Bacteriocinogenic probiotics from chicken gut mucosa as an effective strategy to control *Campylobacter jejuni*

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Thesis submitted to the University of Ottawa

in partial Fulfillment of the requirements for the

M.Sc. degree in Interdisciplinary Health Sciences

The Interdisciplinary School of Health Sciences

Faculty of Health Sciences

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Abstract

Campylobacteriosis is a major global foodborne illness often associated with the consumption of poultry products, a common source of outbreaks and a risk factor for sporadic infections with this pathogen. *Campylobacter jejuni* accounts for 74% of the estimated human campylobacteriosis. Despite the recent ban of antibiotics in poultry as growth promoters, approximately 28% of *C. jejuni* isolates from chickens exhibit antibiotic resistance. Bacteriocinogenic probiotics has been proposed as a promising alternative strategy for reducing the incidence of enteric pathogens in poultry. The main objective of this study was to isolate and identify novel bacterial strains with bacteriocinogenic properties against *C. jejuni* from the cecal mucosa of broiler chickens and characterize their probiotic potential. Of 33 tested bacteria, 15 strains that exhibited the highest antimicrobial activity were identified as *Ligilactobacillus salivarius* based on 16S rRNA Sequencing. The strain *L. salivarius* UO.C249 showed potent antimicrobial activity against *C. jejuni*, as confirmed using micro-dilution assay, agar well diffusion method, and colony counting. Its cell-free supernatant (CFS) exhibited a dose-dependent anti-*Campylobacter* activity, likely due to the presence of bacteriocin-like moieties, as confirmed by protease treatment. Genome and proteome analyses of *L. salivarius* UO.C249 revealed presence of known bacteriocins, including the Abp118. The strain was sensitive to several antibiotics and had a high survival rate in the simulated chicken gastrointestinal tract.

Keywords: *Ligilactobacillus salivarius*, probiotic features, alternatives to antibiotics, bacteriocins, *Campylobacter jejuni.*

Résumé

La campylobactériose est une maladie d'origine alimentaire majeure, souvent associée à la consommation de produits de volaille, une source fréquente d'épidémies et facteur de risque d'infections sporadiques par cet agent pathogène. *Campylobacter jejuni* représente 74 % des cas estimés de campylobactériose humaine. Malgré l'interdiction récente des antibiotiques comme stimulateurs de croissance chez les volailles, environ 28 % des isolats de *C. jejuni* detectés sur le poulet présentent une résistance aux antibiotiques. Le recours aux probiotiques bacteriocinogènes a été proposé comme stratégie prometteuse pour réduire l'incidence des pathogènes entériques chez la volaille. L'objectif principal de cette étude était d'isoler et d'identifier de nouvelles souches bactériennes isolées à partir de la muqueuse cæcale de poulets de chair ayant des propriétés bactériocinogènes inhibant *C. jejuni* et de caractériser leur potentiel probiotique. Sur les 33 bactéries testées, 15 souches bioactives ont été identifiées comme *Ligilactobacillus salivarius* par séquençage de l'ARNr 16S. La souche *L. salivarius* UO.C249 a montré une importante activité antimicrobienne contre *C. jejuni*, confirmée par micro-dilution, diffusion sur gélose et comptage bactérien. Le surnageant a présenté une activité anti-*Campylobacter* dose-dépendante et sensible aux protéases. Les analyses du génome et du protéome de *L. salivarius* UO.C249 ont confirmé la présence de bactériocines connues, incluant Abp118. La souche *L. salivarius* UO.C249 était sensible à plusieurs antibiotiques et avait un taux de survie élevé dans le tractus gastro-intestinal simulé de poulets.

Mots-clés : *Ligilactobacillus salivarius*, probiotiques, alternatives aux antibiotiques, bactériocines, *Campylobacter jejuni*.

Acknowledgements

I would like to commence by expressing my gratitude to Allah for guiding me on this path and providing me with the strength and courage to complete this thesis.

Words cannot adequately convey my appreciation for each person who contributed to my journey.

First and foremost, I extend my deep and sincere gratitude to my supervisor, Professor Dr. Riadh Hammami, for his unwavering support over the past four years. I owe a tremendous debt of gratitude for his invaluable guidance and encouragement since the beginning of my work in his lab as an engineering student with limited knowledge in microbiology. He graciously provided me with the golden opportunity to undertake this remarkable project during my master's degree. Professor Hammami consistently demonstrated patience and generosity with his time, guiding me along the path to success. He took the extra time to explain concepts clearly, provided the assistance I needed, and presented opportunities for my advancement in the field. Working and studying under his direction was a great privilege and honor, and I am profoundly grateful for everything he has done to facilitate my journey.

I also extend my thanks to my Thesis Advisor Members, Professor Dr. Frédérique Tesson, Professor Dr. Maxwell Hincke, and Professor Dr. Chibuike Udenigwe, for their invaluable comments and guidance throughout my research project, along with our OMFARA collaborators. Special appreciation goes to Professor Dr. Wassef Ben Ounis, who has accompanied me throughout these years, offering continuous support and invaluable assistance.

A heartfelt thank you to the members of the Hammami lab, particularly Dr. Basit Yousuf, for his positive support, scientific expertise, valuable suggestions, and dedication to evaluating my work.

Dr. Basit taught me the methodologies to enhance my research skills in the lab and present my work more clearly. Additionally, I express my gratitude to Dr. Saba Miri for her encouragement, enthusiasm, and guidance that significantly contributed to my research and thesis writing. I also thank Dr. Walid Mottawea for his help, extensive knowledge, advice, and teaching of metagenomic techniques, and Dr. Tamer Ahmed for his consistent support and guidance in cell culture techniques. My sincere thanks also go to Dr. Ayman Elsayed, Dr. Galal Ali Farea Esmail, Dr. Ahmed Gomaa, and Dr. Nazila Nazemof for their kindness and support during my research.

I extend warm thanks to my friends and research colleagues, Emmanuel N. Njoku, Yasmina Ait Chait, Luana Leao, Manar Younes, and Salma Sultan, for their wonderful friendship, continual support, valuable assistance, and constant encouraging words throughout this shared journey.

Lastly, I am immensely grateful to my parents for their love, prayers, care, and sacrifices in educating and preparing me for my future. A heartfelt thank you to my sister for her continuous love, words of encouragement, and unwavering support. Without them, I could not have pursued my academic dreams.

Abbreviations

World Health Organization (WHO)

Campylobacter jejuni (*C. jejuni)*

Ligilactobacillus salivarius (*L. salivarius)*

Lactic acid bacteria (LAB)

Short-chain fatty acids (SCFAs)

Antimicrobial resistance (AMR)

Antimicrobial growth promoters (AGPs)

Capsular polysaccharides (CPS)

Gastrointestinal tract (GIT)

Competitive exclusion (CE)

Cell-free supernatant (CFS)

Minimum inhibitory concentration (MIC)

Generally Recognized as Safe (GRAS)

Medium-Chain Fatty Acids (MCFA)

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General Introduction

The poultry industry is a vital economic sector worldwide and poultry meat is a rich source of protein for human nutrition (Alali & Hoface, 2016). However, it can also harbor *Campylobacter*, a gram-negative bacterium that causes campylobacteriosis, the most common zoonotic infection worldwide (Hankel et al., 2022). One of the primary species of *Campylobacter* that infects humans is *Campylobacter jejuni*, which has a spiral morphology and flagella that enable motility, adhesion, and invasion of intestinal epithelial cells (Al Hakeem et al., 2022). *C. jejuni* requires specific growth conditions, such as microaerophilic conditions, an optimal temperature of 42℃ (chicken body temperature), and a pH above 5.0. *C. jejuni* was first detected in 1886 by Escherich, who isolated the bacterium from the colon of dead children and observed it in the stool specimens of children with diarrhea (Al Hakeem et al., 2022). Overuse of antibiotics in the livestock industry has contributed to the emergence of antibiotic-resistant bacteria, posing a global threat to human and animal health. Therefore, researchers are exploring alternative strategies to combat antibiotic resistance and reduce antibiotic dependence, particularly in food animals. Potential alternatives include bacteriophages, probiotics, bacteriocins, and short-chain fatty acids.

As governments have banned or restricted the non-therapeutic use of antibiotics, the poultry industry must shift towards more sustainable and natural alternatives, such as bacteriocin-producing probiotics (Lone, Mottawea, Mehdi, et al., 2021). Probiotics have a strong safety record and are widely used as starter cultures, health supplements, and direct-feed microbes for animal nutrition. Probiotics have been shown to have various beneficial effects, including modulation of the immune system, enhancement of the growth of beneficial commensal bacteria, and

improvement of nutrient absorption (Suresh et al., 2018). The most common probiotics belong to the genera *Bifidobacterium*, *Lactobacillus*, and *Saccharomyces* (M. L. Wan et al., 2018). Probiotics should meet certain criteria, including survival, adherence, and growth in the gastrointestinal tract, and measurable health effects (M. L. Wan et al., 2018). This study aims to investigate the efficacy of bacteriocin-producing bacterial isolates against *C. jejuni* for their potential use as probiotics in poultry production.

Chapter 1:

LITERATURE REVIEW

1. Incidence of Campylobacter jejuni in poultry environment and implication for human health

Poultry is an important source of zoonotic pathogens that can cause economic damage to the poultry industry and are a significant public health concern (Chowdhury et al., 2023). *Campylobacter* is the most common cause of gastrointestinal illness in North America , European Union , United Kingdom, and globally (European Centre for Disease Prevention and Control, 2022). Approximately 250,000 human campylobacteriosis cases have been confirmed in the EU (European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC), 2019). The incidence of *C. jejuni* in industrial broiler farms usually changes, corresponding to the age of the animals (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2020; Elhelw et al., 2022). It is frequently discovered after the third week of life, spreads across the farm, and persists until slaughter, contaminating meat in processing plants (Nothaft et al., 2021; Shreeve et al., 2000). *C. jejuni* can grow in the intestinal epithelium of warm-blooded host species, including humans, swine, cattle, sheep, companion animals, and birds (Barker et al., 2020; Biswas et al., 2019). Poultry is the leading host for *Campylobacter* because of its high body temperature (Beterams et al., 2023; Kers et al., 2018; Tram et al., 2020). Although *Campylobacter* can be found in all commercial bird species, it is particularly prevalent in poultry (Dubovitskaya et al., 2023; Ijaz et al., 2018). *Campylobacter* is present in almost every part of the broiler gut, but it is particularly prevalent in the ceca and cloaca (Pang et al., 2023). *C. jejuni* infection is not considered pathogenic in poultry, but it is a human pathogen of primary importance. Additionally, the multiplication of *Campylobacter* suggests that immunity is ineffective in preventing the spread of infection (Johannessen et al., 2020). In summary, this situation has advantages for *Campylobacter* and does not harm the host (Beterams et al., 2023). *C. jejuni* is commonly found in the microbiome of chickens and originates from various environmental sources, such as soil, water, dust, surfaces,

and air in poultry ecosystems. It is usually transmitted horizontally to chicken flocks because of inadequate biosecurity measures, the presence of wild animals near the chicken house, age and number of chicken houses, slaughter age, environmental factors, respirators, and flies (Śmiałek et al., 2021) **(Figure 1.1)**. *Campylobacter* can be highly prevalent and colonize the chick's intestinal tract early in life, and the bacteria can invade and penetrate intestinal epithelial cells. Evidence shows that human campylobacteriosis outbreaks have increased globally, with *C. jejuni* being the primary contributor (Eriksson et al., 2023). This bacterium is associated with various human gastrointestinal conditions, including inflammatory bowel disease, periodontitis, esophageal disease, functional gastrointestinal disorders, celiac disease, and colon cancer (Kaakoush et al., 2015). In Europe and many parts of the world, *Campylobacter* is recognized as one of the leading causes of foodborne illness, and it is a reported bacterial cause of diarrhea and sepsis in humans worldwide (Eriksson et al., 2023).

