PONTIFÍCIA UNIVERSIDADE CATÓLICA DO PARANÁ
ESCOLA DE MEDICINA E CIÊNCIAS DA VIDA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL

GUSTAVO ZIGOVSKI DE PAULA

Effects of dietary homeopathic additive on the performance, intestinal health, inflammatory response and behavior of nursery piglets

(Efeitos de um aditivo homeopático adicionado à dieta no desempenho, saúde intestinal, resposta inflamatória e comportamento de leitões na fase de creche)

CURITIBA
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Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal, área de concentração Saúde, Tecnologia e Produção Animal, da Escola de Medicina e Ciências da Vida da Pontifícia Universidade Católica do Paraná, para obtenção do título de Mestre em Ciência Animal.

Orientador: Prof. Dr. Leandro Batista Costa

CURITIBA
2023

FICHA CATALOGRÁFICA

Dados da Catalogação na Publicação
Pontifícia Universidade Católica do Paraná
Sistema Integrado de Bibliotecas – SIBI/PUCPR
Biblioteca Central
Luci Eduarda Wielganczuk – CRB 9/1118

Paula, Gustavo Zigovski de

P324e
2023

Effects of dietary homeopathic additive on the performance, intestinal health, inflammatory response and behavior of nursery piglets = Efeitos de um aditivo homeopático adicionado à dieta no desempenho, saúde intestinal, resposta inflamatória e comportamento de leitões na fase de creche / Gustavo Zigovski de Paula ; orientador: Leandro Batista Costa. – 2023.

57 f. ; il. : 30 cm

Dissertação (mestrado) – Pontifícia Universidade Católica do Paraná, Curitiba, 2023

Bibliografia: f. 54-56

1. Veterinária. 2. Estresse. 3. Nutrição. 4. Suíno. 5. Resistência bacteriana. I. Costa, Leandro Batista. II. Pontifícia Universidade Católica do Paraná. Programa de Pós-Graduação em Ciência Animal. III. Título.

CDD. 20. ed. – 636.089

TERMO DE APROVAÇÃO

ATA Nº 0177 E PARECER FINAL DA DEFESA DE DISSERTAÇÃO DE MESTRADO EM CIÊNCIA ANIMAL DO ALUNO GUSTAVO ZIGOVSKI DE PAULA

Aos vinte e oito dias do mês de junho do ano de dois mil e vinte e três, às 13h30, pela sala virtual do Zoom, realizou-se a sessão pública de defesa de dissertação do mestrando Gustavo Zigovski de Paula, intitulada: **"EVALUATION OF A HOMEOPATHIC ADDITIVE ADDED TO THE DIET AND ITS EFFECTS ON THE INTESTINAL HEALTH, INFLAMMATORY RESPONSE, PERFORMANCE AND BEHAVIOR OF NEWLY-WEANED PIGLETS"**. O mestrando concluiu os créditos exigidos para obtenção do título de Mestre em Ciência Animal, Área de Concentração em Saúde, Tecnologia e Produção Animal, segundo os registros constantes na secretaria do Programa. Os trabalhos foram conduzidos pelo Professor orientador e Presidente da banca, Dr. Leandro Batista Costa (PUCPR), auxiliado pelos Professores Doutores Fabiana Ribeiro Caldara (UFGD) e Maicon Sbardella (UFMT). Procedeu-se à exposição da Dissertação, seguida de sua arguição pública e defesa. Encerrada a fase, os examinadores expediram o parecer final sobre a Dissertação, que foi considerada Aprovada.

Prof. Dr. Leandro Batista Costa (Presidente)

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Proclamado o resultado, o Presidente da Banca Examinadora encerrou os trabalhos, e para que tudo conste, eu Caroline Nocera Bertton, confiro e assino a presente ata juntamente com os membros da Banca Examinadora.

Curitiba, 28 de junho de 2023.

Caroline Nocera Bertton

Caroline Nocera Bertton
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Dedicatória

Dedico essa dissertação
ao meu pai e à minha mãe,
que sempre fizeram de tudo por mim.

AGRADECIMENTOS

Os últimos dois anos, para mim, foram de muito esforço, dedicação, medo e superação. A realização desse estudo só foi possível graças ao apoio de diversas pessoas que já conhecia ou que tive o prazer de conhecer durante essa jornada.

Primeiramente, gostaria de agradecer ao meu orientador, Prof. Dr. Leandro Batista Costa, que sempre me fez evoluir e me tranquilizava em diversas situações nas quais achava que algo sairia errado, além disso, sempre foi muito paciente com meus erros. Também gostaria de agradecer aos integrantes do Núcleo de Pesquisa em Animais Monogástricos da PUCPR (Monohub) e do Grupo de Estudos de Animais de Produção (GEAP), que me ajudaram imensamente nesse projeto durante os dias de experimento, principalmente a Isabela Bez e o Luís Fernando Garrido, que no período da manhã conduziram com excelência as atividades na Unidade de Pesquisa de Suínos - UPS da Fazenda Experimental Gralha Azul - FEGA. Meus agradecimentos também para os Profs. Dr. Saulo Henrique Webber e Dr. Jansller Genova pelas análises estatísticas do projeto e ao também Prof. Dr. Ruan Daros pela estatística e pelo auxílio com os testes comportamentais.

Agradeço ao Prof. Paulo Levi Carvalho (UNIOESTE) e seus alunos, por nos auxiliarem na coleta de amostras e demais atividades. Sou grato e tenho muito orgulho de ter recebido o apoio da PUCPR com o programa PIBIC Master durante esse período e também por ter tido a oportunidade de realizar meu estágio obrigatório no Institut de Recerca i Tecnologia Agroalimentàries (IRTA), na Catalunha, sob supervisão de Antoni Dalmau e sua equipe.

Ainda, gostaria de agradecer a Real H – Nutrição e Saúde Animal, pelo financiamento desta pesquisa e também ao PPGCA, e aos professores, que me acolheram da melhor forma possível.

E, por último e mais importante do que tudo para mim, agradeço aos meus pais. Não há palavras que consigam expressar a gratidão que tenho por vocês proporcionarem tudo para mim. Devo ter feito muitas coisas boas na outra vida para cair justamente com vocês; sempre me apoiaram e tiveram paciência comigo, além de me ensinar o que é ser uma família de verdade.

SUMMARY

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THESIS FORMAT

This thesis is composed of 4 chapters. In Chapter 1 is presented the introduction and contextualization of the theme, followed by the hypothesis of this thesis. In chapters 2 and 3, articles are presented that will be submitted to the scientific journals: Livestock Science and Homeopathy Journal, respectively. The formatting of the chapters is not uniform, since each journal follows a different guideline. In chapter 4 is presented conclusion, the impact and future perspectives of the thesis. The same chapter finishes with a brief conclusion. References of the chapter 1 are presented at the end of this thesis.

ABSTRACT

Homeopathy can be an alternative to replace performance-enhancing antibiotics on pig production, acting not only on the animal's development and health, but also on its welfare. The aim of the study was to evaluate the effects of different levels of a homeopathic additive as an alternative to an antibiotic growth promoter on performance, intestinal health and inflammatory response, also, assess their effects on the behavior of nursery piglets. The experiment was double-blind, lasted 37 days and involved 126 animals (castrated males and females) weaned at 21±1.3 days, with an initial weight of 5.62±1.16 kg. The piglets were allotted to six treatments in a completely randomized block design with seven repetitions and three piglets per experimental unit. The treatments were: positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC); negative control – basal diet without additives (NC); basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of homeopathic additive in the feed. Data related to performance were calculated. The occurrence of diarrhea was observed daily and was compared between treatments considering the size of the animals. Blood was collected for analysis of hemogram, serum biochemistry and phagocytic profile. In the end of experiment, one animal per pen was slaughtered for the weighing of organs, analysis of pH of stomach and intestinal contents, as well as collection of intestinal samples for analysis of histopathological alterations. Feces were collected on days D2 and D21 and cecal contents at slaughter for microbiological and antibiogram analysis. Piglets were subjected to behavioral tests starting on day D16 of experiment, where different tests were performed: open field (OF), novel object (NO), sociability, discriminative learning and judgment bias. Also, on three days of the experimental period the piglets' reactivity during the weighing (RDW) was evaluated. For behavioral tests, homeopathic levels and sex were included as factors, as well as its interaction. There was no difference between treatments regarding performance. For diarrhea occurrence concerning the total study period, NC treatment showed lower values for overall animals (small, midsize and large), while the levels 4.5 and 7.5 were equal to PC. The organ weights, pH of digestive contents and serum biochemical parameters showed no difference between treatments. For hemogram, the higher the levels of the additive the higher the values of hemoglobin, mean corpuscular hemoglobin

(MCH) and mean corpuscular hemoglobin concentration (MCHC) ($P<0.05$). Treatment 4.5 exhibited similar results in phagocytic activity of monocytes and a reduction in total and thermotolerant enterobacteria populations in piglet feces, while also increasing lactic acid bacteria, compared to the positive control (PC) ($P<0.05$). All treatments showed less pathological alterations in the duodenum compared to the NC ($P<0.05$). Regarding behavior, vocalizations were lower for females than for males during the OF test ($P<0.05$). For escape attempts, in the RDW test, the higher the level of the homeopathic additive, the lower was the value of the variable for females and higher for males (interaction sex X treatment). There were no statistical differences for the tests of NO, sociability, discriminative learning and judgment bias. Thus, treatment 4.5 kg/ton of the homeopathic additive can be an alternative to the antibiotic growth promoter, as it provided equal or better results than the positive control regarding diarrhea occurrence, resulted in lower populations of total and thermotolerant enterobacteria and higher populations of lactic acid bacteria in the feces. Additionally, the treatment exhibited fewer histopathological alterations in the duodenum when compared to the negative control. It also did not affect the behavior of the piglets in the nursery phase.

Keywords: Antibiotic resistance, Nutrition, Stress, Swine

RESUMO GERAL

A homeopatia pode ser uma alternativa aos antibióticos melhoradores de desempenho, promovendo não apenas o desenvolvimento e a saúde do animal, mas também o seu bem-estar. O objetivo do estudo foi avaliar os efeitos de diferentes níveis de um aditivo homeopático como alternativa a um antibiótico melhorador de desempenho na performance, saúde intestinal e resposta inflamatória, bem como avaliar seus efeitos no comportamento dos leitões na fase de creche. O experimento foi duplo-cego, teve duração de 37 dias e envolveu 126 animais (machos castrados e fêmeas) desmamados aos 21 ± 1,3 dias, com peso inicial de 5,62 ± 1,16 kg. Os leitões foram distribuídos em seis tratamentos em um delineamento de blocos casualizados completos, com sete repetições e três leitões por unidade experimental. Os tratamentos foram: controle positivo - dieta basal + 120 mg/kg de clorohidroxiquinolina (CP); controle negativo - dieta basal sem aditivos (CN); dietas basais com 4,5; 6,0; 7,5 e 9,0 kg/tonelada de aditivo homeopático na ração. Os dados relacionados ao desempenho foram calculados. A ocorrência de diarreia foi registrada diariamente e comparada entre os tratamentos considerando o tamanho dos animais. Foram coletadas amostras de sangue para análise de hemograma, bioquímica sérica e perfil fagocitário. Ao final do experimento, um animal por baia foi abatido para pesagem dos órgãos, análise do pH dos conteúdos estomacais e intestinais, bem como coleta de amostras intestinais para análise de alterações histopatológicas. Foram coletadas fezes nos dias D2 e D21 e conteúdos cecais no abate para análise microbiológica e antibiograma. Os leitões foram submetidos a testes comportamentais a partir do 16º dia de experimento, no qual diferentes testes foram realizados: arena aberta (AB), novo objeto (NO), sociabilidade, aprendizagem e viés de julgamento. Além disso, em 3 dias do período experimental foi avaliada a reatividade dos leitões durante a pesagem (RDP). Para os testes comportamentais, os níveis do homeopático e o sexo foram incluídos como fatores, assim como sua interação. Não houve diferença entre os tratamentos em relação ao desempenho. Para a ocorrência de diarreia durante todo o período de estudo, o tratamento CN apresentou menores valores para o conjunto dos três tamanhos animais (leves, medianos e pesados), enquanto os níveis 4,5 e 7,5 foram iguais ao CP. Os pesos dos órgãos, pH dos conteúdos digestivos e parâmetros bioquímicos

