Re-evaluation of the etiology and pathogenesis of liver abscesses in feedlot cattle: potential involvement of *Fusobacterium varium* and bacteriophages as an intervention tool

by

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B.S., Kansas State University, 2019

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
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KANSAS STATE UNIVERSITY
Manhattan, Kansas

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Abstract

Liver abscesses in cattle are the number one cause of liver condemnations at slaughter and have a significant economic impact on the beef cattle industry. *Fusobacterium necrophorum*, a common ruminal bacterium, has been recognized as the primary causative agent of liver abscess formation in feedlot cattle. Liver abscess formation in beef cattle has been historically attributed to the "ruminitis- liver abscess complex", which hypothesizes that high grain feeding leads to ruminal acidosis and subsequent ruminal epithelial damage, which allows the translocation of *F. necrophorum,* and other liver abscess-causing pathogens, into the liver via portal vein circulation. Recent studies utilizing genomic sequencing-based technologies have revealed that liver abscesses are highly diverse, polymicrobial infections, and have also raised the question of whether the hindgut, specifically the colon, can be a source of liver abscess-causing pathogens. In light of these recent findings, my main research objective was to re-evaluate our understanding of the etiology and pathogenesis of liver abscesses in feedlot cattle, with a focus on the potential involvement of *Fusobacterium varium* in liver abscess development. Additionally, we investigated the potential use of bacteriophages as an antimicrobial alternative for liver abscess prevention.

Fusobacterium varium has recently been reported as the most predominant *Fusobacterium* species in the bovine rumen, as determined by 16S microbiome analysis. Therefore, our first experiment was to develop and validate a real-time quantitative PCR assay for the detection and quantification of *F. necrophorum* subsp. *necrophorum*, *F. necrophorum* subsp. *funduliforme*, and *F. varium* in ruminal fluid samples collected from feedlot cattle. The qPCR primers were designed to target the *hgdA* gene for species-level differentiation and the *lktA* gene for differentiation at the subspecies level. The qPCR assay was conducted on 345

ruminal fluid samples collected from feedlot cattle immediately after slaughter, of which 181 were from cattle with apparently healthy, non-abscessed livers, and 164 were from cattle with liver abscesses. The ruminal fluid samples were strained through cheesecloth to remove large particles and an aliquot of strained ruminal fluid was collected for qPCR analysis. Additionally, strained ruminal fluid samples were enriched using a selective media containing lactate or lysine as the energy source and supplemented with josamycin, vancomycin, and norfloxacin antibiotics. The qPCR assay quantified *F. necrophorum* subsp. *necrophorum* in only 22% and 29% of samples from cattle with healthy and abscessed livers, respectively, with the samples from abscessed livers significantly more likely to contain subsp. *necrophorum* ($P = 0.004$). Interestingly, *F. necrophorum* subsp. *funduliforme* and *F. varium* were found to be prevalent in nearly all samples (98 to 100%), and no significant difference in prevalence was found between cattle with healthy livers and cattle with liver abscesses. Mean concentrations of *F. varium* and both subsp. of *F. necrophorum* were not significantly different between animal status, with mean concentrations of 10^3 to 10^4 CFU/ml.

Our second study was intended as a follow up to further investigate the potential for *F. varium* to be involved in liver abscesses since it was so highly prevalent in ruminal fluid samples. We obtained matched liver abscess, ruminal and colonic epithelial tissue, and ruminal and colonic content samples from 96 steers at the time of slaughter. Samples were homogenized and cultured onto blood agar and selective enrichment media for the presence of *F. necrophorum* and *F. varium*, and the previously described qPCR assay was used for detection and quantification of *F. necrophorum* and *F. varium* in the samples before and after enrichment. Three liver abscess samples (3.1%) yielded *F. varium* by culture-based methods, the first report of isolation of *F. varium* from bovine liver abscesses. Ruminal and colonic epithelial tissue

samples had an *F. varium* isolation rate of 59.4% and 31.3%, respectively. The qPCR assay detected *F. varium* in 10 (10.4%) of samples, with only one of the samples quantifiable prior to enrichment. The total qPCR-based prevalence in ruminal and colonic epithelial tissues was 77.1% and 44.8%, respectively, while ruminal and colonic contents had a total qPCR-based prevalence of 86.5% and 70.1%. These findings showed support for the theory that the colon may be involved in liver abscess etiology.

As a follow-up to this initial study, we investigated the effect of various feeding and management strategies on the prevalence of *F. varium* in liver abscesses or liver tissues and their corresponding ruminal and colonic epithelial tissues. Matched sets of samples were collected from two feedlot studies and one experimental study and subjected to culture-based and qPCR-based methods for *F. varium* detection and quantification. In the first feedlot study of 190 cattle intended to evaluate the effect of tylosin on *F. varium* prevalence, no difference was observed between the tylosin treatment group compared to the non-tylosin fed group in ruminal epithelial tissues, liver abscesses, or healthy liver tissues. In the experimental study, cattle were assigned to either the control treatment (n = 10) or induced ruminal acidosis treatment (n =10), since ruminal acidosis is considered a necessary precursor to liver abscess formation. None of these cattle developed liver abscesses, and no difference in prevalence was observed in liver tissues, ruminal and colonic epithelial tissues, or ruminal and colonic contents between cattle with ruminal acidosis compared to control cattle. The second feedlot study compared samples from 159 cattle subjected to a 2x2 factorial design for the evaluation of two dietary starch levels (CON or HOT) and two management strategies (REG or ERR). Cattle fed a “hot” diet, intended to induce acidosis, and subjected to an erratic feeding schedule had a significantly higher *F. varium* prevalence in colonic epithelial tissues than cattle fed a control diet and subjected to an erratic

feeding schedule ($P < 0.01$). No differences in ruminal or liver abscess *F. varium* were observed, nor was there any difference in *F. varium* concentrations. We also performed whole genome sequencing and analyses on a subset of 14 *F. varium* strains isolated from liver abscesses (n = 5), ruminal epithelial tissue (n = 5), and colonic epithelial tissue (n = 4). Using average nucleotide identity (ANI) comparison, very high levels of genetic similarity were found between strains recovered from the same animal, though no accepted cut-off value for establishing clonality has been set. Previous studies have reported resistance to tylosin in *F. varium* strains recovered from the bovine rumen, however, we did not find any macrolide resistance genes in our strains. These findings of high genetic similarity further emphasized that the hindgut may be a source of bacterial translocation into the liver.

A whole genome sequencing study was then conducted on *F. necrophorum* subsp. *necrophorum* strains recovered from the two previously described feedlot studies, for the purpose of genetically comparing strains recovered from liver abscesses to strains recovered from ruminal and colonic tissues from the same animal, and to compare genomic differences between strains based upon their tissue of origin. A total of 62 *F. necrophorum* subsp. *necrophorum* strains were sequenced and used for bioinformatic analyses. Using core-genome SNP analyses along with ANI analysis, we determined clonal relationships between matched strains recovered from the colon and liver abscess from 2 animals, the ruminal and liver abscess strains from 3 animals, and the matched ruminal, colonic, and liver abscess strains from one animal. When the strains were subjected to AMR screening using StarAMR, it was found that two liver abscess strains carried the *ermB* gene, which encodes resistance to macrolide antibiotics such as tylosin. In feed tylosin administration is the most common liver abscess prevention method, and this was the second report of macrolide resistance in *F. necrophorum*

isolated from bovine liver abscesses. Additionally, relatively high levels of aminoglycoside (17.7%) and tetracycline (24.2%) resistance was observed.

Our final study aimed to investigate the use of bacteriophages as an antimicrobial alternative for liver abscess prevention. Characterization, including host ranging, titer determination, morphological examination, pH stability, and whole genomic sequences was performed on 5 novel bacteriophages previously isolated from untreated city sewage. All 5 phages were found to be highly specific to *F. necrophorum* subsp. *necrophorum*, and were generally stable in pH values ranging from 4-9 for up to 24 hours. Electron microscopy revealed four of the five phages were tailed, while one phage was unable to be visualized. A proof of concept study was conducted to evaluate the efficacy of prophylactic administration of a cocktail of all 5 bacteriophages in preventing liver abscesses caused by *F. necrophorum* in a mouse model. Significant reduction in both morbidity and mortality was observed ($P < 0.05$) in mice treated with the bacteriophage cocktail compared to the negative control group. These findings warrant further investigation into the potential application of bacteriophages as an alternative to tylosin for the prevention of liver abscesses in feedlot cattle.

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Major Professor

Dr. T.G. Nagaraja

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Table of Contents

List of Figures ... xix
List of Tables ... xx
Acknowledgements ... xxii
Dedication ... xxiii
Chapter 1 - Molecular Techniques for the Identification and Characterization of *Fusobacterium necrophorum* at the species and subspecies level ... 1
Abstract ... 1
Introduction ... 2
Ribotyping and Restriction Fragment Length Polymorphism Analysis ... 3
Polymerase Chain Reaction (PCR) Assays ... 7
"-Omics"- based characterization ... 11
Conclusions ... 16
Literature Cited ... 17
Chapter 2 – A real-time PCR assay for the detection and quantification of *Fusobacterium necrophorum* and *F. varium* in ruminal contents of cattle ... 28
Abstract ... 28
Introduction ... 30
Materials and Methods ... 31
Primer and probe design ... 31
Bacterial Culture Preparation ... 32
Assay Specificity Determination ... 32
Assay Sensitivity Determination ... 33
qPCR Assay Running Conditions ... 34
Development and Validation of Enrichment Media ... 34
Ruminal Contents Sample Collection ... 35
DNA Extraction ... 35
Statistical Analysis ... 36
Results and Discussion ... 37
Validation of the qPCR Assay ... 37

Prevalence and Concentrations of Fusobacterium necrophorum and F. varium in Rumen Fluid Before Enrichment 39
Prevalence and Concentrations of *F. necrophorum* and *F. varium* in Ruminal Fluid After Enrichment 40
Cumulative Prevalence of *Fusobacterium* species in the rumen 41
Applications 43
Acknowledgements 44
Literature Cited 50
Chapter 3 Comparative whole genome analyses of *Fusobacterium necrophorum* subspecies *necrophorum* isolates from liver abscesses and matched ruminal and colonic epithelial tissues of cattle 56
Abstract 56
Introduction 58
Methods 60
F. necrophorum isolation 60
DNA extraction 61
DNA sequencing 61
Genome assembly 62
Bioinformatic analyses 62
Results 63
Genome sizes 63
Strain relatedness 63
Phylogenetic trees to visualize strain clustering 63
Average nucleotide identity (ANI) analysis to measure genetic relatedness 63
Single nucleotide polymorphism (SNP) analysis 65
Virulence Genes 66
Antimicrobial resistance genes 67
Plasmids 68
Discussion 68
Conclusions 73
Literature Cited 75

Chapter 4 – First Report of Isolation of *Fusobacterium varium* from Liver Abscesses and Ruminal and Colonic Epithelial Tissues of Feedlot Cattle 91
Abstract 91
Introduction 93
Materials and Methods 94
Sample Collection 94
Sample Processing 94
Enrichment media 95
F. varium isolation 95
F. varium qPCR detection and quantification 96
DNA extraction 96
Quantitative PCR Assay 97
Results and Discussion 97
F. varium in Liver Abscesses 97
F. varium in Ruminal and Colonic Epithelial Tissues 98
F. varium in Ruminal and Colonic Contents 100
Implications and Applications 101
Acknowledgements 101
Literature Cited 105
Chapter 5 *Fusobacterium varium* in liver abscesses, rumen, and colon of cattle: Prevalence, isolation, and characterization 112
Abstract 112
Introduction 114
Materials and Methods 116
Effects of in-feed tylosin administration. 116
Effect of experimentally-induced ruminal acidosis. 117
Effect of dietary starch concentration and feeding management regimen. 117
Processing of healthy and abscessed livers, ruminal contents and epithelial tissues and colonic contents and epithelial tissue samples. 118
Species confirmation and quantification of *F. varium*. 119
F. varium isolation. 119

DNA extraction. 120
Whole genome sequencing 120
Bacterial strains 120
DNA extraction 120
DNA quality control, library preparation, and sequencing. 121
Genome Assembly 122
Bioinformatic analyses. 122
Statistical analysis 122
Results 123
Effects of in-feed tylosin administration on F. varium prevalence in liver tissues, ruminal epithelial tissues, and liver abscesses. 123
Effect of experimentally induced ruminal acidosis on *F. varium* prevalence 125
Effect of dietary starch concentration and feeding management regimen. 126
Whole genome sequencing of 14 F. varium strains 128
Average nucleotide identity Analysis. 128
Identification of Antimicrobial Resistance Genes 129
Virulence factor identification 129
Discussion 130
Conclusions 134
Literature Cited 135
Chapter 6 Characterization of Five Novel Bacteriophages Lytic to *Fusobacterium necrophorum* subspecies *necrophorum* 154
Abstract 154
Introduction 156
Methods 158
Bacteriophage Propagation: 158
Bacterial Cultures 159
Media Preparation 159
Bacteriophage Host Range Determination 160
Bacteriophage Morphology: 161
Bacteriophage Titer Determination: 161

pH stability of bacteriophages ... 161
Bacteriophage survival in cold storage: ... 162
Bacteriophage DNA extraction ... 162
DNA Analysis and Sequencing ... 162
Bioinformatic Analyses ... 163
Evaluation of a bacteriophage cocktail for prevention of liver abscesses in a mouse model ... 163
Bacteriophage isolation from mouse livers: ... 164
Results ... 164
Bacteriophage morphology ... 164
Bacteriophage titers: ... 165
Host Range Determination ... 166
pH and temperature stability ... 166
Genome Assemblies and Annotation ... 167
Bacteriophage cocktail for the prevention of liver abscesses ... 167
Discussion ... 168
Conclusions ... 171
Literature Cited ... 172
Chapter 7 Conclusions ... 185
References ... 190

List of Figures

Figure 2-1 Cumulative prevalence of Fusobacterium species, based on quantitative PCR assay, in rumen fluid, before and after enrichment, of cattle with and without liver abscesses collected at slaughter 49

Figure 3-1 Phylogenetic tree of the *Fusobacterium necrophorum* subsp. *necrophorum* strains isolated from liver abscesses, ruminal and colonic tissues from the 2022-1 feedlot study .. 83

Figure 3-2 Phylogenetic tree of the *Fusobacterium necrophorum* subsp. *necrophorum* strains isolated from liver abscesses, ruminal and colonic tissues from the 2023-3 feedlot study .. 84

Figure 3-3. Heatmap and dendrogram constructed using the pairwise ANI percentages for *F. necrophorum* subsp. *necrophorum* isolates from liver abscesses (LA), rumen tissue (RT), and colonic tissue (CT) from the 2022-1 feedlot study. 85

Figure 3-4 Heatmap and dendrogram constructed using pairwise ANI percentages for *F. necrophorum* subsp. *necrophorum* isolates from liver abscesses (LA), rumen tissue (RT), and colonic tissue (CT) from the 2023-3 feedlot study. 86

Figure 3-5. Pairwise distance matrix and heatmap displaying the number of core-genome SNPs between *F. necrophorum* subsp. *necrophorum* strains from liver abscesses, rumen tissues, and colonic tissues from the 2022-1 feedlot study 87