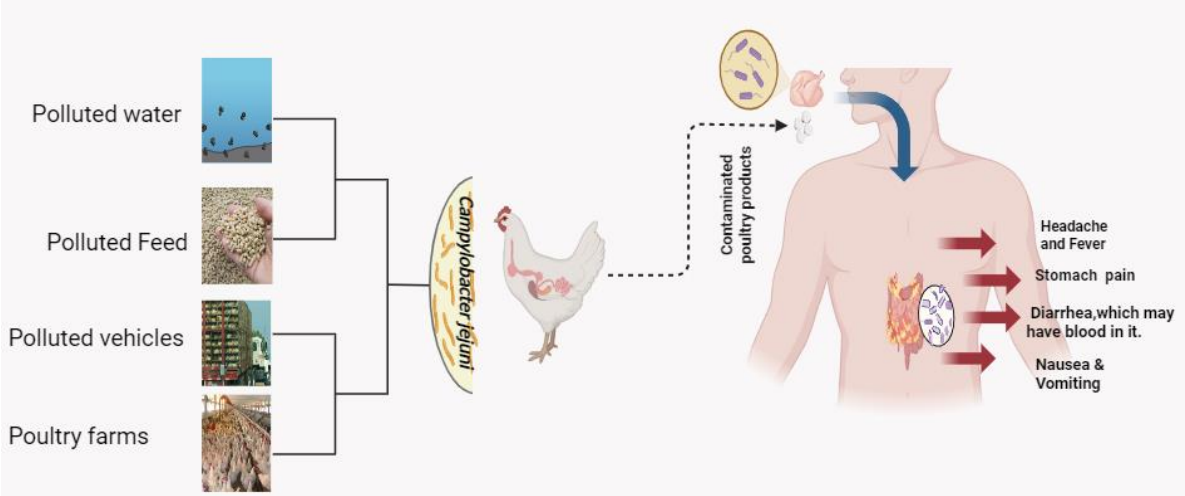

Figure 1.1. Transmission of *Campylobacter* infection to humans from the industrial environment through consumption of poultry products.

2. Challenges of antibiotics use in poultry production

Antimicrobial resistance (AMR) poses a significant risk to the poultry industry and global public health, as antibiotics are commonly used to treat chickens and prevent bacterial infections (Tawyabur et al., 2020). The use of antimicrobials in livestock has contributed to the spread of resistant bacteria, particularly in poultry, and threatens the effective treatment of life-threatening infections in humans (Manyi-Loh et al., 2018). For decades, antibiotics have been used in animal agriculture as growth promoters, medicinal treatments, and metaphylactic/prophylactic medicines (Manyi-Loh et al., 2018). Unfortunately, many of these compounds have been utilized without sufficient caution, primarily because of their remarkable cost-effectiveness (Abreu et al., 2023; Joerger, 2003). The overuse and misuse of antibiotics in livestock have led to increased life-threatening bacterial infections in humans, increased mortality rates, prolonged hospital stays, and higher medical expenses (Hudson et al., 2017; Rodrigues et al., 2022). In 1951, the Food and Drug Administration authorized the use of antibiotics as animal supplements without a prescription. The use of antimicrobial growth promoters (AGPs) in animal feed was first banned in Sweden in 1986, and the European Union (EU) outlawed 25 AGPs from animal production in 2006 (Abreu et al., 2023; Castanon, 2007). Since 2016, the USA has prohibited the use of some significant human medicinal antimicrobials, including AGPs (Abreu et al., 2023; Hudson et al., 2017). Other countries, such as Mexico, New Zealand, and South Korea, have adopted the EU's decision to limit or ban these compounds. Regulations have also been established in the USA, Australia, Japan, and Canada to limit or partially ban several antibiotic-derived compounds (Krysiak et al., 2021). AGPs, antibiotics administered to animals at dosages below those considered therapeutic, aim to alter their gut microbiome and enhance their growth and performance. AGPs have been found to improve animal development rates by reducing competition for nutrients, selecting gut bacteria, and choosing bacteria that are more likely to grow. Some studies support these advantages, stating

that they are crucial in the early stages of production or helpful when there are no optimal hygienic conditions (Abreu et al., 2023; Rushton, 2015), whereas others argue that these practices enhance productivity and stress the importance of maintaining excellent animal husbandry standards in animal production (Hudson et al., 2017). However, using AGPs has also contributed to developing widespread antimicrobial resistance in the gut microbiome (Luiken et al., 2019), leading some states to ban their application in animal production.

3. Alternative Strategies to Control Campylobacter in Poultry

3.1. Essential Oils

Essential oils are naturally occurring aromatic and volatile molecules that are extracted using solvent extraction, steam distillation, or hydro distillation from plant parts such as flowers, buds, stems, leaves, seeds, twigs, roots, fruits, bark, and wood (El-Tarabily et al., 2021) (**Table 1.1**). Essential oils improve chicken productivity by increasing the activity of digestive enzymes, reducing the number of fermentation products, lowering the number of pathogens, improving nutrient digestion, augmenting intestinal accessibility of important nutrients, and enhancing antioxidant capacity and immune functions (Puvača et al., 2022). Essential oils from medicinal and aromatic plants have antibacterial properties that are the basis for multiple applications in the pharmaceutical, nutraceutical, cosmetic, perfume, agronomy, and sanitary industries, among other revenue-generating fields (Swamy & Sinniah, 2015). For example, a combination of garlic and cinnamon extracts, which are abundant sources of essential oils, decreased *Campylobacter* colonization in the cecum by (1 $\log_{10}$ CFU/g) at 3 days post-infection (day 11 of age) (Guyard-Nicodème et al., 2016). Another study investigated the effect of essential oil blends, including thyme-orange essential oils, on controlling *Campylobacter* in poultry carcasses. The authors

showed that 0.5% thyme-orange oil combination significantly reduced *Campylobacter coli (C. coli)* by 4.5 log CFU/mL on breast fillets (Thanissery-Ravindranath, 2013).

3.2. Organic acids

Organic acids are natural compounds with acidic properties that can be defined as weak acids with a carboxylic acid group (R-COOH), including (e.g., lactic, citric, malic, acetic, fumaric, and capric acids) (Ahmad et al., 2022) **(Table 1.1)**. Due to the ban on using antibiotics, organic acids have been used in poultry feeds for a long time as efficient antibiotic substitutes in animal feeds. Organic acids are administered to the drinking water and fed to broiler chickens (Khan et al., 2022). They have been tested as effective additives and antibacterial agents to reduce the prevalence of *Campylobacter* colonization based on their capacity to lower intestinal pH and hence improve gut immunity (Kovanda et al., 2019) and stop attachment between *Campylobacter* and epithelial cell receptors by competitive exclusion. *Campylobacter* generates capsular polysaccharides (CPS) on its cell surface, which may play a significant role in its pathogenicity and contribute to AMR. Sima et al. (2018) have demonstrated that combining organic acid and plant extract supplements can alter the CPS profiles of *C. jejuni* (Sima et al., 2018). This alteration led to the downregulation of critical colonization genes, such as *cetB* and *hcp*, reducing adhesion and invasion of *Campylobacter* in the avian intestinal tract (Sima et al., 2018). The authors suggested that pretreatment with these compounds may interfere with host cell metabolic pathways, thereby aiding in the prevention of bacterial infections.

3.3. Vaccines

Poultry vaccination may be considered a logical starting point and a potentially effective strategy to prevent the initial colonization and prevalence of foodborne pathogens, including *Campylobacter* (Kobierecka et al., 2016). Colonization by *Campylobacter* in young chickens is

not usually observed until 2-3 weeks after hatching, probably because of the availability of maternally derived antibodies against this bacterium, which play a role in the prevention of the early colonization of chick ceca (Connerton et al., 2018). Many vaccination techniques have been developed for poultry against *Campylobacter* using a variety of methodologies, such as whole-cell vaccines, subunit vaccines, crude cell lysate vaccines (Kobierecka et al., 2016; Pumtang-on et al., 2021) (**Table 1.1)**. Some studies have shown that subunit, whole-cell, and crude-cell lysate vaccines have effectively reduced *Campylobacter* colonization in chickens (Hakeem & Lu, 2021). For instance, Cui et al. (2020) showed that immunization of chickens with an enterobactin conjugate vaccine reduced *C. jejuni* colonization in the intestine. In this study, enterobactin conjugate positively affected the development of the immune system and gut microbiota. A high level of systemic anti-enterobactin IgG leads to reduced *C. jejuni* colonization by 3–4 $\log_{10}$ units in the cecum (Cui et al., 2020).

3.4. Bacteriophages

Bacteriophages infect and lyse targeted bacterial cells after binding to specific receptors on bacterial surfaces, such as outer membrane proteins, lipopolysaccharides, and flagellar components (Dowah & Clokie, 2018). There is an interest in using phages as antimicrobial agents worldwide to control *Campylobacter* colonization in poultry and treat several human diseases (Abd-El Wahab et al., 2023) **(Table 1.1)**. In the poultry industry, the use of bacteriophages as therapies (phage therapy) can be viewed as a significant antibiotic substitute (Moye et al., 2018; Wernicki et al., 2017). For instance, dietary supplementation with two field bacteriophages to affect broiler chickens at 37 days of age showed high efficiency in reducing the prevalence of *Campylobacter* by (10^2 CFU/g) at 40 days of age in poultry flocks (D'Angelantonio et al., 2021). Likewise, Wagenaar et al. (2005) showed the potential of phage therapy in controlling *C. jejuni*

colonization in young broilers, either as a preventive or a therapeutic measure. The authors found that the counts of *Campylobacter* reduce 3 $\log_{10}$ after using phage, but it increased again during five days and stabilized at 1 $\log_{10}$ (Wagenaar et al., 2005). Carvalho et al. (2010) reported that a phage cocktail effectively reduced pathogen colonization with approximately 2 $\log_{10}$ reduction of *Campylobacter* counts in Poultry (Carvalho et al., 2010). Similarly, Loc Carrillo et al. (2005) found that increasing the time interval between phage treatment and slaughter from 24 hours to 2-4 days reduces *Campylobacter* from 0.5 log10 to 5 log10 in the cecal contents of broiler chickens (Loc Carrillo et al., 2005). In general, a single bacteriophage strain has not been sufficient to reduce *Campylobacter* colonization in broiler chickens, and phage cocktails have been proven more efficient in controlling *Campylobacter* in broiler chickens in commercial settings.

3.5. Probiotics

Probiotics are defined by the WHO as 'live bacteria that, when provided in adequate amounts, confer a health effect on the host' (Hill et al., 2014). Probiotics help with food digestion, absorption, and regulation of the immune system (Xu et al., 2022). Probiotics can prevent or eliminate harmful bacteria by colonizing and growing in the host gastrointestinal tract (Clavijo & Flórez, 2018). Probiotics secrete various metabolites, such as bacteriocins, organic acids, and hydrogen peroxide, that can protect broilers from harmful microorganisms (Jeni et al., 2021; Nakanishi & Tanaka, 2010; Rubio, 2019). Probiotics maintain a healthy balance, which supports gut health, performance, and resistance to intestinal diseases (Hemarajata & Versalovic, 2013). Probiotics have numerous benefits in poultry, such as increasing feed intake, weight gain, digestibility, digestive enzymes, stress tolerance, pathogen resistance, antioxidant effects, and immunomodulation (Chowdhury et al., 2023). For instance, (Ghareeb et al., 2012) demonstrated that multispecies probiotics containing *Enterococcus faecium*, *Ligilactobacillus salivarius*, and

Limosilactobacillus reuteri significantly reduced cecal colonization by *C. jejuni*, indicating that probiotic products could be used to improve food safety by reducing vertical transmission of human pathogens, such as *C. jejuni*. Likewise, dietary supplements of *Clostridium butyricum* or *Enterococcus faecium* modulated the diversity of broiler cecal microbiome by suppressing the growth of pathogenic bacteria such as *E. coli* and *Clostridium perfringens* and inducing the growth of *Lactobacillus* and *Bifidobacterium* (L. Zhang et al., 2014). *Enterococcus faecium* was also fed as a dietary supplement to broilers, increasing the relative number of SCFA-producing bacteria and enhancing bone development (W. Wang et al., 2020).

Table 1.1. Summary of tested antimicrobial strategies to inhibit *Campylobacter*.

