FEDERAL UNIVERSITY OF UBERLÂNDIA
VETERINARY MEDICINE FACULTY
VETERINARY SCIENCE POST GRADUATION PROGRAM

PAULA FERNANDA DE SOUSA BRAGA

ENHANCING THE CONTROL OF *CAMPYLOBACTER JEJUNI* IN A THERAGNOSTIC APPROACH: FTIR-ATR COMBINED WITH ARTIFICIAL INTELLIGENCE, BINDING-PEPTIDES, AND THE USE OF CHICKEN EMBRYOS AS AN *IN VIVO* MODEL

UBERLÂNDIA- MG
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Tese de Doutorado apresentada ao Programa
de Pós-Graduação em Ciências
Veterinárias como requisito parcial para
obtenção do título de Doutor(a).
Área de concentração: Saúde Animal
Orientador: Profa. Dra. Belchiolina B. Fonseca

UBERLÂNDIA- MG
2024

Ficha Catalográfica Online do Sistema de Bibliotecas da UFU
com dados informados pelo(a) próprio(a) autor(a).

B813 Braga, Paula Fernanda de Sousa, 1987-
2024 ENHANCING THE CONTROL OF CAMPYLOBACTER JEJUNI IN A THERAGNOSTIC APPROACH: FTIR-ATR COMBINED WITH ARTIFICIAL INTELLIGENCE, BINDING-PEPTIDES, AND THE USE OF CHICKEN EMBRYOS AS AN IN VIVO MODEL [recurso eletrônico] / Paula Fernanda de Sousa Braga. - 2024.

Orientador: Belchiolina Beatriz Fonseca.
Coorientador: Murilo Vieira Silva.
Tese (Doutorado) - Universidade Federal de Uberlândia, Pós-graduação em Ciências Veterinárias.
Modo de acesso: Internet.
Disponível em: http://doi.org/10.14393/ufu.te.2024.420
Inclui bibliografia.

1. Veterinária. I. Fonseca, Belchiolina Beatriz,1978-, (Orient.). II. Silva, Murilo Vieira,1988-, (Coorient.). III. Universidade Federal de Uberlândia. Pós-graduação em Ciências Veterinárias. IV. Título.

CDU: 619

Bibliotecários responsáveis pela estrutura de acordo com o AACR2:

Gizele Cristine Nunes do Couto - CRB6/2091
Nelson Marcos Ferreira - CRB6/3074

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ATA DE DEFESA - PÓS-GRADUAÇÃO

Programa de Pós-Graduação em:	Ciências Veterinárias				
Defesa de:	Tese de Doutorado PPGCVET Nº 03/2024				
Data:	05 de junho de 2024	Hora de início:	14:00	Hora de encerramento:	17:00
Matrícula do Discente:	12013VET005				
Nome do Discente:	Paula Fernanda de Sousa Braga				
Título do Trabalho:	Enhancing the control of Campylobacter jejuni in a Theragnostic Approach: FTIR-ATR combined with Artificial Intelligence, Binding- Peptides, and the Use of Chicken Embryos as an In Vivo Model				
Área de concentração:	Saúde Animal				
Linha de pesquisa:	Investigação Etiológica				
Projeto de Pesquisa de vinculação:	Epidemiologia de Zoonoses				

Reuniu-se no Anfiteatro do Hospital Veterinário, Campus Umuarama, da Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Ciências Veterinárias, assim composta: Professores Doutores: Daise Aparecida Rossi (FAMEV/UFU); Marcelo Sebastião Rezende (POLE ALIMENTOS LTDA); Heriberto Fernández Jaramillo (UACh); Mercia Rodrigues Barros (UFRPE); Belchiolina Beatriz Fonseca (FAMEV/UFU), orientadora da candidata.

Iniciando os trabalhos a presidente da mesa, Dra. Belchiolina Beatriz Fonseca, apresentou a Comissão Examinadora e a candidata, agradeceu a presença do público, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de arguição e resposta foram conforme as normas do Programa.

A seguir a senhora presidente concedeu a palavra, pela ordem sucessivamente, aos examinadores, que passaram a arguir a candidata. Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu o resultado, considerando a candidata:

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Documento assinado eletronicamente por **Marcelo Sebastião Rezende**, **Usuário Externo**, em 10/06/2024, às 17:50, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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Referência: Processo nº 23117.034145/2024-88 SEI nº 5455233

DEDICATÓRIA

À Deus, meu motivo de fé para seguir diante das dificuldades.

À minha família, pelo constante apoio e amor incondicional ao longo de toda a minha jornada acadêmica.

À minha orientadora e a todos os professores e amigos que contribuíram de alguma forma para a realização deste trabalho. Suas palavras de encorajamento e incentivo foram fundamentais para que eu chegasse até aqui.

Que esta tese possa contribuir de alguma forma para o avanço do conhecimento em nossa área.

AGRADECIMENTOS

Gostaria de expressar meus sinceros agradecimentos a todas as pessoas que contribuíram de alguma forma para a realização deste trabalho.

Primeiramente, gostaria de agradecer à minha orientadora Bia, por confiar no meu trabalho como sua orientada e por ser minha guia profissional e de vida. Foram anos de total apoio, trabalho e dedicação, sempre disposta a ensinar e contribuir. Muitas vezes abdicou da vida pessoal para me ajudar na pesquisa. Obrigada por ser fonte de inspiração e pelo bem que você faz para a humanidade, com um coração enorme que acolhe a todos, sem distinção. Sem a sua ajuda e orientação, este trabalho não teria sido possível.

Agradeço aos meus pais Maria José e Jair, por terem me dado uma base sólida, pelo apoio incondicional em todas minhas decisões e por sempre reforçarem o quanto me amam, tanto em palavras, quanto em atitudes. À minha irmã Joyce, minha melhor amiga, por ser minha referência desde a infância e por se fazer presente na minha rotina, sempre com palavras de incentivo e melhorando minha autoestima. Ao meu cunhado Saulo, exemplo de disciplina e dedicação, e sobrinhos Luísa e Felipe, por serem as melhores companhias do meu dia a dia.

Ao meu esposo Ricardo, meu melhor parceiro de vida, por me encorajar e apoiar durante todo o processo e por demonstrar seu amor nos mínimos detalhes. Obrigada pelas palavras de incentivo, por acreditar no meu potencial e por comprar minhas batalhas sendo companheiro e me incentivando. Aos meus filhos, Téo e Eva, por serem minhas maiores inspirações da vida, por transbordarem amor e me ensinarem todos os dias a ser uma pessoa melhor por eles. Que eu possa ser exemplo na vida deles, assim como meus pais foram para mim.

À família do meu esposo, Ângela, Gelcimar, Tia Joca, Dedé e todos que são rede de apoio com meus filhos, me permitindo dedicar tempo ao trabalho e à pesquisa, que são pilares importantes para mim. Sem a ajuda de vocês eu não teria conseguido. Obrigada por cuidarem dos meus filhos com tanto amor e afeto.

Aos meus amigos de laboratório (LADOC, LIAVE, NANOS) e de doutorado, que foram grandes presentes que o doutorado me trouxe, especialmente Simone, Lara, Isabelle, Ana Rafaela, Gabi, Thaís, Patrícia, Emília, Fabi, Pedro, Banner, Bruna, Mário, Iara, Luciana, Natassia e todos outros que auxiliaram em coletas e processamento de amostras. Pessoas dedicadas, que acreditam na ciência e trabalham com muita responsabilidade e dedicação. Poder conviver com vocês foi um momento muito especial

na minha vida. Obrigada pelos ensinamentos, parceria, confiança e por continuarem se fazendo presente na minha vida. Espero poder retribuir todo carinho que tiveram comigo.

Aos amigos da BRF, pelo apoio e por terem me confiado um tempo de dedicação exclusiva ao Doutorado, especialmente José Serra e Gabriel Vearick. Esse tempo foi muito importante para que eu pudesse desenvolver minha pesquisa com a dedicação que ela merecia.

Aos professores de Programa de Pós-Graduação da Universidade Federal de Uberlândia, pelos ensinamentos e apoio durante todo o programa.

Além disso, gostaria de agradecer à Capes, que financiou parte da minha pesquisa, tornando-a possível de ser realizada.

Por fim, agradeço a todos os participantes da pesquisa que dedicaram seu tempo e disponibilidade para contribuir com informações e dados importantes para o desenvolvimento deste trabalho.

"Todas as vitórias ocultam uma abdicação". (Simone de Beauvoir)

ASBTRACT

Due to the importance to *Campylobacter* spp. to human health and the high prevalence of *C. jejuni* (CJ) in chickens, this study aimed to seek alternatives to improve the control of CJ through a theragnostic approach, using the chicken embryo as an important pre-clinical model. The work comprises four chapters regarding *Campylobacter jejuni* in poultry. The first chapter provides a literature review of *Campylobacter* spp., highlighting its significance and occurrence worldwide, the pathogenicity of *Campylobacter* in humans and chickens, prevention, and therapy-based *Campylobacter* control, as well as its diagnostic. Also, this chapter describes pathogens' identification by machine learning techniques and phage display technology. The second chapter aimed to evaluate the virulence and infection of strains of CJ isolated from chicken and standard strain isolated from humans to better understand the pathogen-host relationship of CJ and chicken embryo (CE). We also evaluated embryo mortality, weight, gross and microscopic lesions, multiplication of the bacteria in the embryo, macrophage and lymphocyte counting by flow cytometry analysis, cytokine analysis by ELISA, and analysis of histopathological lesions. At low doses, CJ generated lesions in CE, and some strains stimulated the immune system, but the response was strain-dependent, reinforcing the importance of studying the virulence, infection, and immune response of several strains of *C jejuni* and the CE model as of great utility. Chapter three discusses a quick, practical, and reliable new technique to identify CJ - the ATR-FTIR spectroscopy associated with artificial intelligence to detect *Campylobacter jejuni.* This technique was a useful tool to identify CJ in real time, with a specificity of 100% using the Random Forest model. The fourth chapter describes a promising alternative for *Campylobacter jejuni* control through theragnostic approach, using Phage Display-Derived peptides. Besides selecting peptides using the phage display technique, we also performed phages sequencing and the molecular docking to reach the best peptides, which were tested *in vitro*, in an inhibitory assay and *in vivo*, using CE as experimental models. We proved that the peptide is efficient for diagnosis approaches, which is interesting because they can be more specific (since they recognize a specific epitope) and safer, once it is not necessary to handle the pathogen and they may be a potential to inhibit CJ. This thesis brings new potential alternatives to study and control CJ in a theragnostic approach.

Keywords: Foodborne diseases, pathogenicity, machine learning, diagnosis.

SUMARY

PRESENTATION

This thesis was sponsored by Professor Belchiolina Beatriz Fonseca, who perfectly guided all the study. The experiments were performed at the Federal University of Uberlândia, at the Infectious Disease Laboratory (LADOC), at the Incubation Laboratory (LIAVE) and at the Nanobiotechnology Laboratory Prof. Dr. Luiz Ricardo Goulart Filho (IBTEC).

The structure of the study followed the guidance of the Veterinary Medicine Post Graduation Program at the Veterinary Medicine Faculty, with the following sections:

- Theoretical foundation – Literature review of *Campylobacter* spp., highlighting its significance and occurrence worldwide, the pathogenicity of *Campylobacter* spp. in humans and chickens, prevention, and therapy-based *Campylobacter* control, as well as its cultivation and identification. Also, this chapter describes pathogens' identification by machine learning techniques.

- Chapter II – "*Understanding the chicken embryo as a model of Campylobacter jejuni infection beyond death".* Article to be submitted to the periodic "*Research in Microbiology"* with impact score 2.6. The criteria for submission are available at: Guide for authors - Research in Microbiology - ISSN 0923-2508 | ScienceDirect.com by Elsevier

Chapter III – *"ATR-FTIR spectroscopy combined with artificial intelligence can offer an alternative method for detecting Campylobacter jejuni".* Article to be submitted to the periodic "*Applied Microbiology and Biotechnology*" with impact score 5.2. The criteria for submission are available at: Submission guidelines | Applied Microbiology and Biotechnology (springer.com)

Chapter IX – "*Phage Display-Derived Peptides: A promising alternative for Campylobacter jejuni control through theragnostic approach*". Article to be submitted to the periodic "*Biotechnology Journal*" with impact score 5.726. The criteria for submission are available at: https://authorservices.wiley.com/author-resources/book-authors/prepare-your-manuscript/index.html

CHAPTER 1

1 THEORETICAL FOUNDATION

1.1 INTRODUCTION

Foodborne diseases represent a severe public health problem due to their high frequency, mortality, and the significant number of microorganisms that may be involved in a single epidemic event. *Campylobacter spp.* is the most prevalent foodborne pathogen bacterium responsible for causing gastroenteritis worldwide (Bunduruș et al., 2023) and in developed countries it needs to be addressed as high priority on the public health hazards to be covered by inspection of poultry meat (EFSA et al., 2020). In 2022, campylobacteriosis was the second highest number of hospitalizations caused by a zoonotic pathogen after salmonellosis in European Union (EFSA, 2023) and human infections caused by *Campylobacter* are endemic worldwide (Hoffman et al., 2021). Between 2013 and 2022, the number of confirmed campylobacteriosis cases reported in the European Union showed a clear seasonal trend, peaking in the summer months. Annual winter peaks were also observed in January from 2013 to 2022, although peak numbers were lower than those observed during the summer. Luxemburg and Portugal reported a significantly increasing from 2018-2022 (EFSA, 2023). Among the thirteen pathogenic *Campylobacter spp*. known to be related to human infections, *Campylobacter jejuni* (CJ) and *Campylobacter coli* (*C. coli)* are the two main species that are responsible for more than 95% of infections worldwide (Cribb et al., 2022). Authorities in the European Union (EU) regularly publish reports concerning campylobacteriosis cases and outbreaks, and occurrence of this pathogen in food, which is essential to address food safety policies (EFSA, 2023).

Campylobacter spp. colonizes a ubiquitous range of environments, from poultry, companion pets and livestock animals to humans (Bunduruș et al., 2023). Transmission of CJ to humans is usually through food consumption, particularly consumption of raw or undercooked poultry meat, unpasteurized, contaminated milk, or water-based environmental sources and after uptake, CJ colonizes the distal ileum and colon. (Lopes et al., 2021). Despite its extreme vulnerability, *Campylobacter* can survive in broiler farms and be passed from one rearing cycle to the next (Maes et al., 2019). Poor biosecurity and an intensive production system are the main factors in the spread of

infection from infected chicken to others (García e Rovira, 2018). According to Connerton et al. (2018), there is a latency period, which lasts from the time of hatching until around two weeks of age, when *Campylobacter* cannot be detected even if the organism is infected. In chicken, horizontal transmission may be considered the most likely contamination route and this infection is related to contaminated water intake, contact with feces of other infected animals, and contaminated litter (Sahin et al., 2012).

In developing countries, epidemiological data on campylobacteriosis are inconclusive as countries of Asia, Africa and Middle East have not adopted the standard reporting protocols (Kaakoush et al., 2015). *Campylobacter* spp. has not been recognized as a food safety problem yet, probably because surveillance systems are not able to link the prevalence of this agent in foods and the occurrence of foodborne diseases (Havelaar et al., 2009). Kirk et al. (2015) described that one possible reason for the variation of cases among different countries and cities of the same country is the diagnostic method, as *Campylobacter* is not that easy to grow, so many countries are not equipped with those modern sophisticated tools and techniques to perform the proper diagnosis. Another reason could the immune level of population, where people with strong immunity can tolerate the infection without showing any sign and symptoms of the disease (Samapundo, et al., 2015).

Guillain Barré Syndrome (GBS) has been described as a post-infectious immune-mediated disease that occurs because of molecular mimicry wherein immune mechanisms, both cellular as well as humoral, normally directed against microbes, recognize body's own antigens as foreign (Nakano and Kanda, 2016). Infection with CJ is considered the most common cause of GBS (Quino et al., 2022).

In Brazil, there are no legal standards for *Campylobacter* in food and studies aiming to detect this microorganism are scarce comparing to investigations concerning other foodborne pathogens (Silva et al., 2018). According to Silva et. al (2018), between 2002 and 2017, only five foodborne outbreaks involving 37 cases of campylobacteriosis were reported in Brazil, which is the world's largest exporter and the second largest producer of chicken meat (ABPA, 2023).

Considering the importance to *Campylobacter spp*. to human health and the high prevalence of *C. jejuni* (CJ) in chickens, studies are needed to seek alternatives to improve the control of CJ.

2. LITERATURE REVIEW

2.1 SIGNIFICANCE OF *CAMPYLOBACTER JEJUNI* (CJ) AND PATHOGENICITY IN HUMAN AND CHICKENS

CJ infections, which is strongly linked to contaminated retail chicken by several studies (Epps et al., 2013), have the avian reservoir as the predominant source. This bacterium is part of the avian gut microbiome, with high prevalence (10^9 CFU/g) in chicken ceca (Thibodeau et al., 2015), causing no harm to birds. Still, when birds are slaughtered, CJ is released from the intestines and contaminates the meat. Another critical issue is that, as already seen in other studies, *Campylobacter* can survive well on both chicken skin and meat at refrigerated temperatures (Davis and Conner, 2007).

CJ is the leading bacterial cause of human foodborne illness associated with poultry, beef, and pork consumption (Epps et al., 2013) being found in the gut of warm-blooded animals, with poultry species being the major reservoirs (Skarp et al., 2016). The most common human disease caused by CJ is foodborne enteritis (Finsterer, 2022) but the symptoms may vary from person to person, and include diarrhea, fever, vomiting and abdominal pain (Silva et al., 2016). When causing enteritis in humans, *Campylobacter* brings severe consequences for children, the elderly, and the immunocompromised ones (Ushanov, 2018). The effort to reduce *Campylobacter* infections in humans is directly linked to a better understanding of the biological aspects of the pathogen and, particularly, the virulence mechanisms contributing to the pathogenesis of the disease (Javid et al., 2010). Increasing human infection rates are reported during the summer months, reflecting optimized replication rates of *C. jejuni* at higher temperatures or transmissions caused by water activities (Javid et al., 2010). Other complications of CJ infections are GBS (Quino et al., 2022), irritable bowel syndrome (Peters et al., 2021), reactive arthritis, Reiter syndrome (arthritis, urethritis, iritis), spinal abscesses (Fujita et al., 2022), and Achilles enthesopathy (Schönberg et al., 2010).

Since *Campylobacter* colonization is not associated with signs of disease in chickens, the horizontal spread of the pathogen usually remains unnoticed (Epps et al., 2013). However, Awad at el. (2018) suggested that broiler flocks colonized with campylobacters may have an increased incidence of intestinal inflammation, diarrhea, suboptimal growth performance and pododermatitis. Probably because CJ is an organism that cannot multiply outside the animals' intestines and it is sensitive to environmental conditions,

the persistence of campylobacters on the slaughter line after cleaning and disinfection has been little studied (Rasschaert et a., 2020). Some researchers found CJ remained on cleaned and disinfected slaughter line (Peyrat et al. 2008, Kudirkienė et al., 2011, García et al., 2017) and some explanations why this may occur is that organic matter still present after the cleaning process on the machinery may protect CJ during disinfection (Kudirkienė et al., 2011) and may also lead to the formation of biofilms. Although CJ is generally a poor biofilm in initiator it may occur mixed-species biofilms. For example, certain *Campylobacter* strains have been found together with *Pseudomonas aeruginosa*, as the latter protects *Campylobacter* against oxygen stress (The et al., 2019).

A recently identified *Campylobacter* species, *Campylobacter hepaticus*, has been shown to cause spotty liver disease (SLD) around the world (Crawshaw, 2019). SLD manifests as acute infectious hepatitis and is characterized by many multifocal, small necrotic foci on the surface of the liver. It affects mostly free-range layer chickens with up to 15% mortality and 35% reduced egg production. Chlortetracycline has been used as a treatment option during outbreaks, and there are no commercial vaccines available for SLD (Courtice et al., 2018).

For a successful colonization of host organisms, the presence of virulence factors is primordial for *Campylobacter* species. These virulence factors include motility, which confers to the bacteria the ability to move actively in the gastrointestinal tract, being essential for the survival of the microorganism in the digestive system and colonization of the small intestine (Nachamkin et al., 2008). Also, this mechanism is viable due to the flagellum on the cell surface, which in conjunction with the chemotactic mechanism, enables *Campylobacter* to move in the intestinal mucus (Bolton, 2015). The microbial cell moves towards the most favorable environment due to its attraction to chemokines (glycoproteins and mucins), substances that can be found in the intestinal mucous membrane (Chang and Miller, 2006). The ability to adhere to host gastrointestinal epithelial cells is primordial for *Campylobacter* colonization and it is mediated by several adhesins on the bacterial surface (Hermans et al., 2012). This adherence is also regulated by Cytolethal Distending Toxin (CDT), the toxin most studied as virulence factor in campylobacteriosis cases, which is composed of three subunits encoded by the *cdtA*, *cdtB*, and *cdtC* genes, and is associated with the increased virulence of the strain that causes enteritis in humans (Jin et al., 2001). Apparently, all CJ strains have cdt genes, which mostly encode active toxins, but there may be rare exceptions of isolates that do not have these active genes or show mutations, which may affect their molecular identification and

toxin activity (Nishimura et al., 2007). Moreover, these genetic differences may have implications regarding the efficiency of strains on human cells invasion (Al-Mahmeed et al., 2006). Lipooligosaccharides (LOS), capsule and O- and N-linked glycans are polysaccharide structures that confer the microorganism the ability to adhere and invade host cells, as well as function as an immune escape mechanism (Tejero and Galán, 2001).