Figure 3-6 Pairwise distance matrix and heatmap displaying the number of core-genome SNPs between *F. necrophorum* subsp. *necrophorum* strains from liver abscesses, rumen tissues, and colonic tissues from the 2023-3 feedlot study 88

Figure 4-1 Colony morphology of *Fusobacterium varium*, *F. necrophorum* subsp. *necrophorum* and subsp. *funduliforme* on blood agar plate 104

Figure 5-1 Average Nucleotide Index heat map and dendrogram of the 14 matched *Fusobacterium varium* strains isolated from liver, ruminal and colonic tissues of feedlot cattle 147

Figure 6-1 Visualization of bacteriophages as observed using transmission electron microscopy. a) bacteriophage SA2. b) bacteriophage SA3. c) bacteriophage SA4. d) bacteriophage SA11. e) bacteriophage SA14 184

List of Tables

Table 2-1 Species and subspecies of Fusobacterium, genes targeted, and primers and probes sequences used in the four-plex quantitative PCR assay 45

Table 2-2 Prevalence and mean concentrations of Fusobacterium species and subspecies in ruminal contents of cattle with or without abscessed livers abscesses collected at slaughter 46

Table 2-3 Prevalence of Fusobacterium species and subspecies, based on quantitative PCR assay, in rumen fluid after enrichment from cattle with and without liver abscesses collected at slaughter 47

Table 2-4 Mean concentrations of Fusobacterium species, based on quantitative PCR assay, in rumen fluid before or after enrichment from cattle with and without liver abscesses collected at slaughter 48

Table 3-1 Gene matrix indicating the presence or absence of AMR genes, as detected using StarAMR, in *F. necrophorum* subsp. *necrophorum* strains recovered from liver abscesses, ruminal and colonic tissues from the 2022-1 and 2023-3 feedlot studies. Black indicates presence and white indicates absence. 89

Table 4-1. Frequency of Isolation of *Fusobacterium varium* from liver abscesses, ruminal epithelial and colonic epithelial tissues collected from feedlot cattle at slaughter 102

Table 4-2 Prevalence and concentration of *Fusobacterium varium*, based on quantitative PCR assay, before and after enrichment, in liver abscesses, ruminal, and colonic epithelial tissues and contents, collected from feedlot cattle at slaughter 103

Table 5-1 Frequency of isolation of *Fusobacterium varium* from matched liver abscesses, healthy liver tissue, and ruminal epithelial tissues collected at slaughter from beef-on-dairy heifers fed diets with or without probiotic plus zeolite or tylosin 148

Table 5-2 Prevalence and concentration of *Fusobacterium varium*, based on quantitative PCR assay, in matched liver abscesses, healthy liver tissue, and ruminal epithelial tissues collected from feedlot cattle at slaughter either fed or not-fed tylosin 149

Table 5-3 Frequency of isolation of *Fusobacterium varium* from liver tissue and corresponding ruminal and colonic epithelial tissues collected from steers in the control group and in steers with experimentally induced ruminal acidosis... 150

Table 5-4 Prevalence of *Fusobacterium varium*, based on quantitative PCR assay, in liver tissue and corresponding ruminal, ileal and colonic contents and epithelial tissues collected from steers in the control group and in steers with experimentally induced acidosis 151

Table 5-5 Frequency of isolation of *Fusobacterium varium* from liver tissue and corresponding ruminal and colonic epithelial tissues collected from cattle fed diets containing low or high starch level under two feeding management regimens[1] 152

Table 5-6 Prevalence of *Fusobacterium varium*, based on quantitative PCR assay, in liver tissue and corresponding ruminal and colonic contents and epithelial tissues collected from cattle subjected to 4 different management styles 153

Table 6-1. Bacterial strains used in the host ranging experiments for characterization of 5 novel bacteriophages isolated from untreated sewage 180

Table 6-2 Measurement of the 5 novel bacteriophages, as observed under transmission electron microscopy 181

Table 6-3 Results of the host ranging experiments performed using a modified spot on lawn technique. +++ indicates complete lysis, - indicates no lysis 182

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Dedication

This dissertation is dedicated to all of those who believed in me throughout this 5-year journey, I couldn't have done it without you.

To my husband Devin, thank you for never complaining about all of the long hours I spent in the lab (or the smell of rumen fluid that I'd sometimes drag home with me), and for always being willing to set me straight whenever my doubts crept in. You've been with me since the very beginning of this journey, and I could never truly express how much it's meant to be able to do this with you by my side.

To my parents, Tim and Cindy McCormick, thank you for always encouraging me to ask questions, for raising me to know that curiosity is something to be fostered, and teaching me that knowledge is something to seek out continuously. Without your support I never would have had the courage to pursue my degrees.

To all of my friends and family, thank you for your abundance of grace and understanding when I'd have to miss events or gatherings to meet a deadline or finish out a research project, I know it must have been frustrating but you never held it against me and always met me with welcome arms when I'd finally reemerge.

To all my furry creatures who have let me practice presentations to them, dried my tears, and kept me grounded, thank you. Sometimes people are just too much, and the gentle companionship I've been lucky enough to have from my animals made the worst days bearable.

Chapter 1 - Molecular Techniques for the Identification and Characterization of *Fusobacterium necrophorum* at the species and subspecies level

Abstract

Fusobacterium necrophorum is a Gram-negative, anaerobic, rod shaped bacterium commonly found in the oral cavity, gastrointestinal tract, and urinogenital tract of humans and animals. Additionally, *Fusobacterium necrophorum* has been implicated in a variety of necrotic infections, most notably Lemierre's syndrome in humans, and liver abscesses and foot rot in cattle. The accurate identification of *F. necrophorum* is crucial for efficient diagnosis and treatment of disease, and has traditionally relied on its morphological and biochemical characterization. However, molecular-based methods for the identification and characterization of *F. necrophorum* have been developed over the past 50 years, including ribotyping and restriction fragment length polymorphism analyses, polymerase chain reaction (PCR) assays, and various "-omics"-based analyses. These methods provide valuable insights into conserved virulence factors and metabolic pathways, and facilitate in depth understandings of strain diversity and pathogenesis. This review aims to provide a comprehensive overview of the available molecular-based characterization methods for the identification and characterization of *F. necrophorum.* Additionally, we discuss the applications of these methods for the development of therapeutics for the treatment and prevention of *F. necrophorum* infections, and potential future directions for studies on *F. necrophorum* from a molecular perspective.

Key Words: *Fusobacterium necrophorum,* genomics, ribotyping, PCR, bacterial identification

Introduction

Fusobacterium necrophorum is an anerobic, Gram-negative, non-spore forming bacterium often found in the oral cavity and the alimentary and urinogenital tracts, and is responsible for a variety of necrotic infections in humans and animals (Langworth, 1977; Nagaraja and Chengappa, 1998; Riordan, 2007; Hagelskjaer Kristensen and Prag, 2008; Klug et al., 2016). The species is further divided into two subspecies: subsp. *necrophorum*, previously referred to as biotype A, and subsp. *funduliforme*, previously known as biotype B (Shinjo et al., 1991). The subspecies are divided on the basis of biological and biochemical characteristics as well as differences in nucleic acid homology. Subspecies *funduliforme* is considered to be primarily a human pathogen and is most frequently implicated in Lemierre's syndrome, characterized by jugular vein thrombophlebitis and subsequent septicemia (Riordan, 2007), which is a rare sequalae to tonsillitis (Klug et al., 2016; Centor et al., 2022). Subspecies *necrophorum* is most commonly associated with animal infections, particularly liver abscesses, foot rot, and calf-diphtheria in cattle (Langworth, 1977; Scanlan and Hathcock, 1983; Nagaraja et al., 2005; Nagaraja and Lechtenberg, 2007). Along with morphological and biochemical differences, the two subspecies differ in virulence, largely attributed to the variation in leukotoxin production (Tadepalli et al., 2009).

Historically, the identification of *F. necrophorum* has relied on morphological and biochemical characterization. A number of biochemical methods for the identification of *F. necrophorum* have been described, and numerous commercial kits have been developed for these purposes (Natalie Karachewski, 1984; Bennett and Duerden, 1985; Tan et al., 1994a; Downes et al., 1999; Bank et al., 2010). These test kits were once considered to be the gold standard for identification of *F. necrophorum* by measuring enzymatic reactions of isolates recovered from

clinical samples. However, these tests can be insufficient for characterization of novel clinical isolates due to the morphological and biochemical similarities shared between the two subspecies (Marler et al., 1991; Bista et al., 2022).

Alternatively, molecular-based characterization methods have been developed in recent years for the identification of *F. necrophorum* at the species and subspecies level. These identification methods are advantageous to traditional phenotypic or biochemical characterizations due to their rapid turnaround time, low cost, and ease of interpretation (Adzitey et al., 2013). Here, we present a review of the molecular-based characterization methods described in the existing literature for the identification and differentiation of *F. necrophorum* at the species and subspecies levels.

Ribotyping and Restriction Fragment Length Polymorphism Analysis

The concept of ribotyping was initially described in 1986 as a method of identifying and classifying bacteria, and since its inception, numerous ribotyping techniques have been developed. These techniques include conventional ribotyping, automated ribotyping, and polymerase chain reaction ribotyping (Grimont and Grimont, 1986; Kashyap et al., 2020). The molecular genetic basis for ribotyping itself relies on the analysis of restriction fragment length polymorphisms (RFLPs): variations observed in DNA sequences after digestion by various restriction enzymes (Bouchet et al., 2008). After digestion, gel electrophoresis can then be used to visualize the restriction fragments produced by each enzyme, observed as bands of DNA on the gel, and differences in banding patterns can be identified.

The first application of RFLP analysis in *Fusobacterium necrophorum* was the identification, isolation, and characterization of a novel DNA repeat sequence present in the genome of a previously identified *F. necrophorum* strain recovered from a clinical ovine foot rot

sample (Hodgson et al., 1993). The novel repeat sequence was hypothesized to be part of an insertion sequence, as it exhibited high levels of homology with a known *Pseudomonas syringiae* insertion sequence, and also contained a putative transcriptional terminator. Following its identification, the novel repeat was then evaluated for use, in conjunction with the 16S rRNA gene of *F. necrophorum,* to differentiate between isolates of *F. necrophorum* biovars AB and B (now subsp. *funduliforme)* from ovine foot swabs. When this combination was used on 7 *F. necrophorum* biovar AB isolates, each isolate produced distinct DNA banding patterns containing between 4 and 8 bands. These results indicated the assay's potential for use as a highly sensitive diagnostic tool for differentiation at the strain level within *F. necrophorum* biovar AB. Interestingly, when the probe combination was used for RFLP analysis of *F. necrophorum* biovar B strains, no DNA hybridization was observed, indicating that the novel DNA repeat sequence may not exist in biovar B. The observed differences in banding patterns between isolates within *F. necrophorum* biovar AB were hypothesized to be due to strain-specific variations in copy number of the 16S rRNA gene. One major limitation of this study was the lack of inclusion of *F. necrophorum* biovar A (now subsp. *necrophorum*) strains for the analysis. *Fusobacterium necrophorum* biovar AB has been isolated primarily from ovine samples, and because it displays significant similarity to both subsp. *necrophorum* and subsp. *funduliforme*, the lack of inclusion of subsp. *necrophorum* in this study calls into question whether the probes used in the study could accurately distinguish between all three *F. necrophorum* biotypes.

Later studies attempted to further examine the ability of ribotyping and RFLP analysis to distinguish between the two subspecies of *F. necrophorum* in isolates recovered from ruminal contents and liver abscesses in cattle (Okwumabua et al., 1996). Ribotyping was performed on 5

strains of liver abscess origin (2 subsp. *necrophorum*, 3 subsp. *funduliforme*) and 16 strains recovered from ruminal contents of cattle fed a high grain diet (8 subsp. *necrophorum*, 8 subsp. *funduliforme*). Probes for DNA hybridization were designed using the 16S rRNA and 23S rRNA gene sequences of *Escherichia coli*, and endonucleases *Eco*RI, *Eco*RV, *Sal*I, *Pst*I, and *Hae*III were used for digestion of *F. necrophorum* DNA. A total of 9 to11 major DNA bands were observed in all tested *F. necrophorum* isolates after DNA digestion and hybridization. Of these bands, the presence or absence of 4 were able to accurately distinguish subsp. *necrophorum* and subsp. *funduliforme* strains. One 2.6 kb band was only present in isolates of subsp. *necrophorum*, while a 4.5 kb band was only observed in subsp. *funduliforme* isolates. Additionally, one 2.4 kb band was seen from all isolates originating from liver abscesses but none of the ruminal subsp. *necrophorum* strains. A final 5.3 kb band was observed in all subsp. *necrophorum* isolates from liver abscesses, but none from ruminal origin, whereas it was observed in all but three ruminal subsp. *funduliforme* strains. The use of rRNA gene probes proved superior to repeat DNA sequences due to their highly conserved nature, and their presence in multiple copies within bacterial genomes (Grimont and Grimont, 1986). It was concluded from this study that ribotyping is a valuable tool for distinguishing the two subsp. of *F. necrophorum* on a genetic basis, and suggested genomic differences in isolates from different locations within the same animal, which could be a valuable asset towards tracing the origin of a bacterial infection. However, it was highly recommended that future studies include isolates from more diverse sources to assess its applicability on a broader spectrum.

Since the use of rRNA probes showed promise in identification and differentiation of *F. necrophorum*, it was hypothesized that ribotyping could be utilized for investigating clonality of highly genetically similar isolates (Narayanan et al., 1997). Eleven matched sets of *F.*

necrophorum isolates originating from ruminal epithelial, liver abscess, and ruminal contents of cattle were subjected to ribotyping using endonucleases *Eco*I, *Eco*RV, *Sal*I, and *Hae*III, and the probes previously described by Okwumabua, targeting the 16S rRNA and 23S rRNA genes. For the purpose of establishing clonality, bacterial strains differing by one or more DNA hybridization bands were considered to be distinct strains. The ribotyping assay was found to differentiate *F. necrophorum* isolates within each subspecies, according to their location of origin, with liver abscess and ruminal epithelial isolates exhibiting unique banding patterns compared to the ruminal content isolate from the same animal. Using these criteria, clonality was inferred in 8 of the 9 matched sets of *F. necrophorum* isolates from ruminal epithelial and liver abscess origin. This was the first study to attempt to definitively elucidate the origin of *F. necrophorum* in liver abscesses, as the etiology had previously been reliant on the association between ruminal pathology and liver abscess presence (Jensen et al., 1954; Scanlan and Hathcock, 1983). Interestingly, the finding of a 2.6 kb band after double digestion with *Eco*I and *Eco*RV in all tested *F. necrophorum* isolates, regardless of their subspecies, was at odds with previous findings of this band only in subsp. *necrophorum* strains. Therefore, this DNA band is likely insufficient on its own for the differentiation of the subspecies, and should be evaluated in conjunction with other hybridization patterns. This study demonstrated ribotyping as an effective tool for investigating the origin of bacterial translocation into the liver, and establishing clonality between strains from different origins, although very few studies followed. By the early 2000s, the popularity of ribotyping in *F. necrophorum* had largely fallen out of favor due to the rapid development and increasing availability of other sequence-based technologies such as PCR and whole genome sequencing analysis.