Types	Compounds	Target microorganisms	Antimicrobial effects	References
Essential Oils	10 mM cinnamaldehyde	*C. jejuni*	Decrease by at least 5-logs CFU/mL after 8 h incubation.	(Kollanoor Johny et al., 2010)
	Thymol at 0.25 µmol/mL.	*C. jejuni and C. coli*	Reduced by 5 logs CFU/mL in Bolton broth from an initial concentration of 7 log CFU/mL.	(Anderson et al., 2009)
	Combination thyme-orange	*Campylobacter*	Control in poultry carcasses.	(Thanissery & Smith, 2014)
	Combination of garlic and cinnamon extracts	*Campylobacter*	Decreased the colonization in the cecum by (1 log10 CFU/g) at 3 days post-infection.	(Guyard-Nicodème et al., 2016)
	Broiler diets supplemented with 100 mg/kg of 5% carvacrol	*Campylobacter*	Exhibited an impact on the jejunal mucosa.	(Gholami-Ahangaran et al., 2022)
Organic acids	MCFA including Sodium caprate and Sodium laurate (both 1.5%).	*C. jejuni*	Rapidly killed the strain (6.0 log10 CFU/mL in 60s	(Heade et al., 2018)

	Sodium caprylate (1.5%)	*C. jejuni*	Required 60 min to achieve reductions of approximately 4 log10 CFU per ml	(Heade et al., 2018)
	Butyrate at 12.5 mM and pH 6.0	*C. jejuni*	most active, with a bactericidal impact	(Van Deun et al., 2008)
	Acetate and propionate at 50 mM concentration	*C. jejuni*	Inhibited the growth	(Van Deun et al., 2008)
Bacterio phages	A phage cocktails	*Campylobacter*	Effectively reduced pathogen colonization with approximately 2 log_{10} in Poultry	(Carvalho et al., 2010)
Vaccines	Whole-cell vaccines	*C. jejuni*	The vaccine administration reduced colonization from 16% to 93% in the vaccinated group compared with the non-vaccinated one	(Jeong et al., 2022)
	Crude cell lysate vaccines	*C. jejuni*	The subcutaneous route induced the highest immune response in vaccinated broilers and decreased the level by 5.7 log CFU/g in the ceca	(Annamalai et al., 2013)
	Subunit vaccines	*C. jejuni*	Oral delivery reduced the level in layer and broiler chickens by 2.24 log CFU/g and 2.14 log CFU/g, respectively, at 22 days post-infection The vaccinated group had one log CFU/g in the ceca.	(Taha-Abdelaziz et al., 2018) (Singh et al., 2019)
Probiotic s	The supernatant of *Lactobacillus strain* P93	*C. jejuni*	Produced a zone of inhibition of 9 to 15 mm in the well-diffusion agar	(Svetoch et al., 2011)
	L. salivarius K7 (Chicken intestine)	*C. jejuni*	Reduction in colonization by at least 1 million-fold	(Stern et al., 2006)
	L. salivarius NRRL B-30514	*C. jejuni*	1 log CFU/g reduction after 6 h post-inoculation	(Robyn et al., 2012)
	Inoculation of *E. faecalis* strain in ceca	*C. jejuni*	Reduction of C. jejuni level to 1 log CFU/g after 6 h post-inoculation	(Robyn et al., 2012)

3.6. Bacteriocins

Bacteriocins are a group of different, ribosomally synthesized peptides or proteins having a narrow spectrum of antimicrobial activity (Hammami et al., 2013; Soltani et al., 2021). These bacteriocins can be produced by Gram-positive and Gram-negative bacteria (Hammami et al., 2013). Bacteriocins could be bacteriostatic, inhibiting the growth of pathogens, or bactericidal, killing them. The general mechanisms of bacteriocin-mediated pathogen killing include the formation of ion-permeable channels in the cytoplasmic membrane, inhibition of DNA and RNA synthesis and/or cell wall protein synthesis (Lone, Mottawea, Mehdi, et al., 2021). Bacteriocin classification has changed over the years and is based on the structure or the mechanism of action (Lagha et al., 2017). Soltani et al., (2021) provided an updated classification of bacteriocins from both Gram-positive and Gram-negative bacteria into two different classes, the first class: modified bacteriocins (class I) and the second class: unmodified bacteriocins (class II) **(Table 1.2)**.

Table 1.2. Updated classification of bacteriocins (Soltani et al., 2021).

Modified Bacteriocins (class I)	Unmodified Bacteriocins (class II)
• Molecular masses < 5 kDa with post-translational modifications. • More stable to high temperatures, acidic pH, or proteolytic enzyme than class II • Sub-Classes: ✓ Gram- positive bacteria: Lanthipeptides, Sactipeptides, Circular peptides, glycocins (Cotter et al., 2013). ✓ Linear azole(ine)-comprises peptides (LAP) and lasso peptides from both Gram-positive and Gam-negative bacteria. ✓ Nucleotide peptides and siderophore peptides from Gram-negative bacteria (Cotter et al., 2013; Duquesne et al., 2007; Gabrielsen et al., 2014; Norris & Patchett, 2016). ✓ Linaridins (Claesen & Bibb, 2010), thiopeptides (Bagley et al., 2005) and Cyanobactins.	• Molecular Mass 6–10 kDa, with or without disulfide bridges. • Presence of Disulfide bridges increase the stability. • 3 Sub-classes: ✓ Pediocin-like bacteriocins (contains YGNGV consensus sequence). ✓ Non-pediocin-like bacteriocins (without the YGNGV consensus sequence) ✓ Bacteriocins with two peptides.

Although bacteriocins have mostly been used as food preservatives, they are currently considered potential therapeutic antimicrobials. Bacteriocins are recognized as promising antibacterial substances with prospective uses in the food industry and clinical and veterinary sectors. Bacteriocin-producing bacteria have been investigated for their ability to control foodborne infections (Soltani et al., 2021). For example, Stern et al. (2005) demonstrated the efficacy of bacteriocin divercin AS7 in controlling and reducing pathogens strains, such as *Campylobacter* species (Stern et al., 2005). Likewise, L-1077 bacteriocin produced by *Ligilactobacillus salivarius* 1077 (NRRL B-50053) (Svetoch et al., 2011) and SMXD51 bacteriocin produced by *L. salivarius* SMXD51 (Messaoudi et al., 2011) both isolated from chicken ceca have shown a significant reduction in *Campylobacter*. Bacteriocins are typically believed to be nontoxic to mammalian cells and represent a potential solution to this global challenge. Numerous investigations examining various bacteriocins have demonstrated that they have no effect on the integrity of tight junctions of mammalian cells (Soltani et al., 2021). For instance, Belguesmia et al. (2011) reported that treatment of Caco-2/TC7 cells with 10 μg mL^{-1} nisin or enterocin S37 did not result any significant alteration in epithelial integrity (Belguesmia et al., 2011). Still, the current legislation is hindering their approval and acceptance for medical, veterinary, and food applications.

4. Chicken gut microbiome as a source of bacteriocinogenic probiotics

The chicken gut is home to a complex and dynamic microbial community that evolves continuously with diet, environment, antibiotics, and other inducing factors (Ramirez et al., 2020). The five gastrointestinal segments of chickens differ in their bacterial diversity, with five major phyla (Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Cyanobacteria) accounting for more than 90% of the species present (Y. Xiao et al., 2017). The small intestine of broilers harbors a substantially higher number of Firmicutes than other segments (Y. Xiao et al., 2017),

while the cecum has the highest abundance of Bacteroidetes (>50%). The proventriculus has more Proteobacteria than the other four intestinal segments (Y. Xiao et al., 2017). *Lactobacillus* spp., *Enterococcus* spp., and *Bacteroides* spp. are the dominant bacterial genera in all intestinal segments (Mohd Shaufi et al., 2015; Y. Xiao et al., 2017). The ileum and cecum host a diverse bacterial community that is rich in anaerobic bacteriocin-producing bacteria compared to the upper section of the gut. For example, *Lactobacillus* spp. can produce multiple bacteriocins, such as Abp118, a bacteriocin produced by strain *L. salivarius* UCC11 isolated from a human intestine, that was reported to reduce *Listeria monocytogenes* in the liver and spleen of mice (Corr, Li, et al., 2007). Svetoch et al. (2008) also reported that *E. faecium* M74 produces bacteriocin E50–52 active against *Salmonella* in broilers (Svetoch et al., 2008). Moreover, *Bifidobacterium bifidum* NCFB, a strain isolated from feces, produced bacteriocin bifidocin B, which was active against *Listeria* (Yildirim & Johnson, 1998), and a bacteriocin-like antimicrobial substance active against *Salmonella* spp. and *E. coli* (O'Riordan & Fitzgerald, 1998).

5. Mechanism of pathogen control in chicken gut environment by probiotics

Probiotics have a positive impact on the composition and metabolic function of the gut microbiome. The probiotic mechanisms include the protection of the intestinal epithelium from pathogenic microorganisms through competitive exclusion (CE), production of antimicrobial compounds (e.g., organic acids and bacteriocins), and immunomodulation (M. L. Wan et al., 2018) (Figure 1.2).

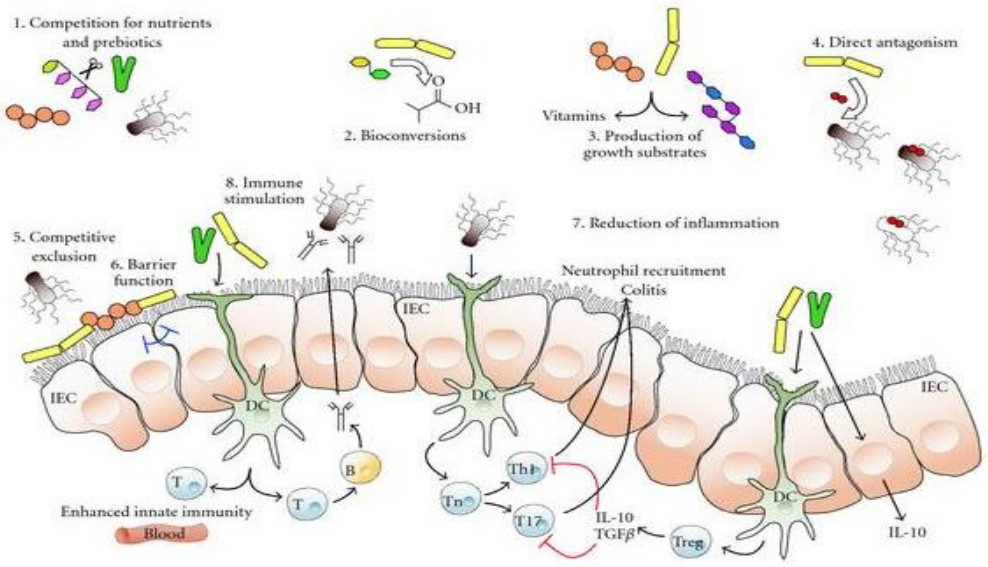

Figure 1.2. Mechanisms of action of probiotics bacteria against pathogens (O'Toole & Cooney, 2008)

5.1. Competitive exclusion of pathogenic microorganisms

CE by probiotics is an antipathogenic mechanism that describes the scenario of competition between the probiotics and pathogen microorganisms for nutrients and adhesion to receptors of the intestinal epithelium. Certain probiotic strains prevent the pathogens from colonizing the gut and inhibit their growth (M. L. Y. Wan et al., 2019). For example, Nishiyama et al. (2014) demonstrated the excellent involvement of *Lactobacillus gasseri* SBT2055 in a CE of *C. jejuni* 81-176, which translates by a decreasing the count of *C. jejuni* cells adhering to epithelial cells of chicks (Nishiyama et al., 2014).

5.2. Immunomodulation

Probiotic bacteria could regulate host immune responses and modulate the functions of dendritic cells, monocytes/macrophages, and lymphocytes (Corr, Gahan, et al., 2007; O'Hara et al., 2006). Probiotics have an impact on the phagocytosis of enteric pathogens by host immunological

phagocytic cells like macrophages (Mathipa & Thantsha, 2017). Taha-Abdelazi et al. (2019) reported the stimulation of macrophages with either a single species or a mixture of *Lactobacillus* spp. (*L. salivarius, L. johnsonii, L. reuteri, L. crispatus, and L. gasseri*), resulting in an increased phagocytosis of *C. jejuni* (Taha-Abdelaziz et al., 2019). *Lactobacilli* could stimulate macrophage activation through increased expression of interferon-γ, IL-1β, IL-12p40, and IL-10 genes. The co-stimulatory CD40, CD80, and CD86 molecules were expressed more strongly in *Lactobacilli*-induced macrophages (Taha-Abdelaziz et al., 2019). Lymphocyte B have also been demonstrated to play a role in the removal of *C. jejuni* and to reduce the number of excreted germs (Lacharme-Lora et al., 2017). It is well known that co-stimulatory molecules take part in the chain reaction of antigenic signal transduction and T and B cell activation. It is possible that the LAB mixture could improve both the non-specific and specific immune responses against *Campylobacter*.

5.3. Production of antibacterial substances

Probiotic strains produce bacteriocins, hydrogen peroxide, and SCFAs, among several compounds with varying spectra of antimicrobial activity, that inhibit the growth of pathogenic microbes and help preserve gut health. Bacteriocins, narrow-spectrum antimicrobial peptides, can either directly inhibit or kill harmful microbes and decrease their capacity to colonize the intestine (Cotter et al., 2013; Y. Zhang et al., 2023). Messaoudi et al. (2011) reported that *L. salivarius* SMXD51, MMS122, and MMS151 were potentially useful bacteriocinogenic bacteria against *Campylobacter* (Messaoudi et al., 2011). Chaveerach et al. (2004) reported that *Lactobacillus* P93 isolate from chicken GIT reduced the proliferation of *Campylobacter* by secreting SCFA and anti-*Campylobacter* proteins (Chaveerach et al., 2004). The interaction between the microbiota and pathogenic bacteria in the intestine is significantly influenced by SCFAs produced by the intestinal microbiota. Although the precise mechanism of some intestinal pathogenic infections remains

unclear, intestinal bacterial metabolites may be crucial for reducing intestinal pathogenic infections (Connolly et al., 2018).

