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FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS

CÂMPUS DE JABOTICABAL

DEVELOPMENT OF ALGINATE MICROPARTICLES COATED WITH HPMCAS CONTAINING THE ANTIMICROBIAL PEPTIDE Ctx(Ile^{21})-Ha FOR APPLICATION AS A NATURAL ORAL ADDITIVE IN LAYING HENS AGAINST SYSTEMIC *Salmonella* Enteritidis INFECTION

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Biotechnology Engineer

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DESENVOLVIMENTO DE MICROPARTÍCULAS DE ALGINATO REVESTIDAS COM HPMCAS CONTENDO O PEPTÍDEO ANTIMICROBIANO Ctx(Ile^{21})-Ha PARA APLICAÇÃO COMO ADITIVO ORAL NATURAL EM GALINHAS DE POSTURA CONTRA INFECÇÃO SISTÊMICA POR *Salmonella* Enteritidis

Discente: Cesar Augusto Roque Borda

Orientador: Prof. Dr. Eduardo Festozo Vicente

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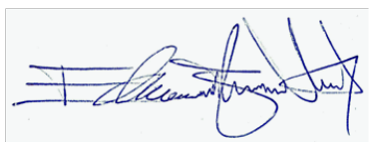

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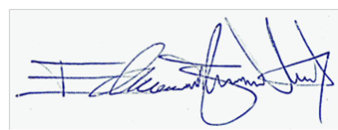

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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Aplicação do peptídeo antimicrobiano Ctx(Ile^{21})-Ha e seus conjugados em diferentes formulações como aditivo nutricional em pintainhas de postura"**, protocolo nº 2588/20, sob a responsabilidade do Prof. Dr. Eduardo Festozo Vicente, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 25 de agosto de 2020.

Vigência do Projeto	01/10/2020 a 01/11/2020
Espécie / Linhagem	Pintainhas de postura / H&N Nick Chick
Nº de animais	90
Peso / Idade	100g – 400g / 0a 28 dias
Sexo	NA
Origem	H&N Avicultura

Jaboticabal, 25 de agosto de 2020.

Fabiana Pilarski

Profª Drª Fabiana Pilarski
Coordenadora – CEUA

AUTHOR CURRICULUM DATA

Cesar Augusto Roque Borda, nascido no dia 15 de maio de 1992 no Peru, filho de Augusto Rodolfo Roque Rivera e Rosalia Borda Campos. Bacharel em Engenharia Biotecnologica com período sanduiche pela *Universidad Católica de Santa María (UCSM), Arequipa - Perú*, título obtido em maio de 2018. Durante a graduação foi líder de grupo de pesquisa ABBC conformada por estudantes sob direção do E-Quipu Santa María, em 2015 ganhou financiamento do Projeto de pesquisa *"Evaluación del efecto hepatoprotector anticancerígeno y anticitotóxico de la Avena sativa"* pela UCSM, RESOLUÇÃO N° 201381-R2015, em 2016 foi convidado para estagiar no "*Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA)*, *Argentina*", temática baseada em caracterização de compostos fenólicos e antioxidantes, no mesmo ano foram ministradas 03 disciplinas de curta duração certificadas pelo E-Quipu Santa María, em 2017, foi convidado novamente ao CIDCA como estagiário de pesquisa em criopreservação em colaboração do Banco de Germoplasma de Balcarce, do Instituto de Tecnologia Agraria (INTA) – Argentina, sendo validado como o período sanduíche. Em 2019 ingressou no PPG em Zootecnia pela Universidade Estadual Paulista (UNESP) e foi bolsista pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) até o dia de defesa da presente dissertação. Em 2019 foi estagiário docente da Faculdade de Ciências e Engenharias - FCE/UNESP da disciplina de Bioquímica e Química Orgânica, e no mesmo ano recebeu a Premiação de Menção Honrosa pela apresentação do trabalho no Evento Renorbio 2019 - II Encontro de Biotecnologia do Nordeste da Rede Nordeste de Biotecnologia - RENORBIO / FIOCRUZ-CE. Atualmente faz parte do grupo de pesquisa "Peptídeos: Síntese, Otimização e Estudos Aplicados – PeSEAp" liderado pelo Prof. Dr. Eduardo Festozo Vicente, orientador desta dissertação.