séricos não apresentaram diferenças entre os tratamentos. No hemograma, quanto maiores os níveis do aditivo, maiores foram os valores de hemoglobina, hemoglobina corpuscular média (HCM) e concentração de hemoglobina corpuscular média (CHCM) ($P<0,05$). O tratamento 4,5 apresentou resultados semelhantes na atividade fagocítica de monócitos e uma redução nas populações de enterobactérias totais e termotolerantes nas fezes dos leitões, além de aumento de bactérias ácido-lácticas, em comparação ao controle positivo (CP) ($P<0,05$). Todos os tratamentos refletiram em menos alterações patológicas no duodeno em comparação com o CN ($P<0,05$). Em relação ao comportamento, as vocalizações foram menores para as fêmeas do que para os machos durante o teste de AB ($P<0,05$). Para tentativas de fuga, no teste de RDP, quanto maior o nível do aditivo homeopático, menor foi o valor da variável para as fêmeas e maior para os machos. Não houve diferenças estatísticas para os testes de NO, sociabilidade, aprendizagem e viés de julgamento. Assim, o tratamento com 4,5 kg/ton de aditivo homeopático pode ser uma alternativa ao antibiótico melhorador de desempenho, pois proporcionou resultados iguais ou melhores que o controle positivo em relação à ocorrência de diarreia, resultou em menores populações de enterobactérias totais e termotolerantes e maiores populações de bactérias ácido-lácticas nas fezes. Ainda, exibiu menos alterações histopatológicas no duodeno em comparação com o controle negativo, além disso, não afetou o comportamento dos leitões na fase de creche.
Palavras-chave: Estresse, Nutrição, Suíno, Resistência Bacteriana

CHAPTER 1

INTRODUCTION AND CONTEXT

Pig farming is a complex activity and encompasses different phases such as reproduction/gestation, maternity, nursery, growth and finishing. During weaning, numerous challenges may limit the piglets' development, including: separation from the sows, fight for dominance and environment/diet change (Campbell et al., 2013). All these issues can compromise the piglets' digestive capacity, providing a deleterious effect on the function of the intestinal barrier (Spreeuwenberg et al., 2001). Bacteria such as *Escherichia coli* and *Salmonella spp.* can develop as a result of improperly digested proteins and other compounds, leading to malabsorption of nutrients and consequent diarrhea (Chamone, 2010). Furthermore, all these adversities can interfere in the animal's welfare, directly impacting the productivity of the herd (Alves et al., 2016).

Since the discovery of the positive effects of antibiotics as growth promoters, their use has been beneficial to farmers, as it improves the feed conversion and prevents infections in the animals (Lillehoj et al., 2018; Castanon, 2007). The mechanism of action of antimicrobials is related to the modulation of the intestinal microbiota and its advantages on animal performance (Lillehoj et al., 2018). Although antibiotics have the potential to treat infections and improve animal growth, their use without prudence may generate consequences such as allergies in hypersensitive individuals (Kasimanickam et al., 2021; Souza et al., 2013), and, besides that, may contribute to foodborne-disease outbreaks and bacterial resistance (Evangelista et al., 2022). Bacteria have a genetic plasticity that allows mutations and sharing of DNA between them, in addition, they are able to modify their target site or produce enzymes that inactivate the effect of the antimicrobial (Munita & Arias, 2016).

Moreover, the consumer market increasingly values a production system that prioritizes both animal health and the environment (Ludwiczak et al., 2021). Consequently, it is noted the demand of consumers for a product free of chemical residues, highlighting, for example, the European production system (Felippelli & Valente, 2009). Since Brazil is one of the largest producers and exporters of pork in the world, sustainable management must be followed. An example of this understanding is the implementation of The National Action Plan on Antimicrobial Resistance in Agriculture, whose goal is the fight against bacterial resistance and the promotion of one health (Dutra et al., 2021).

Also, aiming to circumvent all these problems, researchers have sought alternatives to these drugs that can prevent diseases and decrease the impact of the withdrawal of the antibiotics from swine feed, which would have an impact mainly on newly weaned piglets (Soto et al., 2007). In-depth studies need to be conducted to know the mechanism of action and efficiency of these substances in the field (Lillehoj et al., 2018).

Homeopathy was founded by Samuel Hahnemann in 1828 (Mayer et al., 2016). The therapy consists in treating a disease with substances that can cause the same clinical signs that the patient is feeling, but in minimal doses. Nevertheless, the use of this therapy in pig farming is promising, since homeopathics do not leave residues in animal products and also do not influence bacterial resistance, one of the major advantages of homeopathics (Braccini et al., 2019). Recently, a published article showed that a homeopathic additive enhanced the performance of finishing pigs without negative effects on meat traits and plasma metabolites (Lima et al., 2022).

Currently, our research group has prepared and submitted a systematic review about an alternative therapy to allopathic medicines: homeopathy. It is possible to notice that the studies involving homeopathy as a possible substitute for antibiotics are still scarce. Mathie & Clausen (2015) affirmed that the use of this method in veterinary medicine has not yet been fully elucidated, mainly due to the few studies published, making it difficult to search for evidence. Therefore, this additive was investigated in the present project involving pig performance and behavior in order to contribute with more concrete data to the scientific literature.

Considering the impact that early weaning causes in the piglets and the possible beneficial effects of homeopathics, the additive used in the present project may contribute positively to their performance and behavior of these animals. Therefore, the hypothesis that covers this study is that the additive, when added to the diet of piglets, will result in the improvement of performance, intestinal health, inflammatory response and behavior of the animals.

CHAPTER 2

This manuscript will be submitted to Livestock Science

Beyond antibiotics: unveiling the potential of homeopathic additives on the performance, intestinal health and inflammatory response of nursery piglets

[a]Paula, G. Z., [a]Bez, I. C. C., [a]Garrido, L. F. C., [a]Rodrigues, C. C., [a]Oliveira, A. C. F., [b]Rupolo, P. E., [b]Genova, J. L., [b]Azevedo, L. B. A., [a]Weber, S. H., [b]Carvalho, P. L. O., [a*]Costa, L. B.

[a]Graduate Program in Animal Science, School of Medicine and Life Sciences, Pontifícia Universidade Católica do Paraná, Curitiba, Brazil

[b]Universidade Estadual do Oeste do Paraná, Marechal Cândido Rondon, Brazil

**Corresponding author: Leandro Batista Costa (L. B. Costa)*

Dr. - Associate Professor - Animal Nutrition/Pork Production/Animal Welfare

School of Medicine and Life Sciences - Pontifícia Universidade Católica do Paraná

Imaculada Conceição Street, 1155 - Prado Velho, Curitiba, Paraná, Brazil

Mobile: +55 41 9103-8743

batista.leandro@pucpr.br

3.1 Abstract

Alternatives are sought to replace antibiotics as performance enhancer in swine production and one of them may be homeopathy. The objective of this study was to evaluate a homeopathic additive, replacing an antimicrobial performance enhancer in the diets of nursery piglets on performance, intestinal health and inflammatory response. The experiment was double-blind, lasted 37 days and involved 126 animals weaned at 21±1.3 days, with an initial weight of 5.62±1.16 kg. The piglets were allotted to six treatments in a completely randomized block design with seven repetitions and 3 piglets per experimental unit. The treatments were: positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC); negative control – basal diet without additives (NC); and basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of homeopathic additive in the feed. Data related to performance were calculated. The occurrence of diarrhea was observed daily and was compared between treatments considering the size of the animals. Blood was collected for analysis of hemogram, serum biochemistry and phagocytic profile. In the end of experiment, one animal per pen was slaughtered for the weighing of organs, analysis of pH of stomach and intestinal contents, as well as collection of intestinal samples for analysis of histopathological alterations. Feces were collected on days D2 and D21 and cecal contents at slaughter for microbiological and antibiogram analysis. The Statistics analysis was done using the software SPSS 25. There was no difference between treatments regarding performance. For diarrhea occurrence, concerning the total study period, NC treatment showed lower values for overall animals (small, midsize and large), while the levels 4.5 and 7.5 were equal to PC ($P<0.05$). The organ weights, pH of digestive contents and serum biochemical parameters showed no difference between treatments ($P>0.05$). For hemogram, the higher the levels of the additive the higher the values of hemoglobin, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). Treatment 4.5 exhibited similar results in phagocytic activity of monocytes and a reduction in total and thermotolerant enterobacteria populations in piglet feces, while also increasing lactic acid bacteria, compared to the positive control. All treatments showed less pathological alterations in the duodenum compared to the NC ($P<0.05$). Therefore, treatment 4.5 kg/ton of the homeopathic additive can be an alternative to the antibiotic growth promoter, since it provided equal or better results than the positive control regarding diarrhea occurrence, improved the intestinal microbiota and, additionally, exhibited fewer histopathological alterations in the duodenum when compared to the negative control.

Keywords: Bacterial Resistance, Diarrhea, Microbiology, Pigs

2.2 Introduction

The pig production system is highly intensive, which leads to the early weaning of these animals. In nature, piglets are weaned at 10 to 12 weeks of age, however, depending on facilities, management and sanitary status on commercial farms, they are weaned within 30 days or less (Moeser et al., 2017). The stress caused by this management can result in different problems, including diarrhea, loss of performance and even death (Ma et al., 2021). In addition, bacteria such as *Escherichia coli*, gram-negative rod-shaped bacteria belonging to the family Enterobacteriaceae (Pakbin et al., 2021), can further aggravate diarrhea in piglets. Certain subgroups of *E. coli* have virulence factors that make them capable of causing intestinal diseases (Caldorin et al., 2013). To avoid these problems involving stress and microorganisms, the use of antibiotics has been recurrent in pig production, they are utilized as an element that maintains health and ensures high levels of production in pig farms (Albernaz-Gonçalves et al., 2022).

However, the frequent use of antibiotics in the diet of animals has led to a problem that can affect all society: bacterial resistance. According to the World Health Organization (WHO), bacterial resistance occurs when the microorganism changes in response to the use of these medicines. The concern is such that the European Union banned its use in animal production as a performance enhancer (Dewulf et al., 2022). With these restrictions, studies seeking alternatives to these additives have intensified, aiming to reduce the productive losses of this prohibition.

Homeopathy, a therapy founded by Samuel Hahnemann in 1828 (Mayer et al., 2016), can be seen as an alternative to the allopathic medicines used in animal feed. There are advantages in its employment as the ability not to contaminate animal products or the environment (Braccini et al., 2019). Furthermore, a few studies have highlighted the importance of this therapy in weaned piglets, but they are outdated (Camerlink et al., 2010; Soto et al., 2008). A current study showed that homeopathic additive enhanced the performance in finishing pigs without negative effects on meat traits and plasma metabolites (Lima et al., 2022). It is necessary to emphasize the importance that the use of this additive can bring to the intestinal health of piglets. The action of homeopathy in the digestive system can provide numerous advantages, considering that the intestinal health is fundamental for animals to maintain digestion of feed, nutrient absorption and consequently increase weight gain (Da Silva et al., 2021a).