While the invasion of human epithelia is well described, the attachment and invasion of chicken epithelia by CJ strains is more debatable. There is some evidence that *Campylobacter* can traverse the intestinal epithelium and be recovered from the spleen and liver of young chicks (Young et al., 1999). This invasion, whether transient or sporadic, would require a close interaction between the host and the bacteria. The clear antibody response to *Campylobacter* by poultry (Myszewski & Stern, 1990) indicates that some bacterial/ host cell interaction must take place during the colonization process to initiate the adaptive response. The development of this immunoglobulin response requires the stimulation of innate signals that drive the cell-to-cell interactions required for antigen presentation. The same inflammatory signals that drive adaptive responses have been correlated with intestinal disease in humans (Enocksson et al., 2004) and these are associated with induction of pathology.

2.2 IMMUNE PROFILE OF BIRDS AND EMBRYOS

The immune system of neonates of all vertebrates is characterized by the progressive development of innate and adaptive defenses, and by inadequate immunological memory, predisposing them to microbial infections (Wynn & Levy, 2010). The immune response to a given stimulus varies among species (Hartung et al., 1996) and it is already known that for birds, the pathogenesis and the immune response for some agents may vary according to the age of the affected bird and its immune system maturity (Rautenschlein et al., 2007).

The immune system of birds can be divided into two structures: primary lymphoid system (cloacal bursa and thymus) and secondary lymphoid system (spleen and lymphoid tissues associated with mucous membranes: Harder's gland, intestine, bronchi, Peyer's plaques, Meckel diverticulum, cecal and pineal tonsils) (Oláh and Vervelde, 2008). During embryonic development, the embryo's immature hematopoietic cells migrate from the embryo sac to the embryo's bloodstream and then to the spleen, where red and white blood cells will form. From this, they colonize the primary organs (bursa and

thymus) through chemotactic factors, which attract cells and allow the colonization of these organs by around the sixth day of incubation for the thymus and in bursa, around the tenth day of incubation (Oláh and Vervelde, 2008) until the third week after hatching when the primary and secondary organs mature (Juul-Madsen et al., 2006).

Some markers are efficient in evaluating the immunocompetence of these animals, such as peripheral blood lymphocyte populations. It is known, for example, that the CD4:CD8 ratio is much lower in birds commercially bred, a parameter that indicates lower immunocompetence, such as increased susceptibility to disease (Bridle et al., 2006). In challenging situations, subpopulations of circulating lymphocytes help to understand the pathogenesis and evolution of infections and how to control them.

The knowledge of immunomodulatory properties is essential, particularly regarding those immunomodulatory compounds with a high potential to improve animal health via improved defense against infection (Kiczorowska et al., 2017). Therefore, knowledge about the specific modes of action of immunomodulating compounds is needed to develop diets as alternatives to widely used antimicrobials on farms and to improve the health and welfare of animals, and thereby also humans (Swaggerty et al., 2020).

2.3 THE USE OF CHICKEN EMBRYO AS *IN VIVO* MODEL

The chicken embryo has a long and distinguished history as a primary model system in developmental biology and has also contributed significant concepts to immunology, genetics, virology, cancer, and cell biology, being one of the most versatile experimental systems available (Stern, 2005). It has been so far a widespread animal model used once fertilized eggs are more affordable, easier to be purchased, and available in large numbers throughout the year. Besides, it is essential to decrease the number of born animals in experiments (Sommerfeld et al., 2022) and permit controlled administration of substances and direct observation of embryonic development (Vergara and Canto-Soler, 2012).

The new technologies and resources now usher in a new era for the chick as a system for developmental, genetic, immunological, evolutionary, molecular, physiological, and many other studies (Stern, 2005). Knowledge about the modes of action of immunomodulating compounds such as pathogens, drugs, or feed additives, e.g., probiotics, will allow the development of targeted nutrition strategies, prevent infectious diseases and the usage of antimicrobials, and promote the health of animals (Larsberg et al., 2021).

For assessing the efficacy and toxicity of new drugs and the manufacture of vaccines and biopharmaceuticals, the importance of cell culture technology is indisputable, but the quality of the culture medium, which supports cell survival and proliferation, and cellular functions, directly affects the research results, and researchers usually pay no attention to the possible interactions among the components of the basal medium and the supplements. The fact is that components can interact, and their effects on the cells should be expected to be influenced by those interactions. Also, supplements of biogenic origin, like the serum, can cause variation in the experimental results from batch to batch and carry a risk of microbial contamination of the culture medium. Besides, contaminants such as viruses, bacteria, mycoplasma, and endotoxins can contaminate a culture medium, thus possibly affecting the empirical results (Tatsuma and Asayama, 2017).

Another critical issue is that when using cell culture technology, it is not possible to evaluate an organism's deep response to the tested medication. Chicken embryos provide a technically simple way to study complex biological systems with well-developed vascular structures, in addition to allowing high reproducibility and being cheap and easy to handle, being recognized as an intermediate model that can fill this gap (Sommerfeld et al., 2022).

The embryos of chickens are also useful for viral isolation and titration tests in addition to vaccine technology (Fauzia et al., 2018).

2.4 METHODS TO CONTROL CJ

Campylobacter control in the food chain, especially in chickens' production, has become one of the main targets of efforts in prevention and control, but despite Brazil being the world's largest exporter of chicken meat, information about this agent in poultry production chain is still limited (Silva, 2017). Campylobacteriosis cases are underdiagnosed and underreported and there is no easy access to epidemiological data. The Brazilian legislation has no establishment concerning microbiological standards for *Campylobacter* in food (Brasil, 2009) and there is no regular follow-up of this pathogen in the meat industry, unless it is required by the external customer.

2.4.1 Prevention using alternatives to antimicrobials

The estimated public benefits of a higher reduction of the disease burden of campylobacteriosis and its sequelae are more significant if efforts are made toward controlling *C. jejuni* during the primary production stage (EFSA, 2011); once, unfortunately, there is no vaccination available against *C. jejuni* in birds due to the serological diversity of the pathogen and the short lifespan of broilers (Bishop-Hurley et al. 2010). On-farm biosecurity practices that are usually considered to be an important method for reducing *Campylobacter* infection in chickens, usually achieve up to a 50% reduction of infection in broiler flocks (Gibbens et al., 2001), so, there is no single, effective strategy for controlling the colonization of chickens by *Campylobacter* on-farm, necessitating the development of new specific intervention strategies. Since human campylobacteriosis cases are primarily contracted via the foodborne route, successful control of the disease requires mitigations in both animal reservoirs and the human host.

As an alternative for antibiotics, prebiotics have also been studied for their use to prevent and reduce *Campylobacter* colonization in animals, especially in broiler chickens (Kim et al., 2019). Baurhoo et al. (2009) studied using mannan-oligosaccharide as feed supplement at 2% and saw *Campylobacter* numbers decrease in cecal contents of chicken and litter samples. In another study, when using 1% inulin or 1% oligofructose as feed supplement, *Campylobacter* colonization reduced in large intestine, but remained in the gizzard and small intestine (Chen, 2003). On the other hand, several studies on prebiotic or prebiotic-like treatments did not reveal any significant effects on *Campylobacter* counts in broiler chickens (Rezaei et al. 2015; Park et al., 2017).

Another alternative consists in using probiotics to reduce *Campylobacter* colonization in poultry. To be effective, probiotics must be able to establish in the intestinal tract of inoculated birds. Therefore, the efficacy of probiotics may be affected by factors that influence the establishment, such as the ability to survive low pH in the gastric environment, doses of probiotics, and the route of administration (Dai et al., 2020). Neal-McKinney et al. (2012) studied the production of organic acids by probiotic *Lactobacilli* to reduce pathogens load in poultry and saw that they inhibited *C. jejuni* growth culture media and reduced *Campylobacter* colonization in broiler chickens. Another study found that a probiotic made of *L. johnsonii* altered the gut microbiota and reduced *Campylobacter* colonization in ceca of chickens (Manes-Lazaro et al., 2017).

When evaluating the route of administration, some authors, who tested two different routes (oral and intracloacal), saw that probiotics provided intracloacally, reduced better cecal *Campylobacter* counts when compared to oral route. This may not be a practical way for on-farm application, so these results suggest the need for improved delivery of probiotics into the intestinal tract to increase their efficacy against *Campylobacter* (Arsi et al., 2015). With technology advancing, the study of complex interactions among *Campylobacter*, probiotics, gut microbiome, and the host is possible. These research efforts should guide the targeted development of effective and reliable probiotics in the future (Dai et al., 2020).

Bacteriocins, which are small peptides of bacterial origin (Cotter et al, 2005), are considered a potential alternative for antibiotics (Galvez et al., 2007) and have been explored for mitigating *Campylobacter* in chickens (Svetoch and Stern, 2010). Stern et al. (2005) reported that a bacteriocin (named SRCAM 602) isolated from *Paenibacillus polymyxa* produced more than 7 log unit reduction in *Campylobacter* colonization in chickens when given in feed. The finding that *Campylobacter* was not detectable in any of the bacteriocin-treated chickens suggested that SRCAM 602 might be used as a therapeutic agent to eliminate CJ from chickens. The same author also described a drastic reduction of CJ colonization in chickens compared to the non-treated controls, using bacteriocins OR-7, E-760, purified from *Lactobacillus salivarius,* and E 50-52, purified from *Enterococcus* sp. Further investigation is necessary to evaluate the utility of bacteriocins as a therapeutic agent for *Campylobacter* treatment and even if they are proven to be safe and effective, commercial use requires cost-effective production of bacteriocins in large quantities (Dai et al., 2020).

Several studies have been conducted to develop vaccines against *Campylobacter* colonization in broiler chicken and a great advance was the development of experimental glycoconjugate vaccines constructed by fusing the conserved *C. jejuni* N-glycan to a carrier protein or by linking it to the lipopolysaccharide core of *E. coli.* (Nothaft et al., 2017). The vaccines demonstrated high efficacy in preventing *Campylobacter* colonization in both layer chickens and broiler chickens, inducing IgY antibodies that specifically recognized the N-glycan. Since the vaccines is made of a conserved glycan, they are expected to provide broad protection against different *C. jejuni* strains but this remains to be determined by field trials on commercial farms where chickens are naturally colonized by genetically and antigenically diverse *Campylobacter* strains (Dai et al., 2020). Another potential approach for

preventing or reducing *Campylobacter* colonization in chickens consists in oral administration of hyperimmune antibodies as a prophylactic or therapeutic agent. Laying hens either naturally infected by *Campylobacter* or hyperimmunized with *Campylobacter* antigens produce high-titer anti-*Campylobacter* antibodies which are transferred to egg yolks, as mean to transfer maternal antibodies from layers to young hatchlings. Egg-derived maternal antibodies (IgY) were shown to protect, at least partially, young chickens from *Campylobacter* colonization (Sahin et al., 2003). It is important to mention that antigen selection is especially important considering CJ strains are antigenically diverse and there are many different strains existing in nature. Some studies have also demonstrated potential use of genetically engineered nanobodies for control of *Campylobacter* infection. However, the efficacy of anti-*Campylobacter* nanobodies has not been examined in animal models and their utility as a potential therapeutic approach remains to be investigated in future studies (Dai et al., 2020).

Due to the rising concern with antimicrobial resistance, phage therapy has become an alternative therapy to combat multidrug resistant bacterial pathogens such as *Campylobacter* species (Jackel et al., 2019). Some studies have examined the efficacy of several phages in mitigating chickens' *Campylobacter* colonization. Richards *et al.* (2019) used two *Campylobacter* phages to treat chickens experimentally infected with CJ and saw considerable reduction in *Campylobacter* counts in the intestinal tract throughout the 5-day treatment period, but the most obvious difference was seen 2 days after the initiation of the treatment. Phage therapy may be used as a treatment right before slaughter to reduce the risk of *Campylobacter* transmission via contaminated chicken meat to consumers, since reducing *Campylobacter* counts in the intestinal tract of chickens destined for slaughter will lead to less carcass contamination in the slaughtering process (Dai et al., 2020).

Phage display has been used to generate diagnostic and therapeutic targeting peptides for pathogens and the broad utility of this technology has also been demonstrated by the using selected phage peptides as the capture probe for real-time detection devices (Petrenko, 2008) as well as being incorporated into liposomes for phage targeted drug delivery (Jayanna et al., 2010). Peptides can be used to prevent diseases involving malfunctioning of proteins due to undesirable protein-protein interactions (Nevola, 2015). Many databases and algorithms have been developed in the past specifically in the field

of peptide-based therapeutics (Kumar, 2018). There are more than 200 therapeutics peptides, approved by FDA for the treatment of various diseases (Usmani et al., 2017).

2.5 DIAGNOSIS ASSAYS

Campylobacter spp. diagnosis is still a challenging once this organism is difficult to isolate, grow, and identify (Fitzgerald et al., 2016). Current clinical laboratory practices for *Campylobacter* testing appear to be changing with the increasing availability of new culture independent stand-alone tests for direct detection of *Campylobacter* from stool specimens. Campylobacters are fastidious organisms that require microaerobic conditions for growth, so methods used for stool specimen collection, transport, and culture can have a large effect on sensitivity of testing. Despite the availability of guidelines for the collection, transport, and isolation of *Campylobacter*, procedures used by clinical laboratories may vary (Nachamkin and Murray, 2003).

To diagnose campylobacteriosis, isolation of the agent is the most indicated. *Campylobacter sp.* is typically found in low concentrations in specimens analyzed, except in carcasses of birds recently processed (ISO 10272-1, 2017). The most used culture medium is *Campylobacter* agar base incubated for 36 to 48 hours at 41,5ºC in micro aerobiosis, and in the identification of the agent, there is a thin and curved rod, Gram-negative, a translucent colony of water droplets, smooth, convex, shiny and the growth can be confluent (Andrade et al., 2010). The direct plating technique is more efficient in quantifying *Campylobacter sp.* (Revolledo and Ferreira, 2009). The isolation of *C. jejuni* from some foods and water can be facilitated by using a phase of pre-enrichment with incubation for 4 - 6 hours at 37ºC in a non-selective medium before incubation at 41,5°C (ISO 10272-1, 2017).

Campylobacters require low oxygen tension for multiplication. They are sensitive to concentrations greater than 2% NaCl at 30-35ºC or 1% at 4ºC. The optimal pH is between 6.5 and 7.5 and they are also sensitive to dehydration (Holt, 1994). In this sense, there are several methods to obtain an atmosphere suitable for *Campylobacter* growth, however, due to the practicality of its use, the use of commercially available gas generating envelopes has been adopted, whose approximate atmosphere is 5% to 10% of oxygen and 5% to 12% carbon dioxide. The methodology includes incubating plates in a microaerophilia atmosphere (5% to 6% of oxygen; 10% carbon dioxide; 84% to 85%

nitrogen) required by *Campylobacter* and at high temperatures from 42ºC to 43ºC for selection of thermotolerant species (Brasil, 2011).

For growth in plates, the composition of selective media is an important key for the study of thermotolerant *Campylobacters*. Every medium used for the isolation of *Campylobacter* must contain antibiotics, which is crucial for the recovery of *Campylobacter.* Antibiotics that inhibit mold and yeast are usually included in the media to *Campylobacter.* Amphotericin B is the most widely used antifungal and functions as a substitute for cycloheximide, which is considered toxic for inclusion in microbiological media (Martin et al., 2002).

Most selective media also contain blood, and many include oxygen-trapping agents (superoxide and hydrogen peroxide) to overcome its toxic effects in these species (Brasil, 2011). Since *Campylobacter* species are not able to ferment carbohydrates, peptones are included in the medium as a source of nutrients. The broth Preston (Bolton and Robertson, 1982) and Exeter (Martin et al., 1996) contain meat and peptone extracts. Bolton broth and *Campylobacter* Enrichment Broth – CEB have a peptones-based formulation, along with yeast extract and alpha-ketoglutaric acid, an intermediate of the tricarboxylic acid cycle. Jorge (2005) evaluated the behavior of *C. jejuni* when inoculated in different substratum, as type C pasteurized milk, UHT milk, and Bolton Broth (Oxoid), for different times of incubation and showed that the natural microbiota of type C milk interferes with the proliferation of *C. jejuni*. Besides that, the isolation of this microorganism occurred more easily following 24 hours incubation period than 48 hours, probably due to the shortage of nutrients. Kuana et al., (2007) compared the pre-enrichment (PE) and direct plating (DP) in the identification of *Campylobacter* in cloacae swabs and broiler carcasses and saw that both methods were homogeneous and sensitive for the detection of viable cells of *Campylobacter*. However, direct plating should be recommended, due to its practical usefulness and to the possibility of having the results within 24 hours. This was also seen by Line et al. (2001), who compared two most probable number (MPN) procedures (utilizing different selective enrichment broths and plating media) to the direct plating technique for enumeration of *Campylobacter* from freshly processed (post chiller, post drip) broiler chicken carcasses and concluded that the direct plating method offers a more simple, less expensive, more rapid alternative to traditional MPN procedures for estimating *Campylobacter* populations associated with freshly processed broiler carcasses.

Although identification of *Campylobacter* in plate growth is a viable method, some bacterial pathogens can enter in a dormant stage in which the cells are no longer cultivable but remain viable and virulent. This viable but non-cultivable (VNC) state has been demonstrated in potential health hazard pathogens such as *Salmonella* spp., *Campylobacter jejuni*, *Escherichia coli* and *Vibrio cholerae*.

The standard microbiological techniques for detecting *Campylobacter spp*. are very laborious and time-consuming. These limitations emphasize the necessity of rapid, reliable, and sensitive methods for detecting *Campylobacter* species. Immunochemical assays accommodate such requirements. Compared with DNA-based methods, they do not require expensive and highly sophisticated instrumentation, and it is possible to adapt them for field measurement (Hochel et al., 2007). Also, in the absence of positive cultures, the method of choice for determining a recent *Campylobacter* infection is serology (Ang et al., 2007). ELISA-based methods are the most convenient because of their superior sensitivity compared to complement fixation, as well as their potential for standardization. With an indirect ELISA method, high levels of IgG may occupy binding sites in the ELISA plate, thereby lowering the measured IgA and IgM response (Ang et al., 2007).

Molecular techniques offer rapid detection with improved sensitivity and specificity and include PCR/ real-time PCR, immunoassays (ELISA) and nanotechnology-based methods. The number of reported culture-independent diagnosis tests have been increasing because of the shorter time required to obtain results and the ease of obtaining them (Poonlapdecha et al., 2018).

Dubovitskaya et al. (2023) assessed the suitability of a qPCR method for a rapid quantitative determination of *Campylobacter* spp. at different stages in the poultry production chain and its equivalence with the culture-based method and concluded that the classical culture-based method for food hygiene risk assessment cannot be replaced one-to-one by the qPCR. On the other hand, Dawson et al. (2023) coupled PCR method with a DNA extraction to enumerate *Campylobacter* spp. from poultry gastrointestinal tract samples and conducted three experiments (1. development of a DNA standard curve related to bacterial DNA primers; 2) design of a cell/genomic DNA extraction protocol to isolate *Campylobacter* spp. DNA from complex samples such as poultry feces; and 3) comparison of PCR quantification to standard plate count methodology) which showed that the combination of the standard curve for *Campylobacter* spp. DNA primers, the gradient cell concentration method and DNA extraction techniques with qPCR can be

used to enumerate *Campylobacter* spp. from poultry samples with findings similar those of traditional plate count methodology.

Ito and Kishimoto (2023) developed a direct-qPCR method for determining the viable cell counts of *Campylobacter* spp. using qPCR without DNA extraction from enriched food samples and a sampling method (the wrap procedure) in which the sample is wrapped in a sheet, different from the conventional homogenization procedure and concluded that this method can provide baseline data for the risk assessment *Campylobacter* food poisoning.

2.6 NEW ALTERNATIVES FOR CONTROL OF CAMPYLOBACTER JEJUNI

2.6.1 Pathogens' identification by machine learning techniques

Data science is a broad field of study that focuses on the extraction of information and ideas from data using computational techniques, computers, algorithms, and systems. It is based on deep learning, artificial intelligence, and tools for processing large amounts of data. It is a skill set using mathematics, statistics, programming, and business experience (Zeravan et al., 2023). Machine learning methods are commonly utilized in industrial business to extract valuable insights and solutions from large amounts of data. Machine learning algorithms are an integral part of the market in a variety of very diverse areas, from medical labs to financial firms (Mehyadin and Abdulazeez, 2021).

FTIR spectroscopy is a relatively cost-effective, rapid, convenient, and precise analytical technique that can reflect the DNA structure and composition (Han et al, 2018). FTIR is a method used to obtain an infrared spectrum of emission or absorption of a liquid, solid or gas. An FTIR spectrometer collects the high-resolution spectral data simultaneously over a wide range of spectral data. This technique identifies chemical bonds in different molecules by producing an infrared absorptions spectrum. FTIR spectroscopy has been used as a powerful tool for species identification and differentiation of eukaryotic and prokaryotic cells based on genomic DNA characterization and barcoding (Fredericks et al., 2012). Spectral data from FTIR are so complicated for analysis; therefore multivariate statistical and dimension reduction methods such as principal components analysis (PCA), hierarchical clustering analysis (HCA), partial least squares (PLS) and artificial neural networks (ANN) techniques have

been used for interpretation of the results (Rios et al, 2021). Several researchers used and suggested FTIR assay followed by statistical analysis as a rapid, simple, relatively cheap, precise, sensitive, specific, and convenient method for distinguishing the species of microbial pathogens, isolated from clinical specimens based on their genomic DNA structural differences (Pebotuwa et al., 2020).