“Entre ser o no ser, Yo soy”

Teresa

“Só existem dois dias do ano em que você não pode fazer nada: um se chama ontem e outro amanhã”.

Dalai Lama

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Dedico

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SUMMARY

APLICAÇÃO DO PEPTÍDEO ANTIMICROBIANO Ctx(Ile^{21})-Ha E SEUS CONJUGADOS EM DIFERENTES FORMULAÇÕES COMO ADITIVO NUTRICIONAL EM PINTAINHAS DE POSTURA

RESUMO - A pesquisa por novos compostos que possam aumentar e melhorar o desempenho ou diminuir resíduos químicos em produtos de origem animal torna-se relevante e demandada. Paralelamente, o uso constante de antibióticos sintéticos como promotores de crescimento causa uma grande resistência bacteriana em aves poedeiras, além de rastros e resíduos em seus produtos, os quais podem afetar a sanidade animal e, consequentemente, a saúde humana. Neste sentido, o peptídeo antimicrobiano (PAM) Ctx(Ile^{21})-Ha, extraído e isolado da rã do cerrado brasileiro *Hypsiboas albopunctatus*, é uma molécula muito promissora no controle de bactérias patogênicas. No entanto, os PAMs aplicados em sistemas *in vivo*, podem sofrer degradação ou desnaturação pela presença de proteases ou hidrolise pelo entorno ácido. Assim, este estudo objetivou a síntese e purificação peptídeo Ctx(Ile^{21})-Ha, e o revestimento de micropartículas de alginato com acetato/succinato de hipromelose (HPMCAS), além de sua caracterização fisioquímica. Foi também avaliada a atividade hemolítica e liberação *in vitro* gastrointestinal simulada das micropartículas revestidas. O estudo *in vivo* foi realizado com galinhas de postura recém-nascidas, desafiadas com *Salmonella* Enteritidis, e tratadas com as micropartículas na ração. Foi realizado a contagem bacteriana em fígado, baço e ceco para verificação dos efeitos do peptídeo. Os resultados obtidos mostraram micropartículas termoestáveis, com eficiência de encapsulação acima do 70%, leve liberação gástrica, ótima liberação intestinal, atividade hemolítica do peptídeo diminuída em 95%, além no experimento *in vivo* obteve-se diferença significativa na contagem principalmente no fígado, seguida do baço. Portanto, este estudo demonstrou que as micropartículas revestidas contendo o PAM Ctx(Ile^{21})-Ha possuem alta atividade antimicrobiana e serve de ferramenta para prevenção de infecção sistêmica, sem resíduos contaminantes em comparação com os fármacos convencionais.

Palavras-chave: Aditivos. Aves Poedeiras. Micropartículas. Revestimento entérico. *Salmonella*. Sanidade.

APPLICATION OF THE ANTIMICROBIAL PEPTIDE CTX(Ile^{21})-Ha AND ITS CONJUGATES IN DIFFERENT FORMULATIONS AS A NUTRITIONAL ADDITIVE IN LAYING HENS

ABSTRACT - The search for new compounds that can increase and improve performance or reduce chemical residues in products of animal origin is becoming relevant and in demand. At the same time, the constant use of synthetic antibiotics as growth promoters causes great bacterial resistance in laying birds, as well as traces and residues in their products, which can affect animal health and, consequently, human health. In this sense, the antimicrobial peptide (AMP) Ctx(Ile^{21})-Ha, extracted and isolated from the Brazilian Cerrado frog *Hypsiboas albopunctatus*, is a very promising molecule in the control of pathogenic bacteria. However, AMPs applied in an *in vivo* system can undergo degradation or denaturation due to the presence of proteases or hydrolysis by the acidic environment. Thus, this study aimed at the synthesis and purification of the Ctx(Ile^{21})-Ha peptide, and the coating of alginate microparticles with hypromellose acetate/succinate (HPMCAS), in addition to its physicochemical characterization. Hemolytic activity and simulated *in vitro* gastrointestinal release of the coated microparticles were also evaluated. *In vivo* study was carried out with newly hatched laying hens, challenged with *Salmonella* Enteritidis and treated with microparticles in the feed. Bacterial counts were performed in liver, spleen, and cecum to verify the effects of peptide. The results obtained showed thermostable microparticles, with encapsulation efficiency greater than 70%, slight gastric release, excellent intestinal release, the hemolytic activity of peptide decreased by 95%, and in the *in vivo* experiment a significant difference was obtained in bacterial count, mainly in liver, followed by spleen. Therefore, this study demonstrated that the coated microparticles containing AMP Ctx(Ile^{21})-Ha have a high antimicrobial activity and serve as a tool for the prevention of systemic infections, without contaminating residues compared to conventional drugs.

