
PhD THESIS

Prevalence and characterization of *Clostridium* genus bacteria in animals destined for slaughter- antibiotic resistance in the food chain

(SUMMARY OF PhD THESIS)

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I. INTRODUCTION

In recent decades, concerns for public health and food safety have become increasingly prominent in the context of growing globalization and changes in agricultural and food practices. Within this framework, the bacteria *Clostridium difficile* and *Clostridium perfringens*, two anaerobic organisms with significant pathogenic potential, have drawn the attention of the scientific community and public health authorities worldwide.

These bacteria are associated with various gastrointestinal disorders and have been identified in diverse environments and biological sources, including the gastrointestinal tracts of humans and animals. However, despite their importance in human and animal health, a comprehensive understanding of their prevalence, molecular characteristics, and impact on public health and the food industry remains incomplete.

In this thesis, we aim to explore and investigate the prevalence, virulence, and antibiotic resistance characteristics of *Clostridium difficile* and *Clostridium perfringens* in animal populations in Romania, focusing on cattle, pigs, and broiler chickens. By utilizing a rigorous methodological framework and advanced molecular analysis techniques, we aim to shed light on the dynamics of these bacteria in the agricultural environment and contribute to the development of effective infection control and prevention strategies.

Additionally, we aim to evaluate the potential for zoonotic transmission of these bacteria and identify associated risks for human and animal health. By promoting a One Health approach, which recognizes the interconnectedness of human, animal, and environmental health, we aim to provide an integrated and holistic perspective on issues related to *Clostridium difficile* and *Clostridium perfringens* and contribute to the development of control and prevention strategies based on solid scientific evidence.

This thesis represents a first step towards a better understanding of the dynamics of *Clostridium difficile* and *Clostridium perfringens* infections in the agricultural environment of Romania and has the potential to significantly impact public health and the food industry. Through research and collaboration, we hope to contribute to promoting the health and safety of the population and to the development of sustainable and responsible agricultural and food practices.

II. THESIS STRUCTURE

The thesis titled “Prevalence and Characterization of *Clostridium* Bacteria Tested in Different Meat-Producing Animal Species” is structured into two main parts: “The Current State of Knowledge” and “Personal Contribution.” The first part consists of 24 pages (Chapter 1 and Chapter 2) and represents 21% of the total of the two parts. The second part of the thesis is composed of 8 chapters (Chapters 3, 4, 5, 6, 7, 8, 9, and 10) and the bibliography. This part comprises 88 pages and represents 79% of the total of the two parts.

III. CURRENT STARE OF KNOWLEDGE

The first part of the thesis is structured into two chapters:

Chapter 1 includes general information about *C. difficile* (taxonomy, biological cycle, virulence factors, strain classification, and species prevalence), isolation and identification methods, and antibiotic resistance. It also presents data on community-acquired *C. difficile* infection and *C. difficile* in the food chain.

Chapter 2 presents general data about *C. perfringens* (taxonomy, biological cycle, virulence factors, and the pathological mechanism of the toxins), isolation and identification methods, and aspects of the implication of toxinotype F for human health.

IV. PERSONAL CONTRIBUTION

Work hypothesis:

Clostridium perfringens and *Clostridium difficile*, two anaerobic bacteria with significant pathogenic potential, are present in the farming environments of slaughter animals in Romania, including cattle, pigs, and poultry. These bacteria can also contaminate meat and meat products, as well as the workplace and domestic environments of humans. This observation suggests the possibility of these bacteria being transmitted from animals to humans (potential zoonosis), raising significant public health concerns.

These bacteria are considered to exhibit varying levels of antibiotic resistance, which can influence the treatment of associated infections. Therefore, investigating the prevalence and molecular characteristics of these bacteria, as well as determining their antibiotic resistance profiles, can shed light on the dynamics of associated infections and contribute to their proper management and treatment.