5.4. Interference with virulence gene expression of pathogenic microorganisms

Numerous virulence factors are employed by bacteria to enter host cells in the complex process of microbial pathogenesis, which involves both the pathogen and host (Elbehiry et al., 2023; Finlay & Falkow, 1997). Several virulence factors are associated with bacteria, including flagella, pili, fimbriae, adhesins (e.g., fibronectin, collagen, laminin, integrin, and internalin), and biofilm formation components (such as phospholipids, teichoic acids, nucleic acids, polysaccharides, proteins, capsules, enzymes, poisons, and spikes) (Elbehiry et al., 2023; Kazmierczak et al., 2005). On the other side, the host uses a variety of immune cells to fight pathogens such as phagocytic cells (monocytes\macrophages), lymphocytes, and dendritic cells (Brown et al., 2013; Elbehiry et al., 2023). Probiotics and their metabolites have been demonstrated to be effective in decreasing the virulence of pathogenic bacteria. Likewise, Van Zyl et al. (2020) reported that probiotics may weaken the flagellar motility of intestinal pathogenic microorganisms (Van Zyl et al., 2020). Also, (De Keersmaecker et al., 2006) have shown that *Lacticaseibacillus rhamnosus* GG has a potent inhibitory activity against *Salmonella* Typhimurium due to the production of lactic acid, thus affecting the expression of HilA and InvF virulence factors (Durant et al., 2000). In addition, *Lactobacillus acidophilus* La-5 and *Bifidobacterium longum* NCC2705 cell-free supernatants induced a significant decrease in the expression of virulence genes (*cadF, cdtB, flaA,* and *ciaB)* of *C. jejuni* (Mundi et al., 2013).

6. Application of probiotics in animal production

Probiotics induce the growth of beneficial bacteria, modulate the composition of the gut microbiome, and significantly improve animal health (Liu et al., 2023 ; Morelli & Capurso, 2012). According to recent studies, probiotics as alternatives to antibiotics can address various intestinal issues and increase the health and productivity of chickens by maintaining intestinal homeostasis (Luise et al., 2022; X. Xiao et al., 2021). The beneficial effects of probiotics are influenced by the quantity added and the type of strain used (Kechagia et al., 2013). Currently, *Bifidobacteria* (El-Sharkawy et al., 2020), *Lactobacillus, Enterococcus faecalis* (Shehata et al., 2020), *Bacillus licheniformis* (Zhao et al., 2020), *Bacillus subtilis* (Memon et al., 2022), *Streptococcus thermophilus* (Sihite & Pramono, 2022), and *Aspergillus oryzae* (Chuang et al., 2019) are among the most frequently utilized probiotics in chicken production.

Probiotics exert beneficial effects by manipulating the gut microbiota, which is significant for chicken health (Madlala et al., 2021; Memon et al., 2022). For example, food supplementation with *L. salivarius* CML352 restored intestinal homeostasis by reduction of *Firmicutes*/*Bacteroidetes* ratio, improved *MUC2* expression, and affected late-phase laying hens' intestinal health positively (Xu et al., 2022). Additionally, (Lee & Bak, 2011; Memon et al., 2022) found that dietary supplementation with *Bacillus subtilis* increased the richness of certain commensal genera in chickens infected with *Eimeria tenella*, *such as Clostridium sensu stricto 1, Corynebacterium, Enterococcus, Romboutsia, Subdoli-granulum, Bacillus, Turicibacter, and Weissella.* An increased abundance of these gut microbes increased butyrate production, promoted anti-inflammatory responses, defended against pathogens, and relieved intestinal dysbiosis induced by *Eimeria tenella* (Lee & Bak, 2011; Memon et al., 2022). Acetate and lactate are the main metabolic products of bifidobacteria, and they also prevent the invasion of harmful

microorganisms (Devika & Raman, 2019). In addition, *L. salivarius* CML352 has been shown to reduce fat deposition and improve egg quality in late-phase laying hens (Xu et al., 2022). Another study found that a 4-week treatment with *Clostridium butyricum* and *Bacillus subtilis* decreased the rate of abnormal eggs in 450-day-old Hyline hens from 42.51% to 28.02% (Khogali et al., 2022). The relative abundance of *Ruminococcaceae* was increased by the addition of *Bacillus amyloliquefaciens* CGMCC18230, which improved tibial bone mineralization and growth performance (C. Li et al., 2022). Previous studies have shown that probiotics exert beneficial effects on muscle growth in broiler chickens (Stasiak et al., 2021). Dietary supplementation with *E. faecium* AL41 increased the number of myonuclei per fiber, improved capillarization, and further improved body weight owing to the high intramuscular expression of IGF-1 and lowered MYF5 expression in broilers (Albrecht et al., 2022). Supplementation with a *Clostridium butyricum* and *Bacillus subtilis* mixture improved intestinal development by increasing villus length and the ratio of villus length to crypt depth (Yu et al., 2021). Another study found that the combined supplementation of organic acids and probiotics increased the ability of broilers to digest crude fiber and villus height, while inhibiting the growth of *E. coli* (Ebeid et al., 2021). Probiotic strains, such as *Butyricicoccus pullicaecorum*, produce butyrate, increase feed efficiency, and decrease *Campylobacter* levels in ceca by 1.5 logs (Eeckhaut et al., 2016). Various species of *Lactobacillus* (PCS20, PCS22, PCS25, LGG, and PCK9) decreased *C. jejuni* adhesion, invasion, and translocation to chicken (B1OXI) cell lines (Šikić Pogačar et al., 2020). The combination of *Lactobacillus* species (*crispatus, johnsonii, salivarius, gasseri, and reuteri*) inhibits the production of quorum-sensing autoinducer-2 by *C. jejuni*, leading to a decrease in virulence-associated factors and a reduction in the in vitro invasion of Caco-2 cells (Taha-Abdelaziz et al., 2019). *C. jejuni* 81-176 invasion and survival were also reduced by *E. coli* Nissle 1917 in HT-29

cells (Helmy et al., 2017). *L. salivarius* SMXD51 exhibited significant reductions (0.82–2.81 logs) in *Campylobacter* levels (Saint-Cyr et al., 2017). Additionally, *L. salivarius* SMXD51, an efficient producer of *salivaricin*, was previously linked to induced immunomodulatory effects by enhancing IL-8 production and β-defensin 2 secretion (Saint-Cyr et al., 2017). Another bacteriocin producer, *L. curvatus* DN317, produces *curvaticin*, which demonstrates exceptional bacteriostatic activity against *C. jejuni* (Zommiti et al., 2016). Growing cultures of *C. jejuni* ATCC 33560 were administered 50, 100, and 150 AU/mL of *L. curvatus* supernatant to limit growth. This reduced the viable bacterial count by 23.8 %, 45.5%, and 61.3%, respectively (Zommiti et al., 2016). Another type of bacteriocin obtained from *Enterococci spp*. is *enterocin*, which, when combined with herbal extracts, can be used as a novel ecological strategy to reduce *Campylobacter* in livestock (Ščerbová et al., 2022). Under anaerobic fermentation conditions, *Limosilactobacillus reuteri* may also produce inhibitory substances, such as *reuterin*, which has broad-spectrum antibiotic activity against a variety of foodborne pathogens (Asare et al., 2020). *L. reuteri* exhibited a more significant reduction of *C. jejuni* N16- 1419 during co-culturing from 7.3 logs CFU/mL to just above the detectable limits (1 log CFU/mL) (Asare et al., 2020). Finally, *Bacillus subtilis* slowed the growth of *C. jejuni* by 3.87 to 4.07 logs, and after 48 hours, the growth was below detection limits (Šimunović et al., 2022). *Bacillus subtilis is* a strong probiotic option for *Campylobacter* reduction in chickens, given that all these effects were observed at 42 °C, which is the ideal body temperature for poultry (Šimunović et al., 2022).

Chapter 2:

Hypothesis and Objectives

I hypothesize that bacteriocinogenic strains isolated from the intestinal mucosa of chickens can inhibit the growth of *Campylobacter jejuni* and exhibit potential probiotic properties. Therefore, my main aim is to find a novel probiotic candidate originating from healthy chicken microbiota as an alternative strategy to control *C. jejuni* in the poultry industry.

My specific objectives are as follows:

- Objective 1: Screening and identification of new bacteriocinogenic candidates isolated from chicken intestinal mucosa, selected from the Biobank of N*u*Gut lab (uOttawa), and characterization of their antimicrobial activity.
- Objective 2: Evaluation of probiotic properties of selected strains with high antimicrobial activity against *C. jejuni*.

Chapter 3:

Ligilactobacillus salivarius UO.C249, a novel bacteriocinogenic probiotic candidate from chicken mucosa with anti-*Campylobacter jejuni* properties

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Results from this chapter are submitted to Applied and Environmental Microbiology.

Abstract

Campylobacter is one of the most common causes of foodborne infections worldwide, causing gastroenteritis, and is recognized as one of many causes of diarrheal diseases. This study aimed to explore the ability of bacteriocin-producing bacteria to control and inhibit the growth of *Campylobacter jejuni* ATCC BAA 1153. Bacterial strains were isolated from the intestinal mucosa of broilers and screened *in vitro* against *C. jejuni* ATCC BAA 1153. The cell-free supernatant (CFS) of the selected strain *Ligilactobacillus salivarius* UO.C249 showed potent dose-dependent antimicrobial activity against the pathogen, likely due to the presence of bacteriocin-like moieties, as confirmed by protease treatment. Moreover, genome and proteome analyses revealed the presence of known bacteriocins, including the Abp118 bacteriocin. The genome of *L. salivarius* UO.C249 harbors one megaplasmid with a total length of 203 kb, while the chromosome contained 1.8 Mb. *L. salivarius* UO.C249 was susceptible to several antibiotics, including ampicillin, vancomycin, erythromycin, and chloramphenicol. The strain has a high survival rate in the simulated chicken gastrointestinal tract. The strain showed auto-aggregation percentages of 25 ± 1.4 % and 54 ± 1.7 % after 4 and 8 h of incubation, respectively. Similarly, the coaggregation level with *C. jejuni* was 47 ± 1.6 % after 4 h of incubation. These findings suggest that this bacteriocin-producing strain has potential as a probiotic candidate for reducing *Campylobacter* infections in poultry.

Keywords: poultry, probiotics, *Ligilactobacillus salivarius*, bacteriocinogenic bacteria, antimicrobial activity, bacteriocins, *Campylobacter jejuni*.

1. Introduction

Campylobacter is a major foodborne pathogen causing human gastroenteritis and is commonly transmitted through contaminated food or water (Dewey-Mattia et al., 2018; Inglis et al., 2021). *C. jejuni* can asymptomatically reside in chicken cecum, making poultry a natural reservoir host for the bacteria (Wagle et al., 2020). Controlling the spread of *C. jejuni* in poultry can be challenging while restricting antibiotic use, which endangers the industry's productivity and profitability. Furthermore, the overuse of antibiotics as inhibitory agents for pathogen control in poultry and livestock production has contributed to the development of bacterial resistance (F. Yang et al., 2021), increasing the risk of treatment failure due to the transmission of resistance genes (Yaqoob et al., 2022).

In response to this growing concern, several countries, including Canada and the USA, have banned the use of antibiotics for prophylaxis in poultry farms, prompting the search for innovative alternatives. One promising strategy involves the use of bacteriocinogenic probiotics that inhibit the growth of pathogenic bacteria in poultry. Bacteriocins are antimicrobial peptides produced by certain bacteria that have demonstrated effectiveness against foodborne pathogens (Lone et al., 2022). For example, *L. salivarius* has attracted interest as a potential probiotic for controlling foodborne pathogens (Guerrero Sanchez et al., 2022). Similarly, *L. salivarius,* in combination with *Enterococcus faecium, Pediococcus acidilactici, Bacillus animalis,* and *Limosilactobacillus reuteri,* has been shown to reduce *Salmonella* Enteritidis and *C. jejuni* in the GIT of broilers (Neveling & Dicks, 2021). Bacteriocin-producing bacteria, including *L. salivarius* NRRL B-30514 (producer of bacteriocin OR-7) (Stern et al., 2006), *L. salivarius* NRRL B-50053 (bacteriocin L-1077) (Svetoch et al., 2011), and *L. salivarius* SMXD51 (bacteriocin SMXD51) (Messaoudi et al., 2013) , have been reported to be effective in reducing *C. jejuni* colonization (Messaoudi et al., 2013; Stern et al., 2006; Svetoch et al., 2011). This study aimed to screen and characterize the anti-

C. jejuni potential of novel probiotic candidates isolated from chicken gastrointestinal mucosa and characterize their probiotic potential.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Probiotic strains were isolated from the intestinal mucosa of the cecum of healthy broiler chickens at 3-4 weeks as described previously (Lone, et al., 2021). Fifty samples were randomly selected and cultured in in Man, Rogosa and Sharpe (MRS) broth for 48h at 37 °C under anaerobic conditions. The target pathogenic strain *Campylobacter jejuni* ATCC BAA 1153 was grown under optimal conditions: Mueller Hinton (MH) broth medium for 48h at 42 °C under microaerophilic conditions (O_2 5%, CO_2 10%, N_2) and shaking.