The purpose of this study was to evaluate the effects of a homeopathic additive added to the diet of nursery piglets on the performance, intestinal health and inflammatory response compared with a performance-enhancing antibiotic, evaluating its possible replacement.

2.3 Methods

The experiment was performed at the Swine Research Unit (SRU) located at the Gralha Azul Experimental Farm of the Pontifical Catholic University of Paraná (PUCPR). The study was approved by the Ethics Committee on Animal Use (CEUA) under protocol number: 01720 (2^{nd} version). The experiment was double-blind, lasted 37 days and involved 126 animals (castrated males and females) weaned at 21±1.3 days, with an initial weight of 5.62 ± 1.16 kg, which were assigned to 6 treatments in a completely randomized block design with 7 repetitions and 3 piglets per experimental unit (sex and initial weight were used as blocking criteria). The piglets were housed in suspended pens (1.5m x 1.5m) with semi-automatic feeders, water dispensers and plastic slatted floors. The treatments were composed of six experimental diets: positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC); negative control – basal diet without additives (NC); basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of homeopathic additive in the feed.

2.3.1 Composition of the product

The inclusion of the additive in the diet of animals followed recommendations provided by the manufacturer based on studies of product development. The homeopathic complex is composed of: *Ignatia amara* (30 CH), *Chamomilla* (15 CH), *Silicea terra* (15 CH), *Staphysagria* (15 CH), *Colibacillinum* (9 CH), *Enterococcinum* (9 CH), *Lac defloratum* (9 CH), *Aethusa cynapium* (6 CH), *Artemisia abrotanum* (6 CH), *Calcarea carbonica* (6 CH), *Thyreoidinum* (6 CH), *Thymulin* (6 CH), Vehicle (Calcium Carbonate – 1000g). It was provided by Real H - Animal nutrition and health (Campo Grande, Mato Grosso do Sul, Brazil). Its use is intended for piglets and there is no contraindication. The experiment lasted 37 days and the animals were fed *ad libitum*, until the end of the experimental period. The diets were formulated to meet the piglets' requirements for the pre-initial I (1 to 7 days of experimentation), pre-initial II (8 to 20 days of experimentation) and initial (21 to 37 days of experimentation) phases, following Rostagno et al. (2017) (Tables 2.1; 2.2; 2.3).

Table 2.1 Percentage composition of the experimental diets (Pre-initial I phase).

Ingredients	Treatments[1]					
	PC	NC	4.5	6.0	7.5	9.0
Corn 8.00%	40.667	40.691	39.804	39.508	39.212	38.917
Soybean meal 45.00%	21.021	21.017	21.155	21.200	21.246	21.292
Skim milk powder	14.016	14.016	14.016	14.016	14.016	14.016
Micronized soybeans	10.000	10.000	10.000	10.000	10.000	10.000
Sugar	5.000	5.000	5.000	5.000	5.000	5.000
Fishmeal	3.000	3.000	3.000	3.000	3.000	3.000
Soybean oil	1.954	1.946	2.250	2.351	2.453	2.554
Dicalcium phosphate	1.479	1.479	1.479	1.479	1.479	1.479
Limestone	0.840	0.840	0.839	0.839	0.839	0.838
Lysine sulfate 54.6%	0.714	0.714	0.710	0.709	0.708	0.707
L-threonine 98.0%	0.429	0.429	0.428	0.428	0.428	0.428
DL-methionine 99.0%	0.359	0.359	0.360	0.360	0.360	0.361
L-triptophan 99.0%	0.097	0.097	0.097	0.097	0.097	0.097
Premix[2]	0.300	0.300	0.300	0.300	0.300	0.300
Salt	0.107	0.107	0.107	0.107	0.108	0.108
Chlorohydroxyquinoline	0.012	-	-	-	-	-
Homeopathic additive	-	-	0.450	0.600	0.750	0.900
Total	100.000	100.000	100.000	100.000	100.000	100.000
Analyzed composition						
Dry matter (%)	91.144	90.742	91.869	90.293	91.972	91.692
Ether extract (%)	7.001	6.898	7.225	6.888	6.935	7.111
Calculated composition						
Metabolizable energy (Mcal/Kg)	3.400	3.400	3.400	3.400	3.400	3.400
Crude protein (%)	21.420	21.420	21.420	21.420	21.420	21.420
Total calcium (%)	1.068	1.068	1.068	1.068	1.068	1.068
Available phosphorus (%)	0.528	0.528	0.528	0.528	0.528	0.528
Total sodium (%)	0.224	0.224	0.224	0.224	0.224	0.224
Digestible lysine (%)	1.451	1.451	1.451	1.451	1.451	1.451
Digestible methionine + cystein (%)	0.813	0.813	0.813	0.813	0.813	0.813
Digestible threonine (%)	0.972	0.972	0.972	0.972	0.972	0.972
Digestible tryptophan (%)	0.276	0.276	0.276	0.276	0.276	0.276

[1] Positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC), negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of homeopathic additive in the feed. [2] Vitamin A (min.) 4.000.000UI/kg, vitamin D3 (min.) 800.000 UI/kg, vitamin E (min) 10.000UI/kg, vitamin K3 (min.) 1.600 mg/kg, vitamin B1 (min.) 800 mg/kg, vitamin B2 (min.) 2.000 mg/kg, vitamin B6 (min.) 800 mg/kg, vitamin B12 (min.) 8.000 mcg/kg, niacin (min.) 16 g/kg, pantothenic acid (min.) 8.000 mg/kg, folic acid (min.) 240 mg/kg, biotin (min.) 80 mg/kg, manganese (min.) 30 g/kg, zinc (min.) 40 g/kg, iron (min.) 30 g/kg, copper (min.) 5.000 mg/kg, iodine (min.) 500 mg/kg, selenium (min.) 150 mg/kg.

Table 2.2 Percentage composition of the experimental diets (Pre-initial II phase).

Ingredients	Treatments[1]					
	PC	NC	4.5	6.0	7.5	9.0
Corn 8.00%	50.684	50.707	49.820	49.525	49.229	48.933
Soybean meal 45.00%	19.383	19.379	19.516	19.562	19.608	19.653
Skim milk powder	8.919	8.919	8.919	8.919	8.919	8.919
Micronized soybeans	8.000	8.000	8.000	8.000	8.000	8.000
Sugar	4.000	4.000	4.000	4.000	4.000	4.000
Fishmeal	3.000	3.000	3.000	3.000	3.000	3.000
Soybean oil	1.776	1.768	2.072	2.173	2.274	2.376
Dicalcium phosphate	1.433	1.433	1.433	1.433	1.433	1.433
Limestone	0.745	0.745	0.745	0.744	0.744	0.744
Lysine sulfate 54.6%	0.724	0.724	0.720	0.719	0.717	0.716
L-threonine 98.0%	0.392	0.392	0.391	0.391	0.391	0.391
DL-methionine 99.0%	0.307	0.307	0.308	0.309	0.309	0.309
L-triptophan 99.0%	0.088	0.088	0.088	0.088	0.088	0.088
Premix[2]	0.300	0.300	0.300	0.300	0.300	0.300
Salt	0.232	0.232	0.232	0.232	0.232	0.232
Chlorohydroxyquinoline	0.012	-	-	-	-	-
Homeopathic additive	-	-	0.450	0.600	0.750	0.900
Total	100.000	100.000	100.000	100.000	100.000	100.000
Analyzed composition						
Dry matter (%)	90.021	89.615	91.032	89.765	88.604	88.891
Ether extract (%)	6.935	6.818	6.760	7.929	6.958	6.893
Calculated composition						
Metabolizable energy (Mcal/Kg)	3.375	3.375	3.375	3.375	3.375	3.375
Crude protein (%)	19.870	19.870	19.870	19.870	19.870	19.870
Total calcium (%)	0.973	0.973	0.973	0.973	0.973	0.973
Available phosphorus (%)	0.481	0.481	0.481	0.481	0.481	0.481
Total sodium (%)	0.219	0.219	0.219	0.219	0.219	0.219
Digestible lysine (%)	1.346	1.346	1.346	1.346	1.346	1.346
Digestible methionine + cystein (%)	0.754	0.754	0.754	0.754	0.754	0.754
Digestible threonine (%)	0.902	0.902	0.902	0.902	0.902	0.902
Digestible tryptophan (%)	0.256	0.256	0.256	0.256	0.256	0.256

[1] Positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC), negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of homeopathic additive in the feed. [2]Vitamin A (min.) 4.000.000UI/kg, vitamin D3 (min.) 800.000 UI/kg, vitamin E (min) 10.000UI/kg, vitamin K3 (min.) 1.600 mg/kg, vitamin B1 (min.) 800 mg/kg, vitamin B2 (min.) 2.000 mg/kg, vitamin B6 (min.) 800 mg/kg, vitamin B12 (min.) 8.000 mcg/kg, niacin (min.) 16 g/kg, pantothenic acid (min.) 8.000 mg/kg, folic acid (min.) 240 mg/kg, biotin (min.) 80 mg/kg, manganese (min.) 30 g/kg, zinc (min.) 40 g/kg, iron (min.) 30 g/kg, copper (min.) 5.000 mg/kg, iodine (min.) 500 mg/kg, selenium (min.) 150 mg/kg.

Table 2.3 Percentage composition of the experimental diets (Initial phase).

Ingredients	Treatments[1]					
	PC	NC	4.5	6.0	7.5	9.0
Corn 8.00%	61.106	61.130	60.243	59.947	59.651	59.356
Soybean meal 45.00%	23.686	23.682	23.819	23.865	23.911	23.956
Micronized soybeans	6.000	6.000	6.000	6.000	6.000	6.000
Fishmeal	3.000	3.000	3.000	3.000	3.000	3.000
Soybean oil	2.124	2.116	2.420	2.521	2.623	2.724
Dicalcium phosphate	1.534	1.534	1.534	1.534	1.533	1.533
Limestone	0.678	0.678	0.677	0.677	0.677	0.676
Lysine sulfate 54.6%	0.568	0.568	0.564	0.563	0.562	0.561
L-threonine 98.0%	0.278	0.278	0.277	0.277	0.277	0.277
DL-methionine 99.0%	0.233	0.233	0.234	0.234	0.234	0.235
L-triptophan 99.0%	0.056	0.056	0.056	0.055	0.055	0.055
Premix[2]	0.300	0.300	0.300	0.300	0.300	0.300
Salt	0.422	0.422	0.422	0.422	0.422	0.422
Chlorohydroxyquinoline	0.012	-	-	-	-	-
Homeopathic additive	-	-	0.450	0.600	0.750	0.900
Total	100.000	100.000	100.000	100.000	100.000	100.000
Analyzed composition						
Dry matter (%)	89.378	90.123	89.575	89.497	88.695	89.074
Ether extract (%)	6.631	6.712	6.611	6.109	7.114	6.768
Calculated composition						
Metabolizable energy (Mcal/Kg)	3.350	3.350	3.350	3.350	3.350	3.350
Crude protein (%)	20.550	20.550	20.550	20.550	20.550	20.550
Total calcium (%)	0.910	0.910	0.910	0.910	0.910	0.910
Available phosphorus (%)	0.450	0.450	0.450	0.450	0.450	0.450
Total sodium (%)	0.205	0.205	0.205	0.205	0.205	0.205
Digestible lysine (%)	1.281	1.281	1.281	1.281	1.281	1.281
Digestible methionine + cystein (%)	0.730	0.730	0.730	0.730	0.730	0.730
Digestible threonine (%)	0.833	0.833	0.833	0.833	0.833	0.833
Digestible tryptophan (%)	0.243	0.243	0.243	0.243	0.243	0.243

[1] Positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC), negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of homeopathic additive in the feed. [2]Vitamin A (min.) 4.000.000UI/kg, vitamin D3 (min.) 800.000 UI/kg, vitamin E (min) 10.000UI/kg, vitamin K3 (min.) 1.600 mg/kg, vitamin B1 (min.) 800 mg/kg, vitamin B2 (min.) 2.000 mg/kg, vitamin B6 (min.) 800 mg/kg, vitamin B12 (min.) 8.000 mcg/kg, niacin (min.) 16 g/kg, pantothenic acid (min.) 8.000 mg/kg, folic acid (min.) 240 mg/kg, biotin (min.) 80 mg/kg, manganese (min.) 30 g/kg, zinc (min.) 40 g/kg, iron (min.) 30 g/kg, copper (min.) 5.000 mg/kg, iodine (min.) 500 mg/kg, selenium (min.) 150 mg/kg.