The potential use of FTIR spectroscopy to discriminate, classify and identify microorganisms has been successfully demonstrated in the literature, including many bacterial strains from Gram-positive and Gram-negative species (Cordovana et al., 2021). FTIR method is not only used for bacterial determination, but also supplies information about bacterial metabolism and growth phase (Becker et al., 2006). There is an increased demand for FTIR spectroscopy in food microbiology due to its technical improvement, simplicity of sample preparation, and speed of analysis (Davis et al., 2012). FTIR spectroscopy could be an exquisite alternative to the available analytical techniques in food analysis because of its increased sensibility, resolution, high signal-to-noise ratio, multiple-component analysis, and rapid measurement capabilities (Soto Beltran et al., 2015).

Carranza et al. (2012) demonstrated that the use of a focal-plane-array (FPA) FTIR spectrometer in place of a conventional FTIR spectrometer can enhance the reliability of the FTIR spectral data collected from samples of bacteria. They evaluated the sensitivity and specificity of a novel FPA-FTIR-based method for the differentiation of *C. jejuni* and *C. coli* and concluded that the FPA-FTIR-based method offers a reliable alternative for the identification of *Campylobacter* isolates.

With the advent of mathematical tools, scientists are now able to better predict epidemics, understand the specificity of each pathogen, and identify potential targets for drug development. Artificial intelligence and its components have been widely publicized for their ability to better diagnose certain types of cancer from imaging data (Agrebi and Larbi, 2020).

2.6.2 Phage display: A theragnostic approach

Phage display has been used to generate diagnostic and therapeutic targeting peptides for pathogens including bacteria (Carnazza et al., 2008), fungi (Fang et al., 2006) and spores (Brigati et al., 2004). The broad utility of this technology has also been demonstrated using selected phage peptides as the capture probe for real-time detection

devices (Petrenko, 2008) as well as being incorporated into liposomes for phage targeted drug delivery (Jayanna et al., 2010).

Based on that, Sharon et al. (2010) used a subtractive phage-display protocol to affinity select for peptides binding to the cell surface of a poultry isolate of CJ with the aim of finding peptides that could be used to control this microorganism in chickens and the phage peptides were highly specific, completely inhibiting the growth of two of the four poultry isolates of CJ tested with no activity detected towards other Gram-negative and Gram-positive bacteria.

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OBJECTIVES

1. 1 GENERAL OBJECTIVES

- Enhance the control of *Campylobacter jejuni* in a Theragnostic Approach: FTIR-ATR combined with Artificial Intelligence, Binding-Peptides, and the Use of Chicken Embryos as an *In Vivo* Model.

1.2 SPECIFIC OBJECTIVES

THEORETICAL FOUNDATION

- Perform a literature review of *Campylobacter* spp., highlighting its significance and occurrence worldwide, the pathogenicity of *Campylobacter* in humans and chickens, prevention, and therapy-based *Campylobacter* control, as well as its cultivation and identification.
- Describe about pathogens' identification by machine learning techniques.

CHAPTER II

- Evaluate the virulence and infection of strains of *Campylobacter jejuni* (CJ) isolated from chicken and standard strain isolated from humans to better understand the pathogen-host relationship of CJ and chicken embryo (CE) with an active immune system.
- Evaluate embryo mortality, weight, gross and microscopic lesions, multiplication of the bacteria in the embryo, macrophage and lymphocyte counting by flow cytometry analysis, cytokine analysis by ELISA, and analysis of histopathological lesions.

CHAPTER III

- Develop a sustainable biphotonic platform from ATR-FTIR supported by machine learning algorithms for detection of *Campylobacter jejuni.*

CHAPTER IX

- Select and characterize *Campylobacter jejuni* binding peptides using *Phage Display* technology.
- Confirm selected peptides by ELISA, sequencing, and molecular docking.

- Perform the inhibitory assay to test different CJ strains – *in vitro* experiment.
- Understand the influence of the peptides using chicken embryos as experimental model, challenged with CJ – *in vivo* experiment.

CHAPTER II

Understanding the chicken embryo as a model of *Campylobacter jejuni* infection beyond death

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Abstract

This study aimed to evaluate the virulence and infection of strains of *Campylobacter jejuni* (CJ) isolated from chicken and standard strain isolated from humans to better understand the pathogen-host relationship of CJ and chicken embryo (CE) with an active immune system, which is a way to study complex biological systems with well-developed vascular structures. Then, we evaluated embryo mortality, weight, gross and microscopic lesions, multiplication of the bacteria in the embryo, macrophage and lymphocyte (T and B cells) counting by flow cytometry analysis, cytokine analysis by ELISA, and analysis of histopathological lesions. At low doses, CJ isolated from chicken and standard strain isolated from humans generates discreet or moderate lesions in CE, and some strains can stimulate the immune system, but the response is strain-dependent, reinforcing the importance of studying the virulence, infection, and immune response of several strains of *C jejuni* and the CE model as of great utility.

Keywords: Foodborne pathogens; zoonotic bacteria; innate immune response; human campylobacteriosis

1. Introduction

Salmonella spp. and *Campylobacter* spp. are the bacterial etiological agents most implicated in foodborne infections. *Campylobacter jejuni* (CJ) infections, which have been strongly linked to contaminated retail chicken by several studies [1+], are highly prevalent in commercial broiler flocks. A study showed that 95% of birds could be rapidly colonized with *Campylobacter* spp. once exposed to a single infected seeder bird and remain colonized until market age [2]. This pathogen is highly prevalent in chicken ceca (10^9 CFU/g), often causing no harm to adult birds [3]. When birds are slaughtered, this bacterium is released from the intestines, contaminating the meat and posing a risk to humans [4].

Although CJ causes a self-limiting disease in immunocompetent being humans, it brings severe consequences for children, the elderly, and immunocompromised patients [5]. In addition, extra-intestinal manifestations such as polyarthralgia (i.e., multiple sites of reactive arthritis) or the Guillain Barre syndrome (GBS) might occur [6]. Therefore, the effort to reduce Campylobacter spp. infections in humans is directly linked to a better understanding of the infection mechanisms and host response. However, the molecular bases of virulence mechanisms of CJ are strain-dependent, and assessing the phenotypic characteristics of isolated strains is essential for a better understanding of the pathogen and host relationship. Cell culture studies are efficient in better understanding the virulence and immune response to a strain. However, evaluating an organism's deep response at real time is impossible. So, *in vivo* models are essential to preliminary studies.

Chicken embryos (CE) provide a simple way to study complex biological systems with well-developed vascular structures, in addition to allowing high reproducibility and being cheap and easy to handle [7]. The CE has a long and distinguished history as a primary model system in developmental biology and has also contributed significant concepts to immunology, genetics, virology, cancer, and cell biology, being one of the most versatile experimental systems available [8].

Although in high doses, or infecting CE in the early stages of incubation, CJ can kill the CE [9,10], we know little about the lesions or inflammatory responses caused by this bacterium in CE. To our knowledge, there is no study about the immune response in CE post-CJ infection.

Considering the importance of studying the virulence, infection, and immune response of several strains of CJ and the great utility of the CE model, we aim to evaluate

the virulence and infection of strains of CJ isolated from chicken and standard strains isolated from humans. In this way, we intend to understand better the pathogen-host relationship of CJ and CE with an active immune system.

2. Material and methods

For this experiment, we used a total of 177 eggs (lineage Ross 308) from a commercial broiler hatchery in the region of Uberlândia (MG), being 37 eggs for mortality assay and 140 eggs (divided into two groups of 70 eggs) for virulence and infection analysis. The eggs were taken to the incubation laboratory of the Veterinary Medicine Faculty at the Federal University of Uberlândia, identified with a number and incubated in a hatchery (Premium Ecológica IP30) with medium humidity at 55% and a temperature of 38ºC. The 177 eggs were divided into three trials (the first one using 37 eggs and the next two, with 70 eggs each one) to facilitate material collection and processing.

2.1 Previous test

We first performed a pilot test to understand the mortality rate of bacteria samples better. For a better design of our study, the mortality rate needed to be low to have an ideal number of embryos for laboratory tests. We used a total of 37 eggs divided into the following groups: (i) a total of five (5) CE inoculated with CJ, strain IAL 2383 isolated from humans (Fonseca et al., 2014), (ii, iii, iv) four (4) CE in each group inoculated with CJ, strain C030/30, C046/10, C092/6, respectively isolated from chicken (Peres, 2020), (v) Ten CE inoculated with *Salmonella* Typhimurium (ST) isolated from chicken, (vi) Ten CE as a negative control inoculated with sterile 0.9% NaCl, all inoculated on the 10^{th} day of embryonic development, the eggs were candled to detect and discard those not fertilized or with dead embryos and then were inoculated as described below. We tested a larger number of controls to better understand the number of CE necessary to the next phase of the research.

A hole was aseptically made in the eggshell, and 3.7 logs CFU/CE of bacterial suspensions from an overnight culture diluted in sterile saline solution were inoculated intra-allantoically using a sterile 1 ml syringe with a 27G needle attached. The hole was then covered with polish, and the eggs returned to the incubator. Embryo viability was recorded daily by candling, and the mortality rate was calculated as the mean percentage of embryonic. On the 17^{th} day of incubation, we evaluated the gross lesions and collected

the allantoic fluid to proceed with plating in the CCDA (Charcoal Cefoperazone Deoxycholate Agar) medium (Acumedia) to count CJ in the allantoic fluid.

2.2 Virulence and infection test

Because of the high mortality rate in the pilot test, we decided to use a lower dose of CJ and ST (2.5 logs CFU/CE) via allantoic fluid. The experiment was performed twice to have a replicate and because the number of eggs was too large to perform analyzes in which the samples could not be stored (counting of bacteria and flow cytometry) simultaneously. The total of eggs tested for virulence and infection test was 140 (70 eggs per repetition of the experiment).

On the 10th day of incubation, in every 10 eggs per group, we weighed and identified each egg and performed the inoculation of the following agents: ST (positive control), CJ strains IAL 2383, C030/3, C046/10 and C092/6, a probiotic strain of *Bacillus subtilis* (BS) strain (negative control) [11] and saline solution (negative control) via allantoic fluid. We used the probiotic strain (BS) as a control to evaluate whether the possible alterations caused by CJ were like a probiotic bacterium.

For the inoculated agents, the BS and ST bacteria were grown in nutrient agar (Acumedia) at 37ºC for 24 hours, and CJ's strains were grown in CCDA medium (Acumedia) at 40ºC in a micro-aerobic atmosphere for 48 hours. After the inoculation, the eggs were daily evaluated for viability. On the 17th day of incubation, we weighed the CE without the embryonic annexes and analyzed the lesions. Besides, we collected the blood, the allantoic fluid, and tissue (liver samples).

2.2.1 Weight, gross lesions, and mortality

We used a precision scale (Bel M214-AIH) for weighing the eggs before the agents' inoculation and the embryos on the 17th of incubation. The final embryo weight was adjusted considering every egg with 50g, using the following equation: (50 x embryo weight)/ initial egg weight). Also, gross lesions in allantoic fluid and CE were observed and registered. Embryo mortality was evaluated each day using a flashlight in the dark, so we could see if the embryo was active or dead. We performed the mortality index, weight and gross lesions in all tested CE.

2.2.2 Multiplication of CJ in the CE allantoic fluid

The allantoic liquid was collected using a 5mL syringe in aseptic conditions and sent to a laminar flow for processing. In each repetition of the experiment, we sampled three CE per group to each collection totaling six samples. We used 100µL of each sample and diluted it to 900µL of sterile saline solution, and we performed several serial decimal dilutions for plating. Counting was carried out in duplicate, with ST on nutrient agar and CJ on m-CCDA, after incubation at 36ºC/24hs and 40ºC/48hs in micro-aerobic atmosphere, respectively.

2.2.3 Macrophage and lymphocyte counting by Flow Cytometry Analysis

Total blood was collected from 17-day-old CE in aseptic conditions, using a 5mL syringe for each embryo and collecting around 2mL of blood into a blood test tube containing 50µL of EDTA. In each repetition of the experiment, we sampled 3 (three) CE per group (randomly chosen into each group) totaling six samples. The blood was centrifuged at 200xg for 5 min at room temperature to separate the blood serum. The total leukocyte was collected and added to an erythrocyte buffer lysis (BD) and incubated for 15 min at room temperature. The cells were then centrifuged at 200xg for 5 min at room temperature and incubated with PBS-BSA5% for 10 min at room temperature. After this, we added 2,5 µL of Mouse Anti-Chicken CD8-FITC (Southern Biotech), CD4 Pe-Cy7 (Southern Biotech), and Monocyte/Macrophage-PE (Southern Biotech) and incubated for 1 hour at 4°C in the dark. The isotypes Mouse IgG1-PE (Southern Biotech), IgG2-FITC (Southern Biotech), and IgG1- Pe-Cy7 (Southern Biotech) were used to perform gate and analysis strategy. The cells were washed twice with wash buffer and centrifuged for 5 min at 200xg at room temperature. Next, 100µL of PBS1x was added, and the cells were analyzed by flow cytometry (Attune-Thermo). A negative control with unlabeled cells was used in each test. Cells were analyzed for at least 1,200.000 events at the lymphocytes gate, and data were analyzed using the software provided by Attune-Thermo.

2.2.4 Cytokine analysis by ELISA

Blood serum of three CE of each phase of the experiment (totaling six samples) were obtained from 17-day-old CE in aseptic conditions and stored under -80ºC until processing at the Nanobiotechnology Laboratory at the Federal University of Uberlândia. The levels of Interferon Gamma (IFN-γ), Interleukin-1 beta (IL-1β), and Interleukin 10 (IL-10) in the serum of CE were measured by enzyme-linked immunosorbent assay (ELISA). 96-well microplates (Greiner Bio-One) were sensitized with CE serum (in

duplicate) in 50 mM bicarbonate buffer (pH 8.6) for 1 hour at 37ºC. The microplates were washed three times with PBS-T (0.1M PBS + Tween 20 0.05%), and then, they were blocked with 3% bovine serum albumin (3% BSA) in 0.1M PBS for 1 hour at 37ºC. After this period, the plates were washed three times with PBS-T. The microplates were incubated with the antibodies, rabbit anti-chicken IFN-γ IgG antibody (BioRad), rabbit anti-chicken IL-1β IgG antibody (BioRad), or IL-10 Polyclonal IgG antibody (Thermo), diluted (1:1000) in 3% BSA + PBS for 1 hour at 37ºC. After three washes with PBS-T, the plates were incubated with secondary goat anti-rabbit IgG (Sigma) diluted (1:5000) in 3% BSA + PBS. The plates were washed four 4 times with PBS-T, and the binding of the antibody/antigen was detected by adding 50µL/ well of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Thermo Scientific) in a low-light environment. The addition of 50µL/well of 2 N H2SO4 after 30 minutes stopped the reaction. The absorbance was determined in a plate reader (Titertek Multiskan Plus, Flow Laboratories, USA) at a wavelength of 450 nm. In parallel, different concentrations of recombinant IFN-γ, IL-1β, and IL-10 protein (BD Biosciences, San Diego, CA) were used to construct the standard curve.

2.2.5 Histopathology

Liver samples were collected, and the tissue was preserved in a formalin solution (10%) for processing. The tissues were dehydrated with different ethylic alcohol concentrations (85%, 95%, and 100%, respectively), followed by two xylol (100%) immersions, and then included in liquid paraffin and processed according to the histopathology laboratory routine in identified blades stained with hematoxylin and eosin for histopathological examination at the Pathology Laboratory at the Federal University of Uberlândia. The images were analyzed in an optic microscope (Nikon Y-THM) with a magnification of 40X.

Two experienced pathologists analyzed all slides without knowledge of the treatment group. Then, the lesions were identified and scored for severity, and the slides for the control group were identified and re-evaluated for normality. We used the control samples as a guide for the normal histological appearance. All slides were re-examined compared to the negative control slide to ensure accurate recognition and grading of lesions.

The slides were evaluated semiquantitatively according to the negative control for histological evidence of inflammation lesions and hemorrhage [12], as: 0, corresponding

to normal, with no circulatory for inflammation alterations; 1 – mild; 2 – moderate; 3 – accentuated.

2.2.6 Statistical Analyses

We performed the Kolmogorov–Smirnov test to assess whether the data were parametric. In no parametric date, we normalized the date in the square root (in the case of the TCD4+: TCD8 + ratio). So, we used ANOVA ($p<0.05$) followed by the Tukey test or Kruskal-Wallis to parametric and non-parametric tests, respectively. For association analysis between the number of bacteria and gross lesions, we used the Fisher's test. For ELISA analysis, a relative standard curve was constructed from the absorbance values according to the control (recombinant protein IFN-γ, IL-1β, and IL-10). The data were interpolated using Pade (1.1) or hyperbolic approximant. We used the Wilcoxon tests to evaluate lesion scores, considering a score of 0 (lesions absent) as a standard. We used the software GraphPad Prism 9.0, considering a confidence interval of 95%.

3. Results

3.1 Mortality, bacterial count, Weight, and gross lesions in the pilot test

When we inoculated 3.7 logs CFU/CE of ST or CJ, there was high mortality in CE. Infection by ST resulted in the most increased embryo mortality (80%) (Fig. 1). We found an average of bacteria in allantoic fluid of 2.30; 4.26; 2.49; and 6.22 log CFU/mL in CJ strains IAL 2383, C030/3, C046/10, and C092/6, respectively.

3.2 Low doses of CJ do not result in high mortality or weight change, but CJ multiplies and leads to macroscopic lesions in CE.

When we inoculated a dose of 2.5 log CFU/CE, the mortality caused by CJ was low (table 1). Only one strain (C030/3) showed similar mortality to ST (CP), which at a low infective dose did not lead to high embryonic mortality (table 1).

Regarding the lesions, milky allantoid and urate increases were the most observed lesion in CE inoculated with CJ. CE infected by CJ didn't present greenish or enlargement liver (Table 1). However, an enlarged spleen occurred in two CE inoculated with IAL 2383, and one inoculated with CJ C046/10, and C030/3 (Table 1).

CJ, as well as ST, didn't lead to a decrease in body weight (Fig. 2A). Like ST, all other strains multiplied in the allantoic fluid in the same amount, except for CJ IAL 2383,

whose count was lower than the others (Fig. 2B). In all embryos inoculated with CJ, there was the quantification of the bacteria, except for CJ IAL 2383, in which only one CE out of six tested had the bacteria counted. In the only embryo in which the IAL replicated, the number of bacteria found was 5.17 log CFU/Ml.

We analyzed the association between the number of bacteria in allantoic fluid and gross lesions in CE, considering all groups. The results show an association between the absence of bacteria and the absence of lesions (Fig. 2C) with an odds ratio (OD) between 1.389 to infinity (Fig. 2C).

3.3 CJ do not cause an increase of blood monocytes and lymphocytes CD8+ but some strains can increase lymphocytes CD4+ or CD4+:CD8+ ratio after 7 days of inoculation.

The gating strategy for chicken embryo blood is described in Fig.3A, representing the total leukocytes gate. The CD8-FITC graph, Monocyte/ Macrophage-PE and CD4 PE-Cy7 graphs are represented in Fig.3.B, C and D, respectively. Data represent one biological replicate. A total of 1,200.000 cells were recorded on a Thermo Accuri II flow cytometer.

We found a higher percentage of TCD8+ cells in the blood of CE when compared to TCD4+ ($p<0.05$). We noticed that the percentage of monocytes and lymphocytes TCD8+ didn't change in negative or treated groups (Fig. 3E and 3F). For CD4+ T cells, CE infected by CJ strains IAL 2383 and C030/3 had statistically significant differences from the positive control (ST), and they were like the negative control and group infected by a probiotic strain (BS) (Fig. 3G). However, CE inoculated with strains C046/10 and C092/6 presented a similar level of CD4+ in blood.

We analyzed the lymphocyte CD4+:CD8+ ratio, and the strain IAL 2383 presented a high CD4+:CD8+ (Fig. 3H).

3.4 Different strains of CJ do not change IL-1β and INF-γ but can change IL-10 in CE blood after 7 days of inoculation.

IL-1β in positive control had statistically significant differences ($p<0,05$) among all groups of *Campylobacter* strains, as also BS and the positive control (Fig. 4A); however, the CJ strains infection by C092/6 resulted in decreased IL-10 (Fig. 4B). IFN-γ had no significant differences among the studied groups in this research (Fig. 4C).

3.5 Some strains of CJ can induce discreet or moderate inflammatory changes in CE.

As it is normal to find granulocytes around portal spaces in birds, to facilitate the analysis, we evaluated the extent of the granulocytes around portal spaces using scores: zero (typical score) (Fig. 5B), discreet inflammatory score (Fig. 5C) and moderate inflammatory score (Fig. 5D). The strains CJ IAL, C030/3, and C092/6 induced an inflammatory change in the liver of CE similar to the positive control (ST). The score found was discreet or moderate (Fig. 5A). There was no difference in inflammatory changes seen in CE inoculated with NC, BS, or CJ 046/10 (Fig. 5A).