2.2. Anti-*Campylobacter* activity

2.2.1. Agar well-diffusion method

The agar diffusion method was used, as described by (Hammami et al.,2009), MH agar medium was sterilized and then allowed to cool to 45 °C. An overnight culture of *C. jejuni* (250 µL) was seeded with 25 mL of MH agar, poured into a sterile petri dish, and allowed to solidify for 15 min. In the agar plates, wells of 5 mm diameter were prepared and filled with 80 µL of condensed cell -free-supernatant (CFS) (2x, 4x, and 8x folds) using a rotatory evaporator. The plates were tested in duplicates and incubated at 42 °C for 48 h under microaerophilic conditions. Their diameters were then measured.

2.2.2. Critical-dilution method

CFSs of an overnight culture of the selected strains were prepared in MRS medium, obtained by centrifugation at (10,000 ×g) for 5 min at 4 °C, and filtered through a 0.22 µm pore size. The CFSs were fractionated using a Sep-Pak C18 column (Waters, United States) and eluted with 50% acetonitrile to obtain fraction 50 (F50). Antimicrobial activity was evaluated against the

pathogenic strain using 96-well flat-bottomed plates (VWR, Monroeville, PA, USA), as described previously (Hammami et al., 2009). A total of 100 µL from each CFS and its fraction 50 were added to MH broth and used to perform two-fold serial dilutions, which were then planted with 100 µL of 10^6 CFU/mL *C. jejuni* ATCC BAA1153. The plates were incubated for 48 h, and the absorbance at 600 nm was measured every 10 min using a Stratus microplate reader system (Cerillo system, United States). The antimicrobial activity (AU/mL) = 2^{n} (1.000/100) formula was used to calculate arbitrary units per milliliter (AU/mL), where n is the number of wells inhibited.

2.2.3. Inhibition of C. jejuni growth in the presence of the strain UO.C249 or its CFS

The bactericidal activity of the live culture and CFS of UO.C249 against *C. jejuni* was assessed using a killing assay. In brief, equal volumes of CFS of UO.C249 and 10^7 colony-forming units (CFU) of *C. jejuni* in MH broth were co-incubated at 42 °C overnight under microaerophilic conditions. Equal volumes of 10^7 CFU of UO.C249 and 10^7 CFU of *C. jejuni* in (MH + MRS) broth (1:1) were incubated overnight in a microaerophilic environment. The positive control was the *C. Jejuni* culture. Next, 100 mL of each culture was serially diluted and streaked on MH agar. The plates were incubated for 48 h at 42 °C under microaerophilic conditions and the CFU of *C. jejuni* were counted.

2.2.4. Sensitivity to proteolytic enzymes

A proteolytic reaction was performed using the F50 of UO.C249 treated with Proteinase K (1 mg/mL) for 24 h at 37 °C to assess any modifications in its antimicrobial activity. Thereafter, the antimicrobial activity of treated F50 was analyzed against *C. jejuni* (1:1 volume) in a 96-microwell plate using four-fold microdilution under microaerophilic conditions for 48 h at 42 °C (Miri et al., 2023).

2.3. Identification of Isolated Strains by 16S rRNA Sequencing

The selected probiotic candidates were identified at the species level using 16S rRNA, as described by (Lone, Mottawea, Ait Chait, et al., 2021). Genomic DNA was isolated from an overnight culture of each selected strain using the NucleoSpin Microbial DNA kit (MachereyNagel, Duren, Germany) according to the manufacturer's instructions. The DNA concentration was evaluated using a NanoQuant Plate (Tecan Group Ltd. Männedorf, Switzerland). The 16S rRNA gene was amplified using universal primers 1391-R (5′-GACGGGCGGTGTGTR) and Bact-8F (5′-AGAGTTTGATCCTGGCTCAG-3′) (Millipore-Sigma, Cleveland, OH, United States) using a PCR thermal cycler (Eppendorf, Hamburg, Germany). The total volume of each reaction mixture was 50 µl constituted of 10 mM of Tris–HCl (pH 8.8) with 1.5 mM of MgCl2, 200 mM of dNTP (A, T, C, and g), 0.2 mM of each forward and reverse primers, 10 ng of bacterial DNA, and 5µl of Taq DNA polymerase (0.12 U). The thermal cycling program consisted of an initial cycle of denaturation and polymerase activation at 94 °C for 5 min, followed by 35 cycles of 94°C for 45 s, 54°C for 45 s, and 68°C for 60 s, and a final extension step at 68 °C for 5 min. PCR products were validated by detecting single bands following electrophoresis (60 V for 20 min) on 1,5 % agarose gels (Sigma, Oakville, ON, Canada) in 1X TAE buffer, and visualized by ethidium bromide staining using a gel imaging system (Life Science Research, Bio-Rad Laboratories). The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced at the DNA sequencing facility of the Ottawa Hospital Research Institute (Ottawa, ON, Canada). The resulting sequence data were aligned and analyzed using the Basic Local Alignment Search Tool (BLAST) on the NCBI website.

2.4. Evaluation of probiotic properties

2.4.1. Antibiotic Susceptibility Test

Antibiotic sensitivity tests were performed as previously described (Lone, Mottawea, Ait Chait, et al., 2021). Different classes of antibiotics were tested against the selected probiotic candidate, UO.C249, including ampicillin, vancomycin, gentamycin, streptomycin, erythromycin, tetracycline, and chloramphenicol (all antibiotics were obtained from Alfa Aesar, Mississauga, Canada), except gentamycin, which was purchased from VWR (New York, USA). One hundred microliters of each selected antibiotic (1 mg/mL) were added to 100 µL of MRS medium, followed by serial dilution in a 96-well flat-bottomed microplate. The wells were inoculated with 100 µL of 10^5 CFU/mL of the probiotic strain. The test was performed in triplicate. The absorbance of each well was measured at 600 nm after 24 h of incubation at 37 °C using a Tecan Microplate Reader Spark® (Grödig, Austria). The lowest observed concentration of the test antibiotic determined the minimum inhibitory concentration (MIC) for microorganism growth.

2.4.2. Whole Genome Sequencing of selected probiotic candidate UO.C249

The library was prepared for whole-genome sequencing using Nextera™ DNA Flex Library Prep (Illumina, San Diego, CA, USA). Sequencing of the library was performed as follows (2 × 151 bp) in a 1/20 MiSeq run on the Illumina MiSeq platform (N*u*GUT Research Platform, University of Ottawa, Ottawa, ON, Canada) using a 300-bp MiSeq Reagent Kit v2 (Illumina, San Diego, CA, USA). The quality of the produced reads was verified using FastQC (v. 0.11.9). The sequences were assembled using SPAdes-based assembler unicycler (v. 0.5.0) (Wick, 2016). The assembly process was assessed using the Quast tool (Mikheenko et al., 2018), and the MOB-suite tool (v.3.0.3) was used to predict the plasmid among the draft assemblies (Robertson & Nash, 2018). The assembled contigs were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (V. 6.4.) and visualized using the Proksee server (https://proksee.ca/). Secondary

metabolite genes in the assembled genomes were screened using BAGEL4 and antiSMASH (v.7.0.0 beta) (Lone, Mottawea, Ait Chait, et al., 2021). The assembled contigs were used to recognize and annotate prophage sequences using the PHAge Search Tool Enhanced Release (PHASTER).

2.4.3. Proteomic profiling

CFS of strain UO.C249 was condensed using 4 µL of 100 mM tris (2-carboxyethyl) phosphine solution and incubated for 55 min at 25 °C with shaking at 450 rpm. The sample was then alkylated using 4 µL of 500 mM iodoacetamide in H_2O and incubated for 55 min at 25 °C with shaking at 450 rpm. Proteins were digested using 1.5 L of a 0.3 g/L trypsin/Lys-C solution (Trypsin/Lys-C Mix, Promega V5072) and incubated for approximately 20 h at room temperature. The samples were mixed with 2 µL 100% formic acid, vortexed, and centrifuged at 10,000 ×g for 30 s. The samples were desalted using C18 TopTips columns from Glygen in Columbia, Maryland, and vacuum-dried, according to the manufacturer's instructions. Approximately 5 g of protein from each sample was examined using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) linked to an UltiMate 3000 nanoRSLC (Dionex, Thermo Fisher Scientific) as previously described (Miri et al., 2023). MaxQuant software was used to examine the raw MS files (Cox & Mann, 2008). *L. salivarius* (Taxon ID:1624) peptides were imported from the UniProt database into MaxQuant. Trypsin and LysC proteases were chosen as the digestive enzymes to allow for a maximum of two missed cleavages. Quantitative label-free MaxQuant analysis was used to provide values for label-free protein quantification (LFQ).

2.4.4. Determination of SCFAs production

The production of SCFAs by strain UO.C249 was determined using Gas Chromatography coupled to a Flame Ionization Detector (Miri et al., 2023). Briefly, the supernatant collected from the overnight culture was centrifuged (14,000 × g, 10 min, 4°C), filtered through 0,22 µm pore size,

mixed with the internal standard (2-ethyl butyric acid, 0.5 mM), centrifuged (30000 × g, 30 min, 4°C), and filtered again. Standard solutions of acetic, propionic, butyric, butyric, isovaleric, valeric, ethyl butyric, isocaproic, hexanoic, and n-heptanoic acids were used for identification and quantification.

2.4.5. Auto-aggregation assay

The ability of probiotics to auto-aggregate was assessed as described by (Chen et al., 2020) with some modifications. Briefly, overnight cultures of strains UO.C249 and *Lacticaseibacillus rhamnosus* GG (used as a reference strain) were centrifuged for 10 min at 14,000 × g, and the resulting pellet was collected. Bacteria were washed twice and resuspended in phosphate buffered saline (pH = 7.4). The final optical density of the cell suspension was adjusted to 0.6 at an absorbance of 600. Experiments were performed in triplicate, and data were collected after 4 and 8 h of incubation at 37°C. The percentage of auto-aggregation was determined as follows: A_t is the absorbance at 4 and 8 h, and A_0 is the initial absorbance: auto-aggregation (%) = $(A_0-A_t)/(A_0)$ ×100%.

2.4.6. Coaggregation assay with the pathogenic strain

Coaggregation was determined by mixing equal volumes of selected strains UO.C249 and *Lacticaseibacillus rhamnosus* GG resuspended in PBS and *C. jejuni,* followed by optical density measurements at 0 min and 4 h of incubation. The formula used for the calculation of co-aggregation= $(((A_{prob}+ A_{path})/2-A_{mix})/ A_{prob}+ A_{path}) \times 100$, where A_{prob} represents the absorbance of the probiotic alone, A_{path} represents the absorbance of the pathogen alone, and A_{mix} represents the absorbance of the mixture of pathogen and probiotic strains (Collado et al., 2008).

2.4.7. Survival under gastrointestinal conditions

The survival of the probiotic strain UO.C249 (10^8 CFU/mL) in the simulated broiler GIT was assessed as previously described (Lone, Mottawea, Ait Chait, et al., 2021; X. Yang et al., 2014).

The physiological pH and duration of the corpus (pH 5.4, 30 min), gizzard (pH 2.8, 45 min), and small intestine (pH 6.1, 60 min) were reproduced using simulated gastric juice. SGJ consisted of 2 g L−1 pepsin (Wards Science, St Catherines, Canada) and 2 g L−1 NaCl at pH 1.5. After pH adjustment of the simulated stomach fluid, it was sterilized using a 0.45 m filter, and divided into 50 mL aliquots. Each tube was inoculated with a single strain at a concentration of 10^7 CFU/ml. The drop plate method was used to enumerate samples at 0, 30, 45, and 60 min. The assays were performed in triplicate.

2.5. Statistics

At least three biological replicates and two technical replicates were conducted for each experiment. Statistical analysis was performed using one-way ANOVA for multiples mean comparison using GraphPad Prism 8. Statistical significance was set at $P < 0.05$.