2.3.2 Zootechnical performance and diarrhea occurrence

The animals were weighed using a scale (Universal line, Digi-tron, Curitiba) on days 1, 7, 21 and 35. The quantification of the feed supplied and wasted was performed. The average daily gain - ADG (kg/animal/day), average daily feed intake - ADFI (kg/animal/day) and feed conversion ratio - FCR of the animals were calculated. The occurrence of diarrhea was evaluated daily during the morning by observing the total amount of feces in the pen, and determining how many were liquid (diarrhea) and how many had normal consistency. Data were collected this way to find the percentage of pens with diarrhea within each treatment. Following observation, the pens were cleaned and after 24 hours it was evaluated again. The results of the data collected were divided into three periods: 1 to 7, 1 to 21 and 1 to 35 days of the experiment. Also, the diarrhea data was analyzed considering three animal sizes: small (mean: 4.574 kg), midsize (mean: 5.753 kg) and large (mean: 7.086 kg). : These mean values were based on the average of the blocks in the first day of experiment, considering both males and females. Further analysis was conducted on the overall size.

2.3.3 Blood parameters, inflammatory response and gut health

On D36, after 8 hours fasting, blood samples of one animal per experimental unit, being 7 animals/treatment, totalizing 42 piglets (with the average weight of the block considering D21) were collected by jugular vein puncture to verify blood metabolites, hemogram and absolute count of peripheral blood cells (phagocytosis process).

The blood samples for analysis of biochemical parameters were centrifuged (FANEM Excelsa Baby I Mod. 206) at 3.000 rpm for 10 minutes to obtain plasma, then, approximately 3 mL of plasma of each sample were transferred to previously identified and frozen eppendorf polyethylene microtubes for analysis of urea (Colorimetric-Enzyme method), total protein (Colorimetric-Biuret method), glucose (Enzymatic-Colorimetric method, Trinder), alkaline phosphatase (Kinetic-Colorimetric method), blood ALT (Kinetic-UV method) and AST (Kinetic-UV method). These analyses were determined by spectrophotometry (Bel SPECTRO S05) using specific Gold Analisa Diagnóstica kits.

For determination of absolute proportion and count of peripheral blood cells, 1 mL of blood collected with anticoagulant (EDTA) were used for purification of peripheral blood mononucleated cells (PBMC) by the RBC lysis method by use of ammonium chloride solution. After purification, PBMCs were incubated for 30 minutes (37°C) with 50 μL of a florescent particle conjugate for phagocytosis (pHrodo™ Green Zymosan Bioparticles™ Conjugate for Phagocytosis, from ThermoFisher Scientific, 1mg/mL). Samples were incubated with pHrodo™ for 30 minutes at 37°C and then fixed with 1% paraformaldehyde for 30 minutes at 4°C. Subsequently, the already marked and fixed cells were resuspended with 2 mL of saline phosphate buffer (PBS) + 1% Bovine Serum Albumin (BSA) + 0.01 % Azide sodium, for conservation until reading and analysis by flow cytometry (Citometer - BD FACSCalibur™). For absolute quantification of circulating leukocytes, 50 μL of absolute counting microspheres were added (C36950, CountBright from ThermoFisher Scientific).

2.3.4 Slaughter and collection of samples

On D37, 42 animals (with the average weight of the block considering D21) were slaughtered, after fasting for 12 hours, for data and biological samples collection. The pH measurement of stomach, cecum and colon content was performed using a phmeter (Hanna Instruments, Romania, HI 99163). The final portion of each organ was sealed using twine so that there was no mixing of the contents that would compromise the measurements. After that, the digestive organs (liver, stomach, small and large intestine that were empty), besides the spleen, were weighed to calculate the relative weight of the organs, according to the live weight of the animal measured at D35. The organs were weighed on digital scales (Prix Toledo 2095).

2.3.5 Microbiological analysis and antibiogram

On the second day of the experiment, feces samples were collected directly from the rectum of 10 piglets, as baseline. On the 21st day of the experiment (D21), fecal samples were also collected from 42 piglets (with the average weight of the block considering D21). Also, caecal samples were collected from these 42 piglets after slaughter (D37). The samples were stored in sterile, identified and refrigerated universal collectors for microbiological analysis. The microbiological profile was determined by counting colonies of total and thermotolerant enterobacteria and lactic acid bacteria populations. For this purpose, aliquots of collected feces were homogenized in 0.1% peptone water followed by plating on MacConkey Agar (MAC; for enterobacteria) (Macconkey, 1905) and Man, Rogosa and Sharpe Agar (MRS; for lactic acid bacteria). The inoculated plates were incubated at 37 ºC (total enterobacteria and lactic acid bacteria) and 45 ºC (thermotolerant enterobacteria) for 24-48 hours and the bacterial populations were determined in CFU/g of feces.

To identify the isolated bacteria, three colonies with morphology compatible with *E. coli* were isolated from each sample. For the selection of each one, the study carried out by Barcella (2016) was followed, selecting pink colonies with a bile precipitation halo. These, were subjected to differentiation by inoculation in Rugai with lysine. The tube contained nine biochemical tests present in one test tube: indole reaction, deamination of L-tryptophan, sucrose fermentation, glucose fermentation, urea hydrolysis, gas production, hydrogen sulfide (H2S) production, lysine decarboxylation and motility. After observing the results found in each of the tests, the IDENTAX Bacterial Identification System (v. 12) software was used for identification.

The technique used to determine the susceptibility of *E. coli* was the Mueller-Hinton agar disk-diffusion method. In Petri plates containing Mueller-Hinton agar, 100 µL of a solution containing 108 CFU/mL of each bacterium identified as *E. coli* were inoculated and evaluated separately. After complete drying, 4 discs were placed with the previously chosen antibiotics (2 discs for each antimicrobial). The following antibiotics were used: amikacin (30 µg), amoxicillin/clavulamic acid (30 µg), ampicillin (10 µg), azithromycin (15 µg) ciprofloxacin (5 µg), chloramphenicol (30 µg), streptomycin (10 µg), gentamicin (10 µg), erythromycin (15 µg), sulfazotrim (25 µg), tetracycline (30 µg) and tobramycin (10 µg). After all the processes, the plate was incubated at 37°C for 24h, for subsequent reading of the results.

2.3.6 Histopathological alterations

Immediately after removal of the organs, segments of approximately 3 cm in length from the duodenum (removed 15 cm from the stomach sphincter) and jejunum (removed from the medial portion of the jejunum) were collected, washed with saline solution and stored in 50 mL tubes containing buffered formalin solution. For the analysis of intestinal morphology, the ISI (Scoring System Methodology) methodology adapted from Kraieski et al. (2017) was followed: each histopathological alteration contains an impact factor ranging from 1 to 3; the higher the factor the more harmful is the alteration (Table 2.4). Also, the extent of each lesion or frequency observed, if compared with unaffected tissues, was also evaluated with scores ranging from 0 to 3, being: score 0 - no lesion found, score 1 - alteration of up to 25% of the tissue, score 2 - alteration between 25 and 50% of the tissue, score 3 - alteration of more than 50% of the tissue. For the final value of the ISI methodology, the impact factor of each alteration is multiplied by the respective score, and the result of all alterations are summed.

Table 2.4 Impact factor of each alteration assessed according to the ISI methodology.

Alteration	Impact Factor*
Inflammatory infiltrate	3
Congestion	1
Desquamation	2
Coccidiosis	3
Bacterial clumps	3
Bastonetes	3
Cystic crypts	2
Mucus	1
Necrosis	3
Edema	2

* Adapted from Kraieski et al. (2017).

2.3.7 Statistical analysis

The initial body weight of the piglets was compared using general linear models and it was included as a covariate for zootechnical performance considering NC and homeopathic levels (quantitative variable), also, the positive control was compared with each treatment using Dunnett's test. Relative organ weights, pH of the digestive contents, hemogram, biochemical parameters, flow cytometry and microbiology variables were also analyzed using general linear models (without the covariate) and Dunnett's test. Diarrhea occurrence was analyzed using Chi-square test: the analysis was performed between weight groups (small, midsize and large) within each treatment and the occurrence of diarrhea between treatments within each weight group. Regarding non-parametric variables for flow cytometry, Kruskal-Wallis test was used, followed by Dunn's multiple comparisons post-test and intestinal histopathology data were analyzed by the Mann-Whitney test performing a pairwise comparison between all treatments. All analysis was performed with statistical software SPSS 25 (IBM Corp., 2017). P values less than 0.05 were considered significant.

2.4 Results

Including the initial weight of the animals as cofactor, for all periods, there was no difference between treatments for body weight (BW), ADG, ADFI and FCR (Table 2.5). For the evaluation of diarrhea occurrence (DO), when the different animal sizes are compared, treatments 4.5 and 9.0 maintained the values in the three periods evaluated, while the diarrhea percentage of the other treatments was different as the size changed (Table 2.6). The treatments that had lower values of diarrhea occurrence in the overall animal population on days 1 to 7 were PC, NC, 4.5, and 7.5. On days 1 to 21, the treatments with lower values of diarrhea were NC, 4.5, and 7.5. Throughout the entire experimental period, only NC showed lower values of diarrhea, while the levels 4.5 and 7.5 were equal to PC ($P<0.05$).

Table 2.5 Performance means of nursery piglets that received homeopathic feed additive.

Items	Treatments[1]							
	PC	NC	4.5	6.0	7.5	9.0	SEM[2]	*P-Value*[3]
Initial body weight† (kg)	5.940	6.160	5.670	5.460	5.350	5.170	0.104	0.002
Days 1 to 7								
BW[4] 7	7.290	7.240	6.860	6.460	6.540	6.140	0.127	0.832
ADG[5] (kg/day)	0.193	0.155	0.171	0.143	0.171	0.138	0.009	0.832
ADFI[6] (kg/day)	0.198	0.184	0.207	0.171	0.180	0.162	0.003	0.828
FCR[7]	1.174	1.158	1.325	1.204	1.193	1.306	0.053	0.351
Days 1 to 21								
BW21 (kg)	12.253	12.561	12.039	11.530	11.500	11.169	0.221	0.545
ADG (kg/day)	0.300	0.305	0.303	0.289	0.293	0.285	0.007	0.545
ADFI (kg/day)	0.399	0.383	0.388	0.376	0.371	0.359	0.006	0.125
FCR	1.398	1.266	1.410	1.385	1.331	1.303	0.030	0.613
Days 1 to 35								
BW35 (kg)	21.850	21.580	21.110	20.280	20.270	20.310	0.321	0.291
ADG (kg/day)	0.454	0.441	0.441	0.423	0.426	0.432	0.007	0.291
ADFI (kg/day)	0.657	0.636	0.622	0.617	0.616	0.609	0.006	0.066
FCR	1.473	1.486	1.473	1.487	1.500	1.420	0.022	0.575

[1] Positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC), negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of homeopathic additive in the feed. [2] Standard error of the mean. [3]P-value for linear regression. [4]Body Weight. [5]Average daily gain. [6]Average daily feed intake. [7]Feed conversion ratio. † Linear effect ($P<0.05$) of the homeopathic additive levels on the Initial body weight ($y= - 0.109452x + 6.15606$ $R^2= 0.0859$).