Polymerase Chain Reaction (PCR) Assays

Polymerase chain reaction (PCR) assays were first described in 1986, and have become commonly used diagnostic tools for the specific identification of bacterial pathogens in a wide variety of samples (Zhu et al., 2020). Benefits to using PCR assays over other traditional methods for bacterial identification include high sensitivity and specificity to the target organism, low cost per sample and high throughput capabilities (Mackay, 2004; Zhu et al., 2020). A multitude of PCR assays have been developed for the purpose of identifying and quantifying *F. necrophorum* at the species and subspecies level from a variety of sample types.

Initially, random amplified polymorphic DNA, or RAPD PCR analysis was used on *F. necrophorum* isolates originating from bovine pathological lesions, to differentiate between subsp. *necrophorum* and subsp. *funduliforme* (Narongwanichgarn et al., 2001). The assay utilized four random primers for DNA amplification and the end products were analyzed using gel electrophoresis. The four primers contained G+C contents between 50% to 70%, and had previously been described in studies investigating RAPD DNA fingerprinting (Akopyanz et al., 1992; Williams et al., 1993). Three of the primers failed to differentiate between the two *F. necrophorum* subspecies, however, the primer WIL-2 was able to accurately differentiate 19 *F. necrophorum* isolates at the subspecies level based upon the presence of a 2.4 Kb band observed only in subsp. *necrophorum*. This RAPD PCR assay provided a fast, simple method for the differentiation of the two subspecies of *F. necrophorum*, though it was recommended that additional studies be conducted to enable the selection of species-specific PCR primers for the creation of a more targeted assay.

Following the successful application of RAPD PCR, a one-step duplex PCR assay was developed using specific primers for identification of *F. necrophorum* at the subspecies level (Narongwanichgarn et al., 2003). Additional PCR amplification of *F. necrophorum* was conducted using RAPD PCR, and primer pairs TP1-TP2 and WLF2-WLF1 were selected for use in the one-step duplex PCR assay. Primer pair WLF2-WLF1 was designed based on the previously described WIL-2 primer sequence, while primer pair TP1-TP2 was designed based on the previously described D11344 primer sequence (Akopyanz et al., 1992; Williams et al., 1993). Primer pair TP1-TP2 was observed to generate a 900 bp band specific to the *rpoB* gene, which is conserved between both subspecies of *F. necrophorum*, while primer pair WLF2-WLF1 generated a 250 bp DNA fragment only observed within subsp. *necrophorum* strains, and assigned as a putative hemagglutinin-related protein. Further testing of outgroup strains using the newly developed PCR assay showed a high level of specificity only to *F. necrophorum*. This assay was an improvement on the RAPD PCR due to its ease of standardization and the ability to maximize assay accuracy through the use of specific primers. The ability to utilize both primer pairs in a single one-step assay provided a relatively fast method for identification of *F. necrophorum* at the species and the subspecies level, eliminating the need for conducting sequential tests.

A quantitative, real-time PCR assay was developed based upon the work of Narongwanichgarn et al. for the detection and quantification of *F. necrophorum* in throat swab samples in clinical settings (Aliyu et al., 2004). For this assay, the previously described TP1 primer (Narongwanichgarn et al., 2003) was modified to create primer pair RPO forward- RPO reverse, which returned a 100% identity match with the *F. necrophorum rpoB* gene sequence. For the quantification of *F. necrophorum*, a pair of LightCycler FRET hybridization probes were

designed within the targeted *rpoB* region. Throat swab samples were screened in a clinical setting, and the real-time PCR assay demonstrated a high level of specificity and sensitivity to the *F. necrophorum* species. Due to the inability of this initial *rpoB*- based qPCR assay to differentiate between the two subspecies, a secondary qPCR assay was developed using a primer pair targeting the hemagglutinin-related protein gene (HAEM), for the specific detection and quantification of *F. necrophorum* subsp. *necrophorum*. Samples that were initially positive using the *rpoB* qPCR assay were then subjected to the secondary assay to classify them as either subsp. *necrophorum* (positive) or subsp. *funduliforme* (negative). The two assays performed sequentially resulted in a fast, simple, and sensitive method for the detection of *F. necrophorum* form clinical throat swab samples, without the need for culturing of the samples prior to bacterial identification.

The qPCR assay described by Aliyu was further optimized for use with *TaqMan* chemistry when it was used to validate a novel real-time PCR assay designed based on the *gyrB* gene sequence for the detection of *F. necrophorum* in clinical throat swab samples (Jensen et al., 2007). This was the first development of a *F. necrophorum* PCR assay that utilized a species-specific primer, which generated a 306 bp amplicon in both subspecies, in combination with subspecies-specific probes. Primers were selected based upon published *gyrB* sequences and analyzed for thier specificity to the *F. necrophorum* species. This was the first assay with the capability to simultaneously quantify both subspecies of *F. necrophorum* and displayed a high level of sensitivity, with lower limits of detection (LLOD) between 1.5×10^2 and 1.5×10^3 CFU/sample. The real-time qPCR assay was validated through sequencing of the generated amplicons, revealing extensive homology between the target sequences. This assay was subsequently recommended for use as a screening tool for diagnostics due to its ease of use and

rapid results. The quantification abilities of the assay allowed for nuance in diagnostics, where samples from clinically “normal” patients could be evaluated to investigate baseline bacterial populations.

Another novel PCR was developed for the detecting the *F. necrophorum* species in foot rot samples, utilizing the *lktA* gene sequence, encoding for leukotoxin production, for primer development (Zhou et al., 2009). The *lktA* gene is specific to *F. necrophorum*, with leukotoxin one of the major virulence factors impacting its pathogenicity, making it an ideal candidate for use as a primer. While the ability to identify virulent *F. necrophorum* strains by the presence of the *lktA* gene is appealing from a clinical standpoint, this PCR relied on gel electrophoresis for the evaluation of banding patterns for identification, making it less efficient and more intensive than the previously described real-time qPCR assay (Jensen et al., 2007).

In a further effort to reduce the time to diagnosis of *F. necrophorum* as an infectious agent in clinical veterinary medicine settings, a “Cycliplex” PCR assay was developed to identify *F. necrophorum* in oral samples obtained from captive wallabies. The aim was to optimize isolate characterization to the subspecies level by designing an assay that allowed for multiple reactions to run at once in separate tubes, reducing the labor needed for diagnostics. Five primer pairs were designed for use: Fuso1-Fuso2, a genus-specific primer pair targeting 16S rDNA; GyrB(F)-GyrB(R), a species-specific primer pair that targeted the gyrase B subunit (Jensen et al., 2007); WLF2-WLF1, targeting the hemagglutinin-related protein (Narongwanichgarn et al., 2003); 314F-314R, a primer pair specific to Eubacteria and based upon 16S rDNA; and lktA1-lktA2, targeting the leukotoxin gene. The cycliplex PCR assay differentiated subspecies by a positive Gyrase B gene fragment followed by either positive (subsp. *necrophorum*) or negative (subsp. *funduliforme*) hemagglutinin related protein gene fragment. In the same study, a follow-

up duplex PCR assay was designed for the detection and identification of *F. necrophorum* at the species level using the RpoB(F)-RpoB(R) primer-pair described by Aliyu et al., and the identification of the leukotoxin gene using the lktA1-lktA-2 primer pair described above (Zhou et al., 2009).

From 2013 to 2024, there was no new literature published regarding the development of PCR assays targeting *F. necrophorum*. In 2024, Deters et al. reported the development of a 3-plex real-time qPCR assay for the detection and quantification of *F. necrophorum* subsp. *necrophorum* and subsp. *funduliforme*, and *Fusobacterium varium*: an emerging human and animal pathogen, from bovine rumen fluid samples (Deters et al., 2024b). The assay was developed using a primer pair targeting the *hgdA* (R-2-hydroxyglutarylCoA dehydratase subunit alpha) gene for the differentiation of the *F. varium* and *F. necrophorum* species, with a primer pair targeting the *lktA* gene used for subspecies level differentiation of *F. necrophorum.* The novel assay was found to be highly specific, and sensitivities were 10^3 CFU/ml for each of the three targets. Additionally, the assay was found to be applicable to a variety of sample types, including homogenized epithelial tissues, ruminal and colonic contents, and purulent abscess material (Deters et al., 2024c). This assay demonstrated the potential to include other *Fusobacterium* species in a one-step assay, while maintaining high levels of target specificity and sensitivity, and broadening the potential application of PCR assay for diagnosing *Fusobacterium* infections.

"-Omics"- based characterization

In more recent years, there has been an explosion of interest in "-omics" -based characterization studies for the identification and characterization of bacterial pathogens, thanks to decreasing costs and increasing speeds of analyses (Quainoo et al., 2017). Limiting the

applicability of these technologies thus far is the relatively few complete *F. necrophorum* genomes available compared to many other clinically relevant pathogens. The majority of the available sequences belong to subsp. *funduliforme,* likely due to its more frequent involvement in human bacterial infections (Genbank records last accessed August 2nd, 2024). Whole genome sequencing can provide valuable insight into conserved regions within bacterial genomes, virulence and antimicrobial resistance gene prevalence, and can be used for a more definitive determination of clonality through the use of single nucleotide polymorphism (SNP) or average nucleotide identity (ANI) analyses (Khromykh and Solomon, 2015; Salipante et al., 2015; Amézquita et al., 2020; Rodriguez et al., 2024). Two main types of genome sequencing are available, short-read and long-read, both of which have unique advantages and disadvantages for their application in downstream analyses, as well as hybrid approaches combining the two (Amarasinghe et al., 2020; Hu et al., 2021; Marx, 2023).

The first bovine *F. necrophorum* subsp. *funduliforme* isolate was sequenced in 2014 using 454 sequencing technologies, and the WGS analysis revealed the presence of genes linked to lipopolysaccharide (LPS) biosynthesis, a major virulence factor involved in bovine host response modulation. These genes were absent in the publicly available human subsp. *funduliforme* sequences, revealing key differences within the same subspecies that would likely otherwise have been overlooked using the previously described molecular methods such as RFLP and PCR (Calcutt et al., 2014). Further whole genome sequencing studies were undertaken to compare the virulence factors in subsp. *necrophorum* and subsp. *funduliforme*, in an effort to understand the difference in pathogenicity between the two subspecies (Wright, 2016; Lyster et al., 2019; Umaña et al., 2019a). These studies identified previously unknown nuances in the genomes of both subspecies, providing potential targets for future diagnostic assays and

therapeutics, and resulted in the generation of numerous reference genomes that can be used in future studies for the comparison of novel isolates. As the number of publicly available *F. necrophorum* sequences increased, researchers began utilizing comparative genomics to further characterize the species and subspecies on a genomic level (Umaña et al., 2019c). The majority of these studies focused on virulence gene presence or absence, and investigated whether genes were conserved within species, subspecies, and strains (Umaña et al., 2019c; Bista et al., 2022). The information generated by these comparative genomic studies provide elucidation into potential targets for antimicrobials that can act with a higher level of specificity than traditional antibiotic therapies. Additionally, whole genome sequencing can establish clonality between isolates at a more definitive level than can be accomplished using previously developed methods, which can assist in tracing infections back to their initial sources with a higher level of confidence than previously possible (Deters et al., unpublished data).

With the rise in genomic-based *F. necrophorum* research, a database, Fusobase, was created in 2014 with the intent of facilitating comparative genomic analysis by providing annotation and bioinformatic tools specifically tailored towards *Fusobacterium* research (Ang et al., 2014). This database includes two *F. necrophorum* genomes, both sourced from publicly available Genbank sequences, as reference genomes. Following the creation of Fusobase, which is no longer accessible, another database: FusoPortal was created in 2018 to fill the gap left by the discontinuation of Fusobase (Sanders Blake et al., 2018). FusoPortal contains complete, annotated *Fusobacterium* sequences generated from long read sequencing, including one complete *F. necrophorum* genome. This online repository allowed for visualization of the published genomes, and contained integrated nucleotide and protein blast functions to compare novel sequences with those contained in the repository. These online repositories can create a

much more user-friendly option for conducting comparative genomic analyses focusing specifically on the *Fusobacterium* genus, although they should be used in conjunction with broader, more complete databases to allow for the investigation into novel genes and proteins previously not yet annotated in *Fusobacterium*.

In addition to whole genome sequencing, a select few studies have been conducted utilizing transcriptomics, metabolomics, and proteomics for *F. necrophorum* characterization (Antiabong et al., 2015; Wang et al., 2022; Bista et al., 2023). These additional "-omics" technologies provide an insight into the nuances of bacterial gene expression, metabolite production, and protein structures and functions at a deeper level than can be determined using whole genome sequencing alone.

To date, two transcriptomic studies have been described in *Fusobacterium necrophorum* to evaluate the effect of differing growth conditions on virulence factor expression, and to determine the signaling pathways involved in the apoptosis of macrophages. The first study was conducted to investigate the role of iron restriction in the expression of virulence genes in a sheep isolate of *F. necrophorum* subsp. *necrophorum* (Antiabong et al., 2015). Iron restriction was found to reduce the growth rate of *F. necrophorum* while upregulating numerous stress-specific virulence factors, most notably hemagglutinin and leukotoxin gene expression. Additionally, three other virulence genes, hemolysin, a yebN homolog, and a *tonB* homolog, were notably down regulated during periods of iron starvation. It was also discovered that iron restriction was responsible for promoting metabolism of diverse carbon sources for bacterial growth. These insights into bacterial stress responses can further elucidate the pathways for virulence gene regulation and bacterial persistence. A second transcriptomic study has been published investigating the action of *F. necrophorum* on sheep neutrophils and apoptosis (Wang

et al., 2022). This study uncovered that not only was cell proliferation inhibited by *F. necrophorum* in a time- and dose- dependent manner, but that apoptosis of macrophages and neutrophils was mediated by the activation of death receptor signaling pathways by *F. necrophorum*. Additionally, *F. necrophorum* infection promoted the gene expression of numerous inflammatory cytokines. These findings provided novel insight into the prevention and control of *F. necrophorum* infection from a mechanistic standpoint in the eukaryotic host cell. Finally, two proteomic and lipidomic based studies have been conducted for the characterization of outer membrane proteins specific to *F. necrophorum* and their potential for use in the development of therapeutics (Bista et al., 2023; He et al., 2023). One study characterized the effects of the 43 Kb outer membrane protein (OMP) on protein expression in MAC-T cells to investigate its role in inflammatory cytokine production (He et al., 2023). They found that treatment of cells with the 43 kb OMP resulted in the differential expression of 224 proteins showing a fold change of 1.2, and 34 proteins exhibiting a fold change of 1.5. The proteins affected were determined to be primarily involved in adhesion and regulation of biological processes. Additionally, the 43 Kb OMP of *F. necrophorum* was found to promote expression of pro-inflammatory cytokines through the activation of the NF-κB pathway. These findings shed new light onto the various pathogenic mechanisms employed by *F. necrophorum* during infection. A secondary study conducted by Bista detailed a lipidomic and proteomic approach for further outer membrane vesicle (OMV) characterization to investigate their potential for use as vaccine candidates (Bista et al., 2023). The majority of OMV- associated proteins were OMPs, including the previously described 43 kb OMP, and toxins. Lipidomic analyses revealed several lipid classes in the OMVs, with the findings that these lipid profiles can differ from those of the parent bacteria with the potential for modification to meet functional requirements. These

features may have roles in host-cell targeting and immunogenicity, making them valuable targets for use in vaccine development.