3. Results and discussion

3.1. Characterization of the inhibitory activity of the probiotic strains

Of 33 selected strains isolated from four intestinal tracts, 15 exhibited inhibitory activity against *C. jejuni* (>40 AU/mL). As shown in **(Table 3.1)**, these isolates were identified as *Ligilactobacillus salivarius*. On agar plates, cell-free supernatant (CFS) obtained from *L. salivarius* UO.C249 concentrated at 4× and 8× exhibited large inhibitory zones against *C. jejuni* (Ø = 28 and 33 mm), as illustrated in **(Figure 3.1)**. In the presence of *L. salivarius* UO.C249 cells, the counts of *C. jejuni* were significantly reduced compared to the control **(Figure 3.2A)**. In addition, CFS diluted to 1/2 and 1/4 of *L. salivarius* UO.C249 completely inhibited the growth of *C. jejuni* **(Figure 3.2B)** after 48h of incubation, suggesting the bactericidal nature of the produced compounds. *Lactobacillus* species are generally recognized as safe (GRAS) probiotic strains that have been shown to possess antimicrobial activity against various foodborne bacterial pathogens, including *C. jejuni* (Taha-

Abdelaziz et al., 2019). These strains have been reported to produce various bactericidal molecules, including organic acids, hydrogen peroxide, and bacteriocins.

To assess presence of potential antimicrobial proteinaceous moieties, CFSs obtained from the selected bioactive strains of *L. salivarius* were fractionated using 50% acetonitrile on a C18 chromatography column and referred to as fraction F50. The obtained fraction F50 from the *L. salivarius* UO.C238, UO.C248, and UO.C249 strains exhibited the highest antimicrobial activity against the target microorganism. The residual inhibitory activity of these F50s was evaluated after proteinase K treatment **(Table 3.2)**. As shown in **(Figure 3.3)**, *L. salivarius* UO.C249 exhibited a significant decrease in antimicrobial activity after enzymatic digestion; therefore, *L. salivarius* UO.C249 was sensitive to protease treatment, suggesting the proteinaceous nature of the observed anti-*Campylobacter* activity. Previously, a bacteriocin-like compound produced by *L. salivarius* SMXD51, isolated from chicken cecum, was extremely effective against foodborne *C. jejuni* (Messaoudi et al., 2011). Likewise, *L. salivarius* NRRL B-30514, an isolate originating from the cecum of a commercial broiler (Stern et al., 2006), produced bacteriocin (OR-7) with antibacterial activity against Gram-negative *C. jejuni*.

3.2. Probiotic Potential of *Ligilactobacillus salivarius* UO.C249

Before considering a microorganism for use as a probiotic, it must undergo a battery of tests to assess its suitability and effectiveness. These tests encompass safety evaluations, assessments of survivability within the digestive tract, studies on adherence and colonization, and analyses of its biological effects.

3.2.1. Sensitivity to Antibiotics

The sensitivity of *L. salivarius* UO.C249 to different types of antibiotics is presented in **(Table 3.3)**. *L. salivarius* UO.C249 showed a minimum inhibitory concentration (MIC) of <0.5 μg/mL for ampicillin, vancomycin, erythromycin, and chloramphenicol, indicating the susceptibility of

the strain to the tested antibiotics. In addition, *L. salivarius* UO.C249 was sensitive to gentamycin (MIC = 8 μg/mL) while being resistant to streptomycin (MIC = 128 μg/mL) and cloxacillin (MIC = 16 μg/mL). Overall, the results indicate that the *L. salivarius* UO.C249 strain is highly susceptible to antibiotics. Generally, *L. salivarius* is recognized as a safe bacterium, but numerous strains have been shown to be resistant to antibiotics such as vancomycin, gentamycin, kanamycin, streptomycin, and ciprofloxacin, but sensitive to penicillin and β-lactams (Campedelli et al., 2019).

3.2.2. Whole-Genome, proteome, and metabolome profiling

The generated reads of the probiotic candidate *L. salivarius* UO.C249 were assigned to the genus *Lactobacillus*; the genomic information is summarized in **(Table 3.5)**. A total of 432,254 reads were assembled *de novo*, and the resulting draft genome (including chromosome and plasmids) consisted of 2,035,382 bp, with a GC ratio of 32.72%. Plasmid prediction revealed that the genome of *L. salivarius* UO.C249 harbors one megaplasmid with a total length of 203,737 bp, while the chromosome contained 1,825,924 bp. The megaplasmid, at 203 kb, constitutes 10% of the *L. salivarius* UO.C249 genome, assuming that its chromosome is approximately 2.0 Mb. The annotation results indicated that the chromosome and plasmid harbor 1,793 and 201 genes, respectively, as summarized in **(Table 3.6)**. Notably, five genes encoding bacteriocin-related genes were identified in the sequences of the detected plasmid (Salivaricin B, Bacteriocin-like prepeptide Abp118 bacteriocin alpha, Abp118 bacteriocin beta, and AbpIP induction peptide) based on the PGAP, antiSMASH, BAGEL4, and UniProt BLAST tools, as summarized in **(Table 3.4)** and **(Figure 3.4)**. A previous report on the genome of *L. salivarius* UCC118 revealed the presence of a circular chromosome of 1.8 MB, complemented by a megaplasmid pMP118, whose size 242 kb, on which the genetic determinants for abp118 are located (O'Shea et al., 2011). The production of the Abp118 is megaplasmid encoded, and bacteriocin production might be a competitive advantage for commensal organisms (Y. Li et al., 2007). L-rhamnose is a deoxy-hexose sugar commonly

found in plants as a part of complex pectin polysaccharides that serve as a potential carbon source for plant-associated bacteria (Y. Li et al., 2007). Rhamnose-fermenting ability was previously considered to be a criterion to distinguish *L. salivarius* subsp. *salivarius* from *L. salivarius* subsp. *salicinius*, and the pathway was reported to be present in the megaplasmid of *L. salivarius* UCC118 (Y. Li et al., 2007). The rhamnose fermentation pathway in *L. salivarius* UO.C249 megaplasmid involves rhamnulose-1-phosphate aldolase (rhaD, UO.C249_000138), L-rhamnose isomerase (UO.C249_000139), L-rhamnose mutarotase (rhaM, UO.C249_000140), and rhamnulokinase (rhaB, UO.C249_000141). L-rhamnose mutarotase has not been previously reported in UCC118 (Y. Li et al., 2007), which facilitates the interconversion of the α and β anomers of l-rhamnopyranose, enhancing the rate of rhamnose catabolism. We were unable to find the genes *repE* and *parA* in our plasmid UO.C249, unlike multiple reported *L. salivarius* strains, which have *repA*-type megaplasmids (Y. Li et al., 2007). However, further validation is required to determine the nature of this plasmid.

Besides, proteome analysis of CFS from *L. salivarius* UO.C249 revealed a total of 272 proteins. Proteomic profiling after annotation with the UniProt database showed the presence of distinctive proteins in CFS from *L. salivarius* UO.C249. For instance, the Abp118 bacteriocin alpha peptide has been detected in the UO.C249 proteome. Previous studies have detected Abp118 bacteriocin in *L. salivarius* (O'Shea et al., 2011). For instance, *L. salivarius* UCC118, isolated from the human cecum, produces Abp118 and inhibits *Listeria monocytogenes*. In Addition, *L. salivarius* UO.C249, an ATP-dependent protease secreted in its CFS, was found to be highly expressed in *Lactobacillu*s strains under different environmental conditions (e.g., heat, acid, ethanol) (De Angelis et al., 2016). Proteases have been assumed to control the expression of virulence genes by destroying transcriptional regulators (Butler et al., 2006).

Moreover, the SCFAs production capacity of the probiotic candidate *L. salivarius* UO.C249 was quantified in CFS. As shown in **(Figure 3.5)**, the significant SCFA found in the CFS was acetic acid (24.4 ± 1.2 mM) followed by butyric acid (0.03 mM). In a previous study by Deun et al., butyrate was the most inhibitory SCFA against *C. jejuni*, with 12.5 mM being bactericidal (Van Deun et al., 2008), but unlikely to be active at the concentration produced by *L. salivarius* UO.C249. The mixture of SCFAs (butyrate, acetate, and propionate) was reported to stimulate the expression of the acetogenesis-dependent genes in *C. jejuni*, supporting their potential to attenuate pathogenesis (Luethy et al., 2017).

3.2.3. Survival under gastrointestinal conditions

The tolerance to acidic environment is a significant property for any potential probiotic bacteria. The ability of *L. salivarius* UO.C249 to survive transit in the corpus (pH 5.4, 30 min), stomach (pH 2.8, 45 min), and small intestine (pH 6.1, 60 min) is presented in **(Figure 3.6)**. Despite a slight decrease in cell concentration after 30 min of incubation under crop conditions, *L. salivarius* UO.C249 had a high survival rate with a respective range of 80 % under gizzard, similar to the report by Lone et al. (2021) with a respective range of 76.80-93.98%. Likewise, a recent study from our group reported similar results for *L. salivarius*, which was able to survive after incubation at an acidic pH (2.0) for one hour (Miri et al., 2023).

3.2.4. Auto- and Coaggregation Capacity

The auto-aggregation of a strain is a measure that assesses its capacity to produce the exopolysaccharides, adhere and protect to the epithelial layer against the attachment of pathogenic strains , a desirable probiotic feature. As shown in **(Figure 3.7A)**, the auto-aggregation percentages of the probiotic candidate *L. salivarius* UO.C249 were 25 ± 1.4 % and 54 ± 1.7 % after 4 and 8 h of incubation, respectively. *Lacticaseibacillus rhamnosus* GG, a reference probiotic strain, was used as a positive control and had different indices of 34 ± 1.5 % and 68 ± 1.8 % after 4 and 8 h

of incubation, respectively. Our results concur with those of a recent report by Leska et al. (2022) who noted an auto-aggregation rate of 30-53 % for *Ligilactobacillus* isolates (Leska et al., 2022). In addition, co-aggregation of probiotics with pathogen strains plays a role in establishing a barrier that prevents the attachment of pathogenic bacteria in the intestinal epithelial lining and its exclusion to the lumen.

In this study, *Lactobacillus* strain*s* were evaluated for their ability to co-aggregate with *C. jejuni*. A similar coaggregation level was found for *L. salivarius* UO.C249 (47 ± 1.6 %) and *Lacticaseibacillus rhamnosus* GG (46 ± 1.67 %) with *C. jejuni* as positive control and reference strain after 4 h incubation **(Figure 3.7B)**. The level of aggregation of *L. salivarius* UO.C249 with *C. jejuni* was higher than that previously reported for *Lactobacillus acidophilus* W37 (4.33%), *Lacticaseibacillus paracasei* W56 (13.94%), and *Lactobacillus lactis* W58 (3.42%) (Campana et al., 2017).

4. Conclusion

This study aimed to isolate bacterial strains with inhibitory activity against *C. jejuni,* a major cause of human foodborne illness, from the cecal mucosa of broiler chickens and to characterize their probiotic potential. Of these 33 strains, 15 exhibited antimicrobial activity against *C. jejuni* and were identified as *Ligilactobacillus salivarius*. The most active strain, *L. salivarius* UO.C249, produced bacteriocin, was sensitive to most tested antibiotics, and survived gastrointestinal physicochemical conditions. *L. salivarius* UO.C249 genome was shown to encode bacteriocin genes, including Abp118 bacteriocin, as detected in the megaplasmid sequence and confirmed by proteomic analysis. These findings suggest that bacteriocinogenic probiotics from the chicken gut mucosa could be an effective strategy to control *C. jejuni* in the poultry industry.

Author contributions

M.C. and R.H. conceived and designed the study. M.C. and B.Y acquired the data. M.C., W.M., B.Y., S.M., and G.E. analyzed, and interpreted the data. M.C., S.M., and R.H. drafted or revised the article.

The experiments presented in the manuscript were carried out under my sole responsibility:

- Critical-dilution method.
- Agar well-diffusion method.
- Sensitivity to proteolytic enzymes.
- Inhibition of *C. jejuni* growth in the presence of CFS of the strain UO.C249 (Co-culture).
- Antibiotic Susceptibility Test.
- Determination of SCFAs production.
- Survival under gastrointestinal conditions.
- Auto-aggregation assay.
- Coaggregation assay with the pathogenic strain.

Contribution the other authors have made in terms of experiments:

- Inhibition of *C. jejuni* growth in the presence of the strain UO.C249 and identification of isolated strains by 16S rRNA Sequencing (Mariem Chiba et Basit Yousuf).
- Whole genome sequencing (Basit Yousuf, Galal Ali Farea Esmail and Walid Mottawea).
- Proteomics (Saba Miri).