Keywords: Additives. Enteric coating. Laying hens. Microparticles. *Salmonella*. Poultry health.

CHAPTER 1 – GENERAL CONSIDERATIONS

1. INTRODUCTION

The discovery of new applications brought from Biotechnology area to Animal Production area continues to be an issue that requires a lot of research and dedication. Specifically, poultry sector generates large monetary incomes at the national level, being the United States of America, China and Brazil the largest exporters and producers of this kind of meat. On the other hand, China, USA and India are countries with the highest egg production in the world, and consequently in laying hens' production (FAO 2020). It is known that the main pathogen considered a public health problem is *Salmonella* sp., which it must necessarily be controlled to do not generate discomfort in the consumer (Renu et al. 2020). *Salmonella* sp. transmission is usually due to poor hygiene, by contact with contaminated meat and/or because there is no adequate monitoring of the infected properties (Gaggìa et al. 2010).

In recent years, the use of antibiotics has been severely restricted by regulatory institutions, due to the large number of bacteria mutations that occurred over time. In response, they acquired resistance and made conventional antibiotic compounds almost obsolete. Bell (2014), points out that there is a direct relationship between the use of drugs and their resistance, and that this factor is correlated with sectors of population application, such as country, region, state or municipality. Therefore, the use and study of new biological molecules with capacity for spontaneous or kinetic bacterial inhibition superior to conventional drugs has been recommended, since this factor could prevent these bacteria from acquiring or generating resistance (Gong et al. 2020). Many of these biomolecules can be purchased from different natural sources or synthesized using a natural source as a model.

Antimicrobial peptides (AMPs) are a great example of biomolecules that are present in virtually all living beings. They are a set of linked amino acids that help in the protection and defense of the organism, proliferate, and increase the useful life and/or neutralize the presence of pathogens. AMP Ctx(Ile^{21})-Ha, is a peptide from the skin of the Brazilian Cerrado frog *Hypsiboas albopunctatus*, which has an α-helical amphipathic structure of 21 amino acid residues, is water soluble, and has a positive charge (+3), therefore, it is within the group of cationic antimicrobial peptides (cAMP) and ceratotoxins class (Lorenzón et al., 2012). This peptide has been shown to be relevant due to its antimicrobial activity against public health pathogens with a high affinity for Gram negative bacteria and some fungi, such as *Escherichia coli, Staphylococcus aureus* and *Candida albicans* (Vicente et al 2012). It is a peptide described but not studied in depth, so its application and use as an antimicrobial agent could be very promising.

AMPs use and application are restricted since (i) these AMPs often have a high rate of antimicrobial activity with such a small amount of sample, which implies excessive waste and lack of dose control, (ii) hemolytic activity of these AMPs frequently can be harmful to animal cells and/or (iii) their active form presents biological activity difficulties which depend on the location and conditions of the action site.

To overcome these issues, encapsulation technology was successfully used in food and pharmaceutical technology area to protect bioactive compounds and allow a suitable drug delivery, being an excellent alternative for several research fields, such as Animal Science (Borrmann et al. 2013; Rostami et al. 2019). Microencapsulation, together with biopolymers, can increase the biocompatibility and bioavailability of drugs to treat various diseases. Their molecular structure are easily absorbed. Among techniques at micro or nanoscale, microencapsulation is the most stable (Bratovcic and Suljagic 2019).

For these reasons aforementioned, the present study is based on the application of an antimicrobial peptide known as Ctx(Ile^{21})-Ha, chosen for its potential antimicrobial activity against *Salmonella* sp. It was microencapsulated and coated with cellulose-based biopolymers and applied to laying chickens, to evaluate its effect on this type of formulation as a food additive with efficient pharmacological activity.

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