Table 2.6 Occurrence of diarrhea (%) by size of nursery piglets that received homeopathic feed additive.

Piglet size	Treatments[1]					
	PC	NC	4.5	6.0	7.5	9.0
Days 1 to 7						
Small (%)	9.21 ^{bA}	5.41 ^{bA}	8.93 ^{bA}	19.61 ^{aA}	9.26 ^{bA}	7.69 ^{bA}
Midsize (%)	12.96 ^{aA}	9.30 ^{abA}	5.45 ^{bA}	6.52 ^{abB}	0.00 ^{cB}	15.56 ^{aA}
Large (%)	0.00 ^{bB}	0.00 ^{bB}	6.49 ^{aA}	0.00 ^{bC}	10.20 ^{aA}	10.20 ^{aA}
Overall (%)	8.09 ^{abc}	4.30 ^{c}	6.91 ^{bc}	11.86 ^{a}	7.35 ^{bc}	10.69 ^{ab}
Days 1 to 21						
Small (%)	4.59 ^{bA}	1.96 ^{dA}	4.79 ^{bA}	9.62 ^{aA}	2.51 ^{cdA}	4.49 ^{bcA}
Midsize (%)	8.89 ^{aA}	6.67 ^{abA}	3.82 ^{bcA}	2.99 ^{cdB}	1.74 ^{dA}	8.16 ^{aA}
Large (%)	1.59 ^{bcB}	0.00 ^{dB}	1.90 ^{bcA}	0.88 ^{cdB}	3.82 ^{abA}	5.43 ^{aA}
Overall (%)	5.01 ^{a}	2.00 ^{b}	3.24 ^{b}	6.13 ^{a}	2.70 ^{b}	5.89 ^{a}
Days 1 to 35						
Small (%)	2.82 ^{bcAB}	1.14 ^{dA}	3.64 ^{bA}	5.82 ^{aA}	1.71 ^{cdA}	2.49 ^{bcdA}
Midsize (%)	4.84 ^{aA}	4.15 ^{abA}	2.36 ^{bcA}	1.67 ^{cB}	2.19 ^{bcA}	4.67 ^{abA}
Large (%)	0.84 ^{cdB}	0.00 ^{dB}	1.38 ^{cA}	1.40 ^{cB}	6.51 ^{aA}	3.50 ^{bA}
Overall (%)	2.86 ^{ab}	1.33 ^{c}	2.32 ^{b}	3.75 ^{a}	3.15 ^{ab}	3.48 ^{a}

[1] Positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC), negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of homeopathic additive in the feed. ^{abcd}Different lowercase letters on the same row indicate statistical difference by Chi-square test (P<0.05). ^{AB}Different capital letters on the same column indicate statistical difference by Chi-square test (P<0.05).

The organ weights, pH of digestive contents and serum biochemical parameters showed no difference between treatments (data not shown). Regarding hemogram, it was possible to observe that the higher the levels of the additive the higher the values of hemoglobin, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) (P<0.05) (Table 2.7).

Regarding phagocytic profile, Piglets that received the negative control diet treatment had a higher percentage of monocytes in relation to the total number of circulating leukocytes compared to the last two levels of the additive (P<0.05). For the degree of activity for phagocytic monocytes, levels 6.0, 7.5 and 9.0 of homeopathic additive showed lower values when compared to the positive control. The negative control and the first level of the homeopathic additive were equal to PC (P<0,05). Furthermore, it was possible to identify a difference between the levels: the higher the addition of homeopathic in the feed, the lower the phagocytic activity of monocytes (P<0.05). For the quantification of total undifferentiated leukocytes, animals from the negative control treatment (NC) showed a lower quantity of defense cells when compared to the positive control. Also, the higher the level of homeopathic additive, the greater the amount of leukocytes found (P<0.05) (Figure 2.1).

Table 2.7 Hemogram values of nursery piglets that received homeopathic feed additive.

Items	Treatments¹						SEM²	P-Value³
	PC	NC	4.5	6.0	7.5	9.0		
Red blood cells (Log million/µL)	6.806	6.817	6.823	6.820	6.816	6.829	0.015	0.262
Hemoglobin (g/dL) †	12.214	10.886	11.867	12.257	12.283	12.417	0.425	0.003
Hematocrit (%)	36.429	35.000	37.167	37.286	37.286	37.833	1.325	0.097
MCV (fL) [4]	53.738	54.621	55.389	56.196	54.607	56.451	1.056	0.339
MCH (pg) [5] ††	17.700	16.967	17.933	18.451	18.365	18.576	0.479	0.013
MCHC (%) [6] †††	33.554	31.100	32.436	32.823	32.445	32.924	0.673	0.048
Segmented (%)	45.286	45.143	44.714	45.429	42.286	49.571	4.337	0.723
Eosinophils (%)	1.000	0.857	1.428	0.857	0.714	0.286	0.346	0.222
Monocyts (%)	2.571	1.142	1.857	1.142	1.857	1.571	0.507	0.485
Neutrophils (%)	45.571	45.143	45.000	45.571	42.286	49.714	4.317	0.714
Plasma proteins (g/dL)	6.129	5.800	5.971	5.857	6.114	5.886	0.129	0.326
Platelets (Log mm³)	5.700	5.661	5.648	5.670	5.634	5.680	0.038	0.470

[1] Positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC), negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of homeopathic additive in the feed. [2] Standard error of the mean. [3]P-value for linear regression; [4]Mean corpuscular volume; [5]Mean corpuscular hemoglobin; [6] Mean corpuscular hemoglobin concentration; † Linear effect ($P<0.05$) of the homeopathic additive levels on the Hemoglobin variable ($y= 0.178095x + 10.9838$ $R^2=0.2486$). †† Linear effect ($P<0.05$) of the homeopathic additive levels on the MCH variable ($y = 0.183907x + 17.0677$ $R^2= 0.1772$). ††† Linear effect ($P<0.05$) of the levels of the homeopathic additive on the variable MCHC ($y= 0.195377x + 31.2997$ $R^2=0.1168$).

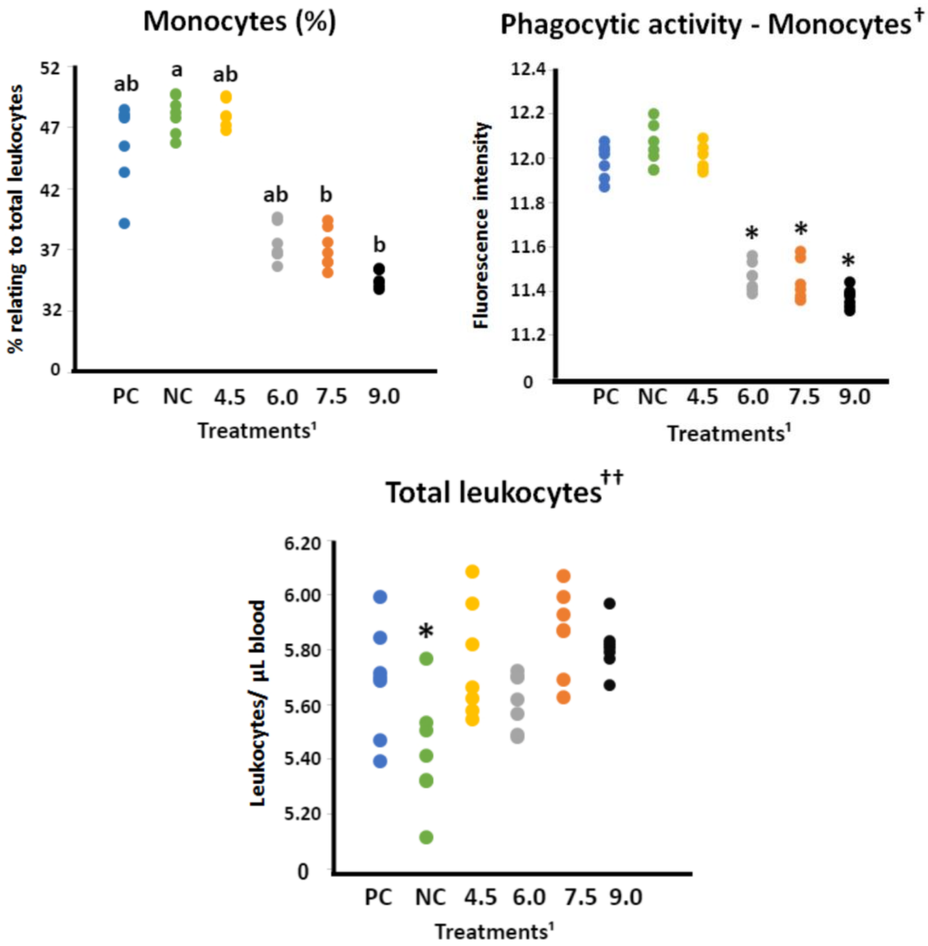

Figure 2.1 Phagocytic profile by flow cytometry in nursery piglets that received homeopathic feed additive.

[1] Positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC), negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of homeopathic additive in the feed. [ab] Different letters represent statistical differences according to the Kruskal-Wallis Test followed by Dunn's multiple comparisons post-test ($P<0.001$). * Statistical difference by the Dunnett test ($P<0.05$) for PC. † Linear effect ($P<0.001$) of the homeopathic additive levels on phagocytic activity of monocytes ($y= - 0.0843791x + 12.1144$ $R^2= 0.7534$). †† Linear effect ($P<0.05$) of the homeopathic additive levels on total leukocytes ($y= 0{,}044186x + 5.45453$ $R^2 = 0{,}3990$).

It was observed an increase in the population of lactic acid bacteria comparing day 21 (D21) with day 2 (D2 - baseline) ($P<0.05$), regardless of the treatment received by the animals (Table 2.8). In addition, animals of groups NC, 4.5, 7.5 and 9.0 also showed an increase of these populations when compared to PC on D21. A further result is that the higher the level of homeopathic, the lower the populations of lactic acid bacteria found. Regarding thermotolerant enterobacteria, there was no difference between D2 and D21 for any groups of animals. For D21, the animals receiving 4.5 and 6.0 kg/ton of homeopathic additive showed lower population than the positive control (P<0.05). For total enterobacteria, piglets from 4.5 treatment showed lower population counts on day 21 compared to baseline. For D21, animals that received 4.5 treatment had a lower population than the positive control (P<0.05). There was no statistical difference (P>0.05) in bacterial culture count for caecal content samples (data not shown).

Table 2.8 Population (mean ± standard deviation Log CFU/g) of lactic acid bacteria and thermotolerant and total enterobacteria from feces collected from nursery piglets that received homeopathic feed additive.