4. Discussion

We inoculated a concentration of CJ of 3.7 logs CFU/CE in the pilot test, and there was a high mortality (40-50%) in CE inoculated with IAL, C030/3, and C092/16 (Fig. 1). We used a positive control ST, which resulted in the most increased embryo mortality (80%) (Fig. 1), similar to other results [13].

Concerning the multiplication of CJ in allantoic fluid in the pilot test, strains C30/3 and C092/6 multiplied in allantoic fluid since we inoculated 3.7 logs UFC/CE and the number of bacteria 7 days after the inoculation was higher (Fig. 1). Regarding IAL 2383 and C046/10, we did not observe an increase in the mean number of bacteria (Fig. 1), but this fact cannot be considered since we only quantified bacteria from recently dead embryos, some live embryos, the bacteria did not grow and then we did not have enough samples.

It is known that *Campylobacter* can kill CE early [10] or at 10 days of incubation at a high infective dose [14,15], and the death of the embryo can be an essential tool to assess the virulence of the strains. However, the response of CE as an *in vivo* model is an exciting study to understand the host-pathogen relationship better. In our research, we intended to understand the changes caused by CJ. So, we inoculated low infective doses (2.5 log CFU/CE). Besides, in the next phase, we quantified bacteria only from live CE.

The fact that the infective dose of 2.5 log CFU/CE resulted in fewer dead CE mortality shows that embryonic death is dose dependent. Milky allantoid and urate increases was the most observed lesion in all CE inoculates with CJ (table 1). These lesions were due to the presence of bacterial infection since the negative control and CE inoculated with a probiotic strain of BS didn't present gross lesions (Table 1). This contradicts another study

that cites that CJ causes a noninvasive fatal infection in CE inoculated at the yolk sac by a lack of dissemination beyond the inoculation site [16] or CJ does not cause macroscopic or microscopic lesions in dead embryos [14].

Urate increase was observed in CE inoculates with CJ strains and the positive control (ST). This result can be explained because the infection increases oxidative stress [17], and oxidative stress could induce uric acid synthesis [18]. We may not confirm the cause of the milky allantoic in infected CE. However, this process can be occurred due to inflammation or a multiplication of the bacteria. Both hypotheses are supported by inflammatory reactions in the histopathological analysis in the liver and because in CE, in which the allantoid was normal, there was no bacterial multiplication (Table 1, Fig. 2C).

CE inoculated with *Salmonella* Pullorum or *Gammacoronavirus* have a decrease in body weight [7]. It appears that CJ colonization may lead to weight loss in 11-day-old grower chickens orally challenged with 0.5mL (4×10^7 / mL per bird) [19]. However, in our study, CJ, as well as ST, did not lead to a decrease in body weight (Fig. 2A). It is possible that the low dose of *Salmonella* or *Campylobacter* inoculum tested in our study led to milder inflammation that could not result in weight loss.

Macroscopic changes were not present in the liver of CE inoculated with *Campylobacter* strains, but some authors have demonstrated that inoculation of suspensions into the allantoic cavity of 10- to 13-day embryonated hens' eggs results in enlarged pale livers with necrotic foci and enlarged spleens [20]. Interestingly, enlarged spleens were one of the lesions we could notice in three of the four CJ strains used in our experiment.

In almost all embryos inoculated with CJ, there was the quantification of the bacteria, except for CJ IAL 2383, in which only 1 CE out of 6 tested had the bacteria counted. In the only embryo in which the IAL replicated, the number of bacteria found was 5.17 log CFU/mL. As IAL 2383 was isolated from diseased humans and has known virulence (Fonseca et al., 2014) and kills CE in the pilot test (at 3.7log UFC/CE), we can hypothesize that in low doses, IAL 2383 could not infect all CE. However, the only embryo it infected there was replication. Another explanation for IAL 2383's lower allantoid multiplication capacity is the embryo's immune response, which monocytes and lymphocyte counts or proinflammatory cytokines cannot confirm. However, there was an increase in the TCD4+:TCD8+ ratio of IAL 2383 compared to other strains and controls, indicating that IAL 2383 infected the CE.

Our result shows that when it comes to monocytes, we found a percentage of 0.062 to 0.14% (Fig. 3E) of the total leucocytes and thrombocytes, similar to other studies in CE at the same age of development and line [7]. We found no statistical difference among the groups when comparing the percentage of monocytes (Fig. 3E). Monocytes have great phagocytic activity and an essential role in antigen processing, which is crucial in removing apoptotic cells during embryo development [21]. Monocytes circulate in the bloodstream for about one to three days and then move into tissues throughout the body [22]. As we analyzed the leukocyte profile after seven days post inoculation of the agents, this could be one reason why we had no differences in monocyte in the bloodstream among the treatments.

Lymphocyte subpopulations in avian blood have been used to describe immunocompetence [23]. Our results showed a high percentage of TCD8+ (ranging the average between 2.29 to 8.09%) (Fig. 3F), higher than TCD4+ (Fig. 3G). We did not find works related to the characterization of CD8+ in CE blood, however, in the bone marrow, the amount of TCD8+ lymphocytes is high [24]. The percentage of CD8αα+ T cells in the spleen and bone marrow were higher in embryos than in young chickens [25]. The high percentage of TCD8 in embryos could be related both to the expression of CD8 on the vast majority of chicken TCR $\gamma\delta^{+}$ cells and the existence of a $CD8^{+}$TCR (TCR0) cell population, which is thought to represent a non-prothymocyte lineage that migrates from the periphery into the thymus early during chicken ontogeny [26].

Researchers have shown that many bacterial species also able to stimulate TCD8+ by a variety of mechanisms [27]. Even in high amounts in the blood, our results do not show the participation of TCD8+ in the immune response to CJ or any of the groups tested. This shows that the TCD8+ profile for the ST pathogen, CJ or BS probiotic is identical to the negative control, contrasting to another study conducted in mice, where both CD8+ and CD4+ T cells from plasma contributed to protection against CJ. However, the predominant role resided in the CD8+ cell subpopulation [28]. In 14-old broilers challenged with CJ (Strain A74C), after 14 days of infection, there was a significant increase in CD8+ T lymphocytes and a significant decrease in CD4+:CD8+ cell ratio in cecal tonsil [29]. In our study is possible that post 7 days of infection, lymphocytes TCD8+ already populated the CE organs and are already stable in the bloodstream.

TCD4 lymphocytes had a smaller cell population than TCD8, ranging from 0.072% in the negative control to 0.283% in the positive control (Fig. 3G). Unlike the other two cell populations, there was an increase in TCD4+ in the positive control. CE

infected by CJ strains IAL 2383 and C030/3 had significant differences from the positive control (ST) (Fig. 3G), and they were similar to the negative control and group infected by a probiotic strain (BS), seeming to have a less response via TCD4+. However, strains C046/6 and C092/6 showed similar levels for both negative control and BS and positive control. The non-increase of TC4+ lymphocytes in bloodstream, even in groups with macroscopic lesions, may suggest a stabilization of the immune system that already delivered TCD4+ to the tissue.

Especially for CE inoculated with CJ C046/6, a level of TC4+ similar to positive control can suggest low pathogenicity of the strain, with resulted in a better response of the immune system since there was non-occurrence of mortality presented by the pilot test (Fig. 1) or increase of microscopic inflammatory changes, which were evaluated according to the amount of granulocytes around the portal space in the liver (Fig. 5A).

In case of CE inoculated with C092/6 the increase of TC4+ can indicate a greater pathogenicity of the strain since the C092/6 killed the CE in pilot test, it resulted in gross lesion (table 1), moderate inflammation (Figure 5) and a decrease of IL-10 (Figure 4C). IL-10 is a key regulatory cytokine in controlling inflammation, and the absence of IL-10 expression in birds indicates that inflammatory responses are poorly regulated, leading to prolonged inflammation [46]. In many species, including chickens, the CD4+:CD8+ cell ratio indicates immune competence [30]. In general, in born animals, a CD4+:CD8+ cell ratio higher than 1 is observed in healthy individuals. In our study, the amount of TCD8+ lymphocytes was greater than TCD4+ in all groups, which can be explained by the fact that the chick begins to develop its defense mechanisms during embryonic life, but immunocompetence only appears a few days post-hatch [31–33].

Although the amount of CD4+ or CD8+ alone did not explain the response of CJ strains 7 days after the infection, the TCD4+:TCD8+ ratio showed an increase in CE infected by IAL 2383 (Fig. 3H). High TCD4+:TCD8+ ratios have been associated with an increase in the immune functional ability of chickens [34] or chronic disease [35]. So, considering that CJ IAL 2383 is pathogenic to humans [36]; it kills the CE at higher infective doses (Fig. 1); in 5 of the 6 inoculated CE; we did not find the multiplication of this bacterium in allantoic fluid and CE infected by IAL 2383 result in macroscopic (table 1) or microscopic lesion (Fig. 5); it is possible that IAL 2383 may stimulate the immune system, and this stimulus leads to a more chronic response compared to the other strains or ST.

Concerning to the CJ C030 we observed a high mortality in CE inoculated with this strain pilot test (Fig. 1) and it led to macroscopic (table 1) or microscopic changes (Fig. 5). However, the lymphocyte and monocyte count tests in bloodstream or cytokine analysis performed in this work were insufficient for a better understanding of the response to this strain.

The IL-1β and IFN-γ are proinflammatory cytokines [37–39], while IL-10 is an anti-inflammatory cytokine [40]. CJ induces the expression of IL-1β and IFN-γ [41,42], however, in our study, even with gross lesions or microscopic inflammatory changes, we found an increase in IL-1β just to ST and even in the positive control, there was no increase of IFN-γ. Probably, in CE, at later times (7 days after the inoculation), there are other actors involved in the immune response of CJ that were not studied in our work.

In conclusion, at a low dose, CJ generates lesions in chicken embryos, and some strains can stimulate the immune system without a defined response pattern, emphasizing that the host-pathogen relationship is strain-dependent. Furthermore, this work shows that the CE may be adequate for studying pathogenicity and response to CJ infection.

5. Conflict of interest

All authors declare that this work was conducted in the absence of any commercial or financial relationship that could constitute a potential conflict of interest.

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7. Figures

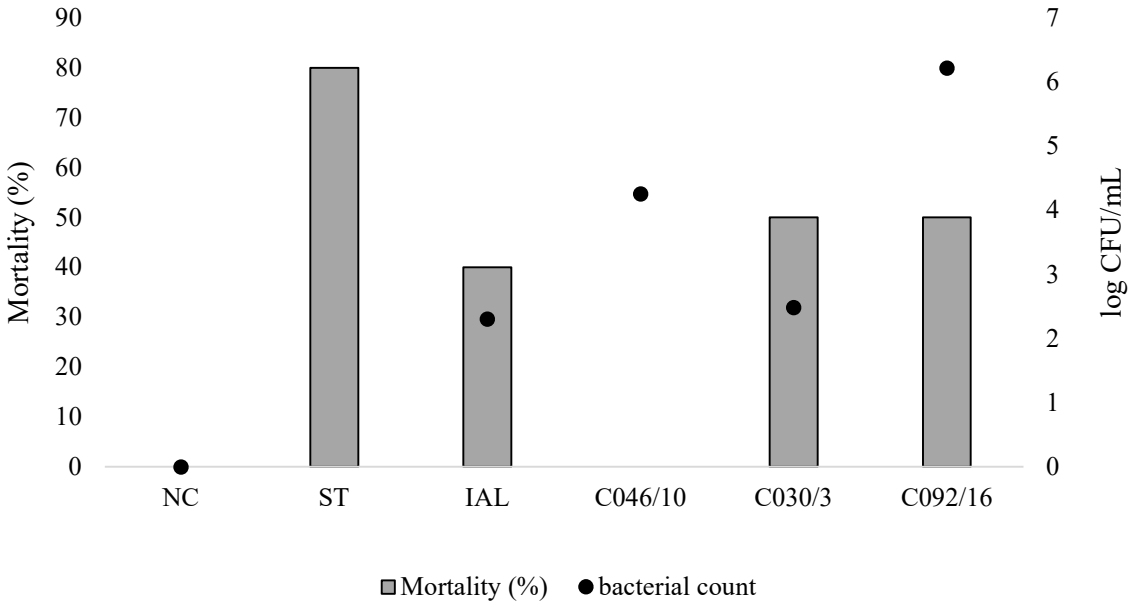

Fig. 1. Mortality and bacterial count in CE on the 17th day of incubation inoculated with different strains of CJ at 10 days of incubation. NC: negative control; ST: *Salmonella Typhimurium*; IAL: CJ strain IAL 2383; C046/10, C030/3, C092/16: CJ strain isolated from chicken. As 90% of the embryos from the positive control (ST) were dead, the bacterial count was not performed in this group. We performed just descriptive statistical analysis.

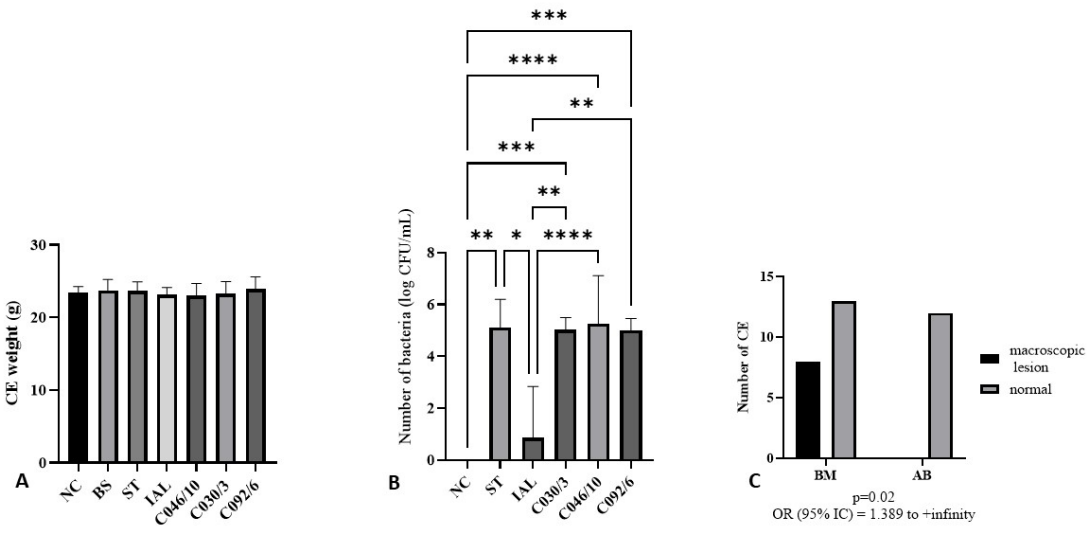

Fig. 2. Embryo mean weight, bacterial count in allantoic fluid, and the association between bacterial count and gross lesion. A: Embryo weight in different groups; B: Correlation between gross lesions and bacterial count in different groups; C. Association between bacteria multiplication and gross lesion in different groups. We used ANOVA and turkey test (A), Kruskal-Wallis test (B) or Fisher test (C). Statistical differences were considered when $p \leq 0{,}05$ (no asterisk: $p > 0.05$; *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$). NC: negative control; ST: CE inoculated with *Salmonella* Typhimurium (ST); IAL: Group of CE inoculated with *Campylobacter jejuni* (CJ) strain IAL 2383; C046/10, C030/3, C092/16: CE inoculated with CJ strain C046/10, C030/3, C092/16 isolated from chicken. CE: chicken embryo; BM: bacteria multiplication; AB: absence of bacteria, OR: odds ratio; IC: confidence interval.

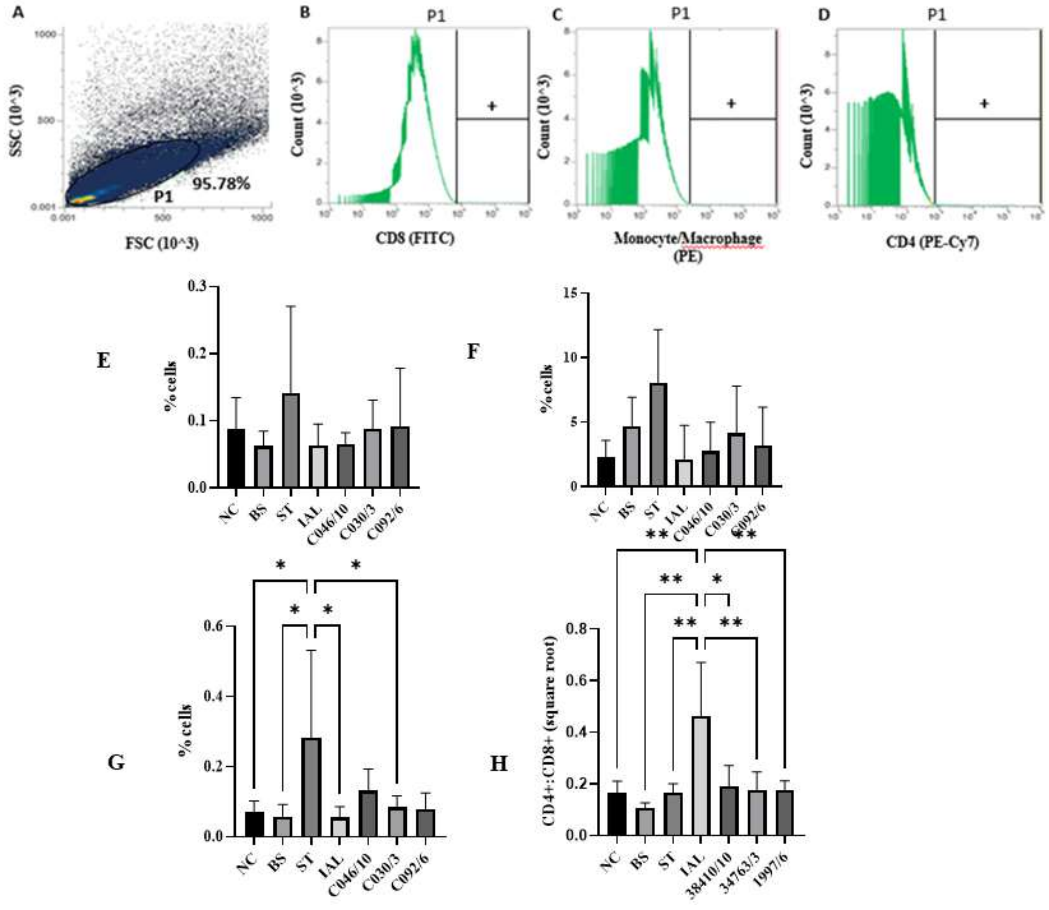

Fig. 3. Example of the gating strategy for chicken embryo blood and mean of Monocytes, Lymphocytes CD8+ and CD4+, and Lymphocyte CD4+:CD8+ ratio in CE blood inoculated with different strains of CJ 7 days after the inoculation. (A) y axes: SSC-A, x axes: FSC-A, the egg-shaped gate represents the total leukocytes gate; (B) y axes: Count cell, x axes: CD8-FITC; (C) y axes: Count cell, x axes:

Monocyte/Macrophage-PE; (D) y axes: Count cell, x axes: CD4-PE-Cy7. Data represent one biological replicate. A total of 1.200.000 cells were recorded on a Thermo Accuri II flow cytometer. (E) Monocytes; (F) Lymphocyte CD8+; (G) Lymphocyte CD4+. (H) TCD4+:TCD8+ ratio. We used ANOVA and Tukey test. The data of the CD4+:CD8+ ratio was not parametric, so we normalized the data using a square rate. * Statistical differences were considered when $p \leq 0,05$ (no asterisk: $p > 0.05$; *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$). NC: negative control; ST: Chicken embryo (CE) inoculated with *Salmonella* Typhimurium (ST); IAL: CE inoculated with *Campylobacter jejuni* (CJ) strain IAL 2383; C046/10, C030/3, C092/16: CE inoculates with CJ strain C046/10, C030/3, C092/16 isolated from chicken.