Conclusions

The evaluation of molecular characteristics of *F. necrophorum* have been used for identification purposes since the 1980s, however the research is lacking both in depth and in breadth compared to other common bacterial pathogens. Ribotyping, also referred to as DNA fingerprinting, was the first molecular-based methodology described for the identification of *F. necrophorum* isolates. The development of PCR assays allowed for the identification, quantification, and differentiation of *F. necrophorum* at the subspecies level, with the benefit of not requiring pure culture samples. These PCR assays were applicable to a variety of sample types, including, but not limited to, liver abscesses, oral swabs, ruminal fluid, colonic contents, foot swabs, and gastrointestinal epithelial tissues. Building upon the success of PCR assays, recent advances in "-omics"- based methodologies have drastically increased the number of *F. necrophorum* genome sequences publicly available. These sequences can be used for the creation of novel diagnostics, as well as references for comparative analysis into the genetic diversity within the species. Two different interactive online *Fusobacterium* repositories have been created to aid in the comparison of *F. necrophorum* sequences, however they both contain small numbers of *F. necrophorum* strains and have not been maintained since their launch. Additionally, a select number of "-omics"-based studies have been conducted for more in depth characterization of *F. necrophorum* gene expression, metabolite production, and protein structures and function. Future studies should aim to apply other "omics" technologies, such as metabolomics, proteomics, and transcriptomics, to better understand the *F. necrophorum* species and its pathogenic potential.

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Chapter 2 – A real-time PCR assay for the detection and quantification of *Fusobacterium necrophorum* and *F. varium* in ruminal contents of cattle

Abstract

Objectives: Develop and validate a quantitative PCR (qPCR) assay for the detection and quantification of the two subspecies of *F. necrophorum*, *necrophorum* and *funduliforme*, and a recently recognized species, *F. varium*, in ruminal contents of cattle collected at slaughter.

Materials and Methods: A qPCR assay with primers and probes designed to target *hgdA*, which encodes for 2-hydroxyglutaryl dehydratase, for *F. necrophorum* (*hgdA-n*) and *F. varium* (*hgdA-v*), and the leukotoxin promotor region, *lktA-n*, and *lktA-f*, for the two subspecies of *F. necrophorum* were developed and validated. A total of 345 ruminal fluid samples were collected in an abattoir immediately after slaughter, which included 181 (52.5%) samples from cattle with apparently healthy, non-abscessed livers and 164 (47.5%) from cattle with abscessed livers. Basal media with lactate or lysine as the major energy source and each with or without josamycin, vancomycin and norfloxacin were used to enrich ruminal samples that were below the lower limit of quantification.

Results and discussion: The subsp. *necrophorum* was quantified in 22% and 29% of samples from cattle with non-abscessed and abscessed livers, respectively ($P = 0.004$). The subsp. *funduliforme* and *F. varium* were prevalent in almost all ruminal samples (98 to 100%) and were not different between cattle with or without abscessed livers. The mean concentrations of the two subspecies and *F. varium* were 10^3 to 10^4 CFU per ml and were not different between cattle with abscessed or non-abscessed livers.

Implications and Applications: A major finding of the study was that only a small proportion of cattle ruminal contents harbored the subsp. *necrophorum*, which suggested that it is not a normal member of the ruminal microbial community. In contrast, the subsp. *funduliforme* and *F. varium* were prevalent in all ruminal contents tested. Because *F. varium* is a pathogen, the question whether the species contributes to the development of liver abscesses needs to be investigated.

Keywords: Polymerase chain reaction, rumen, cattle, *Fusobacterium necrophorum*, *Fusobacterium varium*

Introduction

Fusobacterium necrophorum is a normal ruminal inhabitant, contributing to lactic acid fermentation and amino acid fermentations. The species is the primary causative agent of liver abscesses in feedlot cattle (Scanlan and Hathcock, 1983; Nagaraja and Chengappa, 1998), which result in a significant economic impact to the beef cattle industry (Brown and Lawrence, 2010; Reinhardt and Hubbert, 2015). The *F. necrophorum* species is divided into two subspecies: subsp. *necrophorum* and subsp. *funduliforme* (Shinjo et al., 1991) which differ in morphologies, biochemical characteristics, and virulence (Tadepalli et al., 2009). Differences in virulence between the two subspecies are attributed to higher production of leukotoxin by subsp. *necrophorum,* which is more frequently associated with liver abscesses (Tan et al., 1992; Amachawadi and Nagaraja, 2022).

Historically, the determination of ruminal *F. necrophorum* concentrations in ruminal contents has relied on culture-based methods, with concentrations in the range of 10^3 to 10^6 MPN/ml (Wada, 1978; Shinjo, 1979; Tan et al., 1994). Because both subspecies are lactate fermenters and produce indole, the culture method does not distinguish between the two *F. necrophorum* subspecies. Recently, *Fusobacterium varium* was reported to be the dominant *Fusobacterium* in the rumen (Schwarz et al., 2023), where its role appears to be similar to *F. necrophorum* in that it utilizes lactate, ferments amino acids, and produces indole (Donahue, 1990; Bailey and Love, 1993; Olsen, 2014), suggesting that the previous *F. necrophorum* abundance estimation may have included *F. varium*.

Quantitative PCR (qPCR) assays have been reported for the identification and quantification of *F. necrophorum* both at the species and subspecies levels (Narongwanichgarn et al., 2001; Narongwanichgarn et al., 2003; Jensen et al., 2007; Antiabong et al., 2013; Sanmillan et al.,

2013). None of these PCR assays were designed to differentiate *F. necrophorum* and *F. varium*, therefore, our objective was to develop and validate a qPCR assay for the detection and quantification of *F. necrophorum* at the species and subspecies level and *F. varium* in ruminal contents of cattle. Additionally, enrichment media were developed and evaluated to detect *F. necrophorum* and *F. varium* in ruminal contents that were below the detection limit of the qPCR assay.

Materials and Methods

No live animals were used in the study; therefore, no Institutional Animal Care and Use Committee approval was required. All samples were collected post-harvest at a commercial abattoir.

Primer and probe design

The following genes were used for targeted detection and quantification: *hgdA*, which encodes 2-hydroxyglutaryl dehydratase, an enzyme involved in the glutamate fermentation, for *F. necrophorum* (*hgdA-n*) and *F. varium* (*hgdA-v*) and the promotor region (*lktA-n,* and *lkt-f*) of the leukotoxin operon (*lktBAC*) to differentiate the two subspecies of *F. necrophorum* (Zhang et al., 2006). The primers and probes for *hgdA-n, hgdA-v*, *lktA-n,* and *lktA-f* were designed based on evaluation of sequences from the NCBI GenBank database available at the time (56 *lktA* sequences and 47 *hgdA* sequences; (Benson et al., 2013). Sequences from the database were aligned using ClustalX version 2.1, and viewed in BioEdit version 7.1.3.0 for primer and probe selections. Primer and probe candidates with the most matched target sequences that did not match closely related non-target sequences were chosen for further analyses (Table 2-1). The selected primers and probes were synthesized by and obtained from Integrated DNA Technologies (Coralville, IA).

Bacterial Culture Preparation

Fusobacterium necrophorum subsp. *necrophorum* strains 430A and 90A, and subsp. *funduliforme* strains 16B and 45B, of bovine liver abscess origin, and *F. varium* strains CT7 and WL1 isolated from ruminal contents (Schwarz et al. 2023) were used initially for optimization and validation of the qPCR assay. Bacterial isolates, stored in pre-reduced, anaerobically sterilized (PRAS) Brain-Heart Infusion (BHI) agar slants at -80° C, were streaked onto blood agar plates (Tryptic soy agar with 5% sheep blood; Remel Inc., Lenexa, KS) and incubated anaerobically at 37°C for 48 hours. Species and subspecies identities were confirmed by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry analysis for *F. varium* and subsp. *necrophorum*, and for subsp. *funduliforme*, which is not identifiable by MALDI-TOF, a commercial biochemical kit, RAPID-ANAII (Remel Inc., Lenexa, KS), was used (Tan et al., 1994).

Assay Specificity Determination

The specificity of the assay was determined using several strains of the target species and subspecies of *F. necrophorum* and *F. varium*, other species of *Fusobacterium*, and a number of other Gram-negative and Gram-positive bacterial species. A total of 28 *Fusobacterium* species and strains were used for this assay, including seven subsp. *necrophorum* (390A, 430A, 439A, 61A, 46A, 2016-13 11A, and 88A) and six subsp. *funduliforme* (2018-13 7B, 2018-13 16B, 2018-13 35B, 2018-13 45B, FN-H3, and FN-H4). The other species of *Fusobacterium* tested included *F. varium* (strains WL1, 27725, CT7, and 2021-07 AM). *F. russii* (2 strains), *F. gastrosuis* (2 strains), *F. ulcerans* (3 strains), *F. nucleatum* (1 strain), *F. naviforme,* H335 (1 strain), *F. equinum* (1 strain), and *F. gonidiaformans* (1 strain). Additionally, a total of 49 other bacterial species (strains) were tested, which included: *Trueperella pyogenes* (1 strain), *Listeria*

monocytogenes (1 strain), *Streptococcus pyogenes* (1 strain), *Serratia marcescens* (3 strains), *Morganella morganii* (2 strains), *Salmonella enterica* subsp. *enterica* (7 serotypes: *S.* Typhimurium (3 strains), *S.* Bareilly (1 strain), *S.* Derby (1 strain), *S.* Enteritidis (2 strains), *S.* Infantis (1 strain), *S.* Lubbock (1 strain), *S.* Reading (1 strain), *Enterococcus faecium* (1 strain), *Enterococcus faecalis* (2 strains)*, Enterococcus casseliflavis* (1 strain), *E. coli* 074 (1 strain), *E. coli* O26 (1 strain), *E. coli* O45 (1 strain), *E. coli* O103 (1 strain), *E. coli* O111 (1 strain), *E. coli* O121 (1 strain), *E. coli* O145 (1 strain), *E. coli* O157 (1 strain), *E. coli* O104 (1 strain), *E. coli* (ATCC 25922), *Campylobacter jejuni* (1 strain), *C. coli* (1 strain), *C. fetus* (1 strain), *C. lari* (1 strain), *C. upsaliensis* (1 strain), *C. hyointestinalis* (1 strain). *Actinobacillus pleuropneumoniae* (1 strain), *Bordetella bronchiseptica* (1 strain), *Enterobacter aerogenes* (2 strains), *Klebsiella pneumoniae* (1 strain), *Mannheimia haemolytica* (1 strain), *Pasteurella multocida* (1 strain), *Proteus mirabilis* (1 strain)*, Proteus vulgaris* (1 strain), *Pseudomonas aeruginosa* (1 strain)*,* and *Pseudomonas stutzeri* (1 strain).

Assay Sensitivity Determination

Sensitivity of the qPCR assay was determined using pure cultures of *F. necrophorum* subsp. *necrophorum* (strains 2016-13 430A and 2016-13 90A), *F. necrophorum* subsp. *funduliforme* (strains 2018-13 16B and 2018-13 45B), and *F. varium* (strains WL1 and CT7). Briefly, a single isolated colony from a blood agar plate was inoculated into 10 ml of PRAS-BHI broth and incubated at 37°C until a late logarithmic phase growth was reached (approx. 12 h). The culture tube was mixed by vortexing and 300 µl was inoculated into 10 ml of PRAS-BHI broth and incubated at 37°C until an absorbance of 0.4 at 600 nm. An aliquot of the culture was serially diluted in PRAS-BHI and 100 µl of the appropriate dilutions (10^{-4}, 10^{-5} and 10^{-6}) were spread-plated on blood agar plates and incubated anaerobically at 37°C for 24 h to determine bacterial

concentration (CFU/ml). All serial dilutions and spread plating were performed in an anaerobic glovebox (Thermo Fisher Scientific, Waltham, MA). Initial concentrations of the pure-cultures used were 5.43 x 10^7 CFU/ml (2016-13 430A), 5.67 x 10^7 CFU/ml (2016-13 90A), 1.81 x 10^8 CFU/ml (2018-13 16B), 2.36 x 10^8 CFU/ml (2018-13 45B), 9.23 x 10^7 CFU/ml (WL1) and 2.68 x 10^8 CFU/ml (CT7). One milliliter aliquot of the undiluted culture was transferred to a 2-ml microcentrifuge tube, boiled for 10 min and centrifuged at 9,300 RCF for 5 min to extract bacterial DNA. Serial dilutions of the DNA extract were then made with sterile distilled water for use in the qPCR assay to determine the lower limit of detection (LOD) for each bacterial target.

qPCR Assay Running Conditions

The PCR reactions were carried out in 96-well plates containing 20 µl of reaction mix composed of the following: 10 µl of IQ Multiplex Powermix (2X) (Bio-Rad, Hercules, USA), 1 µl of primer mix (at 5 pM/µL for each primer), 1 µl of *F. necrophorum* subsp. *necrophorum* probe (5 pM/µl), 1 µl of *F. necrophorum* subsp. *funduliforme* probe (3 pM/µl), 1 µl of *F. necrophorum* probe (1.25 pM/µl), 1 µl *F. varium* probe (5 pM/µl), 3 µl double-distilled H_2O, and 2 µl of template DNA. Probe concentrations were optimized for each target gene. The final assay running conditions were 95° C for 5 min followed by 45 cycles of 95° C for 15 sec and 60° C for 40 sec. Positive (DNA mix of the two subspecies and *varium*) and negative controls (nuclease-free water) were included in all PCR runs. Assays were performed with the BioRad CFX96 Real-Time System.

Development and Validation of Enrichment Media

A PRAS basal medium (peptone, yeast extract, and salts solution; PY) with sodium lactate (100 mM; Thermo Fisher Scientific; PY-La) or lysine (100 mM; Thermo Fisher Scientific; PY-

Ly) as the major energy source, each with or without selective antibiotics, was prepared. The selective antibiotics (Brazier et al., 1991) included were josamycin (3 µg/ml), vancomycin (4 µg/ml) and norfloxacin (1 µg/ml; PY-La JVN and PY-Ly JVN). The pH of the medium before autoclaving was 7.2 (± 0.1).

Ruminal Contents Sample Collection

Sample collection period was from November 2021 to April 2022. Ruminal content samples were collected from 345 cattle originating from 12 feedlots at a commercial beef processing facility in the Midwest. Rumens were identified as belonging to cattle with apparently healthy, non-abscessed livers (n= 181) or with abscessed livers (n= 164). Approximately 45 ml of ruminal contents were collected into a 50 ml screw-capped centrifuge tube. Samples were tightly capped and transported to the Anaerobe Laboratory for immediate processing. Each sample was strained through 3 layers of cheese cloth and 1 ml of strained ruminal fluid was inoculated into 9 ml of each of four enrichment media (PY-La, PY-La JVN, PY-Ly, and PY-Ly JVN) and incubated at 37°C for 24 h. Remaining strained rumen fluid was aliquoted in duplicate into 2-ml microcentrifuge tubes and frozen at -20°C for the qPCR assay.