	Treatments[1]						SEM[2]	P-Value[3]
	PC	NC	4.5	6.0	7.5	9.0		
Lactic acid bacteria (Log CFU/g)								
D2			6.65 (±2.12)^{B}					-
D21†	8.82 (±0.64)^{A}	9.83 (±0.66)^{A}*	9.63 (±0.64)^{A}*	9.30 (±0.62)^{A}	9.70 (±0.64)^{A}*	9.43 (±0.62)^{A}*	0.147	0.048
Thermotolerant enterobacteria (Log CFU/g)								
D2			7.38 (±2.85)					-
D21	8.53 (±1.33)	7.84 (±1.35)	6.54 (±1.38)*	7.12 (±1.33)*	7.73 (±1.34)	8.30 (±1.35)	0.334	0.255
Total enterobacteria (Log CFU/g)								
D2			7.83 (±2.11)^{B}					-
D21	8.29 (±1.31)^{B}	7.71 (±1.29)^{B}	5.86 (±1.57)^{A}*	6.86 (±1.30)^{B}	7.84 (±1.30)^{B}	8.06 (±1.32)^{B}	0.368	0.401

[1] Positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC), negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of homeopathic additive in the feed. [2] Standard error of the mean. [3] P-value for linear regression. ^{AB}Statistical difference by the Dunnett test (P<0.05) for baseline. * Statistical difference by the Dunnett test (P<0.05) for PC. † Linear effect (P<0.05) of the homeopathic additive levels on the lactic acid bacteria ($y = -0.0396821x + 9.80066$ $R^2 = 0.0795$).

Regarding the susceptibility of *E. coli* found in feces from D2 sampling, out of 10 feces collected, only one of these detected the growth of *E. coli*. Bacterial resistance for *E. coli* was observed to eight antimicrobials, which were ampicillin, azithromycin, ciprofloxacin, chloramphenicol, erythromycin, streptomycin, sulfazotrim and tetracycline. In the samples from D21, the highest percentage of resistance was found for the antimicrobial tetracycline (PC; NC; 4.5; 6.0 and 7.5 = 100% resistance), only the last homeopathy level showed a rate of 85.7% resistance. In the collection of caecal content, a total of 12 *E. coli* were identified, and none was identified in the animals that received the first level of the homeopathic additive. When analyzing the results, a great resistance to antimicrobials is observed. Among the 12 antibiotics used, *E. coli* showed 100% resistance to 4 of them (amoxicillin, erythromycin, streptomycin and tetracycline).

As for the histopathology data, it was possible to observe that all treatments showed less pathological alterations in the duodenum compared to the NC, as shown in Table 2.9.

Table 2.9 Means of histopathological alterations in nursery piglets that received homeopathic feed additive.

Items	Treatments[1]					
	PC	NC	4.5	6.0	7.5	9.0
Duodenum						
Pathological alterations	8.000 [b]	16.571 [a]	5.290 [b]	8.290 [b]	5.430 [b]	5.430 [b]
Jejunum						
Pathological alterations	4.857	12.571	11.000	8.714	5.429	4.714

[1] Positive control – basal diet + 120 mg/kg of chlorohydroxyquinoline (PC), negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of homeopathic additive in the feed. [ab] Different letters on the same row indicate statistical difference by Mann-whitney test (P<0.05).

3.5 Discussion

Due to the growth-promoting properties of the antimicrobials, it was expected that the animals in the positive control group would show some improvement over the negative control regarding performance, which did not occur. Sbardella et al. (2016) stated that the efficacy of these medications varies according to the pig raising conditions, with better results being obtained in commercial farms than in experiments. Furthermore, other studies have shown that chlorohydroxyquinoline did not present positive effects on the performance of weaned piglets (Cairo et al., 2018; Gois et al., 2016). The authors justified that this result was mainly due to the strict sanitary control of the facilities, which resulted in a lower level of challenge for the animals. Andrade et al. (2016) also did not observe an improvement in animal performance using this antibiotic when compared to dietary nucleotides. Regarding the homeopathic additive, our findings do not align with those of Lima et al. (2022): they reported positive performance results in finishing pigs after 97 days of experimental period. Our study focused on piglets, which possess a less mature intestinal tract compared to adult animals (Santos et al., 2022), this distinction is a possible reason for the discrepancy in results, since some of the homeopathic compounds were similar.

Regarding diarrhea, levels 4.5 and 9.0 showed properties capable of maintaining diarrhea values regardless of animal size: their effects do not only benefit large animals, as is the case for PC and NC. However, when the results of the overall animals are observed, it is noticeable that the NC treatment presents advantages over both additives. This outcome once again prompts us to contemplate the conditions under which the animals were being kept. The facilities are unable to fully represent an environment of a commercial farm, since given the prolonged vacancy, the environment lacked the challenges necessary to fully unlock the potential of the additives. Even with favorable results of the negative control, levels 4.5 and 7.5 of the homeopathic additive also showed satisfactory values with regard to overall animals, moreover, both levels were equal to or even better than the positive control in certain periods of the experiment. As in the present study, Fortuoso et al. (2017) used Enterococcinum (9 CH) along with other compounds in order to verify its effects against diarrhea. In the experiment it was shown that neonatal diarrhea in cattle reduced 50%.

The variables hemoglobin, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) presented a difference with regard to the levels of homeopathic: the higher the level, the higher the values found, however, It can be stated that the findings are within the reference interval for the domestic pig (Brooks et al., 2022). It must be noted that the main problem concerning blood analysis is the stress that handling can cause to the animals, which can easily change the values, being the largest source of hematologic variation (Brooks et al., 2022). Although most published intervals are comparable, the ranges for most elements of porcine blood are quite wide. In addition, when the animal is handled under high stress, there is an increase of neutrophils in the blood, which did not occur in the present study.

Regarding total and thermotolerant enterobacteria, the 4.5 treatment allowed a reduction in the number of colonies when compared to the positive control. Homeopathics may have the ability to alter the microbiota, since in the study by Vacaras et al. (2023), which investigated whether there is a correlation between microbiota and multiple sclerosis, a treatment containing *Ignatia, Calcarea Carbonica* and other compounds modified intestinal colonies in human patients. A substance that most probably could have assisted the piglets was Lac defloratum (9 CH), skimmed cow's milk. Its introduction into homeopathy occurred in 1879 by Samuel Swan (Wegener, 2003) and its potential against gastrointestinal disorders has already been tested in infants by Raak et al. (2019), obtaining positive results. As for the bacterial population in the feces, comparing D2 with D21, there was a significant increase in the population of lactic acid bacteria in all treatments. The greater bioavailability of nutrients, due to the adaptation in the consumption of solid diet, could be one of the possible explanations for the observed increase, since, according to Collier et al. (2003), lactic acid bacteria are present mainly in the small intestine, where the greatest absorption of nutrients occurs. Furthermore, the lower population of lactic acid bacteria for the PC in comparison to the other treatments is directly related to the broad-spectrum property of the antibiotic, which influences both populations of beneficial and harmful bacteria (Gois et al., 2016).

Regarding bacterial resistance, tetracycline showed the highest rate among the 12 antimicrobials tested, reaching 100% resistance in many cases and presenting as the lowest level of resistance 85.7%. In the same proposal, Moredo et al. (2017) describe the high resistance (62%) of *E. coli* to antibiotics commonly used in pig farming, highlighting resistance to ampicillin, streptomycin, chloramphenicol and tetracycline. The same occurs with the study of Brito and Tagliari (2000), when finding less than 30% of sensibility to the antimicrobials streptomycin, tetracyclines and chloramphenicol. The present study corroborates with both studies described, being the resistance values of *E. coli* even higher for the antimicrobials tested. It is important to highlight that homeopathic additives do not promote bacterial resistance, which is one of their major advantages (Braccini et al., 2019).

The finding that all the homeopathic treatments and the positive control showed lower pathological alterations in the duodenum compared to the negative control proves the beneficial properties of additives (Sales et al., 2015). The homeopathic provided to the treated animals contained Colibacillinum (9 CH), a substance already used in a study demonstrating the improvement it causes in the intestinal tract, leading to a lower incidence risk of digestive problems (Da Silva et al., 2021b).

As a limitation of the experiment, we can cite the initial weight of the piglets, which, even after randomization, presented disparity between treatments. This inaccuracy was fixed by adding the initial weight as a covariate in the statistical model. In addition, a point that should also be taken into account is that the animals did not go through challenging situations, which did not allow the additives to show their full potential. Likewise, there are other conditions that can influence these values, such as aspects concerning the sow, the pen, the litter and the piglet (Johansen et al., 2004).

3.6 Conclusion

The present study showed that the use of the lower level of the homeopathic additive (basal diet with addition of 4.5 kg/ton of homeopathic additive) can be a good alternative for the substitution of antibiotics, as it provided equal or better results than the positive control (basal diet + 120 mg/kg of chlorohydroxyquinoline) regarding diarrhea, resulted in lower populations of total and thermotolerant enterobacteria and higher populations of lactic acid bacteria in the feces compared to the PC and, additionally, exhibited fewer histopathological alterations in the duodenum when compared to the negative control (basal diet without additives).

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CHAPTER 3

This manuscript will be submitted to the Homeopathy Journal

Dietary homeopathic additive shows different behavioral effects on castrated male and female nursery piglets

[a]Paula, G. Z., [a]Bez, I. C. C., [a]Garrido, L. F. C., [a]Catoia, M. R. R., [a]Daros, R. R., [a]Monteiro, K. M., [b]Carvalho, P. L. O., [a*]Costa, L. B

[a]*Graduate Program in Animal Science, School of Medicine and Life Sciences, Pontifícia Universidade Católica do Paraná, Curitiba, Brazil.*

[b]*Universidade Estadual do Oeste do Paraná, Marechal Cândido Rondon, Brazil*

**Corresponding author: Leandro Batista Costa (L. B. Costa)*

Dr. - Associate Professor - Animal Nutrition/Pork Production/Animal Welfare

School of Medicine and Life Sciences - Pontifícia Universidade Católica do Paraná

Imaculada Conceição Street, 1155 - Prado Velho, Curitiba, Paraná, Brazil

Mobile: +55 41 9103-8743

batista.leandro@pucpr.br

3.1 Abstract

Homeopathy is an alternative therapy widely utilized nowadays. However, studies involving its use in the behavior of production animals are still scarce. The aim of the present study was to evaluate the effect of dietary homeopathic additive on the behavior of male and female nursery piglets. The experiment was double-blind, lasted 37 days and involved 105 animals weaned at 21±1.3 days, with an initial weight of 5.56±1.15 kg. Piglets were allotted to five treatments in a completely randomized block design with seven repetitions using three piglets per experimental unit. The treatments were: negative control – basal diet without additives (NC); basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of homeopathic additive in the feed. Piglets were subjected to behavioral tests starting at 37 days old (D16 of experiment), where different tests were performed: open field (OF), novel object (NO), sociability, discriminative learning and judgment bias. Also, on three days of the experimental period, the piglets' reactivity during the weighing (RDW) was evaluated. Homeopathic levels and sex were included as factors, as well as its interaction. For the OF and NO data, linear regression analysis was performed; for the RDW test, mixed linear regression, and for sociability and discriminative learning a controlled two-way Anova was applied. Vocalizations were lower for females than for males during the OF test (P<0.05). For escape attempts, in the RDW test, the higher the level of the homeopathic additive, the lower was the value of the variable for females and higher for males (interaction sex X treatment). There were no statistical differences for the tests of NO, sociability, discriminative learning and judgment bias. Our study indicates that the sex of the piglet may play a role in influencing the response of the additive.

Keywords: Behavioral tests, Fear, Pigs, Stress

3.2 Introduction

Homeopathy is one of the most widely used complementary or alternative therapies in several European countries as a form of treatment for a variety of illnesses[1]. Its use in animals has been studied, but mostly to verify its efficacy against diseases: few are the studies that provide it as an additive in the diet of pigs, and even fewer are the studies that compare behavior between females and castrated males piglets.

The homeopathy treatment consists in treating a disease with substances that can cause the same clinical signs that the patient is feeling, but in minimal doses; it has even been used to treat behavioral deviations in humans[2]. An advantage of homeopathics is that since they are ultra-diluted of animal, vegetable and mineral components, their residues do not contaminate the environment or meat[3], making them perfect for use as an animal additive and possibly behavior modulator.