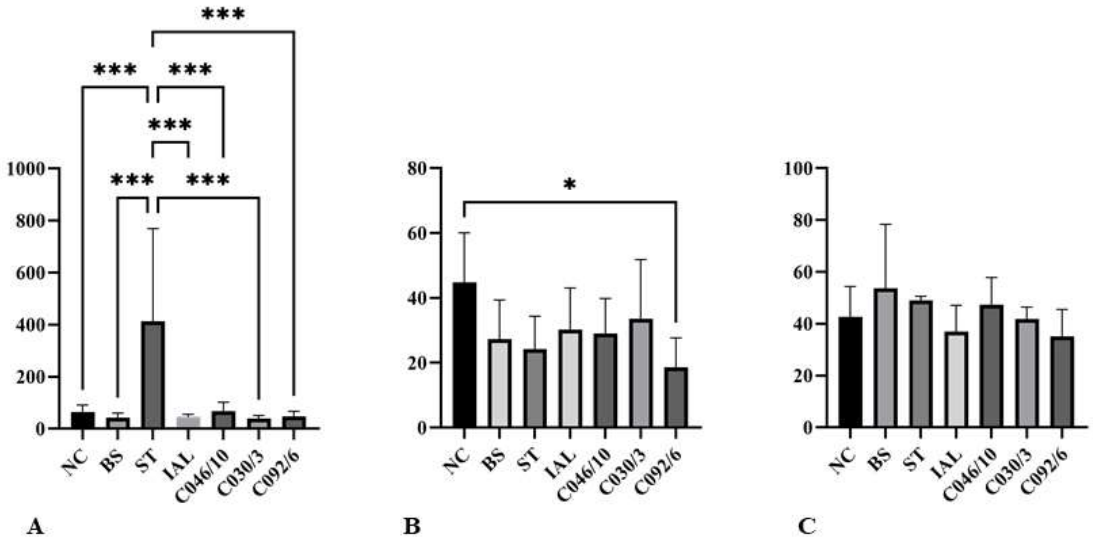

Fig. 4. Mean of interleukines IL-1β, IL-10 and INF-γ among the different treatments**.** A: IL1-β; B: IL-10; C: Interferon-γ. Statistical differences were considered when $p \leq 0,05$ (no asterisk: $p > 0.05$; *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$). NC: negative control; ST: Chicken embryo (CE) inoculated with *Salmonella* Typhimurium (ST); IAL: CE inoculated with *Campylobacter jejuni* (CJ) strain IAL 2383; C046/10, C030/3, C092/16: CE inoculates with CJ strain C046/10, C030/3, C092/16 isolated from chicken.

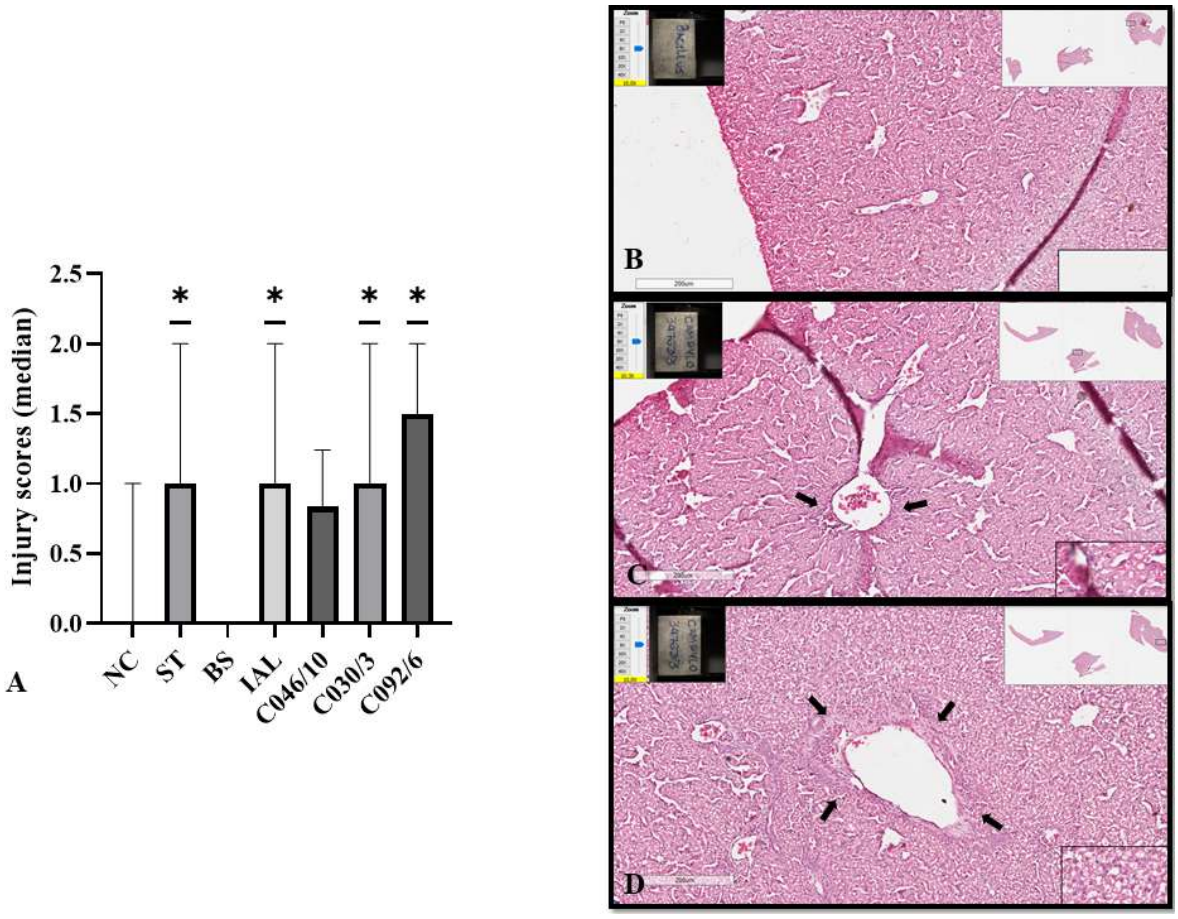

Fig. 5. Median of scores of lymphocytic infiltrates in liver of CE inoculated with different strains of CJ. A. Median of lymphocytic infiltrates scores in the liver of CE inoculated at 10 days of incubation and evaluated at 17 days of incubation. B. Score zero (normal score). C: discreet inflammatory score. D. moderate inflammatory score. The arrow shows perivascular lymphocytic infiltrates. Statistical differences were considered when $p \leq 0{,}05$ (no asterisk: $p > 0.05$; *: $p \leq 0.05$). NC: negative control; ST: Chicken embryos (CE) inoculated with *Salmonella* Typhimurium (ST); IAL: CE inoculated with CJ strain IAL 2383; C046/10, C030/3, C092/16: CE inoculates with CJ strain C046/10, C030/3, C092/16 isolated from chicken. PS: It is normal to find infiltrates of perivascular lymphocytes in birds, then we classified the infiltrates by score using the Wilcox test.

8. Tables

Table 1

Mortality (%) and gross lesions in the different groups

		NC	BS?	ST	IAL	C046/10	C030/3	C092/6
	Mortality (%)	0.0 (0/20)	0.0 (0/20)	5.0 (1/20)	0.0 (0/20)	0.0 (0/20)	5.0 (1/20)	0.0 (0/20)
Lesions (%)	**Urate increase**	0.0 (0/20)	0.0 (0/20)	10.5 (2/19)	10.0 (2/20)	15.0 (3/20)	10.5 (2/19)	5.0 (1/20)
	Liver enlargement	0.0 (0/20)	0.0 (0/20)	5.3 (1/19)	0.0 (0/20)	0.0 (0/20)	0.0 (0/19)	0.0 (0/20)
	Greenish liver	0.0 (0/20)	0.0 (0/20)	10.5 (2/19)	0.0 (0/20)	0.0 (0/20)	0.0 (0/19)	0.0 (0/20)
	Milky allantoid	0.0 (0/20)	0.0 (0/20)	21 (4/19)	35 (7/20)	15 (3/20)	10.5 (2/19)	5.0 (1/20)
	Small embryo	0.0 (0/20)	0.0 (0/20)	5.3 (1/19)	0.0 (0/20)	0.0 (0/20)	0.0 (0/19)	0.0 (0/20)
	Enlarged spleen	0.0 (0/20)	0.0 (0/20)	0.0 (0/19)	10.0 (2/20)	5.0 (1/20)	5.3 (1/19)	0.0 (0/20)

NC: negative control; ST: Chicken embryo (CE) inoculated with *Salmonella* Typhimurium (ST); IAL: CE inoculated with *Campylobacter jejuni* (CJ) strain IAL 2383; C046/10, C030/3, C092/16: CE inoculated with *Campylobacter jejuni* (CJ) strain C046/10, C030/3, C092/16 isolated from chicken. We performed just descriptive statistical analysis.

CHAPTER III

ATR-FTIR spectroscopy combined with artificial intelligence can offer an alternative method for detecting *Campylobacter jejuni*

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Abstract

Campylobacter is one of the most common bacterial causes of human gastroenteritis in the world and considering it is difficult to isolate, grow, and identify, this organism diagnosis is still a challenging. In this study, we used attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) with artificial intelligence (AI) to detect *Campylobacter jejuni* (CJ). Infrared spectra were recorded from 245 CJ (positive samples) and 246 not CJ (negative samples) bacteria after removing data from noisy sample readings. The CJ samples for positive control were consisted of CJ strain 1997/6, CJ strain 35528/8, CJ strain 39259/3, CJ strain 9591/6 and CJ strain IAL. The negative samples were consisted of E. *coli* ST131, *Staphylococcus aureus*, *Campylobacter coli* strain 1, *Campylobacter coli* strain 2, and *Salmonella* Enteritidis. The data were divided randomly across training data (291 samples) and external validation data (200 samples) for the construction of the *Campylobacter* database. We tested three trained algorithm models: Randon Forest (RF), Gradient Boosting and Neural Network. All models showed high accuracy and area under the curve (AUC). However, RF presented the best results in both cross and external validation. Cross validation by RF showed sensitivity, specificity, accuracy, positive likelihood ratio (LR+) and AUC of 99.31%, 99.32%, 99.31%, 144.9 and 99.9% respectively. In external validation the sensitivity, specificity, accuracy and AUC reached 100%, which proves the success of the model. Considering the need for rapid identification of CJ, the ATR-FTIR along with AI is a useful tool to identify CJ in real time.

Key words: Artificial intelligence, campylobacteriosis, infrared spectroscopy, diagnostic.

1 Introduction

Campylobacter is an important foodborne pathogen to human, and it is considered the most common bacterial cause of human gastroenteritis in the world. The World Health Organization (WHO) estimates that 550 million people fall ill every year from gastroenteritis, of which 220 million are children (WHO 2020). The EFSA (European Food Safety Authority) considers the need to address *Campylobacter* spp. as high priority on the public health hazards to be covered by inspection of poultry meat, specially, the specie *Campylobacter jejuni* (CJ) (EFSA al., 2020). In 2022, campylobacteriosis was the second highest number of hospitalizations caused by a zoonotic pathogen after salmonellosis in UE (EFSA, 2023). In animals, during the same period, the overall proportion of positive units in the EU was highest in turkeys (71.9%), followed by broilers (18.1%), being the other ones, cats and dogs, bovine, small ruminants, and pigs (EFSA, 2023). Human infections caused by *Campylobacter* are endemic worldwide, and in the recent years, the incidence of campylobacteriosis prevailed throughout North America, Europe and Australia and remained at stable levels (Hoffman et al., 2021).

The epidemiological study of *Campylobacter* is essential since, studies have evidenced a possible association between campylobacteriosis and global climate change (Kuhn et al., 2020). This is because *Campylobacter* has a characteristic seasonality with cases increasing sharply in the summer (Lake et al., 2019). Furthermore, when causing enteritis in humans, *Campylobacter* brings severe consequences for children, the elderly, and the immunocompromised ones (Ushanov, 2018). Besides, CJ infection is correlated with more severe complications as neurological diseases and reactive arthritis. Most cases of campylobacteriosis occur as self-limiting enteritis, but in some cases, this agent can lead to the Guillain-Barré Syndrome (GBS), an autoimmune disease characterized by the loss of the myelin sheath. This syndrome triggers an acute inflammation of the peripheral nerves, which impairs the conduction of the nerve stimulation from the brain to the muscles and vice versa (Hughes and Cornblath, 2005).

Campylobacter spp. diagnosis is still a challenging once this organism is difficult to isolate, grow, and identify (Fitzgerald et al., 2016). To diagnose campylobacteriosis, isolation of the agent is the most indicated. The standard microbiological techniques for detecting *Campylobacter spp*. are laborious, expensive, and time-consuming. These limitations emphasize the necessity of rapid, reliable, and sensitive methods for detecting *Campylobacter* species, especially CJ due to association with GBS.

Fourier transform infrared (FTIR) spectroscopy is a phenotypic method traditionally used in chemistry to determine the molecular composition of a wide range of sample types. It is a rapid, non-destructive, simple, inexpensive, and high-throughput analytical tool, based on the differential vibrational modes of distinct chemical bonds when exposed to an infrared beam (Griffiths and Haseth, 2007). FTIR spectroscopy can be used to rapidly differentiate and identify pathogenic bacteria. It probes the total composition of a given biological sample, such as a colony of microorganisms, in one single experiment. FTIR-based bacterial typing might not only be useful for quick and reliable strain typing but also to help understanding the diversity, evolution, and host adaptation factors of key bacterial pathogens or subpopulations (Novais et al., 2019).

An FTIR image can be acquired mainly in three different configurations: transmission, reflection or attenuated total reflection (ATR). In special, ATR has been shown to be a highly versatile imaging mode because the sampling path length does not depend on sample thickness and hence sample preparation is often not required (Kazarian and Chan, 2006).

The potential FTIR based methods for microbial identification and classification have been shown by several authors (Novais et al, 2019; Naumann et al., 1991; Mariey et al., 2001; Beekes et al., 2007) and the application of FTIR spectroscopy to the identification of *Campylobacter* strains specifically has been previously studied by Mouwen et al. (2006).

Attenuated total reflectance–Fourier transform infrared (ATR-FTIR) spectroscopy is a simple, label free, non-invasive, non-destructive analytical technique that can characterize the biochemical profile of a sample without extensive sample preparation. By interrogating biological samples with infrared light, it is possible to elucidate a specific biochemical fingerprint. ATR-FTIR spectroscopy has therefore demonstrable potential as a powerful diagnostic tool (Baker et al., 2014). Then, this study aimed to evaluate the potential of ATR-FTIR spectroscopy associated with artificial intelligence to detect *Campylobacter jejuni*.

2 **Material and Methods**

2.1 Samples preparation

The samples preparation was conducted at the Infectious Diseases Laboratory – UFU (LADOC-UFU). For this study, we worked with four CJ strains (1997/6; 35528/8,

39259/3, 9591/6) isolated from chicken and one CJ strain IAL, isolated from humans (Fonseca et al., 2014) as the positive samples. For negative control, we used two *Campylobacter coli* strains, avian pathogenic *E. coli* (ST131), *Salmonella* Enteritidis (confirmed by commercial microarray PCR test Check & Trace *Salmonella* (Bio-Rad Laboratories, Inc)) isolated from chickens and *Staphylococcus aureus* isolated from dogs at LADOC-UFU. The bacteria were grown according to their specifications. *Campylobacter* strains were grown in CCDA (charcoal cefoperazone deoxycholate agar) medium (Acumedia) at 40ºC in a micro-aerobic atmosphere for 48 hours and the others were grown in nutrient agar (Acumedia) culture medium at 37ºC for 24 hours.

After growing, colonies were collected from the agar plates with a sterile loop and diluted in 1000µl of MilliQ water to measure the absorbance (OD) in a spectrophotometer at 600nm until reaching an OD of 2.9. Each analysis was performed in two repetitions, and a minimum of fifty readings per bacterium was analyzed for potential errors or noise in the two pieces of equipment used. After the analysis, for cross validation, excluding data from noisy sample readings, a total of 145 CJ samples were obtained for positive control, being 29 samples CJ strain 1997/6 (20%), 37 CJ strain 35528/8 (26%), 30 CJ strain 39259/3 (21%), 19 CJ strain 9591/6 (13%) and 30 CJ strain IAL (21%). For the negative control, we obtained a total of 146 samples, being 29 *E coli*. (20%), 29 *Staphylococcus aureus* (20%), 27 *Campylobacter coli* strain 1 (18%), 33 *Campylobacter coli* strain 2 (23%), and 28 *Salmonella* Enteritidis (19%). For the external validation group, 200 samples were used, being 100 positive control samples, consisting of 19 samples of CJ strain 1997/6 (19%), 11 samples of CJ strain 35528/8 (11%), 19 samples of CJ strain 39259/3 (19%), 31 samples of CJ strain 9591/6 (31%) and 20 samples of CJ strain IAL (20%). As negative control samples, we used a total of 100 samples, being 21 *E. coli.* (21%), 21 *Staphylococcus aureus* (21%), 22 *Campylobacter coli* strain 1 (22%), 15 *Campylobacter coli* strain 2 (15%), and 21 *Salmonella* Enteritidis (21%).

2.2 Infrared Spectra

For each sample, 2 µL were applied directly to the crystal of a portable FTIR spectrophotometer coupled with an ATR unit (Agilent Technologies, Agilent Cary 630, Santa Clara, CA, USA) with two separate pieces of equipment. This approach ensured that the multivariate analysis models considered potential sources of variance in the sampling procedure. The internal reflection element in the ATR unit was a diamond disk. The samples were naturally dried, and absorbance readings were performed in the spectral

range of 650 to 4000 cm^{-1}, with a resolution of 2 cm^{-1}, with 32 scans. Before each infrared analysis, the air spectrum was used as a background. The spectrum was recorded by absorbance.

2.3 Data segregation for use in Artificial Intelligence (AI) and multivariate analysis

For the multivariate analyses with the data, the Orange 3.34.1 software was used, and the spectra were processed to obtain the best algorithm models for classifying the groups as positive and negative. Sensitivity, specificity and accuracy calculations were performed using the MEDCALC online platform.

The datasets were randomly divided into two parts: training data and testing data. Training data is used to train the machine learning models, enabling them to predict test results. External validation data, or test data, is then employed to accurately and efficiently assess the performance of the trained algorithm (Uçar et al., 2020). Then, the spectra generated from the sets of the cross and external validation were then utilized in Artificial Intelligence analysis. In summary, for data of the absorbance readings of the bacterial samples, the region of 650 – 1800 cm^{-1} was selected, which was treated with Gaussian smoothing, baseline correction with Rubber band model, normalization of the maximum-minimum model. The dates were analyzed by principal components (PCA). The spectral data passed through preprocessing techniques to correct the baseline, and then chemometric models were built and validated, whereby possible spectral biomarkers as well as sensitivity and specificity metrics could be obtained. After the creation of the artificial intelligence algorithms, the prediction models were trained with cross-validation with folds of 10 in a Training Confusion Matrix, and the three best models, which were random forest (RF), gradient boosting and neural network, were then subjected to external validation.

3 Results and Discussion

Data of the absorbance readings of the bacterial samples ate the region 650 – 1800 cm^{-1} was corrected and normalized (the average profiles are shown in figure 1). Visually, it is possible to separate the average spectral profile from positive and negative control samples in both training (Figure 1A) and external validation (Figure 1B) data, specially between regions 1100 – 1600 cm^{-1}.

In the training data, three components were used, reaching 96% of explained variance and for the external validation data and 95% for the cross validation. There is a visible separated group, however this division is not perfect since some spectral regions show no difference between positive and negative samples (Figure 2).

To obtain meaningful and reliable information, the IR spectra within the fingerprint region were processed through specific computational techniques, known as chemometrics. The spectral data initially undergo preprocessing techniques to correct the baseline and to remove possible physical variations not related to characteristics of target bacteria, and then chemometric models are built and validated, whereby possible spectral biomarkers as well as sensitivity and specificity metrics can be obtained (Morais et al., 2019).

After the creation of the artificial intelligence algorithms, the prediction models were trained with cross-validation with folds of 10 in a Training Confusion Matrix, and the three best models, which were random forest (RF), gradient boosting and neural network, were then subjected to external validation. All the models showed high accuracy; however, RF presented the best results in both cross and external validation (Table 1). Cross validation had sensitivity, specificity, accuracy and positive likelihood ratio (LR+) of 99.31%, 99.32%, 99.31% and 144.9 respectively. And in external validation the sensitivity, specificity and accuracy reached 100% (Table 1) which proves the success of the model. The area under the curve (AUC) showed excellent results for the three models in training data (Figure 4A, B, C) and validation data (Figure 4D, E, F). For both, cross and external validation, RF also has the best AUC (Figure 4A and D).

Infrared (IR) spectroscopy is a powerful technique to investigate biological materials (Baker et al., 2014). The interaction of IR with the biochemical molecules that make up a tissue sample generates a spectrochemical fingerprint, allowing one to extract both quantitative and qualitative information. Our study proved results with high AUC for the three models.

RF modeling has been one of the most popular research methods in data mining area and information to the biological field (Liu et al., 2012). For the RF modeling, the external validation data reached a perfect result (Table 1, Figure 4A, D). RF possess high classification accuracy and tolerate outliers and noise well (Breiman, 2001); however, the other models used also presented high accuracy, specially, the high specificity. Gradient boosting employs gradient descent algorithms to minimize errors in sequential models (Zeravan et al., 2023) and is a commonly employed approach for ensemble instruction. It

reduces both variance and classification bias (Choubin et al., 2019). This modeling represents one of the most promising methodological approaches for data analysis developed in the last decades (Mayr et al., 2014, Bentéjac et al., 2020). In the validation test the gradient boosting presented specificity of 100% (Table 4) which is of great value for the objective of our work and again proves the success of our results. model performs nonlinear mapping from the input layer to the output layer using an extensive number of parameters which are tuned to allow flexibility. The model is capable of handling missing values and supports multioutput problems. In our work the model showed high sensibility (99%) and specificity (99%) although the results were lower compared to RF and gradient boosting.