Table 3.1. Anti-*Campylobacter* activity of cell-free supernatant (CFS) and their respective fraction 50 (AU/mL) on *Campylobacter jejuni* ATCC BAA 1153 using microplate method.

Sample Id	Strain identity		
		CFS	F50
UO.C200	*L. salivarius*	40	20
UO.C201	NI	20	20
UO.C202	NI	20	20
UO.C203	NI	>10 *****	10
UO.C204	*L. salivarius*	40	20
UO.C205	NI	20	20
UO.C206	NI	>10 *****	10
UO.C207	*L. salivarius*	40	20
UO.C208	*L. salivarius*	40	20
UO.C209	NI	20	>10 *****
UO.C210	*L. salivarius*	40	20
UO.C211	NI	>10 *****	>10 *****
UO.C212	*L. salivarius*	40	20
UO.C213	NI	20	>10 *****
UO.C214	*L. salivarius*	40	20
UO.C215	NI	10	10
UO.C216	NI	10	10
UO.C217	NI	10	10
UO.C218	NI	10	10
UO.C219	NI	10	10
UO.C220	NI	10	10
UO.C221	*L. salivarius*	40	20
UO.C222	*L. salivarius*	40	20
UO.C223	NI	20	>10 *****
UO.C224	NI	20	20
UO.C225	*L. salivarius*	40	20
UO.C226	*L. salivarius*	40	20
UO.C227	NI	20	20
UO.C228	*L. salivarius*	40	20
UO.C229	NI	20	>10 *****
UO.C238	***L. salivarius***	**>10 ***	**40**
UO.C248	***L. salivarius***	**40**	**40**
UO.C249	***L. salivarius***	**40**	**40**

*: **AU <20**
The assay was performed in triplicates.

Table 3.2. Residual antimicrobial activity of fraction 50 (AU/mL) of the most potent strains against *Campylobacter jejuni* ATCC BAA 1153 after proteinase K treatment using a microplate method.

Strains	F50 (CTRL¯)	F50 (+K)
	AU/mL	AU/mL
L. salivarius UO.C238	40	40
L. salivarius UO.C248	40	40
L. salivarius **UO.C249**	40	20

The assay was performed in triplicates.

Table 3.3. Sensitivity of *Ligilactobacillus salivarius* UO.C249 to the tested antibiotics.

		***L. salivarius* UO.C249**	
Antibiotics	**Breakpoints**	MIC (μg/mL)	Susceptibility
Ampicillin	4	<0.5	S
Vancomycin	n.r	<0.5	S
Gentamycin	16	8	S
Streptomycin	64	128	R
Erythromycin	1	<0.5	S
Chloramphenicol	4	<0.5	S
Cloxacillin	0.5≥	16	R

MIC: Minimal Inhibitory Concentrations. Breakpoints: Reference MIC for wild type strain as determinant by Clinical Laboratory Standard Institute (CLSI) (Laulund et al. 2017). The assay was performed in triplicates.

Table 3.4. Identification of the bacteriocin-related genes harbored by *Ligilactobacillus salivarius* UO.C249 plasmid.

Genes	Gene translation	NCBI PGAP	UniProt	antiSMASH	BAGEL4	Manual validation
Gene (1)	MNNNFIQVDKKELAHIIGGRNSYDYIDSGKFGYDI GCTIANTKFFKRLRHSNQNICS	Bacteriocin	Salvaricin B	ND*	Salivaricin	Nonfunctional salvaricin B
Gene (2)	MLKKLWETWLDGGFIRGKKRYVIAPVIWALLIPL GIWLFGNEEMSYLDYIQTPKMIIVTIFCLVGGSTLL YLLDTAMKVNKHMKG	Bacteriocin	Bacteriocin-like prepeptide	ND	ND	Bacteriocin-like prepeptide
Gene (3)	MMKEFTVLTECELAKVDGGKRGPNCVGNFLGGL FAGAAAGVPLGPAGIVGGANLGMVGGALTCL	Blp family class II bacteriocin	Abp118 bacteriocin alpha peptide	Salivaricin CRL1328 α peptide	**Salivaricin**	Salivaricin CRL1328 α
Gene (4)	MKNLDKRFTIMTEDNLASVNGGKNGYGGSGNRW VHCGAGIVGGALIGAIGGPWSAVAGGISGGFTSCR	Blp family class II bacteriocin	Abp118 bacteriocin beta peptide	Salivaricin CRL1328 β peptide	ND	Salivaricin CRL1328 β
Gene (5)	MKFEVLTEKKLQKIAGGATKKGGFKRWQCIFTFF GVCK	Bacteriocin	AbpIP induction peptide	ND	Planosporicin	salivaricin induction peptide

Table 3.5. *De novo* assembly of the sequences of the strain *Ligilactobacillus salivarius* UO.C249 using SPAdes-based unicycler assembler.

Strain	16S rRNA Identity	Genome Size (bp)	GC ratio	Contigs	Largest contig (bp)	N50	L50	Closest Neighbour
UO.C249	*L. salivarius*	2,054,375	37%	90	195	62293	10	*L. salivarius*

Table 3.6. Whole genome draft sequence overview annotated by NCBI Prokaryotic genome annotation pipeline (PGAP).

Bacterial Identifier	**UO.C249**	
Organism identity	*Ligilactobacillus salivarius*	
Genomic Feature	Chromosome	Plasmid
Genes (Total)	1,793	201
CDSs (total)	1,745	200
Genes (coding)	1,725	192
CDSs (with protein)	1,725	192
Pseudo Genes	20	8
RNAs	48	1
CRISPR Arrays	3	ND

ND: Not Detected

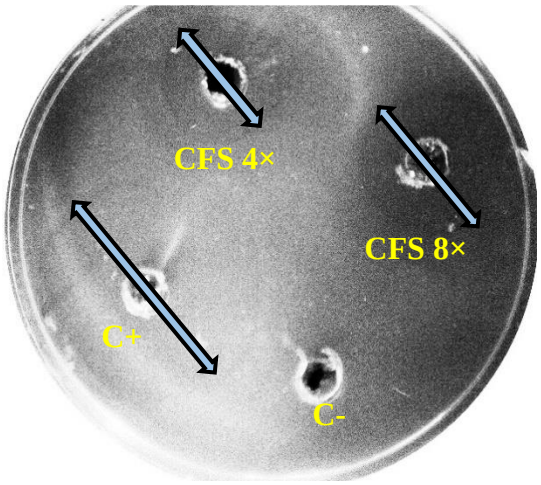

Figure 3.1. Inhibition of *Campylobacter jejuni* ATCC BAA1153 by cell-free-supernatant (CFS) recovered from *Ligilactobacillus salivarius* UO.C249 using agar well diffusion method. CFS was tested at 4× and 8× fold concentration. Sterile water was used as negative control C $^{-}$, and Ampicillin was used as positive control. The assay was performed in duplicates.

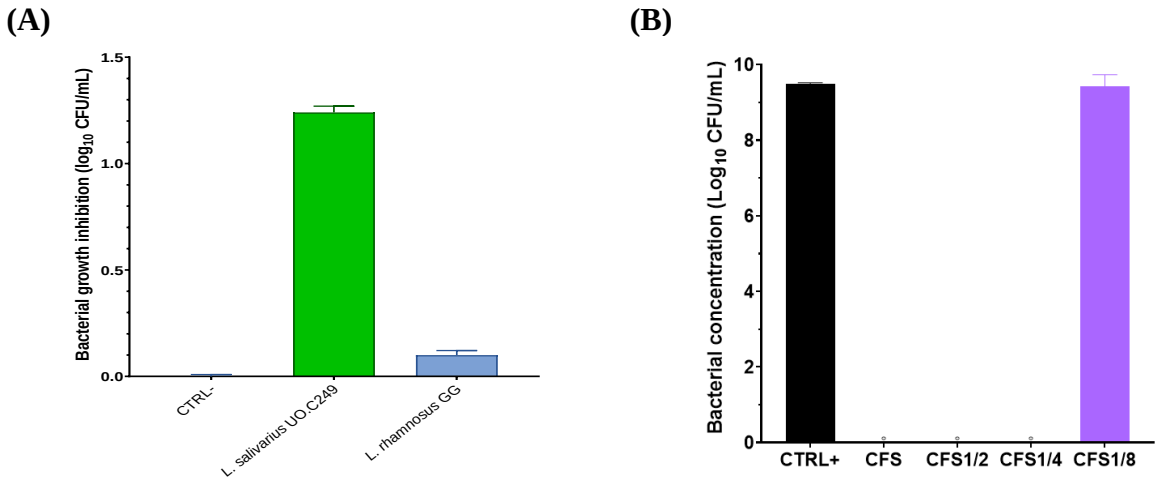

Figure 3.2. Antimicrobial effect of *Ligilactobacillus salivarius* UO.C249(**A**) and its CFS (**B**) at different dilutions (non-diluted, 1/2, 1/4, and 1/8) against *Campylobacter jejuni* ATCC BAA 1153 after 48 h incubation, as determined by colony-counting methods. The assay was performed in triplicates.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

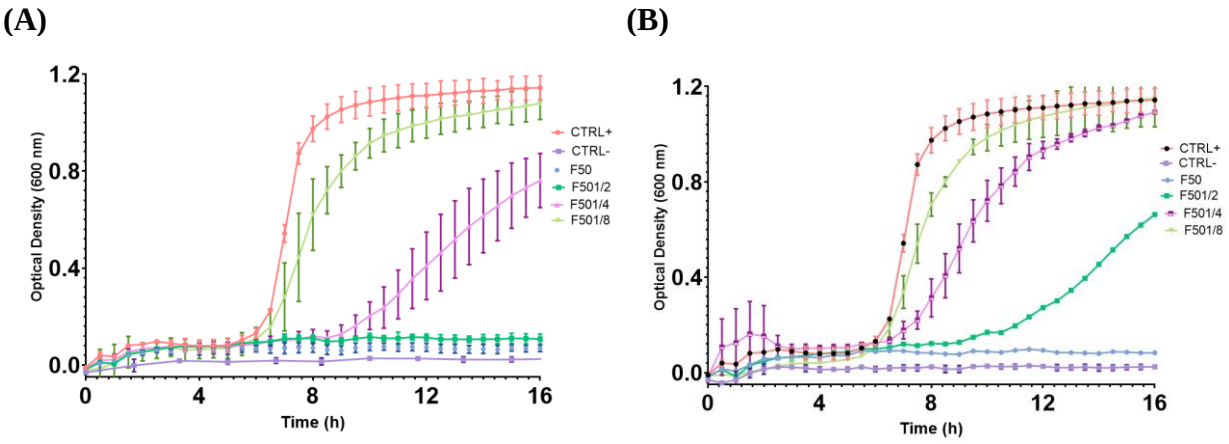

Figure 3.3. Dose-response growth inhibition of *Campylobacter jejuni* ATCC BAA 1153 by fraction F50 obtained from *Ligilactobacillus salivarius* UO.C249 in the absence (A) or presence (B) of proteolytic enzymes. The assay was performed in triplicates.

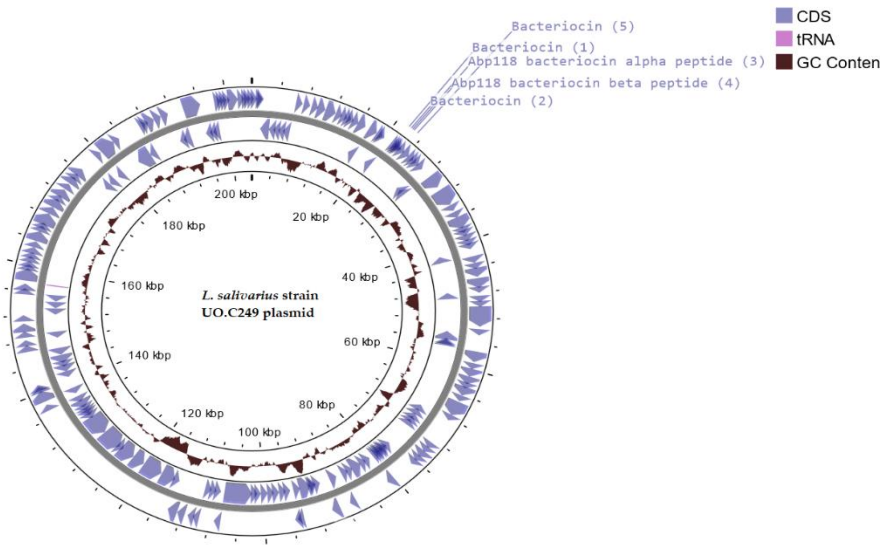

Figure 3.4. Visual presentation of the identified plasmid in the genome of *Ligilactobacillus salivarius* UO.C249, including the detected putative bacteriocin genes. The outer tracks in light blue indicate CDS genes on the forward and complement strands, the track in dark purple refers to the GC ratio, and the inner circle is the plasmid size. Figure illustrates the location of five bacteriocin-related genes located between 21634 and 23163 pb of the plasmid sequence.