DNA Extraction

DNA was extracted from rumen fluid samples, before and after enrichment, using the GeneClean Turbo Kit (MP Biomedicals, Solon, USA). Briefly, 1 ml aliquots of samples were boiled for 10 min, centrifuged at 9,300 x g for 5 min and 100 µL of the supernatant was added to 500 µl of GeneClean Turbo Salt Solution in a 2 ml microcentrifuge tube and gently mixed. The suspension was transferred to a GeneClean Turbo cartridge and centrifuged at 14,000 x g for 5 seconds. The flow-through in the catch tube was discarded and 500 µl of GeneClean Wash solution was added to the cartridge, which was again centrifuged at 14,000 x g for 5 seconds.

The flow-through was again discarded and the cartridge was centrifuged a third time at 14,000 x g for 4 minutes. Cartridges were removed from the catch tube and placed into a new collection tube, 30 µl of GeneClean Turbo Elution solution was added, and tubes were incubated at room temperature for 5 min. Post-incubation, the cartridge was centrifuged at 14,000 x g for 1 minute to elute the DNA. Eluted DNA was stored at -20°C until use.

Statistical Analysis

Targeted species or subspecies prevalence in ruminal contents were considered positive when PCR assay was positive pre- or post-enrichment and negative when PCR assay was negative pre- and post-enrichment. The binary outcome of prevalence was analyzed using the logit linear mixed model. Fixed effects of the model included liver status (non-abscessed or abscessed), enrichment method (none, PY-La, PY-La JYN, PY-Ly, PY-Ly JVN), and their interactions. Random effects included feedlot and animal nested within feedlot. The back-transformed least-squares means (i.e. rates) and differences (i.e. log odds ratios) were used to assess the fixed effects. For samples that were PCR negative pre-enrichment, the binary outcome of PCR results of enriched samples were analyzed using the logit linear mixed model. Fixed effects of the model included liver status, enrichment method (PY-La, PY-La JYN, PY-Ly, PY-Ly JVN), and their interaction. Random effects included animal nested within feedlot. The back-transformed least-squares means and differences were used to assess the fixed effects.

For samples that were PCR negative pre-enrichment, the numeric outcome of qPCR contained a sizable portion of left censored values and was analyzed using the log-normal frailty model (i.e. a linear mixed model for log transformed responses with censoring). Measurements below LLOD were left censored and contributed to the target likelihood function with the normal cumulative distribution function at the log of LLOD. Measurements above LLOD contributed to the target

likelihood function with the probability density function at the log of CFU values. Fixed effects of the model included liver status, enrichment method, and their interaction. Random effects included animal nested within feedlot and the error term. The transformed least-squares means and differences in $\log_{10}$(CFU/ml) were used to assess the fixed effects. For *F. necrophorum* ssp. *necrophorum*, methods with PY-Lys and PY-Lys JVN were excluded from the statistical modeling due to high portions of below-LLOD samples.

The numeric outcome of qPCR pre-enrichment was left-censored and therefore underwent the log-normal frailty modeling. Fixed effects of the model included liver status and feedlot. Random effects included animal nested within feedlot and the error term. Feedlot was treated as a fixed effect to avoid non-convergence of likelihood maximization. The transformed least-squares means and differences in $\log_{10}$ (CFU/ml) were used to assess the fixed effects. Disregarding results of the test for interaction, the effect of liver status was evaluated within each enrichment method and the effect of enrichment method was evaluated within a given liver status. All tests were two-sided and were conducted at the 0.05 level. No multiplicity adjustment was applied. Statistical analyses were performed via Statistical Analysis Software (SAS version 9.4; Cary, NC) GLIMMIX and NLMIXED procedures.

Results and Discussion

Validation of the qPCR Assay

Of all the bacterial species and strains used for specificity determination, none yielded amplification except strains of *F. necrophorum* subsp. *necrophorum*, *F. necrophorum* subsp. *funduliforme*, and *F. varium*. All *F. necrophorum* and *F. varium* strains used for specificity determination resulted in amplification of the qPCR primers and fluorescence of the respective targeted probes. The lower limits of detection (LOD) of the qPCR assay with pure cultures were

5.5 x 10^2 CFU/ml for *F. necrophorum* subsp. *necrophorum*, 1.5 x 10^3 CFU/ml for *F. necrophorum* subsp. *funduliforme*, and 1.8 x 10^3 CFU/ml for *F. varium*. The primers and probes targeting *hgdA-fn* gene to detect and quantify *F. necrophorum* at the species level was initially used for specificity studies and subsequently was not included in the assay.

Molecular detection methods such as random amplified polymorphic DNA-PCR (Narongwanichgarn et al., 2001; Narongwanichgarn et al., 2003), ribotyping (Narayanan et al., 1997), conventional PCR assays (Narongwanichgarn et al., 2003; Jensen et al., 2007; Antiabong et al., 2013; Sanmillan et al., 2013) and real-time PCR assays (Jensen et al., 2007; Brooks et al., 2014) have been developed to detect at the species level and to differentiate the two subspecies. The genes that have been targeted in PCR assays include 16S rDNA (genus-specific), *haem* (hemagglutinin), *rpoB* (RNA polymerase subunit B), *gyrB* (gyrase subunit B), and *LktAB* (leukotoxin A and B region). However, none of the PCR assays were designed to differentiate *F. necrophorum* and *F. varium.*

The PCR assay reported in our study targeted the promoter region (*lktA*) of the three genes operon of the leukotoxin (*lktABC*), which differs in sequence and length between the two subspecies (Zhang et al., 2006), and *hgdA*, which encodes for 2-hydroxyglutaryl dehydratase, an enzyme involved in glutamate fermentation, to differentiate *F. necrophorum* and *F. varium*. The *gyrB* gene, which has been used to detect and quantify *F. necrophorum* (Jensen et al., 2007), was not used because of the limited sequence information available for the *gyrB* gene from *F. varium.* Also, the two ruminal *F. varium* isolates that have been whole genome sequenced did not contain the leukotoxin gene (Schwarz et al., 2023).

DNA extraction of ruminal contents used in the study was based on boiling to lyse the cells and the lysate and the DNA was purified by GeneClean Turbo Kit. We have utilized this method

for PCR-based detection and quantification of foodborne pathogens in feces of cattle (Noll et al., 2015). It is possible that a different extraction method may have given us a higher DNA yield (Vaidya et al., 2018).

Prevalence and Concentrations of Fusobacterium necrophorum and F. varium in Rumen Fluid Before Enrichment

Fusobacterium necrophorum subsp. *necrophorum* was detected in quantifiable concentrations in 22% of samples from cattle with non-abscessed livers, and in 29% of samples from cattle with abscessed livers. This difference was statistically significant ($P = 0.004$; Table 2-2). The prevalence of subsp. *funduliforme* was much higher than subsp. *necrophorum*, reaching 94% and 91% of ruminal content samples from cattle with non-abscessed livers and cattle with abscessed livers, respectively (Table 2-2). *Fusobacterium varium* was detected in 49% of ruminal content samples from cattle with non-abscessed livers and in 63% of ruminal content samples from cattle with abscessed livers, but the difference was not significant ($P = 0.73$). The mean concentrations of the two *F. necrophorum* subspecies and *F. varium* were in the range of 10^3 to 10^4 CFU per ml of strained ruminal fluid (Table 2-3). The concentrations were not different between ruminal contents collected from cattle with non-abscessed or abscessed livers.

Thus far, the enumeration of *F. necrophorum* concentrations in ruminal contents has relied upon culture methods based on bacterial growth in a selective medium containing the antibiotics bacitracin, gentamycin, and streptomycin, with lactate as a major carbon source and indole production as an indicator of *F. necrophorum* growth (Tan et al., 1994). However, this method enumerated *F. necrophorum* at the species level and did not differentiate the two subspecies, and more likely, and possibly erroneously, included *F. varium* because of similar biochemical activities (lactate utilizer and indole producer) to *F. necrophorum* (Schwarz et al., 2023), thus,

confounding the true prevalence of *F. necrophorum* in the rumen. A major advantage of our novel qPCR assay was that it differentiated the two subspecies and *F. varium.*

Prevalence and Concentrations of *F. necrophorum* and *F. varium* in Ruminal Fluid After Enrichment

Because the limits of quantification of the two subspecies of *F. necrophorum* and *F. varium* were 10^2 to 10^3 CFU per ml or g, an enrichment step was deemed necessary to identify samples that contained concentrations below the limits of quantification. Both *F. necrophorum* and *F. varium* have been shown to utilize lactate and lysine as energy sources (Tan et al., 1994; Russell, 2005; Olsen, 2014; Schwarz et al., 2023). Therefore, a basal medium composed of peptone and yeast extract with either lactate or lysine as the major carbon source and each with or without the three antibiotics, josamycin, vancomycin and norfloxacin, were used. The JVN antibiotics collectively inhibit a number of anaerobic and facultative Gram-negative and Gram-positive bacteria, thereby promoting the growth of *Fusobacterium* species (Brazier et al., 1991). Although all three targeted bacteria were enriched by lactate and lysine, the lactate-JVN broth was the most effective enrichment medium for subsp. *necrophorum,* and both lactate- and lysine-JVN broths were equally effective in enriching the subsp. *funduliforme* and *F. varium.* Lysine has been used to enrich fusobacteria from ruminal contents (Russell, 2005), however, lysine supplementation of cattle diets did not enrich the population of *Fusobacterium* in the rumen, possibly because of pH-dependent inhibition by fermentation acids (Russell, 2006). In those studies, isolates of *F. necrophorum* were not differentiated into subspecies.

Ruminal fluid samples that were negative by qPCR assay were subjected to enrichment in each of the four selective media containing either lactate or lysine as the major energy substrate, and each with or without the addition of josamycin, vancomycin, and norfloxacin (PY-la, PY-La

JVN, PY-Ly, and PY-Ly JVN, respectively). The evaluation of enrichment media, based on lactate utilization and lysine degradation, intended to enrich both subspecies of *F. necrophorum* and *F. varium* resulted in noticeably different results between the three targeted bacteria. In the case of *F. necrophorum* subsp. *necrophorum*, the number of samples positive was higher ($P < 0.05$) in medium containing lactate (107/257; 41.6%) compared to the lysine medium (3/257; 5.1%) with or without JVN (Table 2-3). The PY-La medium with JVN tended to enrich more samples than the medium with no JVN, although the difference (30 vs. 13%) was significant only with ruminal contents from cattle with no liver abscesses. No noticeable enrichment of subsp. *necrophorum* was apparent after enrichment in lysine medium, with or without the antibiotics. However, with subsp. *funduliforme* and *F. varium*, there were no differences in enrichment with media containing the two energy sources or selective antibiotics (Table 2-3). Although enrichment of the samples in the four media detected more samples as positive, the concentrations were below the lower limit of detection for subsp. *necrophorum* (Table 2-4). The concentrations of subsp. *funduliforme* and *F. varium* after enrichment ranged from 10^3 to 10^6 CFU per ml of strained ruminal fluid. The concentrations of subsp. *funduliforme* were 2 log units higher in lactate medium with JVN than without JVN, and concentrations were not different between lysine media with or without JVN. The concentration of *F. varium* enriched in lactate medium with and without JVN, and in lysine medium concentration with or without JVN were similar (Table 2-4).

Cumulative Prevalence of *Fusobacterium* species in the rumen

Pre- and post- enrichment data were combined to determine the cumulative prevalence of each of the three targeted organisms (Figure 2.1). The cumulative prevalence of subsp. *necrophorum* was 39% of samples from cattle with non-abscessed livers and 43% of samples

from cattle with abscessed livers. In contrast, the prevalence of subsp. *funduliforme* was detected in almost 100% of samples from cattle with non-abscessed or abscessed livers. Similarly, *F. varium* was present in nearly all samples from cattle with non-abscessed and abscessed livers (97% and 98%, respectively).

A major, and surprising, finding of this study was that the subsp. *necrophorum* was not prevalent in all ruminal samples tested and less than 50% of cattle sampled for detection and quantification were positive. An obvious limitation of the study was that these observations were based on one-time sampling of ruminal contents at slaughter, therefore, it is unknown whether an animal negative for subsp. *necrophorum* at the time of slaughter was positive at any time during the finishing period. The fact that a proportion of cattle with abscesses were negative for subsp. *necrophorum* at the time of slaughter suggests that the cattle were likely positive sometime during the finishing phase. A longitudinal study with repeated sampling is needed to understand the dynamics of *Fusobacterium* population prevalence and fluctuations. The higher prevalence of subsp. *necrophorum* in the rumen of cattle with liver abscesses compared to the rumen of cattle with no abscessed livers may be interesting, but not likely meaningful. The samples were collected at slaughter, but liver abscess development happened long before the sample collection. Therefore, any link between ruminal prevalence and concentration at the time of slaughter to liver abscess presence is not relevant. Also interesting is that a proportion of cattle that had no liver abscesses were positive for subsp. *necrophorum*.

The prevalence based on quantifiable subsp. *necrophorum* was even lower than mere prevalence based on detection. Thus far, a relationship between ruminal concentration of *F. necrophorum* and liver abscesses has never been demonstrated, and whether other factors may work together with *F. necrophorum* to cause liver abscesses is unclear. Alternatively, subsp.

funduliforme, which is less virulent than subsp. *necrophorum* and less frequently isolated from liver abscesses, was present in nearly all samples, albeit in low concentrations ($10^3 – 10^4$ CFU/ml). Therefore, subsp. *funduliforme,* and not subsp. *necrophorum,* is more likely a normal member of the ruminal microbial community.

Inclusion of *F. varium* in the present study was because of the recent finding by Schwarz et al. (2023) that *F. varium* is the dominant ruminal *Fusobacterium* species. The prevalence of *F. varium* in high proportion (98%) of ruminal contents of cattle tested supports the finding of Schwarz et al (2023), who used culture methods with selective agar and 16S rRNA metagenomic analysis, and identified *F. varium* in every ruminal sample evaluated and under conditions intended to selectively enumerate *F. necrophorum*. *Fusobacterium varium* is a known human and animal pathogen (Lee et al., 2022), and has the ability to invade epithelial cells (Ohkusa et al., 2002), but its association with ruminitis and liver abscesses in cattle has not been investigated. A preliminary genomic analysis of two *F. varium* isolates from the rumen revealed the presence of virulence genes related to the pathogenic human *F. varium* strains associated with active invasion of mammalian cells (Schwarz et al., 2023).

Applications

A novel qPCR assay to detect and quantify the two subspecies of *F. necrophorum* and a recently recognized *Fusobacterium* species, *F. varium,* in ruminal contents of cattle was developed and validated. The assay was used to detect and quantify the three-targeted bacteria in ruminal contents of cattle collected at slaughter. A major finding of the study was that only a small proportion of cattle ruminal contents harbored the subsp. *necrophorum,* which suggested that it may not be a normal member of the ruminal bacterial community. In contrast, the subsp. *funduliforme* and *F. varium* were prevalent in almost all ruminal contents tested. Because *F.*

varium is a known pathogen with the ability to invade tissues, the question whether the species contributes to the development of liver abscesses needs to be investigated. Additionally, this qPCR assay could be utilized to investigate the presence and concentration of *Fusobacterium necrophorum* or *varium* in clinical samples, such as those collected from oral or urogenital samples, respiratory secretions, and hoof swabs. This would allow for a more rapid identification of *Fusobacterium* species and subspecies in infections, which would facilitate prompt initiation of antibiotic therapy and better clinical outcomes.