Usually, the gold standard according to 10272–2: 2017 (ISO 10272, 2017) is culture–based detection method for *Campylobacter* spp. that requires several days (5–7 days) for sampling, culture enrichment, enumeration, and biological or molecular confirmation, which is time–consuming. Furthermore, false–negative result could be interpreted when *Campylobacter* spp. encounter limiting environmental conditions which leads the cells to be in a viable but non–culturable (VBNC) state (Zhao et al., 2017). Our results showed that ATR-FTIR spectroscopy associated with AI is a useful tool to detect CJ simply, quickly and practically. Considering CJ is one of the main causes of the campylobacteriosis and its association with the serious GBS, the CJ identification in real time is essential and our work proved that this is possible. Although there are tests to identify CJ rapidly after the isolation, as PCR and mald-tof, the ATR-FTIR can provide some advantages. PCR methods are expensive and labor-intensive (Natsos et al., 2019) and/or time-consuming. Even MALDI-TOF mass spectrometry with high accuracy, to CJ the level of accuracy is 99.4% (Bessède et al., 2011), which is lower than our result in the RF.

It is important to also consider that CJ is a fastidious bacterium and hard to cultivate. And, although the direct detect of CJ was not our aim, our study brings light to the possible potential tool do detect the pathogen using algorithm, which can be easily used in the field and other studies about this approach should be performed in the future. An interesting next step would be testing of the efficiency of ATR-FTIR and AI using as samples, swabs collected directly from the feces, carcasses during the slaughtering and environment, for an instance. However, for this approach it is necessary to differentiate live and dead CJ and other works showed this is possible using FTIR (Sundaram et al., 2012).

Our study shows the potential of using ATR-FTIR to identify CJ in real time in a practical way with sensitivity and specificity of 100%.

4 Conclusions

This work describes a quick, practical, and reliable new technique to identify CJ. Considering the need for rapid identification of CJ, the ATR-FTIR along with AI is a useful tool to identify CJ in real time, with a specificity of 100% using the RF model. Further studies need to test other kind of samples, such as swabs collected directly from the feces or carcasses during the slaughtering.

Figure 1

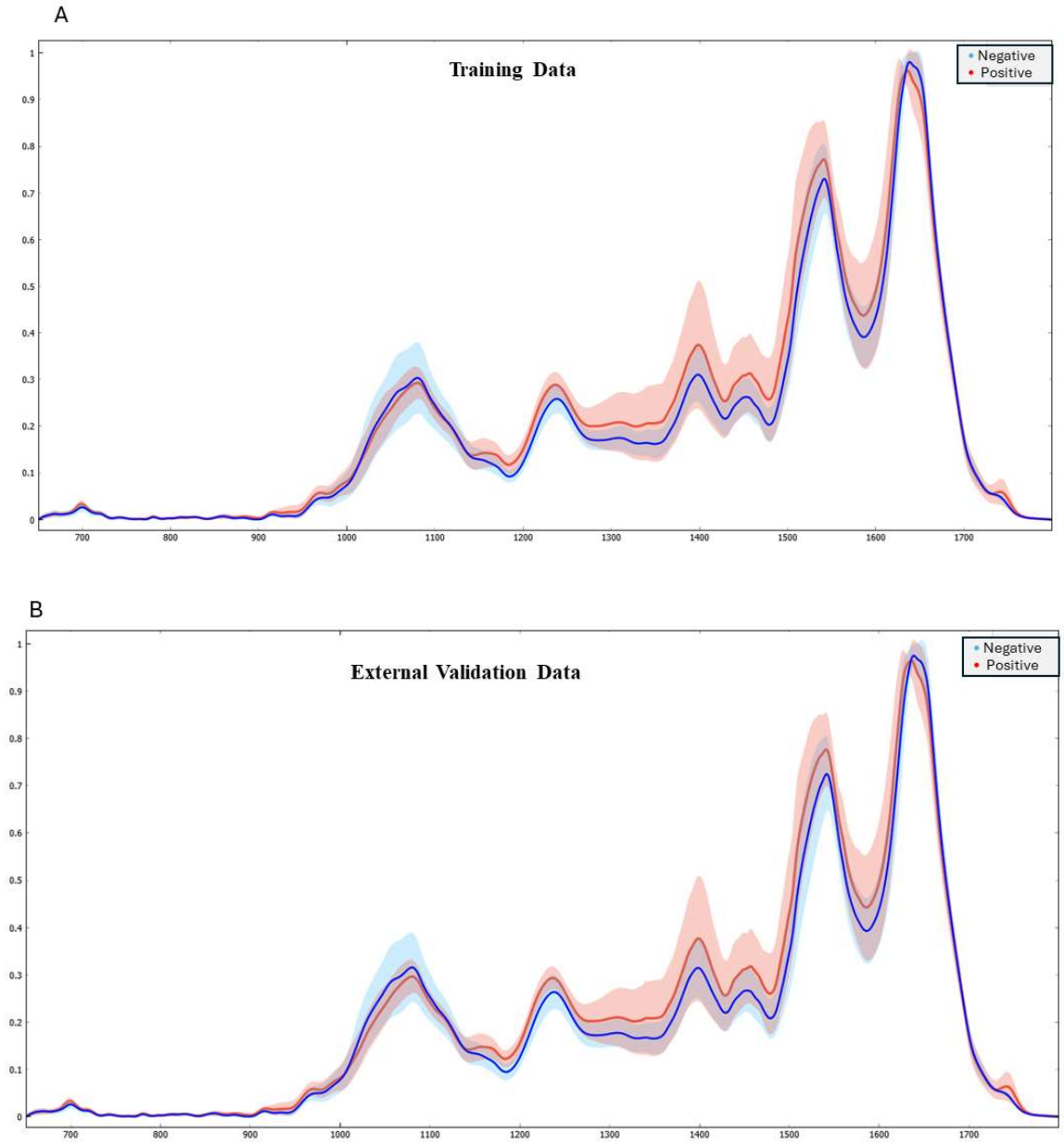

Figure 1 – The average spectral profile of the sample groups for training (A) and external validation (B) data is presented. In red are the averages of the IR spectra for the positive group (CJ and IAL strains), and in blue are the averages for the negative groups (other bacteria).

Figure 2

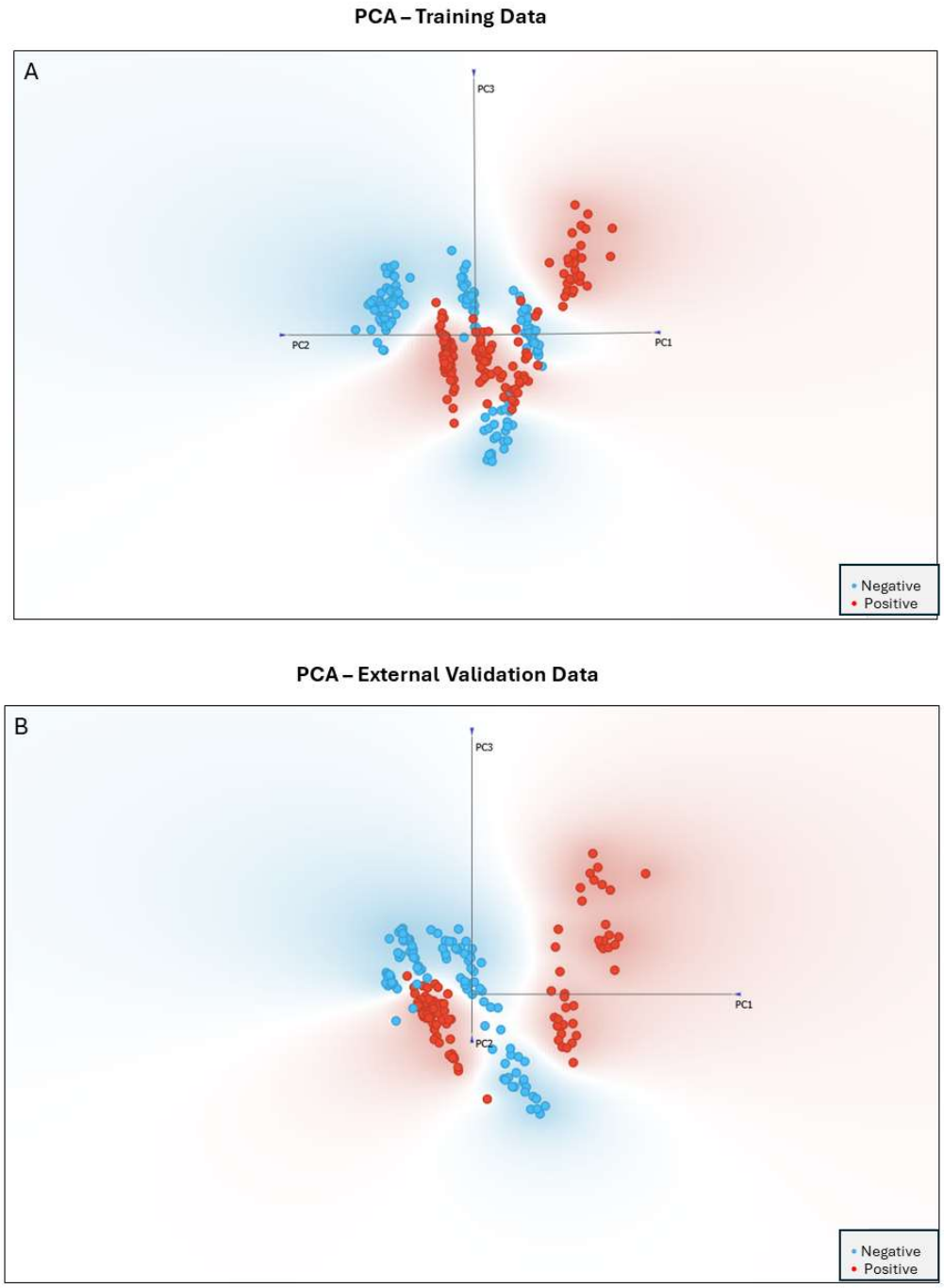

Figure 2 – Graphs of principal component analysis (PCA) for training data and external validation data are presented. A) 96% explained variance was found for the training data. B) 95% explained variance was found for the external validation data. Positive samples are represented in red, and negative samples are represented in blue.

Supplementary Figure 3 – Training Confusion Matrix and External Validation

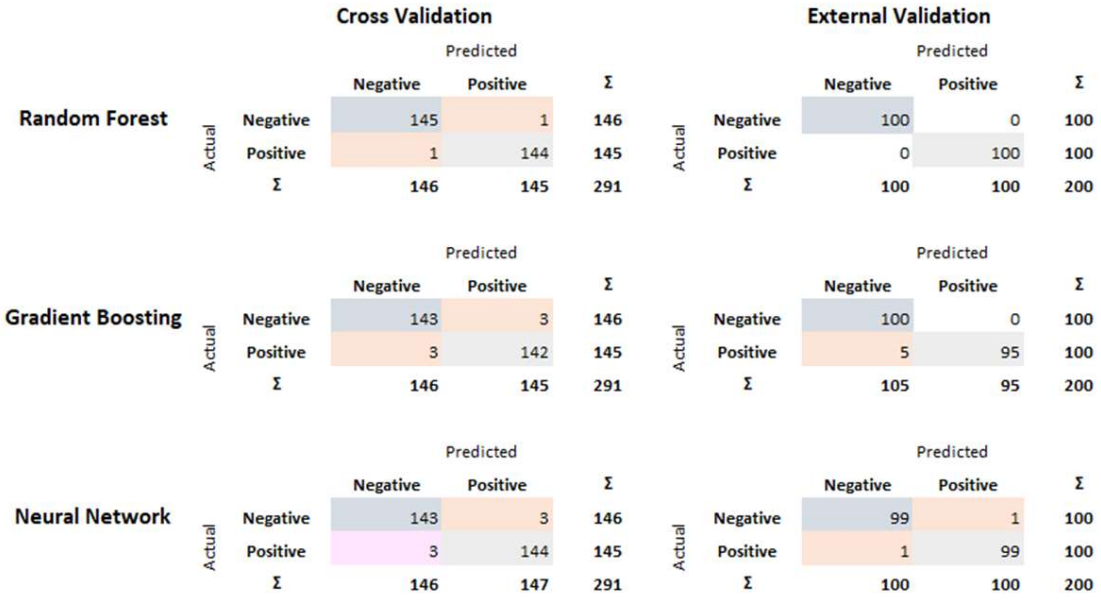

Table 1 - Result: sensitivity, specificity, positive likelihood ratio and accuracy of all models from cross and external validation.

Model	Statistic	Cross Validation		External Validation	
		Value	95% CI	Value	95% CI
Random Forest	Sensitivity	99.31%	96.22% to 99.98%	100.00%	96.38% to 100.00%
	Specificity	99.32%	96.24% to 99.98%	100.00%	96.38% to 100.00%
	Positive Likelihood Ratio	144.99	20.56 to 1022.50		
	Accuracy	99.31%	97.54% to 99.92%	100.00%	98.17% to 100.00%
Gradient Boosting	Sensitivity	97.93%	94.07% to 99.57%	95.00%	88.72% to 98.36%
	Specificity	97.95%	94.11% to 99.57%	100.00%	96.38% to 100.00%
	Positive Likelihood Ratio	47.66	15.55 to 146.10		
	Accuracy	97.94%	95.57% to 99.24%	97.50%	94.26% to 99.18%
Neural Network	Sensitivity	99.31%	96.22% to 99.98%	99.00%	94.55% to 99.97%
	Specificity	97.95%	94.11% to 99.57%	99.00%	94.55% to 99.97%
	Positive Likelihood Ratio	48.33	15.77 to 148.13	99	14.08 to 696.03
	Accuracy	98.63%	96.52% to 99.62%	99.00%	96.43% to 99.88%

Figure 4

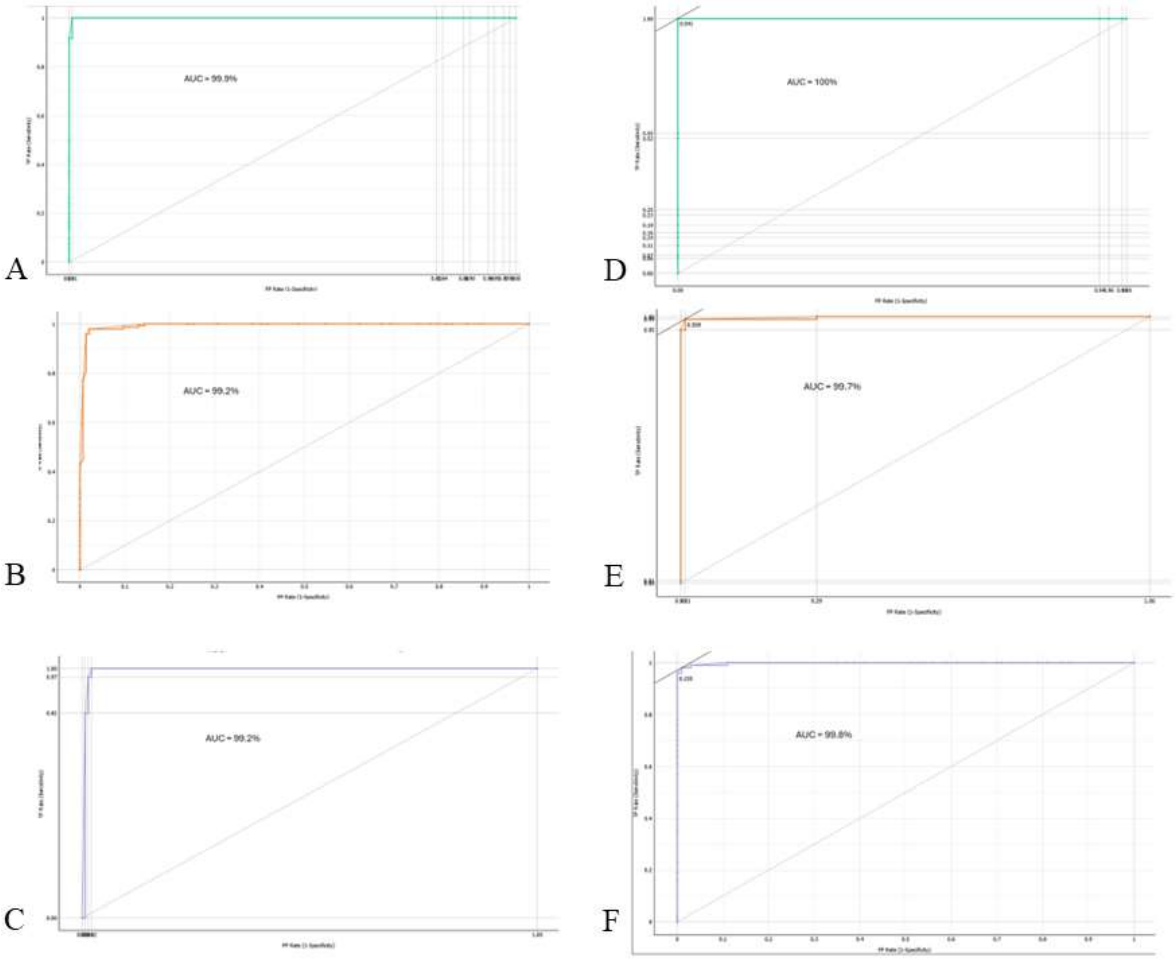

Figure 4 – Roc curve showing the area under the curve (AUC) values for the training data (A, B, C) from the three best prediction models are presented: (A) Randon Forest model result, (B) Gradient Boosting model result, and (C) Neural Network model result from. D, E, F: external validation data from the three best prediction models are presented: (A) Randon Forest model result, (B) Gradient Boosting model result, and (C) Neural Network model result.

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CHAPTER IX

Phage Display-Derived Peptides: A promising alternative for *Campylobacter jejuni* control through theragnostic approach

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ABSTRACT

Considering the importance of *Campylobacter jejuni* (CJ) for human health and the need of new theragnostic approaches, this study aimed to select and characterize specific biding peptides from CJ by using phage display, to control this bacterium. We performed peptides selection using the bioppaning technique and a total of 23 phages were selected using CJ as the target. These clones were sequenced and tested via screening phage-ELISA, where we considered with significant binding effect those phages with higher absorbance compared to a wild phage (M13). To help choosing the best peptide, we performed the molecular docking and based at the binding strength. After molecular docking, we selected five peptides and performed a second screening phage-ELISA, which gave us the two best choices. Then, we followed a protocol of *in vitro* assay to evaluate the minimal inhibitory concentration (MIC) using two different CJ strains and the peptides alone and mixed. An *in vivo* test was then performed in chicken embryos with 10 days of incubation, testing five study groups with 11 embryos each one: (i) Negative control – Inoculated only with saline solution, (ii) Inoculated with CJ IAL 2383 and peptide B6, (iii) Inoculated with CJ IAL 2383 and Peptide C5, (iv) Inoculated with CJ IAL 2383 and Peptide B6 and C5, (v) Positive control (PC) – Inoculated with only IAL strain 2383. The inoculation was via allantoid and on the 16^{th} day of incubation, the mortality was evaluated, chicken embryos were weighed, and around 1000µL of allantoid liquid was collected from each embryo, so that the allantoid was diluted, and 10 µL of each dilution were plated in CCDA medium and incubated at 41ºC in a micro-aerobic atmosphere for 48 hours. Then, grown colonies were counted. We observed the immunoreactivity of CJ with B5 and C5 phage. Besides, phages B6 and C5 inhibited CJ by approximately 90-97% when we tested a high initial dose of CJ. When testing the inhibition rate of C5 for an initial low-dose inoculum, the inhibition rate was 100%. The test in chicken embryo showed that B6 was able to inhibit CJ multiplication, although with low performance, but C5 was not. Then this work proves that peptides B6 and C5 can be explored by a theragnostic approach, especially diagnostic, although the presentation and delivery of the peptides B6 and C5 should improve to be used in animals.

1 INTRODUCTION

Campylobacter spp. is one of the most implicated agents in foodborne infections and in developed countries it needs to be addressed as high priority on the public health hazards to be covered by inspection of poultry meat (EFSA et al., 2020). According to the Centers for Disease Control (CDC), there are about 1.3 million cases of *Campylobacter* infection each year in the United States alone. This leads to an economic cost between $1.3 to $6.8 billion annually (Kaakoush et al., 2015). *Campylobacter* organisms are also a significant cause of traveler's diarrhea, particularly in Thailand and other areas of Southeast Asia (Swierczewski, 2017). In humans, *Campylobacter* brings severe consequences for children, the elderly, and the immunocompromised ones (Ushanov, 2018). The symptoms may vary from person to person, and include diarrhea, fever, vomiting and abdominal pain (Silva et al., 2016). A global concern about *Campylobacter* infections is its correlation with more severe complications as neurological diseases and reactive arthritis. Most cases of campylobacteriosis occur as self-limiting enteritis, but in some cases, this agent can lead to the Guillain-Barré Syndrome (GBS), a serious autoimmune disease characterized by the loss of the myelin sheath (Hughes and Cornblath, 2005). According to Leonhard et al. (2022), the most common bacterial agents that trigger GBS are *C. jejuni* (CJ) and *Mycoplasma pneumoniae.*

In 2022, campylobacteriosis was the second highest number of hospitalizations caused by a zoonotic pathogen after salmonellosis in European Union (EFSA, 2023) and human infections caused by *Campylobacter* are endemic worldwide (Hoffman et al., 2021). In poultry flocks, horizontal transmission may be considered the most likely contamination route, and this infection is related to contaminated water intake, contact with feces of other infected animals, and contaminated litter (Sahin et al., 2012).

Phage display has been used to generate diagnostic and therapeutic targeting peptides for pathogens and the broad utility of this technology has also been demonstrated by the using selected phage peptides as the capture probe for real-time detection devices (Petrenko, 2008) as well as being incorporated into liposomes for phage targeted drug delivery (Jayanna et al., 2010).

