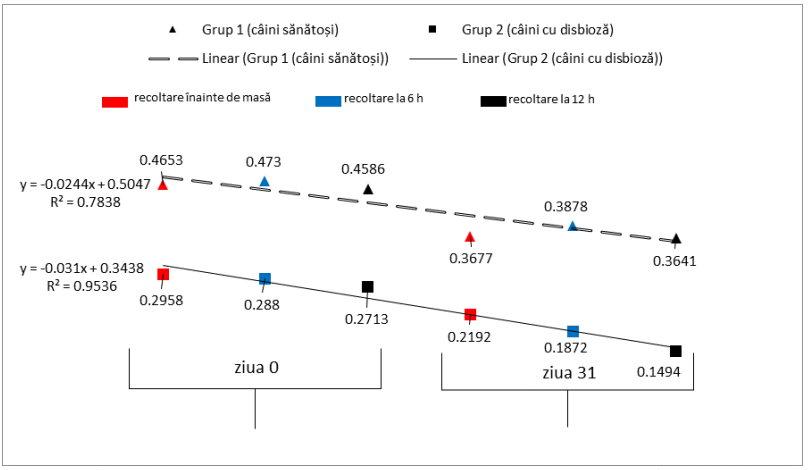

Fig. 2 Dynamics of mean endotoxin concentrations and linear regression (EU / mL) for healthy dogs and dogs with dysbiosis (before and after probiotic treatment)

Evolution of intestinal inflammatory markers: dosing of inflammatory markers was performed for group 2 (dogs with apparent dysbiosis). At the same time, the correlation between the evolution of endotoxemia and the values recorded for sCD14 and IL-1β was established (Table 3). Changes in sCD14 values are a decrease after probiotic treatment, which is directly related to the evolution of serum endotoxemia values. IL-1β marker values remained relatively constant throughout the study.

Table 3

Serum endotoxin, soluble CD14 and IL-1β response pre- and post-probiotic administation

Parameter	**Before probiotic administration (Day 0)**			**After probiotic administration (Day 31)**		
	Pre-meal	After meal		Pre-meal	After meal	
		6h	12 h		6h	12 h
Endotoxemia (EU/mL)	0.2958 ±0.168	0.2880 ±0.200	0.2713 ±0.170	0.2192 ±0.241	0.1872 ±0.145	0.1494 ±0.070
Soluble CD14 (pg/mL)	23.6028 ±6.99	23.2065 ±7.275	23.3054 ±6.011	21.1645 ±2.110	20.5592 ±0.993	20.7246 ±1.1389
IL-1β (ng/mL)	0.0447 ±0.015	0.0410 ±0.014	0.0486 ±0.015	0.0410 ±0.009	0.0458 ±0.007	0.0453 ±0.010

Assessing the impact of probiotics on digestion

Fecal score: in most of the dogs included in the study, the consistency and volume of the faeces improved during probiotic treatment, the smell of the samples did not improve significantly, the sounds produced in the digestive tract were absent, and flatulence was decreased. There was a significant change in the amount of feces, which decreased considerably after treatment.

The study of food digestibility by quantifying the apparent digestibility coefficient shows a remarkable improvement in digestion capacity for all components studied (dry matter, fat, cellulose, non-nitrogenous extractive substances) except proteins where the values decreased for one of the subjects. The increase in digestibility is statistically significant ($P < 0.001$) for dry matter, protein and nitrogen free extract substances (NFE).

Recommendation

We recommend increasing the focus in veterinary practice on intestinal dysmicrobism and natural postprandial endotoxemia and the use of probiotics to counteract their negative effects.

We suggest the administration of probiotics during the meal, in order to diminish the negative influence of the acidic pH on the viability of the bacteria. We sustain the administration of probiotics at the same time with antibiotics, not at the end of antibiotic therapy, to counteract the negative effects induced by the state of dysmicrobism.

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