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Table 2-1 Species and subspecies of Fusobacterium, genes targeted, and primers and probes sequences used in the four-plex quantitative PCR assay

Target species and subspecies	Gene target	Primer sequence (5'-3')	Probe sequence (5'-3')	Amplicon size
Fusobacterium necrophorum	*hgdA-n*	Forward: CTTTTTCCAATACGGTAGATACTCC Reverse: CCTGTCAATTCTTCCAACTGC	5'TexasRed-X-TGGATTATTTGATTGGACAGTTCGA-Iowa Black RQ	94 bp
Fusobacterium necrophorum subsp. *necrophorum*	*lktA-n*	Forward: GCTTTGGAAGAAGCCAAACA Reverse: AATGCTTCCATTCGGATTCA	FAM-TGGAATCATTCCAGTAGATGGAAAAG-ZEN™/3'IB®	93 bp
Fusobacterium necrophorum subsp. *funduliforme*	*lktA-f*	Forward: AAAGACGCTCAAAATAGCAAAGTT Reverse: TTTGGATTCAACGGAATCTTG	MAX-TTGTTCCACAACAGGATGGGAGTA-ZEN™/3'IB®	80 bp
Fusobacterium varium	*hgdA-fv*	Forward: TTCAAATACAGTGGATACACCAGAA Reverse: AATTCTTCTAATTGTTTGATTGCATAA	Cy5-AGTGGATTATCTAATCGGACAATTTGA-Iowa Black RQ	84 bp

Table 2-2 Prevalence and mean concentrations of Fusobacterium species and subspecies in ruminal contents of cattle with or without abscessed livers abscesses collected at slaughter

Species or subspecies	Liver abscess status	Prevalence, No. positive/total (%)	Mean concentration (CFU/ml)
F. necrophorum subsp. *necrophorum*	Non-abscessed	40/181 (22)	6.22×10^3
	Abscessed	48/164 (29) *	6.91×10^3
F. necrophorum subsp. *funduliforme*	Non-Abscessed	170/181 (94)	1.41×10^4
	Abscessed	149/164 (91)	2.24×10^4
F. varium	Non-Abscessed	88/181 (49)	2.71×10^4
	Abscessed	103/164 (63)	2.06×10^4

*Differs from non-liver abscessed cattle at $P = 0.004$

Table 2-3 Prevalence of Fusobacterium species and subspecies, based on quantitative PCR assay, in rumen fluid after enrichment from cattle with and without liver abscesses collected at slaughter

Species or subspecies	Liver abscess status	No. positive after enrichment in (%): Peptone yeast extract-lactate	Peptone yeast extract-lactate plus JVN[1]	Peptone yeast extract-lysine	Peptone yeast extract-lysine plus JVN
F. necrophorum subsp. *necrophorum*	Non-abscessed	19/141 (13)[a]	42/141 (30)[b]	3/141 (2)[c]	4/141 (3)[c]
	Abscessed	19/116 (16)[a]	27/116 (23)[a]	3/116) (3)[b]	3/116 (3)[b]
F. necrophorum subsp. *funduliforme*	Non-abscessed	9/11 (82)[a]	11/11 (100) [a]	10/11 (91)[a]	10/11 (91)[a]
	Abscessed	8/15 (53)[a]	14/15 (93)[b]	9/15 (60)[a]	10/15 (67) [a,b]
F. varium	Non-abscessed	84/93 (90)[a]	87/93 (94)[a]	87/93 (94)[a]	87/93 (94)[a]
	Abscessed	56/61 (92)[a]	57/61 (92)[a]	55/61 (90)[a]	57/61 (93)[a]

[1] JVN = Josamycin (3 µg/L), Vancomycin (4 µg/L), Norfloxacin (1 µg/L)

[a,b,c] Means within rows not sharing the same superscripts are significantly different (P > 0.05)

Table 2-4 Mean concentrations of Fusobacterium species, based on quantitative PCR assay, in rumen fluid before or after enrichment from cattle with and without liver abscesses collected at slaughter

Species or subspecies	Liver abscess status	Concentration after enrichment (CFU/ml) in: Peptone yeast extract-lactate	Peptone yeast extract-lactate plus JVN[1]	Peptone yeast extract-lysine	Peptone yeast extract-lysine plus JVN[1]
F. necrophorum subsp. *necrophorum*	Non-abscessed	< LLOD	< LLOD	n/a	n/a
	Abscessed	< LLOD	< LLOD	n/a	n/a
F. necrophorum subsp. *funduliforme*	Non-abscessed	3.2×10^{4a}	1.3×10^{6b}	2.8×10^{4a}	4.8×10^{4a}
	Abscessed	1.7×10^{3a}	1.4×10^{5b}	1.8×10^{3a}	3.8×10^{3a}
F. varium	Non-abscessed	2.3×10^{5a}	4.4×10^{6b}	2.8×10^{6b}	3.4×10^{6b}
	Abscessed	4.8×10^{5a}	8.1×10^{6b}	3.7×10^{6b}	5.1×10^{6b}

[1] JVN = Josamycin (3 µg/L), Vancomycin (4 µg/L), Norfloxacin (1 µg/L)

< LOD: Avg. concentration was lower than the lower limit of detection (due to inclusion of all samples subjected to enrichment, not just those that yielded positive results)

[a,b]Means within rows not sharing superscripts are significantly different.

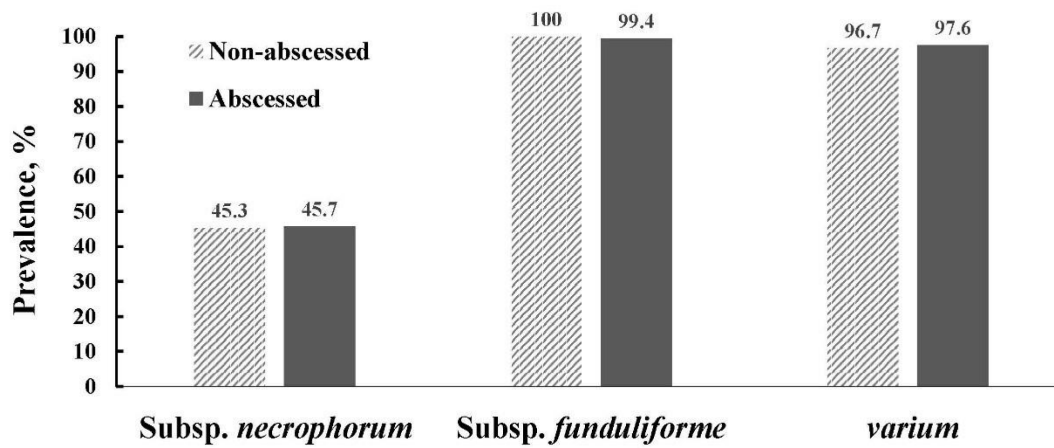

Figure 2-1 Cumulative prevalence of Fusobacterium species, based on quantitative PCR assay, in rumen fluid, before and after enrichment, of cattle with and without liver abscesses collected at slaughter

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Chapter 3 Comparative whole genome analyses of *Fusobacterium necrophorum* subspecies *necrophorum* isolates from liver abscesses and matched ruminal and colonic epithelial tissues of cattle

Abstract

Fusobacterium necrophorum is a Gram-negative, anaerobic bacterium commonly found in gastrointestinal tract of cattle, where it contributes to fermentation of lactate and amino acids. The species is split into two subspecies: subsp. *necrophorum* and subsp. *funduliforme*, the former being the primary causative agent of liver abscesses in cattle. Liver abscesses are a major economic concern to the beef cattle industry, and many studies have been conducted in an attempt to elucidate the etiology and pathogenesis of their development. Historically, *F. necrophorum* has been hypothesized to translocate through the ruminal epithelium to enter and colonize the liver via portal vein circulation secondary to rumen epithelial damage, although recent research findings have suggested the hindgut as a potential source of liver abscess pathogens. Therefore, our objective was to conduct whole genome sequencing (WGS) analysis for genetic comparison of *F. necrophorum* strains recovered from matched ruminal epithelial, colonic epithelial, and liver abscess samples from the same animal. A total of 64 *F. necrophorum* strains from ruminal epithelial tissues (n = 17), colonic epithelial tissues (n = 16) and liver abscesses (n = 31) from two independent feedlot studies were subjected to WGS and bioinformatic analyses including core genome SNP analysis, ANI analysis, and virulence and antimicrobial resistance gene identification. High levels of genetic similarity were observed between multiple sets of matched colonic and liver abscess strains, as well as matched ruminal and liver abscess strains. Numerous virulence genes were identified within the genomes, with

major roles in toxin production, adhesion, metabolism, and bacterial persistence. The leukotoxin gene, *lktA*, a major virulence factor involved in the development of liver abscesses, was present in all genomes. Antimicrobial resistance genes encoding resistance to tetracycline and aminoglycoside antibiotics were observed in 24% and 18% of genomes, respectively. The *ermB* gene, encoding resistance to tylosin phosphate, was observed in the genomes of two liver abscess strains but none of the strains from ruminal or colonic tissue origin. The findings of high genetic similarity between ruminal and liver abscess strains reinforces the concept of the "ruminitis-liver abscess complex", while the findings of high genetic similarity between colonic and liver abscess strains highlight the need for future research investigating the potential for the colon to provide a source of bacterial liver abscess-causing pathogens. Future research should also be conducted to investigate the clinical relevance of the antimicrobial resistance genes identified in the genomes, and our results emphasize the need for more research into antimicrobial alternatives for liver abscess prevention.

Key Words: *Fusobacterium necrophorum*, whole genome sequencing, cattle, liver abscesses, antimicrobial resistance, virulence genes

Introduction

Fusobacterium necrophorum is a Gram-negative, anaerobic bacterium commonly found in the bovine rumen. As a member of the ruminal bacterial community, *F. necrophorum* has roles in lactate and amino acid fermentations, particularly fermentation of lysine and tryptophan (Langworth, 1977; Russell, 2006; Tadepalli et al., 2009). *Fusobacterium necrophorum* is also considered an opportunistic pathogen, implicated in numerous necrotic infections in cattle, including foot rot, calf-diphtheria, and most notably, liver abscesses (Tan et al., 1996; Nagaraja and Chengappa, 1998). Of the two subspecies of *F. necrophorum*, subsp. *necrophorum* is considered more virulent and more frequently involved in liver abscesses than subsp. *funduliforme* (Shinjo et al., 1991; Tadepalli et al., 2009). Liver abscesses are the most common cause for liver condemnation in fed cattle at slaughter, and cattle with severe abscesses (scored as A+), exhibit reduced animal performance and decreased carcass yield (Brown and Lawrence, 2010; Harris et al., 2018). The increased feeding of highly fermentable grains to feedlot cattle during the finishing period can result in acidosis and subsequent ruminitis, compromising the integrity of the ruminal epithelium. It has long been an accepted etiology that liver abscesses form as a result of *F. necrophorum*, specifically subsp. *necrophorum*, infiltration through the damaged ruminal epithelium into portal vein circulation, where the bacteria are then transported to, and colonize in, the liver to form abscesses (Jensen et al., 1954; Scanlan and Hathcock, 1983; Nagaraja and Chengappa, 1998).

Numerous studies have demonstrated a positive correlation between ruminal wall pathology at slaughter and liver abscess incidence (Jensen et al., 1954; Rezac et al., 2014). Additionally, DNA finger-printing analysis, or ribotyping, of *F. necrophorum* strains isolated from the ruminal wall were clonally identical to strains from liver abscesses of same animal,

supporting the pathogenesis that *F. necrophorum* in liver abscesses originates from the rumen (Narayanan et al., 1997). However, liver abscesses have also been observed in cattle with seemingly normal ruminal epithelium (Bester et al., 2016).

In cattle fed high-grain diets, ruminal microbial and fermentative changes associated with acidosis have received a great deal of attention (Nagaraja and Titgemeyer, 2007). Recent research has resulted in the increased recognition that post-ruminal starch flow due to high grain diets results in the development of hindgut acidosis (Gressley et al., 2011). The microbial changes associated with dysbiosis and the accumulation of toxic end-products in the hindgut, such as endotoxin and biogenic amines, are similar to those experienced in the rumen (Li et al., 2012; Plaizier et al., 2012). The presence of *F. necrophorum* subsp. *necrophorum* in colonic contents and tissues of cattle has been previously shown (Jennings et al., 2021). In contrast to the rumen, colonic fermentation is inherently less buffered and the colonic epithelium is much thinner (single cell layer) than the ruminal epithelium (four cell layers). Therefore, the colonic epithelium has been theorized to be more susceptible to damage caused by acidotic conditions.

Because of the critical knowledge gap regarding the role of the hindgut in liver abscess pathogenesis, our primary objective was to determine whether *F. necrophorum* in liver abscesses originates from the colon in addition to the rumen. We performed whole genome sequencing (WGS) analyses on *F. necrophorum* subsp. *necrophorum* strains isolated from liver abscesses to genetically compare them with strains isolated from matched ruminal tissues, colonic tissues, or both, from the same animal. Secondarily, we aimed to compare genomic differences, including virulence genes, antimicrobial resistance (AMR) genes, and mobile genetic elements between subsp. *necrophorum* strains of liver abscesses, ruminal and colonic tissues.

Methods

F. necrophorum isolation

A total of 64 strains of *F. necrophorum* subsp. *necrophorum*, 31 from liver abscesses, 17 from ruminal epithelial tissues, and 16 from colonic epithelial tissues, were utilized for WGS. The liver abscesses, ruminal and colonic tissue samples were collected from commercial abattoirs at the time of slaughter. The samples were part of two independent studies designed to further delineate etiology and pathogenesis of liver abscesses (Salih et al., unpublished data; (Schneid et al., 2024)). In both studies, sections of livers with intact abscesses and matched ruminal and colonic tissues were collected in commercial slaughter facilities and shipped overnight on ice to the Anaerobe Laboratory in the College of Veterinary Medicine for bacteriological analyses. In study 1, conducted in 2022, samples were collected from 96 cattle originating from feedlots that did not feed tylosin during the finishing period. These cattle originated from 15 feedlots located throughout the midwestern United States, with 1 to 19 cattle sampled from each feedlot. The second study, conducted in 2023, was designed to determine the effects of starch level in the diet (low [49.1%] vs. high [64.4%]) and feeding management regimens (consistent vs. randomized variations in feed quantity and feed delivery time) (Schneid et al., 2024).

Liver abscesses were surface sterilized by searing using a flame-heated spatula. After surface sterilization, liver abscesses were excised using a sterile scalpel and approximately 5 g of capsule tissue and purulent material was collected. Ruminal and colonic tissue samples were rinsed in distilled H_2O to remove any particulate matter then a sterile scalpel was used to collect approximately 5 g of epithelial tissue from each sample. All tissues were weighed, suspended in 45 ml of sterile phosphate buffered saline (PBS), and homogenized using a Nutribullet blender

(Nutribullet, California US). A sterile cotton swab was used to spot-inoculate the tissue homogenate onto 1 plate each of blood agar (TSA supplemented with 5% sheep's blood; Remel, Lenexa, KS), PY-La JVN agar, and PY-Ly JVN agar (Deters et al., 2024), and a sterile plastic inoculating loop was used to streak for isolation from the spot inoculum. Inoculated plates were incubated anaerobically for 48 h at 37°C. Presumptive *F. necrophorum* subsp. *necrophorum* colonies (flat, whiteish yellow in color) were picked from the initial plates and streaked for isolation onto blood agar plates. The subspecies confirmation was by a real time qPCR assay (Deters et al. 2024a).