Objectives:

1. Investigation of the prevalence and characteristics of *Clostridium difficile* and *Clostridium perfringens* bacteria in animal populations in Romania, focusing on major meat-producing species.
2. Molecular characterization of isolated strains, determination of antibiotic resistance levels and resistance genes, and assessment of virulence factors to understand pathogenic mechanisms and antibiotic resistance profiles.
3. Evaluation of the zoonotic transmission potential of these bacteria and promotion of the One Health approach by recognizing the interconnectedness of human, animal, and environmental health in the context of *Clostridium difficile* and *perfringens* infections.
4. Comparison of findings from these studies with other similar studies to assess trends and variations in *Clostridium* prevalence and characteristics across different environments and geographic regions, evaluating the zoonotic transmission potential of these bacteria and identifying associated risks to human and animal health

Chapter 3, titled " Prevalence, characterization and antibiotic resistance of *Clostridium difficile* bacteria isolated from cattle and swine," aimed to:

1. Investigate the prevalence of *Clostridium difficile* bacteria in the feces of cattle and swine of different age categories. This will provide insight into the spread of the bacteria among slaughter animals, particularly the young ones.
2. Determine the antibiotic sensitivity of the isolated strains, providing useful information about potential antibiotic resistance and its impact on the treatment and control of infections.
3. Evaluate the presence of antimicrobial resistance determinants and identify antibiotic resistance genes (such as tetM, tetW, ermB) to gain a more detailed understanding of resistance mechanisms in *Clostridium difficile* strains.

Study Results: In total, 24 (12.5%) strains of *C. difficile* were isolated from the 192 samples analyzed, with the highest rate of positive isolation in fecal samples collected from piglets (25%). Regarding the virulence gene profiles, most isolates recovered in this study were toxigenic (10% of the isolates from piglets carried the tcdA, tcdB, and cdtA/B genes, while 85% were positive only for tcdA and tcdB). Among the isolates detected in fecal samples collected from beef calves, one carried the tcdA and tcdB genes. Therefore, the predominant profile in this study was *C. difficile* tcdA+tcdB+.

All *C. difficile* isolates recovered in this study were found to be susceptible to vancomycin, except for one isolate from cattle feces that exhibited resistance to metronidazole. A significant proportion of isolates from pigs (50%) showed resistance to levofloxacin, a third-generation fluoroquinolone. Interestingly, 60% of the isolates recovered from piglet fecal samples also exhibited resistance to tetracycline, and 35%

of these contained the tetW gene. Four isolates from pigs showed resistance to erythromycin, and among these, two included the erm(B) gene. Out of the 24 *C. difficile* strains isolated from piglets, calves, and adult cattle, 4 (20%) (from the 20 isolates from piglets) were resistant to 3 of the antibiotics analyzed (tetracycline, erythromycin, and levofloxacin). Four strains from piglets were resistant to two antibiotics (tetracycline and levofloxacin), one strain from cattle was resistant to levofloxacin and metronidazole, and one strain from calves was resistant to tetracycline and levofloxacin. Among the strains isolated from piglets, six showed resistance to a single antibiotic (mostly tetracycline, and two to levofloxacin), while six strains from the 20 isolated from piglets and two from the three isolated from calves were sensitive to the antibiotics used in the study. Almost all isolates identified in fecal samples from piglets (except for one case), as well as one isolate from a beef calf, which exhibited resistance to the antimicrobials tested in the study, were also toxigenic.

Chapter 4, titled " Prevalence, characterization and antibiotic resistance of *Clostridium perfringens* bacteria isolated from cattle and swine," aimed to :

1. Investigate the prevalence of *Clostridium perfringens* bacteria in the feces of cattle and swine from different age categories.
2. Determine the antibiotic sensitivity of the strains isolated from these animals.
3. Evaluate the presence of antimicrobial resistance determinants in *Clostridium perfringens* isolates.