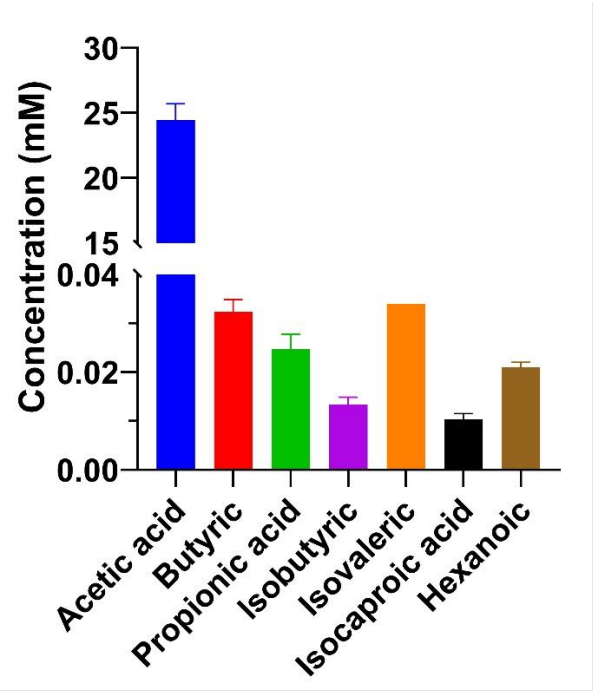

Figure 3.5. Quantification of short-chain fatty acid production in CFS produced by *Ligilactobacillus salivarius* UO.C249.

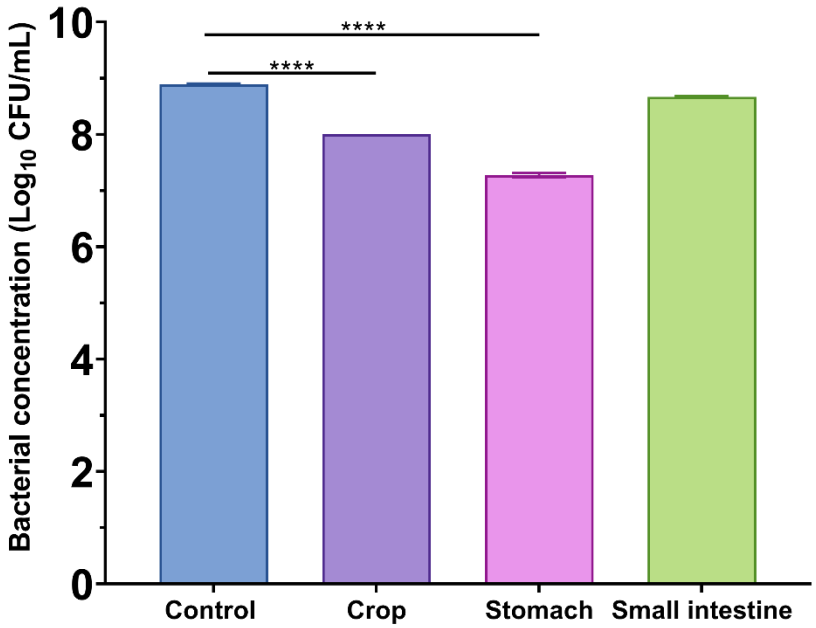

Figure 3.6. Survival of *Ligilactobacillus salivarius* UO.C249 under simulated chicken gastrointestinal physicochemical conditions. The assay was performed in triplicates.

**P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001*

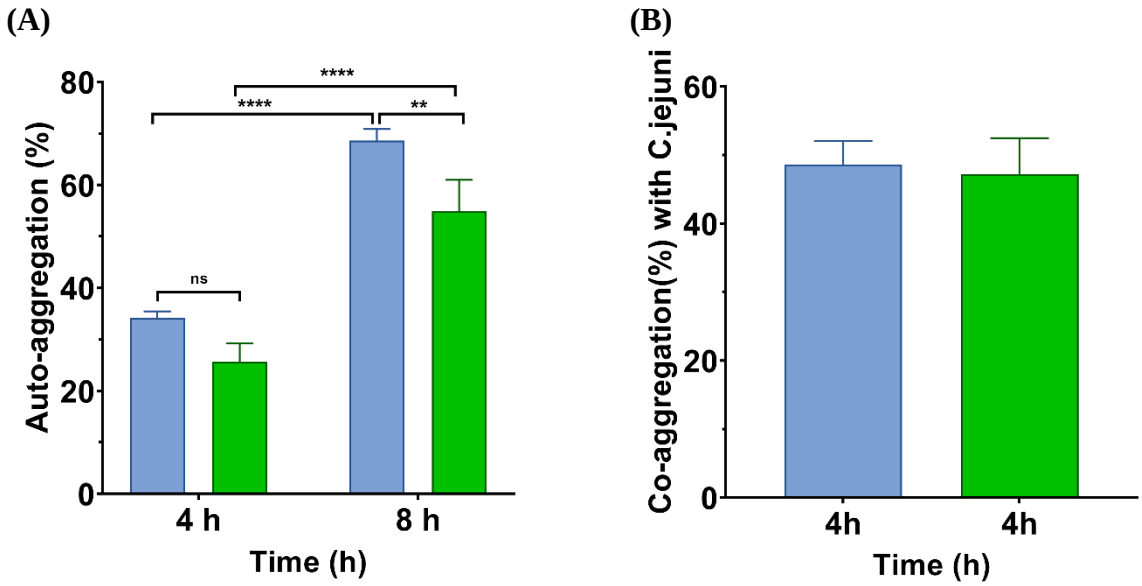

Figure 3.7. Determination of the auto-aggregation (A) and coaggregation (B) capacity of *Ligilactobacillus salivarius* UO.C249 (green) *and Lacticaseibacillus rhamnosus* GG (blue) with *Campylobacter jejuni* ATCC BAA 1153 after 4 and 8 h of incubation. The assay was performed in triplicates. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$

Chapter 4: General Discussion and Conclusion

Probiotics are at the forefront to be excellent candidates for efficient antibiotic substitutes to prevent and control *C. jejuni* colonization in broiler chickens (Lone et al., 2022). The present study aimed to characterize the antimicrobial activity and evaluate the probiotic properties of novel strains isolated from the chicken gut mucosa. Our results revealed that the CFSs of isolated *Ligilactobacillus salivarius* (15/33) exhibited antimicrobial activity against the target pathogen. Lactobacilli were previously reported to exhibit antimicrobial activity through the production of bacteriocins, organic acids, and hydrogen peroxide (Micciche et al., 2018). Following exposure of *L. salivarius* UO.C249 CFS to the proteolytic enzyme, a notable decrease in the inhibitory activity was detected, suggesting the proteinaceous nature of the observed anti-*Campylobacter* activity. Therefore, *L. salivarius* UO.C249 was selected for its potential use as a bacteriocinogenic probiotic because of its high inhibitory activity and sensitivity to proteolytic enzymes. Whole-genome sequencing of the probiotic candidate *L. salivarius* UO.C249 and proteomic analysis of its CFS revealed the presence of plasmid-encoded Abp118 bacteriocin. *Ligilactobacillus* strains, such as *L. salivarius,* are predominantly associated with the gut mucosa rather than the lumen content because of their high adaptation to this particular niche through adhesion and CE mechanisms (Adhikari & Kwon, 2017). Therefore, these lactobacilli possess inherent advantages beyond their GRAS status and possess a head start in exhibiting potential probiotic properties (Adhikari & Kwon, 2017).

Previous studies have detected Abp118 bacteriocin in *L. salivarius,* and multiple *L. salivarius* strains obtained from various sources produce bacteriocins (Barrett et al., 2012; Messaoudi et al., 2011; O'Shea et al., 2011; Vera Pingitore et al., 2009). For instance, *L. salivarius*

UCC118, a strain isolated from the ileal region of the human GIT, produces Abp118, a small heat-stable bacteriocin that is active against the foodborne pathogen *L. monocytogenes*. *L. salivarius* CRL 1328, an isolate from a healthy human vagina, produces the bacteriocin salivaricin CRL 1328 (Vera Pingitore et al., 2009). Likewise, *L. salivarius* DPC6502 from a porcine intestinal probiotic produces a two-component bacteriocin salivaricin P (Barrett et al., 2012). *L. salivarius* NRRL B-30514, an isolate originating from the cecum of a commercial broiler (Stern et al., 2006), produces bacteriocin OR-7, which has antibacterial activity against *C. jejuni*. A bacteriocin-like compound produced by *L. salivarius* SMXD51, previously isolated from chicken cecum, was extremely effective against *C. jejuni* (Messaoudi et al., 2011). Our results showed that *L. salivarius* UO.C249, isolated from chicken intestinal mucosa, produced Abp118 bacteriocin and showed *in vitro* activity against *C. jejuni*, implying that Abp118 could be responsible for the observed inhibition.

Lactobacillus is a non-pathogenic organism that is generally recognized as a safe bacterium. Our probiotic candidate, *L. salivarius* UO.C249, demonstrated susceptibility to ampicillin, vancomycin, erythromycin, and chloramphenicol, but resistance to gentamycin, streptomycin, and cloxacillin. Several *Lactobacillus* species have been described as resistant to antibiotics such as vancomycin, gentamycin, kanamycin, streptomycin, and ciprofloxacin, but sensitive to penicillin and β-lactams (Campedelli et al., 2019). During the selection of a probiotic candidate, tolerance to the acidic environment is a major factor that affects the survival of probiotic bacterial strains in the gastrointestinal tract (Lone et al., 2022). Our results indicated that the bacteriocinogenic strain *L. salivarius* UO.C249 exhibited a high survival rate in the stomach and small intestine of chickens, indicating that they can survive physiological pH conditions, in accordance with the study described by Miri et al. (2023), making them useful as probiotics in

chicken feed. In this study, we found a relative value of 54% for autoaggregation with *L. salivarius* UO.C249. These results were in accordance with those reported by Leska et al. (Leska et al., 2022), who observed 30-53% autoaggregation of *Lactobacillus* isolates. Co-aggregation is another desirable feature of probiotics and may be crucial in the gastrointestinal system by reducing pathogen adherence to the host tissue. The ability of *Lactobacillus* to inhibit the attachment of *C. jejuni* to epithelial cells has been reported in several studies (Campana et al., 2017; Tareb et al., 2013). In this study, the co-aggregation of *L. salivarius* UO.C249 with *C. jejuni* was higher than that previously observed for *L. acidophilus* W37 (4.33%), *Lactobacillus paracasei* W56 (13.94%), and *Lactobacillus lactis* W58 (3.42%) (Campana et al., 2017). An essential step in disease development is the adherence of pathogenic intestinal bacteria to intestinal epithelial cells. Pathogenic bacteria attach to the adhesion sites of intestinal epithelial cells, create tight junctions, multiply, and produce enzymes and toxins that harm the host. Additionally, they produce a thick biofilm of harmful bacteria, which increases damage to host cells (Jamal et al., 2018). The findings of this study revealed a novel bacteriocinogenic probiotic strain isolated from chicken mucosa that could inhibit the growth of *C. jejuni*. We identified the bacteriocinogenic strain *Ligilactobacillus salivarius* UO.C249 with highly effective cell-free supernatants against *C. jejuni*.

Our aim was to identify bacteriocin-producing bacterial strains obtained from the mucosa of the gastrointestinal tract of broiler chickens and to determine their probiotic characteristics, both have been achieved. We identified multiple strains, including a bacterium with inhibitory activity against *C. jejuni* (ATCC BAA 1153). Our selected strain showed potential probiotic properties and the ability to tolerate acidic pH conditions. Our antibiotic susceptibility test results correlate with the expected results for lactic acid bacteria, such as the inherent resistance of lactobacilli to

vancomycin, gentamycin, and erythromycin. Finally, our strains contained genes involved in bacteriocin production. Additional investigation of this strain is necessary before its potential application in the poultry industry. For example, *L. salivarius* UO.C249 could be studied for its efficacy against *Campylobacter ex vivo* in the presence of the chicken microbiome and then assessed *in vivo*. Finally, our project exhibits different disciplines in the way it is rooted in the concepts and methods of molecular biology and microbiology. It contains aspects of bioinformatics and adds to the literature on alternative strategies to control *Campylobacter* and the applications of probiotic strains in poultry.

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