DNA extraction

Fusobacterium necrophorum subsp. *necrophorum* strains (n= 64) were grown in pre-reduced, anaerobically sterilized brain heart infusion broth (PRAS-BHI; Difco, US) supplemented with 0.05% cysteine HCL at 37°C for approximately 6 h until growth reached a late log phase (0.6 absorbance at 600 nm). The DNeasy PowerLyzer Microbial Kit (Qiagen) was used as according to manufacturer instructions for DNA extraction from broth cultures. Extracted DNA was checked for purity and concentration using a NanoDrop spectrometer, then frozen at -20°C until DNA sequencing.

DNA sequencing

DNA quantification, library preparation, and sequencing were done by CosmosID (Germantown, MD) using established protocols. Briefly, DNA library preparation was done using the Nextera XT DNA Library Preparation Kit (Illumina) and IDT Unique Dual Indexes with a total DNA input of 1 ng. Genomic DNA was fragmented using proportional amounts of Illumina NexteraXT fragmentation enzyme. Unique dual indexes were added to each sample, followed by 12 PCR cycles to construct genomic libraries. DNA libraries were purified using

AMpure magnetic beads (Beckman Coulter) and eluted into QIAGEN EB buffer (Qiagen). After purification, libraries were quantified using a Qubit 4 fluorometer and the Qubit dsDNA HS Assay Kit (Thermofisher Scientific). Libraries were sequenced on Illumina Novaseq platform 2x150 bp.

Genome assembly

For quality control, Trim galore version 0.6.7 was used to trim adapters and perform quality trimming on the raw FastQ files. Paired-end reads were used for subsequent assembly and analysis, with unpaired reads retained. Flash version 2.2.00 was used to merge paired-end reads to obtain interleaved outputs for assembly. Genomes were assembled using SPAdes version 3.15.3 using the careful option with default parameters. Following assembly, genome qualities were checked using QUAST version 5.0.2.

Bioinformatic analyses

Parsnp was used to align the core genomes of the samples while taking into account potential recombination events and variation that exists naturally within genomes, to infer phylogenetic placement and genome relatedness. FastTree2 was used for the creation of phylogenetic trees based upon the core-genome SNPs generated by Parsnp. Average nucleotide identity between genomes was determined using FastANI (v1.33), and a heatmap of the resulting values was created in R studio with the Heatmaply package. The presence of plasmids and/or antimicrobial resistance genes within the genomes was determined using StarAMR (0.10.0). Single nucleotide polymorphisms were identified by Parsnp and displayed as a distance matrix between sample strains. Virulence gene identification was completed via a manual search of the Blast database, using previously identified potential virulence genes (Bista et al., 2022).

Results

Genome sizes

A total of 64 *F. necrophorum* subsp. *necrophorum* strains were submitted for whole genome sequencing. After sequencing, one strain was identified at *F. varium* as according to GTB-Tk classifications (2022-1-CT59), and one genome did not meet quality control standards (2023-3-RT195). The remaining 62 genomes were assigned to the *Fusobacterium necrophorum* species using GTB-Tk as a classifier. The estimated genome size of the 62 *F. necrophorum* isolates ranged from 2.47 Mb to 2.73 Mb in length, with an average genome size of 2.57 Mb.

Strain relatedness

Phylogenetic trees to visualize strain clustering

Fusobacterium necrophorum strains were split into two groupings: “2022-1” (n=28) and “2023-3” (n= 34) according to the study the strains were isolated from. Regarding strains from the 2022-1 study, matched colonic and liver abscess isolates clustered most closely to each other from animals 23, 48, 82, 61, 66, 54, 81, 85, and 31, while colonic and liver abscess isolates recovered from animals 87, 39, 49, 59, 48, and 38 were less closely clustered by animal (Figure 3-1). Additionally, strains 2022-1 CT49 and 2022-1 RT48 clustered together away from the other strains. Within the strains from the 2023-3 study, the matched colonic and liver abscess isolates clustered most closely to each other from animals 496 and 663, and the ruminal and liver abscess isolates clustered most closely to each other in animals 212, 151, 164, 518, 517, and 42 (figure 3-2). Strains 2023-3 RT71 and 2023-3RT56 formed their own unique cluster.

Average nucleotide identity (ANI) analysis to measure genetic relatedness

Average nucleotide identity (ANI) analysis was performed using FastANI on all 61 isolates, separated by the previously described strain groupings (2022-1 and 2023-3). The R

package “Heatmaply” was used to generate a heatmap of the pairwise ANI comparisons for each cohort. The generated heatmaps are provided in figures 3-3 and 3-4, with the ANI percentages rounded to the nearest 0.1% for clarity. In the 2022-1 cohort, strain CT59 was included, which was reported to belong to the *F. varium* species using GTDB-Tk classification. Within the 2022-1 strains (not including strain 2022-1-CT59, which was an outlier) the ANI pairwise comparison values ranged from 96.173% to 99.9947% (figure 3-3). A distinct cluster of very high genetic similarity was seen within the 2022-1 group, composed of the liver abscess and colonic epithelial strains from animals 81, 82, and 85. The ANI pairwise comparison values within this cluster ranged from 99.9578% to 99.9947%. Previously published literature suggests the use of an ANI value of >99.99% for establishment of clonal relationships between bacterial strains (Rodriguez et al., 2024), and using these guidelines clonality was inferred between strain pairs LA87-LA39, CT85-LA81, LA82-CT82, CT39-CT38, CT82-CT85, CT48-LA48, CT81-CT82 and CT81-LA82. Of these clonal pairs, only pairs LA82-CT82 and CT48-LA48 were matched isolates recovered from the same animal.

Within the 2023-3 strains, pairwise ANI values ranged from 96.0966% to 99.999%. The 2023-3 strains displayed distinct clustering between the liver abscess and ruminal epithelial isolates from animals 151, 187, and 212, along with the ruminal epithelial isolates from animals 189, 195, and 222, in which the % ANI values ranged from 99.93% to 99.9933%. A second cluster was also observed in the 2023-3 strains heatmap, consisting of the liver abscess isolates from animals 56, 204, 195, and 171 in which ANI% values ranged from 99.97% to 99.9836%. Applying the same guidelines for inferring clonality that were used for the 2022-1 group, clonality was inferred between strain pairs RT212-LA151, LA517-RT517, RT189-RT151,

RT222-RT151, RT195-LA151, and RT212-RT222. Within these clonal strains, only strain pair LA517-RT517 were recovered from the same animal.

Single nucleotide polymorphism (SNP) analysis

Parsnp was used to align the core genomes of the *F. necrophorum* strains, followed by variant (SNP) calling for both groups of strains (2022-1 and 2023-3). The results from this SNP analysis are presented in figures 3-5 and 3-6. As with the ANI results, a cluster of high genetic similarity, as evidenced by a low number of SNPs was observed within the strains recovered from the colonic tissue and liver abscesses from animals 81, 82, and 85 in the 2022-1 group. Within the 2022-1 group, 8 matched sets of strains (animals 23, 31, 48, 54, 61, 81, 82, and 85) isolated from the colonic epithelial tissue and liver abscess from the same animal differed by 45 or fewer SNPs. The matched colonic tissue – liver abscess strains from animals 23, 31, 54, 81, and 82 differed by 25 or fewer SNPs. An additional 2 matched sets of colonic tissue - liver abscess strains, from animals 38 and 66, differed by 75 and 79 SNPs, respectively. A large gap was observed in SNP differences between approximately 100 and 1,000 SNPs, suggesting that overall, there was a large amount of genetic diversity present between the *F. necrophorum* strains from different locations and between animals. Strains 2022-1-CT49 and 2022-1-RT48 were observed to be highly dissimilar to any other strains recovered from the 2022-1 study, with over 55,000 SNPs observed when compared to the other genomes. Interestingly, they were most similar to each other, although they still displayed over 20,000 SNP differences.

The strains isolated from the colonic tissues of animals 81, 82, and 85 were found to be closely related to each other, with only between 15 and 29 SNP differences observed. The same was found for the liver abscess strains from these animals, which only contained between 22 – 39 SNP differences.

Within the *F. necrophorum* strains recovered from the 2023-3 group, 2 of the 3 matched sets of colonic tissue – liver abscess strain genomes differed by only 10 SNPs (animals 496 and 663). Seven matched sets of ruminal epithelial – liver abscess strain genomes differed by fewer than 40 SNPs. The liver abscess and ruminal epithelial tissue strains from animal 2023-3-164 had 0 SNP differences between them, therefore confirming clonality. Interestingly, these isolates were not considered to be clonal based on ANI guidelines. The matching ruminal tissue – liver abscess strains from animals 2023-3-518, 2023-3-42, and 2023-3-517 differed by only 11, 2, and 7 SNPs, respectively. A cluster of high genetic similarity was also observed between strains 2023-3-LA151, 2023-3-RT189, and 2023-3-RT212. Additionally, it appeared that strains isolated from liver abscesses tended to be more genetically similar (have fewer SNP differences) to other strains isolated from liver abscesses than to strains isolated from ruminal or colonic epithelial tissues. Another set of two ruminal strains: 2023-3-RT56 and 2023-3-RT71, were closely related to each other with only 39 SNP differences, but were genetically very dissimilar to the rest of the 2023-3 strains with over 56,000 SNP differences from each. While there has been no generally accepted number of core-genome SNP differences allowed for the inference of clonality, these results lend further support and complexity to the findings of the ANI and Min-hash analyses.

Virulence Genes

A Blast search was conducted using a previously created custom database containing 46 virulence genes with potential relevance to *Fusobacterium* species (Bista et al., 2022). Using the results from this blast search, a heatmap was created using the percent identities of the virulence factors within each genome. Twenty-six virulence genes were identified within the 2022-1 group, and 28 virulence genes were identified within the 2023-3 group. Three virulence genes that code for RhuM family protein, adhesion protein FadA and the Type II secretion system

protein GspD, were only found in strains from the 2023-3 group. The autotransporter adhesion aidA was found in one genome from the 2022-1 group: 2022-1-CT49, and none of the genomes in the 2023-3 group. Eleven of the 29 virulence genes were identified in all 61 genomes, including *lktA*, which codes for leukotoxin, one of the most well-known virulence factors in *F. necrophorum* with 96.8% - 100% identity. Two genes that code for chaperonins, DnaJ and DnaK, were also present in all genomes. The other virulence genes found in all the genomes had roles in secretion, adhesion, transport, substrate utilization/metabolism, and the vapD protein, hypothesized to be involved in bacterial persistence. The activator protein for another toxin often associated with *F. necrophorum* infections was present within 26 (76.5%) of the 2023-3 group genomes, and 11 (40.7%) of the 2022-1 group genomes. There was no apparent correlation between tissue of origin for the *F. necrophorum* strains and virulence genes presence. Additionally, aside from the GspD protein gene, which was found in only one strain (2023-3-RT222), and the AidA gene, none of the virulence genes were exclusive to strains from any one tissue of origin.

Antimicrobial resistance genes

The *F. necrophorum* genomes were screened for the presence of antimicrobial resistance (AMR) genes using StarAMR. The results are presented in table 3-1. Overall, 6 AMR genes were identified within at least one genome within the samples set. The *ant(6)-Ia* gene, encoding for aminoglycoside resistance, was present with 100% identity in the genomes of two strains: 2022-1 LA39 and 2022-1 LA87, both of which were recovered from liver abscesses. The *aph(3')-III* gene, which is another AMR gene encoding aminoglycoside resistance, was only found within genomes from the 2023-3 study: 2023-3 LA151, 2023-3 RT151, 2023-3 LA187, 2023-3 RT187, 2023-3- RT189, 2023-3 RT195, 2023-3 LA212, 2023-3 RT212, and 2023-3

RT222, all with 100% identity. Macrolide resistance, encoded by the *erm(B)* gene, was found with 100% identity in two strains recovered from liver abscesses in the 2022-1 study: 2022-1 LA39 and 2022-1 LA87, both of which also carried *ant(6)-Ia*. Three genes encoding tetracycline resistance, *tet(O), tet(M), and tet(40)* were identified within the study genomes. The *tet(O)* gene was most abundant, contained within 15 (24.2%) of the genomes with 99.7% to 100% identity, of which 4 were from the 2022-1 group (2 liver abscess strains and 2 colon tissue strains) and 11 were from the 2023-3 group (4 liver abscess strains and 7 rumen tissue strains). Nine genomes, all from the 2023-3 group, carried the *tet(M)* gene with 98.12% identity (3 liver abscess strains and 6 rumen tissue strains), while one strain from the 2022-1 group, 2022-1 CT49, carried the *tet(40)* gene with 99.8% identity. Forty-seven (75.8%) of the *F. necrophorum* genomes contained no AMR genes.

Plasmids

The presence of plasmids within the *F. necrophorum* genomes was also analyzed using StarAMR. No plasmids were found in any of the 2022-1 cohort strains, however, 9 of the 34 genomes (26.5%) from the 2023-3 study contained the repUS43 plasmid with a 100% identity map and 100% sequence overlap (2023-3LA187, 2023-3LA212, 2023-3LA151, 2023-3RT187, 2023-3RT189, 2023-3RT195, 2023-3RT212, 2023-3RT222, and 2023-3RT151).

Discussion

This is, to date, the most comprehensive whole genome comparison study on *F. necrophorum* subsp. *necrophorum*, with the goal of furthering the understanding of liver abscess pathogenesis, particularly whether the colon can serve as a source of bacterial translocation to the liver. This study compared 62 *F. necrophorum* subsp. *necrophorum* strains isolated from ruminal epithelial tissues (n= 16), colonic epithelial tissues (n= 16), and liver abscesses (n= 31)

to analyze genetic similarity between strains recovered from the same animal, as well as compare differences in virulence genes, AMR genes, and mobile elements between strains from each of the three tissue types.