Study Results: The study obtained 14 (14%) *Clostridium perfringens* isolates from the 100 samples analyzed, with most identified in fecal samples collected from pigs (12 isolations). All *Clostridium perfringens* isolates recovered in our study were found to be susceptible to vancomycin, rifampicin, and lincomycin. A significant portion of the isolates exhibited resistance to tetracycline (71.4%), penicillin (64.2%), erythromycin (42.8%), and enrofloxacin (35.7%). In the context of Romania, there is currently very limited data available regarding the prevalence of *Clostridium perfringens* isolated from animal sources

Chapter 5: "Prevalence and Characterization of *Clostridium* Bacteria Isolated from Broiler Chickens" aimed to:

1. Determine the prevalence of *C. difficile* in fecal samples and cecal contents from broiler chickens.
2. Investigate the toxin gene profiles (tcdA, tcdB, cdtA, and cdtB) of the isolated *C. difficile* strains.
3. Compare the results with similar studies to evaluate variability and trends in the prevalence and distribution of *C. difficile* in broiler chickens.
4. Analyze the potential public health implications and zoonotic transmission risk if *C. difficile* is detected in broiler chickens.

Results: The study found a prevalence rate of 15%, with *C. difficile* isolated from 25% of fecal samples and 5% of cecal content samples. The toxin gene profile showed that all six isolates were positive for the *tcdA* and *tcdB* toxin genes, which are associated with the production of toxins A and B. However, none of the isolates were positive for the binary toxin genes *cdtA/B*.

Chapter 6: titled "Research on *Clostridium perfringens* in Broiler Chickens", aimed to:

1. Investigate the prevalence of *Clostridium perfringens* in fecal samples and intestinal contents from broiler chickens.
2. Isolate and identify *Clostridium perfringens* strains using appropriate culture media and standardized laboratory techniques.
3. Characterize the molecular aspects of *C. perfringens* isolates, including toxinotyping and antibiotic resistance profiles.
4. Compare the results with similar studies to evaluate variations in the prevalence and characteristics of *C. perfringens* in the poultry industry.
5. Analyze the potential impact of *C. perfringens* contamination in the poultry industry on public health and the risk of foodborne illnesses in humans.
6. Suggest management and control strategies to prevent and reduce *C. perfringens* contamination in broiler farms and during slaughter, including promoting hygiene practices and monitoring antibiotic resistance in the isolated strains.

Results: The research found no *C. perfringens* isolates in the fecal and intestinal content samples from broiler chickens. Similar studies have shown varying isolation rates of *C. perfringens* from fecal, intestinal content, meat, and meat products.

Chapter 7: General Conclusions and Recommendations

General Conclusions:

1. The studies revealed a significant prevalence of *Clostridium difficile* in fecal samples collected from cattle, pigs, and broiler chickens, with higher incidence in piglets and broiler chickens. *Clostridium perfringens* was also identified in most fecal samples from pigs, highlighting the importance of closely monitoring these bacteria in animals intended for human consumption due to their potential impact on public health.
2. A significant proportion of *C. difficile* and *C. perfringens* isolates were toxigenic, carrying genes associated with toxin production (*tcdA*, *tcdB* for *C. difficile*, and *cpa* for *C. perfringens*). This underscores the risk of toxin-mediated infections and the necessity for rigorous surveillance and control measures to prevent the spread of these virulent strains.
3. Antibiotic sensitivity tests indicated varying degrees of antibiotic resistance, with significant resistance to tetracycline and levofloxacin among *C. difficile* and *C. perfringens* isolates from piglets and pigs. Identifying resistance genes such as *tetW*

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and *ermB* provides valuable insights into the genetic basis of resistance and emphasizes the importance of prudent antibiotic use in veterinary and human medicine to minimize the risk of selecting multi-resistant strains.

4. The studies contribute valuable data to the One Health perspective, highlighting the interconnection between human, animal, and environmental health. The spread of *Clostridium* bacteria between animals and humans suggests the need for an integrated approach to monitoring and managing infections, considering potential zoonotic transmission.

5. The results emphasize the importance of implementing control and prevention measures in the food industry to reduce the risk of spreading these bacteria and causing foodborne illnesses.

Recommendations:

1. Implementation of surveillance and control measures: Develop and implement effective surveillance programs for *Clostridium difficile* and *Clostridium perfringens* in farm animal populations, particularly those supplying meat. These programs should include regular monitoring of prevalence, virulence, and antibiotic resistance profiles.

2. Promotion of responsible antibiotic use: To reduce the risk of selecting antibiotic-resistant strains, promote and implement responsible antibiotic use practices in veterinary medicine and the food industry. This may include guidelines for judicious antibiotic prescription and local and regional resistance monitoring.