Sequential advancements in displayed peptide technology have been achieved by fusing phage display peptide libraries into phage coat proteins. Screening procedures for phage display readily recognize a variety of binding affinity peptides, which are alternatives to antibodies. Displayed peptides have been used for specific receptors in

various biosensors in recent years because of advantages such as high affinity, low procedure cost, high purity and mass production, easy design and modification at the molecular level, and increased resistance to the sophisticated detection environment (Ping et al., 2021). In the past decade, phage display has become the most prevalent peptide display system (Wu et al., 2016).

Peptides can be used to prevent diseases involving malfunctioning of proteins due to undesirable protein-protein interactions (Nevola, 2015). Many databases and algorithms have been developed in the past specifically in the field of peptide-based therapeutics (Kumar, 2018). There are more than 200 therapeutics peptides, approved by FDA for the treatment of various diseases (Usmani et al., 2017).

In that connection, it is mandatory the effective control of CJ in poultry without antimicrobial, using diagnostic, control, and preventive methods, a theragnostic approach. In this sense phage display technology is a useful and safe tool since we can select highly specific biding peptides. Therefore, this study aimed to select and characterize specific biding peptides from CJ by using phage display, to control this bacterium in a theragnostic approach.

2 MATERIAL AND METHODS

2.1 Samples preparation

This study was conducted at the Infectious Diseases Laboratory - UFU (LADOC), at the Nanobiotechnology Laboratory Prof. Dr. Luiz Ricardo Goulart Filho (UFU) and at the Poultry Incubation Laboratory – UFU (LIAVE). *Campylobacter* strains CJ IAL 2383 isolated from humans (Fonseca et al., 2014) and CJ 1997/6 isolated from broilers (Peres, 2021) were grown in CCDA (charcoal cefoperazone deoxycholate agar) medium (Acumedia) at 41ºC in a micro-aerobic atmosphere for 48 hours and after growing they were confirmed by Gram staining and PCR techniques.

2.2 Peptides selection – biopanning

The selection of peptides displayed in M13 phages that bind to CJ IAL 2383 antigens was performed using the phage display technique. Microplate 96-well, high binding (Greiner Bio one, Kremsmunster, Austria) was coated with 150 µL of CJ (OD 600 ~0.5) in 0.1 M carbonate/bicarbonate buffer ($NaHCO_3$, pH 9.6) at 4 °C overnight. The plate was blocked for 1 h at 37 °C with phosphate-buffered saline-bovine serum albumin 5%

(PBS-BSA) (Bovogen, East Keilor, Australia) and washed five times with phosphate-buffered saline plus 0.1% of Tween 20 (PBS-T 0.1%). Then, we performed 3 rounds of selection. In the first round, 10 µL of 1×10^{11} phage particles from the Ph.D.-C7C library (New England Biolabs, Beverly, MA, USA) was incubated with 140 µL of PBS per well per 1 h at 37 °C by shaking to select the phages that bind to the CJ. After incubation, the plate was washed ten times with PBST 0.1% to minimize the nonspecific binding of phages. Next, the first-round high-affinity phages were eluted by incubating for 10 min with 150 µL of 0.2 M glycine (pH 2.2) at room temperature (RT) and then neutralized with 22.5 µL of 1M Tris- HCl (pH 9.1). In the next step, we amplified the phage clones (100 µL) using *E. coli* ER 2738 (ECR) (New England Biolabs, Beverly, MA, USA) as a host cell (5h at 37ºC with gentle shaking). Then, we precipitated them using 20% polyethylene glycol (PEG)/2.5 M NaCl and suspended them in PBS.

We performed the next round (second round) similarly to the first round but using the first round's selected phages to incubate with CJ. After the incubation, colonies infected by phage were visualized in blue and titrated in Luria-Bertani (L.B.) medium containing IPTG (200 mg/mL) and X-Gal (20 mg/mL). The subsequent round was maintained the same amount of input (1×10^{11} pfu of phages) (Barbas, 2001). At the end, a total of 23 phages were selected using CJ as the target.

2.3 Peptides selection

We selected a total of 23 clones that were sequenced and tested via screening phage-ELISA. From phage-ELISA, we considered with significant binding effect those phages with higher absorbance compared to the wild phage (M13). To help choosing the best peptide, we performed the molecular docking and based at the binding strength. After molecular docking, we selected five peptides and performed a second screening phage-ELISA, which gave us the two best choices. In both phage-ELISA techniques we used CJ IAL 2383 in OD 0.5.

2.3.1 Sequencing

Phage DNA from clones selected after the third round of biopanning was isolated from 1 mL of ECR culture in early-log phase (OD600 ~0.03) overnight by precipitation with 1/6 volume of PEG/NaCI (20% w/w, polyethylene glycol 8000) and iodide buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 4 M NaI). The phage DNA was precipitated with absolute ethanol, followed by a wash with 70% ethanol and resuspended in 20 µL of

Milli-Q water (Barbas, 2001). Electrophoresis was performed on a 0.8% agarose gel stained with red gel solution to verify DNA quality. Twenty-three individual clones selected were submitted to DNA sequencing, which was carried out by using BigDye® Terminator v3.1 Cycle Sequencing Kit on ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, California) at BPI Biotecnologia® (Botucatu, São Paulo, Brazil). Amino acid sequences were deduced according to the nucleotide sequences and analyzed using the translate tool from ExPASy-SIB Bioinformatics Resource Portal (http://web.expasy.org/translate/).

2..3.2 Screening Phage-ELISA

In the first Phage-ELISA, all 23 selected clones were tested. In the second Phage-ELISA, we tested only 3 phages with high binding energy to CadF and FlaA protein and that reacted in ELISA (A8, B6, C5) and 2 aleatory phages that reacted in ELISA but binding just one protein (CadF) (H7 and H9 clones). Besides, with the objective of evaluating specificity, we also used as control, a pool of bacteria from SPF (Specific Pathogen Free) birds' microbiota. To extract this microbiota, 100 of SPF bird feces were used, which underwent a dilution process in distilled water in a ratio of 1:5 and later were filtered on filter paper of up to 12 µm of diameter. After filtration, 40 mL was distributed in 50 mL tubes for centrifugation at 7000 rpm for 10 minutes and washed twice. After centrifugation, the supernatant was discarded, and the pellet was resuspended in carbonate bicarbonate buffer (pH 8.6) and adjusted to OD 0.5 at 600nm. After that, 50uL were used to sensitize each well of the ELISA plate.

ELISA plates were coated with 50 µL/well of CJ IAL 2383, total microbiota of SPF bird's intestine or wild phage (M13 phage – 1 x $10^{11|}$ pfu/well) as a negative control (NC) in 0.1 M x carbonate/bicarbonate buffer ($NaHCO_3$, pH 9.6) at optical density (OD 0.5) - 600nm at 4ºC overnight. Another NC was tested using just antibody anti-M13 without phage or CJ. To each selected clone, we performed the test in triplicate. After washing once with PBST 0.1%, the wells were blocked for nonspecific sites with 270 µL of BSA blocking buffer, 5% in PBS, incubating for 1 h at 37 °C. After washing the wells three times, 50 µL of selected phage clones (1×10^{10} pfu/well) were added in PBST 0.1% and incubated at 37 °C for 1 h. We washed the wells five times with PBST 0.1% and added 50 µL per well of 1:5000 anti-M13 HRP conjugate diluted in PBST 0.1% and incubated at 37 °C for 1 h to detect the phage clones binding to CJ. Then the wells were washed six times and 50 µL of TMB substrate was added and incubated at R.T. for 5–15 min. Finally,

the reaction was stopped using 20 µL of 2 M H_2SO_4 and plates were read at 450 nm using a microplate reader.

We performed the analysis in triplicate, and we used the ANOVA followed by the Tukey test compared each group with the M13 phage considering $p<0.05$. The GraphPad Prism 10.2 was used.

2.4.2 Molecular docking

To predict the three-dimensional structure of the peptides, the computational tool PEP-FOLD3 (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3) was used (LAMIABLE et al., 2016). The three-dimensional models of FlaB and CadF (NCBI IDs: YP_002344726.1 and WP_002869202.1) proteins were constructed by homology using the YASARA model software via the default macro hm_build.mcr. We used these proteins because they are considered highly antigenic in CJ (Monteville et al., 2003). For FlaA protein, the crystallographic structure PDB ID 6X80 was used. The three-dimensional models constructed by homology were validated using PROCHECK software (LASKOWSKI et al., 2006) and ProSA-Web (WIEDERSTEIN et al. 2007). The three-dimensional structures were subjected to energy minimization using the YASARA software. The global molecular docking experiments were performed using the AutoDock Vina tool (4.2.5.1) implemented in the YASARA structure software through the macro dock_runscreening.mcr, performing a total of 100 runs per ligand. The results of the interactions between the target proteins and the peptides were selected according to the best values of binding energy (Kcal/mol) and dissociation constant (pM) for further analysis.

2.5 Synthetic peptide synthesis

Based in phage-ELISA and the docking we chose two CJ-binding clones with better immunoreactivity to CJ and good results in docking biding in FlaA and CadF. These two clones had their amino acid sequences used in the design for the chemical synthesis of peptides performed by GenScript USA Inc. (Piscataway, NJ, USA). Each peptide (B6 and C5) was constructed with 20 residues, containing: NH_2 – AC-sequence of peptide-GGGS-AETVESCL – COOH in the C-terminal. Between the resides 2-10 there is a disulfide bridge (forming a looping between the cysteines, and in the N-terminal region acetylation with 4-aa spacer (GGGS) and 6-aa part of the phage p3 (AETVESL). Peptides were aliquoted in microtubes at 2mg/mL for later use and kept at -80°C.

2.6 *In vitro* Inhibition assay

For this step, we performed one experiment with CJ strain IAL 2383 and CJ 1997/6 to evaluate the potential of the peptides in inhibition assay. We tested the peptides at 1000µg/mL, 500µg/mL, 250µg/mL, 125µg/mL, 62.5µg/mL, 31.25 µg/mL, 13.625µg/mL and 7.8125µg/mL in one plate of 96 wells. In summary, the higher concentration (1000µg/mL) was inoculated in the first well and the serial dilutions in Muller Hinton broth (MH) plus 1% yeast extract (MHEL) at base 2 was performed in the next wells with the final volume of 180µL. Then, we added 20µL of bacterial solution (Bact + MHEL) at 8 logUFC/mL concentrations. For negative control, we used 200µL of pure MHEL and for positive control we did not add the peptide. The plates were incubated at 41ºC in a micro-aerobic atmosphere for 48 hours. After this step, each well was serially diluted, being 900µL of MHEL broth + 100µL of peptide + bacteria solution. We plated the dilutions in drop of 10µL at CCDA agar (Acumedia) added to 5% of yeast extract and incubated at 41ºC in a micro-aerobic atmosphere for 48 hours. Finally, the grown colonies were counted, and the counting was noted for statistical analysis.

As we had a success with the first experiment, we followed with another test using only peptide C and CJ IAL 2383, but in a low initial inoculum of CJ (4 UFC/µL), in triplicate. In this experiment, just the highest doses of the peptides were tested (1000µg/mL, 500µg/mL, 250µg/mL, 125µg/mL and 62.5 ug/mL). In the first test we performed descriptive analysis with mean and standard deviation. In the second test, we performed the statistical analysis using the nonparametric Wilcoxon test t, considering the mean of inoculated bacteria (4 logUFC/mL). The Graphpad Prism 10.20 was used, and we considered $p<0.05$.

2.7 *In vivo* test in chicken embryos models

For this step, the strain IAL 2383 was grown in CCDA medium at 41ºC in a micro-aerobic atmosphere for 48 hours and after growing the colonies were confirmed by Gram staining. We used 55 embryonated eggs from *Gallus gallus*, line Ross with 10 days of incubation, provided by a commercial hatchery. We used the candling technique to certify the embryos were viable and each egg was enumerated and weighted, and the weights were registered. For the assay, we tested five study groups with 11 embryos each one: (i) Negative control – Inoculated only with saline solution, (ii) Inoculated with CJ IAL 2383 and peptide B6, (iii) Inoculated with CJ IAL 2383 and Peptide C5, (iv) Inoculated with

CJ IAL 2383 and Peptide B6 and C5, (v) Positive control (PC) – Inoculated with only IAL strain 2383. For groups ii, iii, iv and v, the inoculation concentration was 4 log UFC/embryo via allantoid. In groups 2 and 3, the concentration of peptides was 250µg/mL and for group 4, the concentration of peptides was 125µg/mL each peptide. After inoculation, the eggs were incubated in an automatic incubator (Premium ecologica®) under controlled temperature of 37ºC and 55% humidity. On the 16th day of incubation, the mortality was evaluated, chicken embryos were weighed, and around 1000µL of allantoid liquid was collected from each embryo, so that the allantoid was diluted four times in saline solution (0,85%), and 10 µL of each dilution were plated in CCDA medium and incubated at 41ºC in a micro-aerobic atmosphere for 48 hours. Then, grown colonies were counted.

We performed the ANOVA followed by Tukey test to evaluate the weigh and chi-square to assess mortality comparing each group with the positive control. For the analysis of the CJ count in embryos, the number of the initial inoculum and the amount of CJ recovered from the embryo were divided by the weight of the egg. This is because there is a variation between the weight of the egg and in consequently the embryo, that can interfere with the analysis. Thus, we obtained the amount of inoculum/gram of the embryo. Thus, the data was transformed into a logarithm. The Kolmogorov-Smirnov test was used to assess whether the data were parametric. After that, the test of the difference between means (paired t-test) was performed, comparing the initial inoculum (adjusted by the initial weight of the egg) with the amount of CJ isolated from the embryo (also adjusted for the initial weight of the egg). A significant multiplication of the bacterium was considered a $p<0.05$ using the GraphPad Prism 10.2 program.

3 RESULTS AND DISCUSSION

3.1 Peptides selected by phage display proved to be CJ-specific ligands showing stable binding by ELISA and molecular docking.

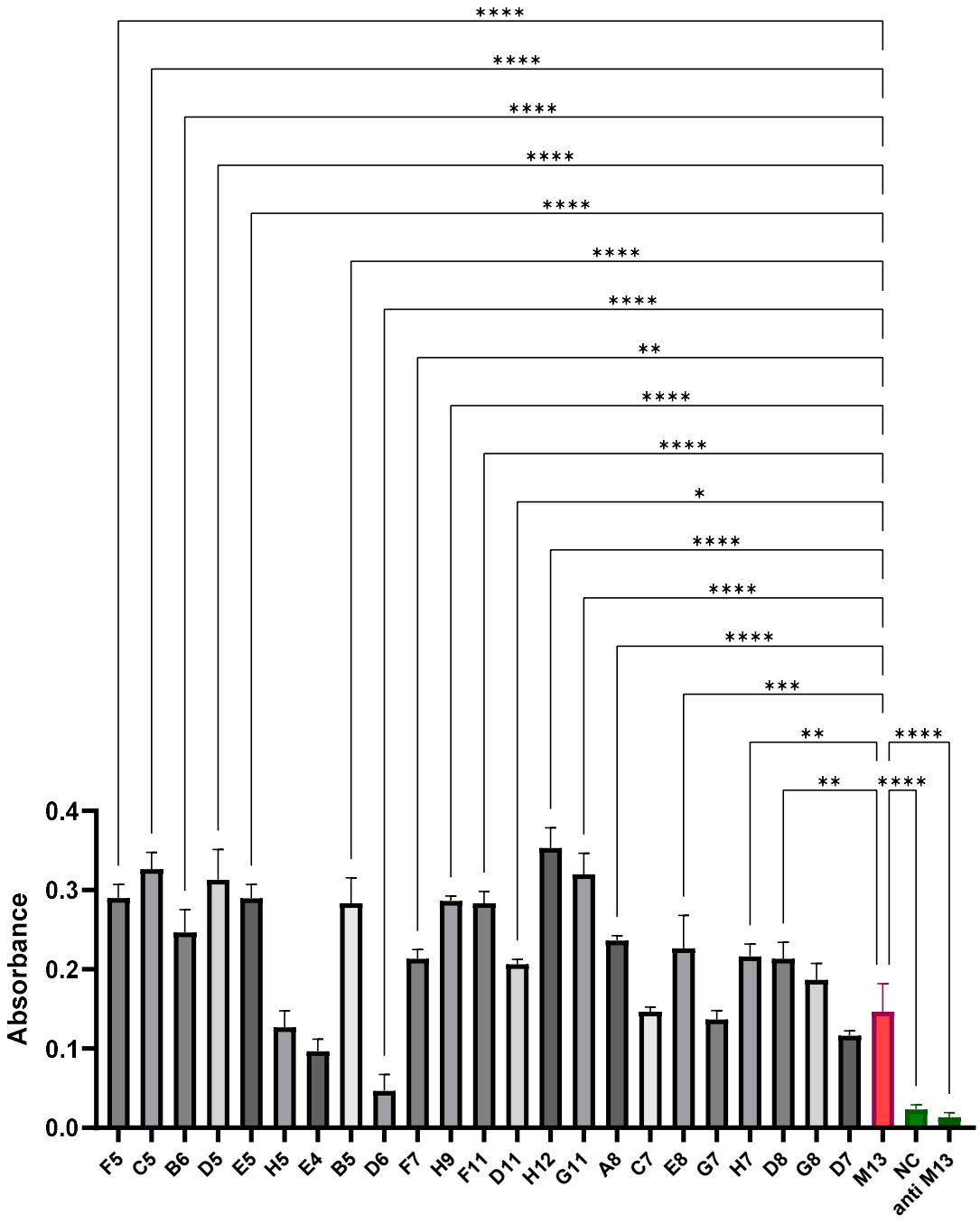
Twenty-three blue colonies were isolated which means that there was substrate X-Gal hydrolysis and the expression of the β-galactosidase gene present only in bacteria ER2738 infected by the virus. After sequencing, all peptides were considered viable (data not shown because patent is not available).

When we used selected bacteriophage displaying the CJ biding-peptides in ELISA, six clones showed immunoreactivity like M13 and so, they were discarded. A total of 17

clones reacted in phage-ELISA which indicating the binding between CJ and peptides (Figure 1). However, we also observed cross-reactivity with the wild phage (M13) used as a control, which probably happened because although M13 is an *E. coli* phage, it may have regions like other phages such as CJ phage that allow cross-reactivity. However, phages that present CJ-binding peptides reacted more compared to M13.

ELISA test qualitatively analyzes the selectivity of phages for the target and determine whether the selected peptide chain has affinity by comparing the absorbance of the phage clone to the binding target, the negative control, or the target similar substance (Qiu et al., 2019). So, our results proved the success of the phage, and we had 17 candidate peptides to be used in the diagnosis and prevention of CJ.

Figure 1 – Immunoreactivity of selected phages in binding to CJ IAL 2383 by Phage-ELISA.



F5, C5, B6, D5, E5, H5, E4, D6, F7, H9, F11, D11, H12, G11, A8, C7, E8, G7, H7, D8, G8, D7: Phage displaying CJ-binding selected by phage display. M13: we tested the CJ with the phage M13 (wild phage, without displayed peptide), NC: just M13 and anti M13, anti M13: just CJ and antibody anti M13. We performed ANOVA followed by the Tukey test compared each group with the M13 phage considering $p<0.05$. The asterisks show that the phage is larger than M13. In red, there is M13 reactivity. Axe X it is each selected phage. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

After sequencing, all 23 peptides were considered viable, and analyzed through molecular docking. For molecular docking, a ranking of the selected peptides was performed for binding of the three following high antigenic CJ proteins: FlaA, FlaB and CadF. A cut-off around 8 kcal/mol (Binding energy) was used to select good ligands in the virtual process (Table 1).

Given the complex structure of Fla A, we performed the simulations with the whole protein (complex) and its monomers (subunit). Another detail is that we did not consider the glycosylation sites of the protein. In general, CadF showed more interaction sites followed by FlaA, and FlaB was practically not interesting. We ranked the results and arbitrarily believed that the minimum cut-off to be considered should be 7 kcal/mol, especially those that also presented a higher dissociation constant. Dissociation constant (DC) is most important parameter to understand chemical phenomenon such as biological activity and absorption, so is the key parameter in drug development and optimization (Guzide and Hakan, 2010) and the lower the DC means the better the peptide.

Numerous docking methods have been developed in the past for structural determination of protein-peptide complexes (Zhou et al., 2018) and all these docking methods can be used to predict the interaction between protein and peptides (Agrawal et al., 2019).

Table 1 – Ligand, Efficiency (Kcal/mol), Binding energy (Kcal/mol) and Dissociation constant of the selected peptides (with binding strength greater than 7.8) in the protein complex.