Piglets may express different types of behaviors, some of which may be reflections of stressful situations. Stress is a reaction that can be triggered by the weaning phase of the animals. It can occur due to the large amount of stimuli that the brain interprets, thus responding in an exaggerated way[4]. Events such as separation from the sows, fight for dominance and environment/diet change[5] are examples of challenging scenarios. The provision of a suitable feed additive could decrease not only the stress and fear of the animals, but also bring positive consequences with regard to their exploratory behavior and learning ability. In mice, studies have already been performed in which anxiolytic and emotional response modulating properties of homeopathics have been discovered[6,7].

In this study, we aimed to determine the effect of the homeopathic additive on the behavior and whether sex can influence the outcome. To this end, behavioral tests were used, which have the potential to assess some aspects of animal welfare, such as emotional state[8].

3.3 Methods

The experiment was performed at the Swine Research Unit (UPS) located at the Gralha Azul Experimental Farm of the Pontifical Catholic University of Paraná (PUCPR). The study was approved by the Ethics Committee on Animal Use (CEUA) under protocol number: 01720 (2nd version). The experiment was double-blind, lasted 37 days and involved 105 animals (54 castrated male and 51 female piglets) weaned at 21±1.3 days, with an initial weight of 5.56±1.15, which were assigned to five treatments in a completely randomized block design with 7 repetitions and 3 piglets per experimental unit (distributed according their weight and sex).

The piglets were housed in suspended pens (1.5m x 1.5m) with semi-automatic feeders, water dispensers and plastic slatted floors. From the time of arrival of the animals at the facility until the start of the study with the experimental diets (four days), all piglets received an isonutritive post-weaning diet to ensure that they would begin the experiment in good condition. From the fifth day on, each animal group received its specific treatment. The treatments were composed of five experimental diets: negative control – basal diet without additives (NC), and basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of homeopathic additive in the feed. The inclusion of the additive in the diet of animals followed recommendations provided by the manufacturer, based on studies of product development.

3.3.1 Composition of the product

The homeopathic complex is composed of: *Ignatia amara* (30 CH), *Chamomilla* (15 CH), *Silicea terra* (15 CH), *Staphysagria* (15 CH), *Colibacillinum* (9 CH), *Enterococcinum* (9 CH), *Lac defloratum* (9 CH), *Aethusa cynapium* (6 CH), *Artemisia abrotanum* (6 CH), *Calcarea carbonica* (6 CH), *Thyreoidinum* (6 CH), *Thymulin* (6 CH), Vehicle (Calcium Carbonate – 1000g). Most of these compounds are indicated as anxiolytics, antidepressants, as well as immunomodulators and were provided by Real H - Animal nutrition and health (Campo Grande, Mato Grosso do Sul, Brazil). Its use is intended for piglets and there is no contraindication. The experiment lasted 37 days and the animals were fed *ad libitum*, until the end of the experimental period. The diets were formulated to meet the piglets' requirements for the pre-initial I (1 to 7 days of experimentation), pre-initial II (8 to 20 days of experimentation) and initial (21 to 37 days of experimentation) phases, following Rostagno et al. (2017)[9].

3.3.2 Pre-dietary treatments observations

After the piglets have being placed in the pen, while receiving the basal feed and based on the standard time of dominance establishment[10, 11], social interactions between them during the first 72 hours were recorded. During two minutes, for each pen, agonistic interactions were observed. They are categorized as negative interactions and fighting between animals, and the author, receptor and the winner was registered. Scan sampling, known as instant observation of positive interactions and common piglet behaviors (sleeping, exploring the pen, eating and drinking) was also performed. Both observations were done by the same two researchers with previous training for recognizing cited behaviors. A total of 10 rounds of observations were conducted over the course of 72 hours (20 minutes in total per pen).

At the end of the first and second day, the amounts of lesions on the animals were recorded. The count of recent skin lesions was also used to evaluate the intensity of negative interactions. Through these parameters, negative interactions, counting the animals that started and won more fights, and also had fewer lesions, it was possible to define the dominant and subordinate animals of each pen.

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3.3.3 Behavioral tests

Behavioral tests were applied when the piglets reached 37 days of age (D16 of experiment). The following tests were performed: open field, novel object, sociability, discriminative learning and judgement bias. In the open field test, the dominant piglet of each pen (total n=35) was taken to a structure that served as an ante-room of an unknown environment (arena). The arena was built specifically for behavioral tests during the project, and had an area of 9.00

m² (Figure 3.1). In addition, nine quadrants of 1 m² each were marked on the floor.

Figure 3.1 Arena used for behavioral tests.

After the release of the animal to the arena, during two minutes, different behaviors of the animal were observed by the researchers: quantity of quadrants explored, time spent exploring the arena and vocalizations. Then, the animal was taken back to its pen. On the same day of the open field test, in the afternoon, the novel object test was performed with the same animals. For the novel object test, an object, that had never been seen by the piglets (conventional blue cement feeder), was placed in the arena (Figure 3.2). For two minutes, the researchers observed frequency and duration of contact with the object.

Figure 3.2 Novel Object test.

On D17 of the experiment, a sociability test was also applied with a total of 30 piglets. In this test, two dominant piglets, from the same treatment, but different pens, were placed in the arena. In the center of the arena was a feeder with chopped apples. The behavioral test lasted three minutes and the researchers observed fighting, escape attempts, negative interactions and eliminatory behaviors. From D23 onwards, discriminative learning was initiated, and the judgement bias test was performed on D35.

The discriminative learning test was performed in two phases and both lasted 2 minutes for each session/piglet. For this test, the 35 dominant animals were used. During phase one, a feeder (containing apple slices) was put on one side of an arena and the opposite side remained empty. When the piglet was released in the arena, the following variables were recorded: latency and type of interaction with the feeder/apple (did not touch the feeder; touched the feeder; ate the apple). In order for the piglet to move on to the next phase, it had to meet the criterion of three consecutive sessions eating the apple or touching the feeder. During phase two, two feeders were inserted in the same arena on opposite sides, one of them had apple slices inside it (positive reinforcement), and the other had no content (positive punishment). If the animal encountered the empty feeder, an aversive crumpling sound was performed as punishment. The following variables were recorded: latency, type of interaction with the feeder and number of punishments. In order for the piglet to move on to the judgement bias test, it had to meet the criterion of three consecutive sessions eating the apple or touching the feeder without receiving punishment. For the judgment bias test, only one feeder (containing apple slices) was inserted between where it was located positive reinforcement and positive punishment. The researchers observed the latency and type of interaction with the feeder.

Also, the piglets' reactivity was evaluated while being weighed during the animals' feed change on days 7, 21 and 35 of the experiment. On these days, the animals were placed in a large plastic box that was on a scale so that the observers could assess the animal's behavior for one minute. The description of all evaluated behaviors can be found in the Table 3.1.

Table 3.1 Description of the behaviors observed during the behavioral tests.

Tests	Behavior	Description
Open Field	Explored quadrants	Number of quadrants explored (quantity)
Open Field	Time exploring the arena	Time spent exploring the arena (seconds)
Open Field and Reactivity during weighing	Vocalizations	Screams, squeals or grunt-squeals (quantity)
Novel Object	Frequency	Number of times the piglet touched the object (quantity)
Novel Object	Duration of contact	Contact time with the object (seconds)
Sociability	Fighting	Fight between two piglets (seconds)
Sociability	Negative interactions	Ear or tail biting (quantity)
Sociability	Eliminatory behaviors	Defecating or urinating (quantity)
Sociability and Reactivity during weighing	Escape attempts	Number of times the animal jumped/ raised its front legs against the sides of the weighing scale (quantity)
Discriminative learning (phase one and two) and Judgement bias	Latency	Time to reach the feeder (seconds)
Discriminative learning (phase one and two) and Judgement bias	Type of interaction with the feeder	Interaction or lack of interaction with the feeder/apple (didn't touch the feeder; touched the feeder; ate the apple)
Discriminative learning (phase two)	Number of punishments	Number of times the punishment was applied (quantity)
Reactivity during weighing	Resistance to movement	No movement of any portion of the piglet's body visible for a period > 2s. The duration comprises the beginning of the standstill to any movement of the body (seconds)

3.3.4 Statistical analysis

For all behavioral tests, homeopathic levels and sex were included as factors, as well as its interaction. For the behaviors in the Open Field and Novel Object tests, linear regression was performed. For the reactivity test during weighing, mixed linear regression was performed. For the tests of sociability and discriminative learning, a controlled two-way Anova analysis for repeated measures was performed, P values less than 0.05 were considered significant. R Core Team (2020)[12,13] statistical software was used for all behavioral tests.

3.4 Results

The Table 3.2 shows the results regarding all tests performed during the experimental period. Concerning vocalizations, during open field test, there was a statistical difference between sex: castrated male piglets exhibited a higher mean vocalization compared to females, regardless of the treatment, with 38.5 vocalizations for males and 19.7 for females. For the variable escape attempts, on the reactivity during weighing test, there was a statistical difference for the interaction between treatment and sex, in which the higher the level of the homeopathic additive, the greater was the number of escape attempts by males and lower for females (Figure 3.3).

For the sociability test, only the variable “negative interactions” was analyzed. For the variables “escape attempts”, “fights” and “eliminatory behaviors”, in the sociability test, we did not have enough data that allowed statistical comparisons, since these behaviors were not consistently observed. Regarding the discriminative learning test, the variables “number of attempts” and “number of punishments” were analyzed, and there was no statistical difference between treatments ($P>0.05$). Of the 35 animals, only 12 reached the learning criteria, passing to the judgment bias, presenting an average of 10.6 attempts to reach this threshold. For the judgment bias test, we did not have enough data that allowed statistical analysis, since a minimal number of animals reached this phase.

Table 3.2 Mean values (± standard error) from behavioral tests performed on nursery piglets that received homeopathic feed additive.

Items	Treatments[1]					*P-value*		
	NC	4.5	6.0	7.5	9.0	T[2]	S[3]	TxS
Open Field								
Vocalizations (Quantity)*	41.2 (±8.5)	18.8 (±8.5)	25.5 (±8.5)	36.5 (±8.5)	27.5 (±8.5)	0.479	0.011	NS
Explored quadrants (Quantity)	14.4 (±2.5)	17.3 (±2.5)	13.7 (±2.5)	19.7 (±2.5)	14.0 (±2.5)	0.277	0.264	NS
Time exploring the arena (Seconds)	48.9 (±5.9)	59.6 (±5.9)	51.2 (±5.9)	61.2 (±5.9)	49.4 (±5.9)	0.497	0.339	NS
Novel Object								
Frequency (Quantity)	1.7 (±0.5)	0.8 (±0.5)	1.1 (±0.5)	1.4 (±0.5)	1.4 (±0.5)	0.907	0.751	NS
Duration of contact (Seconds)	7.6 (±4.1)	5.0 (±4.1)	6.1 (±4.1)	12.8 (±4.1)	10.0 (±4.1)	0.800	0.857	NS
Reactivity During Weighing								
Escape attempts (Quantity)**	0.6 (±0.3)	1.1 (±0.3)	0.9 (±0.3)	0.8 (±0.4)	0.8 (±0.4)	0.038	0.021	0.004
Vocalizations (Quantity)	9.9 (±2.1)	14.9 (±2.1)	17.04 (±2.2)	11.2 (±2.2)	13.25 (±2.1)	0.201	0.560	NS
Resistence to movement (Seconds)	53.3 (±1.0)	52.1 (±1.0)	50.3 (±1.0)	50.9 (±1.0)	50.2 (±1.0)	0.235	0.096	NS
Sociability								
Negative interactions (Quantity)	2.1 (±1.3)	3.0 (±1.3)	1.3 (±1.3)	3.8 (±1.3)	1.6 (±1.3)	0.754	0.287	NS
Discriminative learning behavior								
Number of attempts on phase 1 (Quantity)	3.9 (±0.5)	4.6 (±0.5)	4.4 (±0.5)	3.8 (±0.5)	4.1 (±0.5)	0.925	0.370	NS
Number of attempts on phase 2 (Quantity)	10.2 (±0.5)	9.6 (±0.7)	10.8 (±0.6)	9.0 (±0.6)	8.9 (±0.6)	0.268	0.872	NS
Number of punishments (Quantity)	3.3 (±1.0)	2.8 (±1.2)	2.8 (±1.0)	3.3 (±1.0)	2.1 (±1.1)	0.964	0.778	NS

[1]Negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of homeopathic additive in the feed. [2]The effects of treatments. [3]The effects of sex. *Significant statistical difference for sex. **Significant statistical difference between treatment x sex.