3. Education and awareness: Provide education and raise awareness among animal health professionals, farmers, and food industry workers about the risks associated with *Clostridium difficile* and *Clostridium perfringens* infections. Information should include preventive measures such as proper hygiene and correct food handling practices.

4. Investment in further research: Continue and expand research into the ecology, epidemiology, and public health impact of these bacteria. Investing in additional research could lead to more effective strategies for controlling and preventing infections with *Clostridium difficile* and *Clostridium perfringens*.

5. Promotion of the One Health approach: Collaboration between sectors including human health, animal health, and environmental protection is crucial for addressing issues related to *Clostridium difficile* and *Clostridium perfringens*. Promoting the One Health approach could facilitate the exchange of information and expertise between different fields, leading to more effective and integrated solutions.

6. Initial analysis basis for future monitoring: This study represents the first analysis of *C. difficile* prevalence in animals destined for slaughter and consumption in our country, providing a foundation for future antimicrobial resistance monitoring of *C. difficile* in food animals, food products, and the environment in Romania. A more comprehensive future study, including human *C. difficile* isolates, is

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needed to evaluate their potential contribution as a source of resistant *C. difficile* strains and as a reservoir for antimicrobial resistance determinants.

6. Research gaps and future directions: While the studies provide essential information on the prevalence and characteristics of *Clostridium* bacteria in animal samples, further research is needed to elucidate specific transmission routes and reservoirs of these strains. Exploring the prevalence and resistance profiles in the food supply chain would contribute to a comprehensive understanding of the public health impact. Continued research is essential to developing effective strategies for controlling and preventing contamination and associated diseases in the poultry and pork industries.

7. Practical implications: For veterinary and public health specialists: These findings offer useful insights into managing *Clostridium* spp. infections and underscore the need for ongoing efforts to prevent and control the spread of these bacteria in the food chain. **For the Food Industry:** Implementing strict control and hygiene measures at all stages of meat production and processing is crucial to minimizing contamination risk and preventing zoonotic infections.

These conclusions highlight the significance of monitoring and controlling *Clostridium* bacteria in animal populations, particularly those intended for human consumption, to protect public health and ensure food safety.

Chapter 8: Originality and innovative contributions of the thesis

The research is entirely original, based on a specific methodological framework and unique experiments. The main innovative contributions are as follows:

1. Exploring the prevalence and virulence of *Clostridium difficile* and *Clostridium perfringens*: The thesis investigates the prevalence, virulence, and antibiotic resistance characteristics of *Clostridium difficile* and *Clostridium perfringens* among cattle, pigs, and broiler chickens. This research provides valuable data on the distribution of these bacteria in animal populations and the associated risks to human and animal health.

2. Genomic characterization of *Clostridium difficile* strains: The thesis contributes to the genomic characterization of *Clostridium difficile* strains isolated from fecal and cecal content samples from farm animals. This genomic analysis offers a deeper understanding of the genetic diversity of the bacterium and potential mechanisms of virulence and antibiotic resistance.

3. Evaluation of zoonotic transmission risk: By investigating the presence and characteristics of *Clostridium difficile* and *Clostridium perfringens* in animal feces, the thesis addresses the risk of zoonotic transmission of these bacteria. This risk evaluation is crucial for understanding the interconnectedness of human, animal, and environmental health in the context of pathogenic bacterial infections.

4. Identification of antibiotic resistance genes: The thesis identifies antibiotic resistance genes in *Clostridium difficile* and *Clostridium perfringens* strains, contributing significantly to understanding the molecular mechanisms involved in antibiotic resistance in these bacteria. This information can be useful for developing more effective control and prevention strategies for associated infections.

5. Promoting the One Health approach: By highlighting the interconnectedness of human, animal, and environmental health in the context of *Clostridium difficile* and *Clostridium perfringens* infections, the thesis promotes the One Health approach. This integrated approach is essential for effectively managing public health issues and the risks associated with these bacteria.

6. International Recognition: At the international level, the thesis contributes to the advancement of knowledge in the field due to the publication of research results in prestigious high-impact journals, thereby consolidating the importance and relevance of the studies in the domain of animal and public health.