Ligand	**Efficiency (Kcal/mol)**	**Binding energy (Kcal/mol)**	**Dissociation constant [pm]**	**Peptide**	**Protein**
3	0.1363	9.132	202374.25	B6	CadF
3	0.1334	8.94	279829.25	B6	FlaA_Complex
10	0.1549	8.831	336349.8125	D09	CadF
4	0.1323	8.731	398191.6563	C5	CadF
1	0.1741	8.532	557135.8125	A8	FlaA_Complex
22	0.1701	8.506	582129.125	H07	CadF

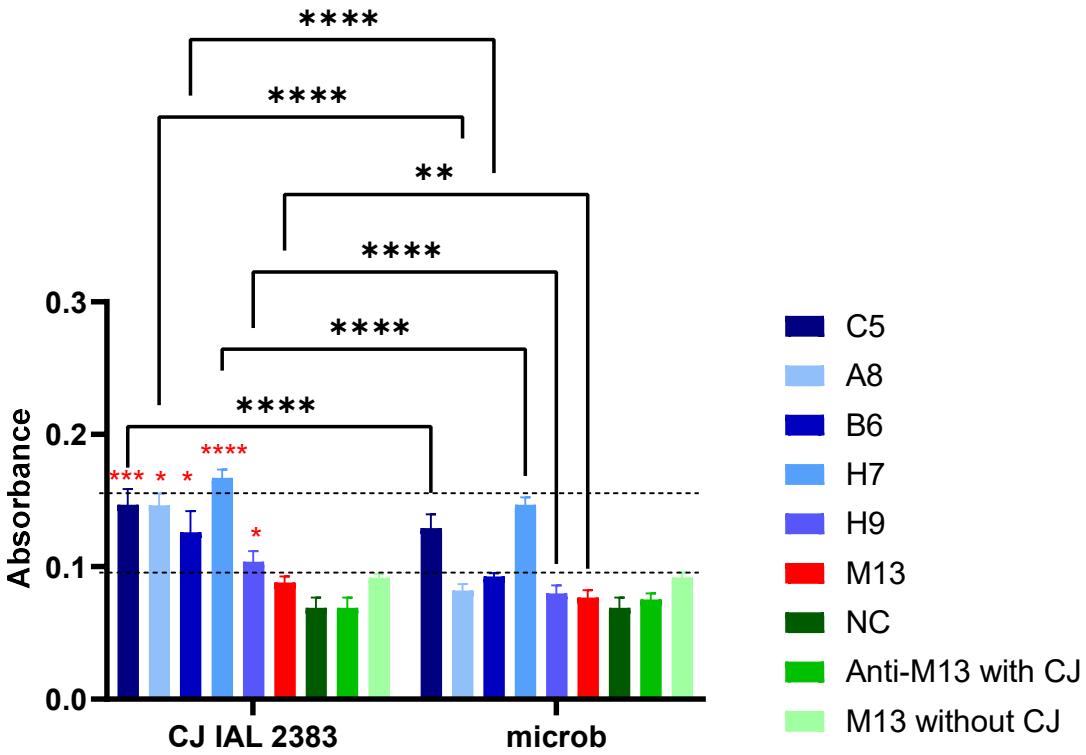
4	0.1283	8.468	620688.5	C5	FlaA_Complex
19	0.1348	8.355	751110.4375	G08	CadF
6	0.1323	8.334	778210.375	D05-2	FlaA_Complex
8	0.1462	8.334	778210.375	D07	FlaA subunit
24	0.1508	8.295	831159.5	H12	CadF
1	0.1693	8.294	832563.5625	A8	CadF
6	0.1313	8.27	866981.0625	D05-2	CadF
5	0.1247	8.232	924408.625	C07	FlaA subunit
12	0.1706	8.188	995672.3125	E04-2	CadF
8	0.1431	8.158	1047385.875	D07	FlaA_Complex
9	0.1431	8.157	1049155.125	D08	CadF
16	0.1626	8.13	1098072.375	F07	CadF
2	0.1683	8.08	1194762.25	B02-2	CadF
24	0.1463	8.049	1258939.25	H12	FlaA_Complex
9	0.1411	8.045	1267467.375	D08	FlaA_Complex
5	0.1208	7.974	1428829.75	C07	CadF
23	0.1397	7.964	1453150.5	H09	CadF
17	0.1624	7.957	1470420.875	F11	CadF
10	0.1396	7.957	1470420.875	D09	FlaA_Complex
2	0.1647	7.906	1602600.125	B02-2	FlaA_Complex

After performing molecular docking, we selected five peptides to second phage-ELISA, three with the highest binding strength to CadF and FlaA (A8, B6 and C5) and two just CadF binding (H07 and H09). We proved the reactivity of the phages to the CJ. The microbiota reacted as expected with all phages, including M13, but less than the bacterium IAL 2383. (Figure 2). Probably, the immunoreactivity between microbiota may be explained because there is a diversity of bacteria in the intestine including the Proteobacteria (Kollarcikova et al., 2019), which may have epitopes common to CJ, since they are from the same phylum.

Also, for this research, peptides B6 and C5 were chosen because they were selected in phage-ELISA and showed low dissociation constant and high binding energy at the molecular docking, along with high antigenic CJ proteins (FlaA and CadF) binding (Table 1). The interactions between the peptide and the bacterium protein were evaluated using

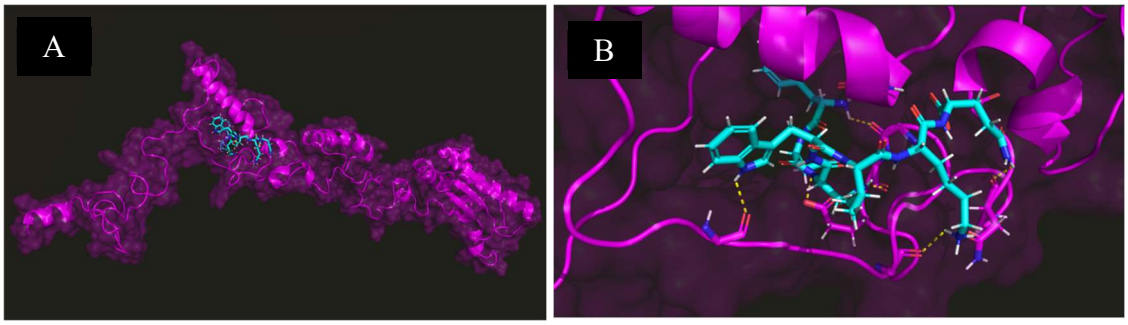
molecular docking images, where it is possible to see the different affinity levels between them.

Figure 2 - Immunoreactivity of phages selected through molecular docking to binding CJ by Phage-ELISA

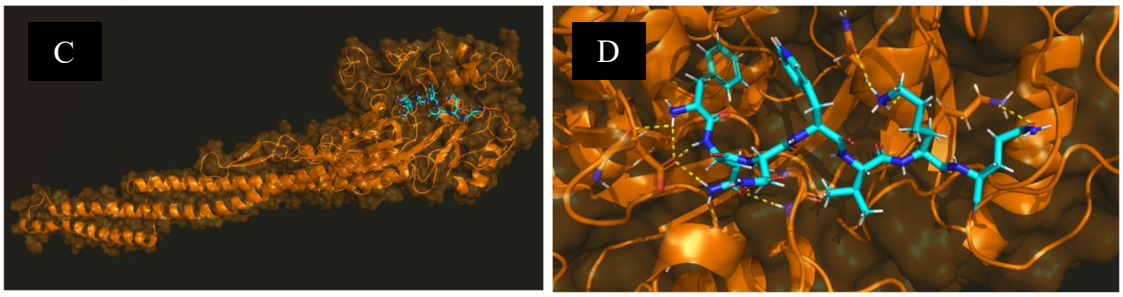


CJ IAL 2383: *Campylobacter jejuni* IAL 2383; microb: Total gut microbiota of SPF birds. C5, A8, B6, H7, H9: Selected phage displaying CJ binding-peptides. M13: wild phage. We performed ANOVA followed by the Tukey test compared each group with the M13 phage considering $p<0.05$ to CJ. We performed paired test t compared the immunoreactivity between the same phage form CJ IAL 2383 and microbiota. Asterisks in red show that there was a significant difference among the clones and M13 to binding to CJ. Asterisks in black show that there was a significant difference among each clone with CJ and microbiota. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

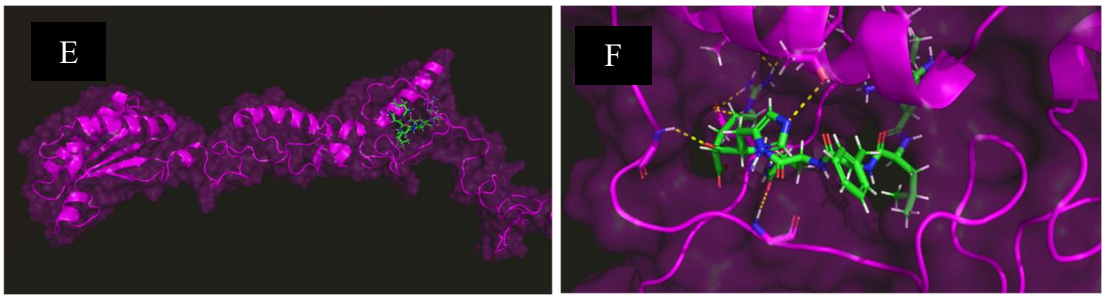
Figure 3 – Molecular docking of peptides B6 and C5 with proteins CadF and FlaA showing binding energy



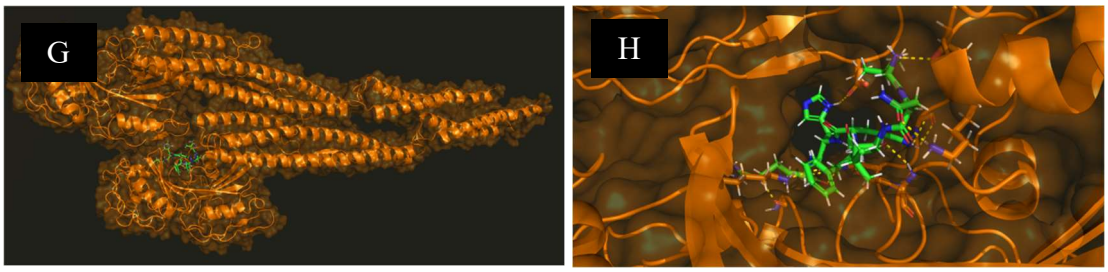
Molecular docking of peptide B6 with protein Cad F.



Molecular docking of peptide B6 with protein FlaA.



Molecular docking of peptide C5 with protein CadF.



Molecular docking of peptide C5 with protein FlaA.

Molecular docking of peptide B6 with protein Cad F or the protein FlaA. Molecular docking of peptide C5 with protein CadF or the protein FlaA. In purple the protein Cad F

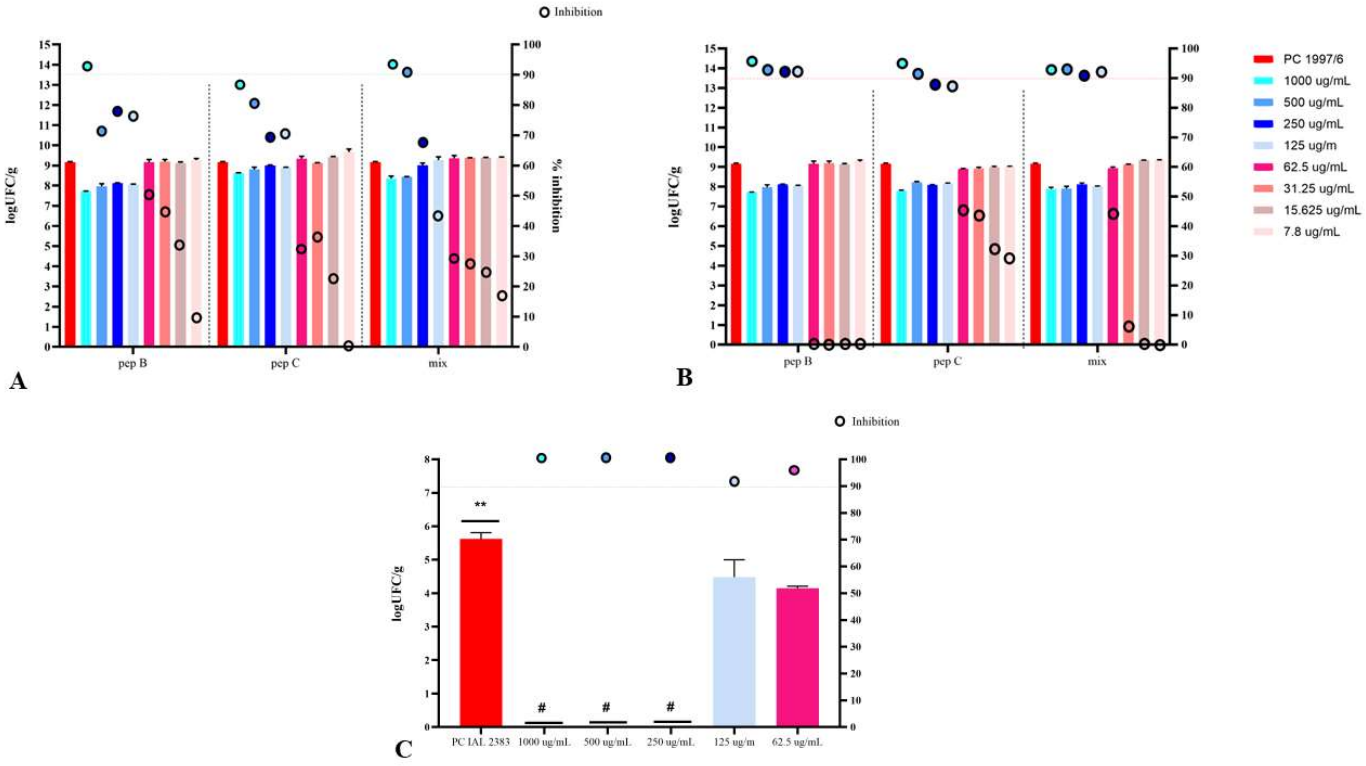
and in brown the protein FlaA. In blue we can see the peptide B6 (A-D) and in green, the peptide C5 (E – H) biding to the protein.

3.2 Peptides B6 and C5 can inhibit *Campylobacter jejuni*

Comparing to the positive control, peptide B6 was the one with the most bacteria reduction power. It reduced CJ IAL 2383 in 93.93% at 1000ug/mL while peptide C5 did not reach 90% reduction (Figure 4A). To the strain CJ 1997/6 peptide B6 decreased CJ from 125 ug/mL (92.02% of reduction) with 96% of reduction at 1mg/mL. Peptide C reduced bacteria from 250µg/mL (91.43% of reduction, Figure 4B). At 1mg/mL peptide C decreased CJ in 95.8% (Figure 4B). When using both peptides (mix), the reduction of CJ IAL 2383 was from 500ug/mL with 91.72% of reduction reaching 93% of reduction at 1000ug/mL. To the mix, the results of reduction to strain 1997/6 started from 125ug/mL (92.71% of reduction) and at peptide at 1mg/mL decreased CJ in 94.73% (Figure 4A).

Considering the worst result of the C5 comparing to B6 we performed another analysis with C5 in triplicate, but with CJ initial inoculum of 4 UFC/mL (Figure 4C) to evaluate if the initial inoculum changed the results. In this case, the peptide C5 reduced bacteria growth in 100% from concentration of 250µg/mL and 97.62% at 62.5 ug/mL. This show that the peptide has high power of inhibit the CJ when the initial dose is low.

Figure 4 – Average of *Campylobacter jejuni* amount and percentage of bacteria reduction after treatment with different concentrations of *C. jejuni* biding-peptides B6 and C5



A – Test of peptides B6 and C5 in reducing *Campylobacter jejuni* (CJ) strain IAL 2383 (A) or 1997/6 (B). In the graph we can see the number of bacteria (log UFC/mL) and the % of reduction. In A and B, the initial inoculum of CJ was 8log UFC/mL. The tests were performed in duplicate, and we performed a descriptive statistical analysis. C: We tested the peptide C to inhibit CJ IAL 2383 (C). The analysis in C was performed in triplicate and we the nonparametric Wilcoxon test t, considering the mean of inoculated bacteria (4 logUFC/mL). The Graphpad Prism 10.20 was used, and we considered $p<0.05$. Asterisks in show that there was a significant difference among each clone with CJ and microbiota. ** $p \leq 0.01$. The symbol # means that the Wilcoxon test does not provide a p value since the three replications were identical (zero).

When we tested peptides B6, C5 or a mixed and CJ IAL 2383 the percentage of death was like all groups. From 11 embryos tested, one embryo died in the negative group and in group inoculated with peptide B, C, mix and positive control died four, zero, two and two respectively. There was not changed in the weight of chicken embryo (Figure 5A). These results were expected since when a low dose of CJ is inoculated in chicken embryo at 10 days of incubation, the mortality is low (date not published yet).

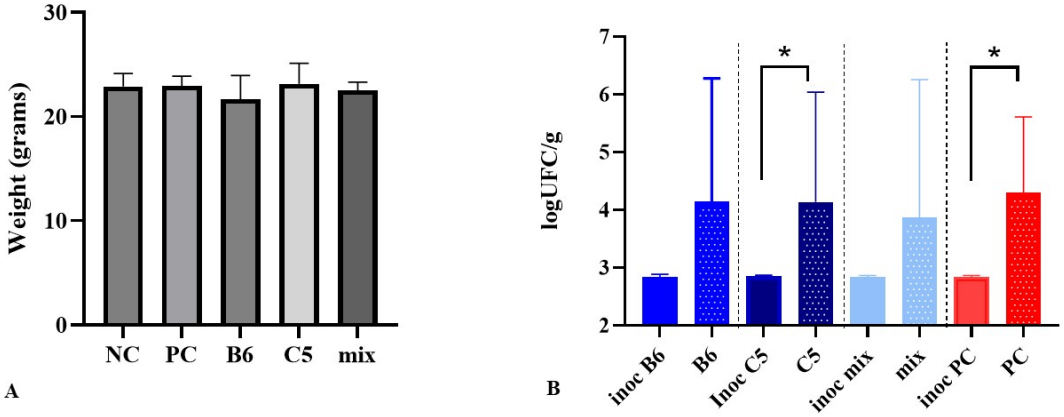
The peptides did not inactivate the CJ, but B6 and mix inhibited the multiplication, while peptide C5 did not present effect on the reduction of CJ (Figure 5B). Peptides are short molecules that can inactivate or reduce the infectivity of bacteria. In our work we proved that peptide C5 had *in vitro* effect but not *in vivo.* Even though B6 reduced CJ, the result could be better since we proved the stable binding seen in the docking and inhibition *in vitro*. There are several hypotheses to understand our results. First, we synthetized conformational peptides with the looping between the cysteines, however, a linear peptide might be more interesting to *in vivo* test. Second, peptides are short molecules, and, in some cases, it is necessary to stabilize peptides with PEGylation techniques, anchoring them in larger proteins or other delivery techniques (Pasut and Veronese, 2012; Santos et al., 2023). Lastly, although peptides are relatively stable, its stability can be disturbed in biological organisms. In chicken embryos, for example, the presence of allantoid may offer a harmful environment for the peptide, especially because besides respiratory interchange of oxygen and carbon dioxide, the allantoid also serves as reservoir for the waste products excreted by the embryo, mostly urea at first, and mainly uric acid later (Ribatti, 2016). Then, methods to improve our molecule seems to be necessary.

Even we did not find an excellent result *in vivo*, we proved the peptide is efficient to diagnostic approaches. The use of peptides is interesting to diagnostic because they can be more specific (since they recognize a specific epitope) and safer, once it is not necessary to handle the pathogen. So, peptides B6 or C5 should be tested in prove like fast tests as chromatographic tests, automated ELISA, electrochemical biosensors, and biosensors associated with artificial intelligence.

The stable binding in a bacterium is a property that can only be defined operationally. The ability to inhibit a pathogen remains anchored in the biological rather than the chemical realm. So, although *in silico* and *in vitro tests* are essential to select a peptide, it is necessary *in vivo* tests. In this sense, the chicken embryo is an interesting pre-clinical model, once it provides a simple way to study complex biological systems with well-developed vascular structures, in addition to allowing high reproducibility and being cheap and easy to handle (Sommerfeld et al., 2022). The chicken embryo has a long and distinguished history as a primary model system in developmental biology and has also contributed significant concepts to immunology, genetics, virology, cancer, and cell biology, being one of the most versatile experimental systems available (Fonseca et al., 2021).

Although many diverse antimicrobial peptides have been discovered, relatively few have been tested against CJ (Lohans et al., 2015). Here B6 and C5 presented good results *in vitro* but *in vivo* it is still necessary to search for better results.

Figure 5 - Amount of *Campylobacter jejuni* from allantoid fluid and weigh of chicken embryos treated with peptide B6, C5 or mix



B. Amount of *C. jejuni* (CJ) in allantoic fluid of chicken embryo treated with peptide B6 or C5 or mix of them. Inoc: Initial inoculum. A. Weight of chicken embryo in different groups. The data of CJ amount were adjusted for embryo weight and transformed into logarithms. The Kolmogorov-Smirnov test was performed followed by the paired t-test, comparing the initial inoculum/egg weight at 10 days of incubation with the amount of CJ/egg weight isolated from the embryo. PC: Positive Control. * Indicates $p<0.05$. The dotted vertical lines only separate the groups.

CONCLUSIONS

Peptides are efficient resources for diagnostic approaches, which is interesting because they can be more specific (since they recognize a specific epitope) and safer, once it is not necessary to handle the pathogen. Our work presents two peptides (B6 and C5) with the high ability to recognize CJ and inhibit its multiplication, which can be explored in a theragnostic approach. However, there is a need to test techniques to make peptides more constant to be used to inhibit CJ.

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