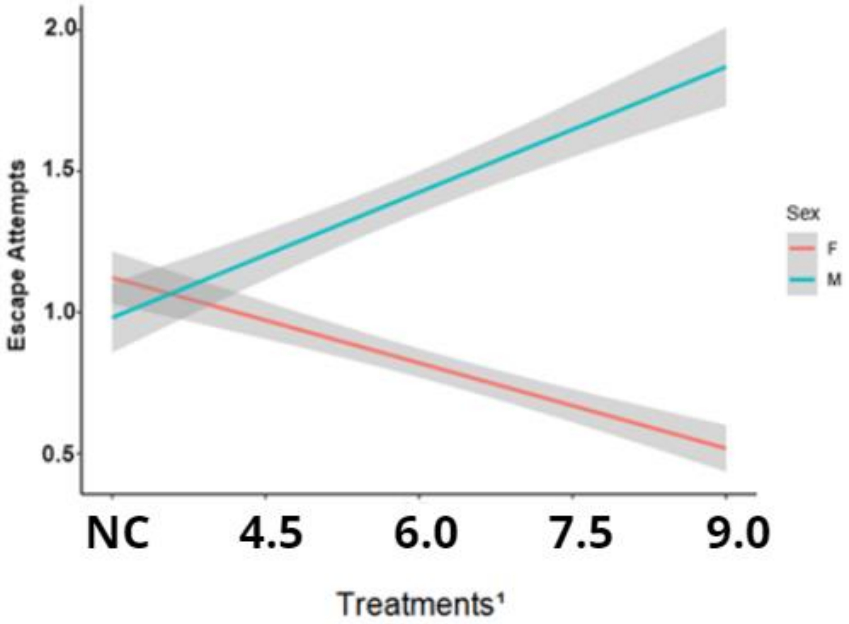

Figure 3.3 Interaction between dietary homeopathic feeding and sex for escape attempts for reactivity during weighing test performed on nursery piglets.

[1] Negative control – basal diet without additives (NC), basal diets with 4.5; 6.0; 7.5 and 9.0 kg/ton of homeopathic additive in the feed

3.5 Discussion

The use of the homeopathic feed additive, on its own, did not affect the behaviors assessed during the tests applied to the nursery piglets. An interaction between the homeopathic feed additive and sex was found for reactivity during weighing: the higher the level of the additive, the lowest was the number of escape attempts by females, and the opposite occurs with the males. The males were castrated, and testosterone, the sex hormone that decreases with the removal of the gonads, possibly did not influence the outcome. Also, regarding sexual maturity, for the females is around 5 months of age, and, for males, around 8 months[14]. Our study is not the first to report a sex-related difference in the behavior of pigs. It has been cited by authors that factors such as age and sex may influence behavioral responses[15]. Fleming & Dilger (2017)[16] presented results showing that males and females piglets behave differently in response to a new stimulus in a novel object test and that this outcome could be due to different brain development. Similar findings concerning behavioral disparity between sex were reported by Mieloch et al.

(2020)[15], but involving adult pigs. Female piglets, in a study that aimed to explore social behaviors and identify early social styles in suckling piglets, were considered more inactive when compared to castrated males, since males were responsible for greater behavioral expressions, such as agonistic and social interactions[17].

The homeopathic complex possibly potentiated the behaviors related to inactivity of females, influencing fewer escape attempts, since it contains *chamomille*. This plant has sedative, analgesic and anxiolytic effect[18], and can also be used in cases of disorders of the gastrointestinal[19]. *Chamomille* was already used in mice at a dilution of 6 CH to verify its stress modulating properties[20]. In the aforementioned study, the homeopathic accelerated the recovery of basal behavior in animals subjected to stressful situations.

Calcarea carbonica and *Staphysagria* are substances that are present in the homeopathic complex and are also related to anxiolytic and antidepressant properties[21,22]. An investigation conducted on rodents to assess the effects of Staphysagria highlighted its considerable antidepressant attributes[22]. This compound's ability to act as an antidepressant is linked to the presence of flavonoids, leading to heightened serotonin (5-HT) levels[22]. Researchers have already indicated that 5-HT has a significant connection with impulsive behaviors, and a deficiency in the 5-HT system can lead to aggression, fear and anxiety[23].

Regarding the open field test, it was possible to observe that females showed fewer vocalizations than males, which is further evidence for the sex effect in behavioral tests[15]. As for the homeopathic additive, the components did not bring negative impacts to the piglets, regardless of the dilution. The results linked to exploration variables in the open field test corroborate with Marzotto et al. (2012)[7]. The authors compared in mice the effects of *Ignatia amara,* a remedy made from the *Strychnos ignatii* seeds, which is present in the additive used in our study, applied intraperitonially with an allopathic drug in order to verify some emotion-related symptoms. The researchers found that the time spent in the center of the arena was not influenced for either the homeopathic or the allopathic group.

Regarding the sociability test, in which piglets were intentionally mixed to verify agonistic interactions and fights, it was not possible to identify differences between all the treatments or sex. Performing this test can reveal that there was no impact on the piglets' aggressiveness from the additive, even when animals from different pens shared a space (arena) with a feeder containing chopped apples. Therefore, more studies involving homeopathy should be carried out, since it is known that the way of obtaining the active compound coming from plant extracts, as well as the dilution used, the interaction with other compounds present in the additive and even in the feed, besides the animal species studied and experimental conditions can influence the effects of the tested compounds on the animals[24].

3.6 Conclusion

The homeopathic additive used in the present study did not affect the behavior of the piglets in the nursery phase on its own. With the results obtained in this study, it is difficult to measure whether there were any positive consequences on the animals' welfare, since this topic encompasses several aspects. The sex of the animal has been shown to be a factor influencing the response of the additive on behavior, since higher amounts of the homeopathic complex decreases the escape attempts of the females and increases for males.

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CHAPTER 4

CONCLUSION/IMPACT AND FUTURE PROSPECTS

Livestock production is extremely important for the global supply of animal protein. Besides feeding a large part of the population, pig farming employs thousands of workers, which consequently promotes the country's development. To make pig farming more sustainable and environmentally friendly, it is necessary to study ways to mitigate already known problems and management practices. The use of antimicrobials as performance enhancers affects not only global health through bacterial resistance, but also the environment through contamination of soil and effluents. The homeopathy additive studied in this project with the aim of verifying changes in performance, intestinal health, inflammatory response and behavior of piglets in the nursery phase may be a viable replacement for this class of drugs. The first level of the homeopathic additive provided equal or better results than the positive control regarding diarrhea, resulted in lower populations of total and thermotolerant enterobacteria and higher populations of lactic acid bacteria in the feces compared to the chlorohydroxyquinoline treatment group and, additionally, exhibited fewer histopathological alterations in the duodenum when compared to the negative control. It also did not affect the behavior of the piglets in the nursery phase. Further studies need to be carried out involving different periods of feed consumption, other types of homeopathic compounds and different phases of swine production to better understand the effects of homeopathy in the animal body and its yet undiscovered effects.

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ANNEXE

1 – Guidelines for publication of the first article:

https://www.elsevier.com/journals/livestock-science/1871-1413/guide-for-authors

2 – Guidelines for publication of the second article:

https://web.thieme.com/media/ita/Homeopathy_authors_instructions_FINAL+.pdf

3 – Animal ethics committee

Pontifícia Universidade Católica do Paraná
Pró-Reitoria de Pesquisa, Pós-Graduação e Inovação
Comissão de Ética em Pesquisa no Uso de Animais

PARECER CONSUBSTANCIADO DA CEUA

TÍTULO DA PESQUISA	AVALIAÇÃO DO ADITIVO NATURAL (HOMEOSUIS CRECHEPLUS®) NA DIETA E SEUS EFEITOS SOBRE O BEM-ESTAR, SAÚDE INTESTINAL, RESPOSTA INFLAMATÓRIA E DESEMPENHO DE LEITÕES RECÉM-DESMAMADOS. EVALUATION OF A NATURAL ADDITIVE (HOMEOSUIS CRECHEPLUS®) IN FEED AND ITS EFFECTS ON WELFARE, INTESTINAL HEALTH, INFLAMMATORY RESPONSE AND PERFORMANCE OF WEANED PIGLETS		
Nº DO PARECER / VERSÃO	01720 – 2ª versão		
PESQUISADOR RESPONSÁVEL	Leandro Batista Costa		
ESPECIE DO ANIMAL	*Sus scrofa domesticus*	Nº DE ANIMAIS	125
NOME COMUM DO ANIMAL	Suínos	Nº SISBIO (animais de vida livre)	*Não se aplica*
SEXO / IDADE / PESO	21 dias (5 – 7 Kg)	ATIVIDADES (animais de vida livre)	*Não se aplica*
ORIGEM DO ANIMAL	Granja Comercial, Sapopema (Ivaí - PR	GP TAXONÔMICOS (animais de vida livre)	*Não se aplica*
DATA DE INICIO DA PESQUISA	26/03/202	LOCAL (IS) (animais de vida livre)	*Não se aplica*
DATA DE TÉRMINO DA PESQUISA	01/05/2020	Nº SISGEN	*Não se aplica*

CONSIDERAÇÕES SOBRE A PESQUISA	Não há.
CONSIDERAÇÕES SOBRE OS TERMOS DE APRESENTAÇÃO OBRIGATÓRIA	Todos os termos de apresentação obrigatória foram submetidos e estão de acordo.
RECOMENDAÇÕES	Não há recomendações.
CONCLUSÕES OU PENDÊNCIAS E LISTA DE INADEQUAÇÕES	Nada a declarar.
CONSIDERAÇÕES FINAIS	O colegiado da CEUA certifica que este protocolo que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto homem), para fins de pesquisa cientifica, encontra-se de acordo com os preceitos da Lei nº 11.794/2018 e Decreto nº 6.899/2009, e com as normas editadas pelo CONCEA sendo aprovado pela CEUA - PUCPR em reunião de colegiado. Se houver mudança do protocolo o pesquisador deve enviar uma emenda à CEUA descrevendo de forma clara e sucinta, a parte do protocolo a ser modificado e as suas justificativas. Se a pesquisa, ou parte dela for realizada em outras instituições, cabe ao pesquisador não iniciar antes de receber a autorização formal para a sua realização.
SITUAÇÃO DO PARECER	APROVADO

CURITIBA, 19 de MARÇO de 2020

PROF. DR. SÉRGIO LUIZ ROCHA

Coordenador CEUA-PUCPR

A validade deste documento pode ser confirmada pelo e-mail ceua@pucpr.br informando o número do parecer.