Recently, there has been an increased focus on the response of the hindgut to ruminal acidosis, and whether it may play a role in liver abscess development (Gressley et al., 2011; Li et al., 2012; Sanz-Fernandez et al., 2020). The colon lacks much of the buffering capacity exhibited in the rumen, and due to its structure has the potential for increased susceptibility to epithelial damage due to acidotic conditions (Amachawadi and Nagaraja, 2022). Thus, we aimed to investigate whether *F. necrophorum* isolated from liver abscesses can originate from the colon. The 2022-1 group was composed of 13 matched sets of *F. necrophorum* strains isolated from the colonic epithelial tissue and liver abscess of the same animal. When a phylogenetic tree was conducted for the strains using Parsnp, which aligns the core genomes of a bacterial species and analyzes the variations observed between them, it was found that in 8 of the 13 matched sets, the corresponding colonic and liver abscess strain were genetically similar. This high level of genetic relatedness was further demonstrated when core genome SNP analyses were conducted on the genomes. The same 8 sets of isolates displayed high levels of genetic similarity, with fewer than 45 SNP differences between the colonic tissue and liver abscess isolate from each animal. While no threshold for clonality has been established for *F. necrophorum* when using cgSNP analyses, other studies on bacterial outbreak tracing have suggested that the SNP threshold can be variable depending on the duration of the outbreak as well as the mutation rate of the bacteria being studied (Coll et al., 2020; Miro et al., 2020; Duval et al., 2023). Therefore, it becomes difficult to determine a definitive cut-off value for clonality. We observed that a subset of 5 our 8 highly genetically similar matched sets of strains differed by 25 SNPs or less. When ANI was used to

analyze genetic similarity, we saw that 3 of the 8 sets of highly genetically similar strains as determined by cgSNP analysis were also very highly similar using ANI percent identity values. It has been previously suggested that an ANI value of >99.99% be used as a threshold value for establishing clonality (Rodriguez et al., 2024), and using those guidelines, clonality could be inferred on two of the three matched strains sets. Within the 2023-3 group, 2 of the matched sets of strains from colonic epithelial tissue and liver abscesses differed by only 10 SNPs, although the ANI analyses did not show clonal levels of genetic similarity. A lack of definitive guidance on using WGS-based analyses for the establishment of clonality amongst bacteria hinders the ability to confirm clonality on the remaining 5 sets of matched strains, however their high levels of genetic similarity cannot be overlooked. Combining the results of all three analyses of genetic similarity, clonality was confirmed between two sets (15.4%) of *F. necrophorum* strains isolated from matched colonic epithelial and liver abscesses from the same animal, supporting the hypothesis that the colon can be a source of liver abscess-causing pathogens. Future research should investigate the genetic similarity between strains recovered from the ruminal epithelium, the currently accepted location for bacterial translocation into portal circulation, and those recovered from the colonic epithelium, to further delineate the implication of the hindgut in liver abscess formation.

We also investigated the genetic similarities between ruminal epithelial and liver abscess strains from matched animals in the 2023-3 group to further investigate the rumen as the source of liver abscess-causing pathogens. While the “ruminitis-liver abscess complex” has been widely accepted since its introduction 70 years ago (Jensen et al., 1954), the majority of the supporting evidence relies on the association between ruminal wall pathology and the presence of liver abscesses at slaughter (Scanlan and Hathcock, 1983; Rezac et al., 2014). A ribotyping study was

able to establish clonal connections between *F. necrophorum* strains recovered from the ruminal wall and liver abscesses of cattle (Narayanan et al., 1997), however, no WGS-based studies have been published to support these findings. The cgSNP based analyses reported high levels of genetic similarity between five matched sets of ruminal epithelial and liver abscess strains. Of these, one set had 0 SNP differences, and a further three matched sets differed by 11 or fewer SNPs. Interestingly, there was more discrepancy between the SNP analyses and the ANI analyses within the 2023-3 group, as the ANI only implied clonality between 1 of these highly genetically similar strain pairs. Using such a high threshold for clonality by ANI percentage, and without a well-established guideline for cgSNP-based clonality, it is highly possible that we are underestimating the number of strain pairs that are indeed clonal. However, these results showcase the high level of genetic similarity between ruminal epithelial and liver abscess strains from the same animal, further supporting the "ruminitis-liver abscess complex" hypothesis.

Aside from genetic relatedness between matched sets of strains, we also investigated the presence of virulence genes within the *F. necrophorum* genomes, and whether there were any patterns observed in strains according to their tissue of origin. A previously described, custom database containing virulence factors potentially relevant to *Fusobacterium* was utilized for this analysis (Bista et al., 2022). Of the 29 total virulence genes identified, three (10.3%) were unique to the 2023-3 group, and 1 (3.4%) was unique to a single genome within the 2022-1 group. However, 11 of the 29 (37.9%) virulence genes were identified in all 62 *F. necrophorum* genomes. These conserved virulence genes are primarily involved in functions like metabolism, secretion, persistence, and protein folding (Koebnik et al., 2000; Singh et al., 2007; Henderson et al., 2013; Beites et al., 2021). Additionally, the *lktA* gene, which encodes for leukotoxin production, was present in all 62 genomes. Leukotoxin production has been the major virulence

factor attributed to liver abscess formation in beef cattle (Tan et al., 1996; Pillai et al., 2021), and has been previously reported to be conserved amongst the majority of *Fusobacterium* species (Bista et al., 2022). Additionally, the gene for the virulence factor hemagglutinin was identified in all *F. necrophorum* genomes. Hemagglutinin has been observed to be crucial for the attachment of *F. necrophorum* to ruminal epithelial cells and hepatocytes, making it another virulence factor largely associated with the development of liver abscesses and resulting in the classification of *F. necrophorum* as an active tissue invader (Nagaraja et al., 2005). We had hypothesized that there may be differences in the virulence genes in the genomes of strains recovered from liver abscesses compared to those from ruminal or colonic epithelial tissues, but this was not observed within this sample set. It is likely that numerous strains of *F. necrophorum* are present in the gastrointestinal tract, as evidenced by ribotyping studies conducted on ruminal content and ruminal epithelial *F. necrophorum* strains (Narayanan et al., 1997), however, it is still unclear what makes some strains able to translocate into and colonize the liver.

A recently published study reported the first finding of macrolide resistance gene, *erm(B),* in two *F. necrophorum* strains originating from liver abscesses in beef cattle (Schwarz et al., 2024), along with resistance to tetracyclines, thus we investigated the presence of AMR genes in our genomes. The genomes of two strains recovered from liver abscesses contained the *erm(B)* gene, responsible for encoding resistance to macrolide antibiotics. Historically, tylosin phosphate, a macrolide antibiotic, has been the most widely used liver abscess preventative in feedlot cattle, and is commonly fed throughout the duration of the finishing period (Nagaraja and Lechtenberg, 2007; Reinhardt and Hubbert, 2015). There has been increasing concern over the use of in-feed antibiotics in food animals due to their potential for furthering the development of antimicrobial resistance (Angulo et al., 2009; Harris et al., 2017), and the recent findings of

tylosin resistance in *F. necrophorum* strains recovered from liver abscesses emphasize the need for the development of antimicrobial alternatives for liver abscess prevention. In addition to macrolide resistance, we found that a large proportion of *F. necrophorum* strains carried genes responsible for tetracycline resistance. Tetracycline antibiotics are commonly given to cattle for both prophylaxis and treatment of disease, making the rise in resistance amongst *F. necrophorum* strains particularly worrisome. Finally, we also saw a high prevalence of aminoglycoside resistance within the genomes of our *F. necrophorum* strains. Aminoglycosides are one of the oldest classes of antibiotics, and widely used in both veterinary medicine and human medicine for the treatment of severe bacterial infections (Nowacka-Kozak et al., 2023). Our study did not include the investigation into phenotypic resistance to tylosin, which would be of interest to determine. Similarly, we did not look into phenotypic resistance to any of the other classes of antibiotics encompassed by the AMR genes found within our genomes. Phenotypic resistance data is therefore needed in future studies to determine the clinical relevance of the AMR gene in *F. necrophorum* strains.

Conclusions

This was the largest whole genome comparison study of *F. necrophorum* subsp. *necrophorum* isolates to date, encompassing 62 strains isolated from bovine ruminal epithelial tissues, colonic epithelial tissues, and liver abscesses. We found high levels of genetic similarity between strains recovered from ruminal and liver abscesses within the same animal, reinforcing the “ruminitis- liver abscess complex” hypothesis first described in 1954 (Jensen et al., 1954), and also from colonic tissues highlighting the potential for the colon to serve as a source of bacterial liver-abscess causing pathogens. Additionally, we discovered that many virulence genes involved in toxin production, adhesion, metabolism and persistence were detected in strains.

Finally, we observed a relatively high prevalence of antimicrobial resistance genes, especially those encoding resistance to aminoglycoside and tetracycline antibiotics within the *F. necrophorum* genomes, regardless of their tissue of origin, but further research is needed to determine the clinical relevance of these findings. Overall, the whole genome analyses of *F. necrophorum* strains from liver abscesses, ruminal and colonic tissues provide evidence that colon also serves as a source of the pathogen to reach the liver to cause abscesses. Therefore, any novel interventions to prevent liver abscesses should target colon, in addition to the rumen.

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Figure 3-1 Phylogenetic tree of the *Fusobacterium necrophorum* subsp. *necrophorum* strains isolated from liver abscesses, ruminal and colonic tissues from the 2022-1 feedlot study

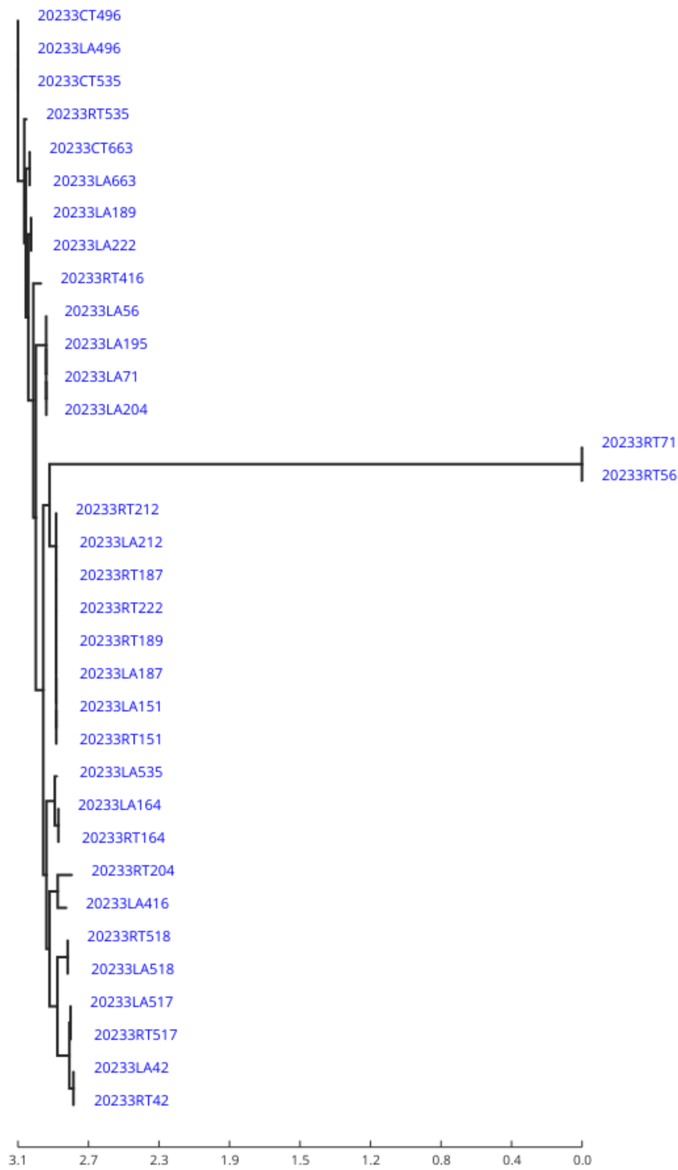

Figure 3-2 Phylogenetic tree of the *Fusobacterium necrophorum* subsp. *necrophorum* strains isolated from liver abscesses, ruminal and colonic tissues from the 2023-3 feedlot study

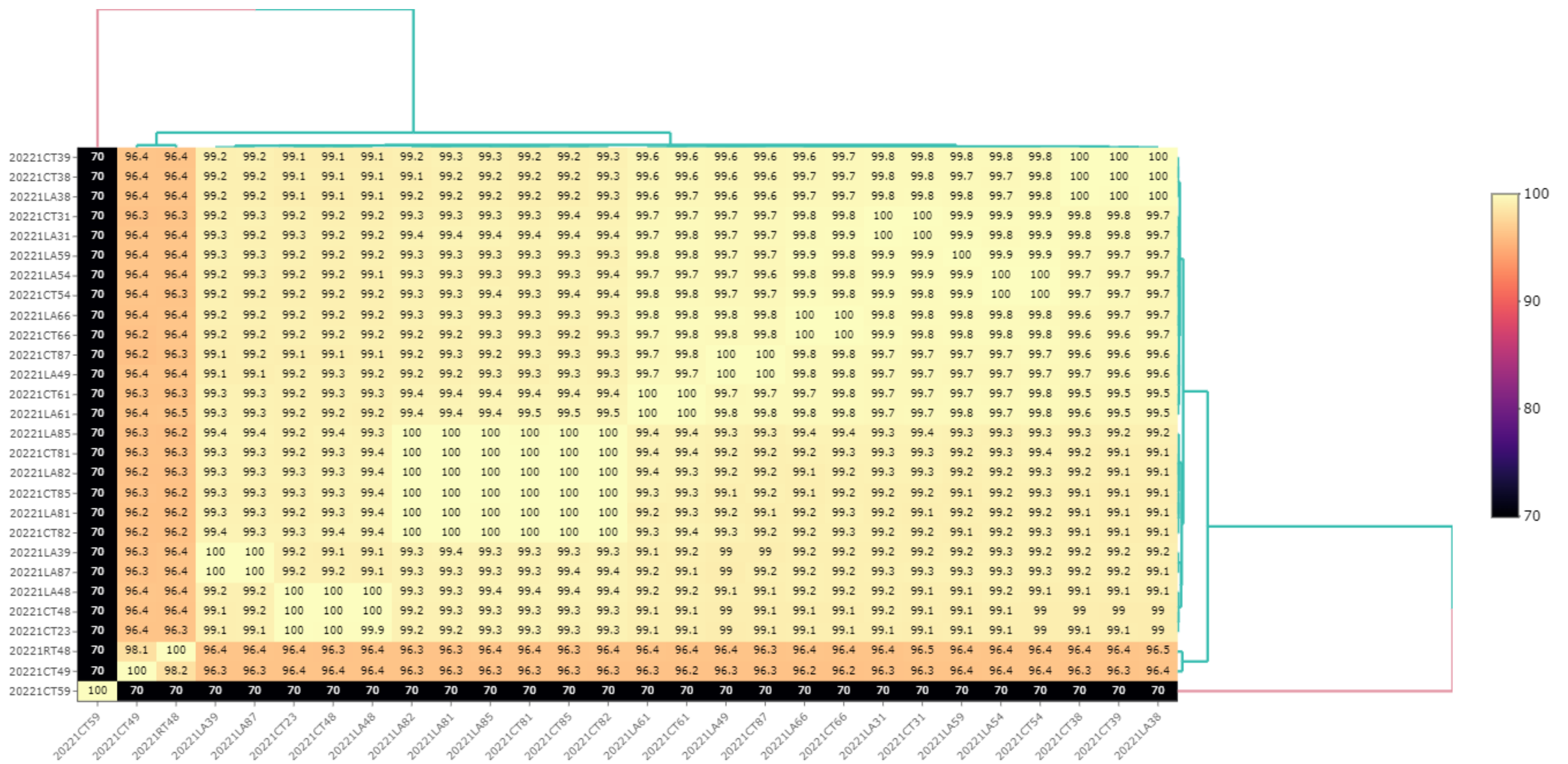

Figure 3-3. Heatmap and dendrogram constructed using the pairwise ANI percentages for *F. necrophorum* subsp. *necrophorum* isolates from liver abscesses (LA), rumen tissue (RT), and colonic tissue (CT) from the 2022-1 feedlot study.

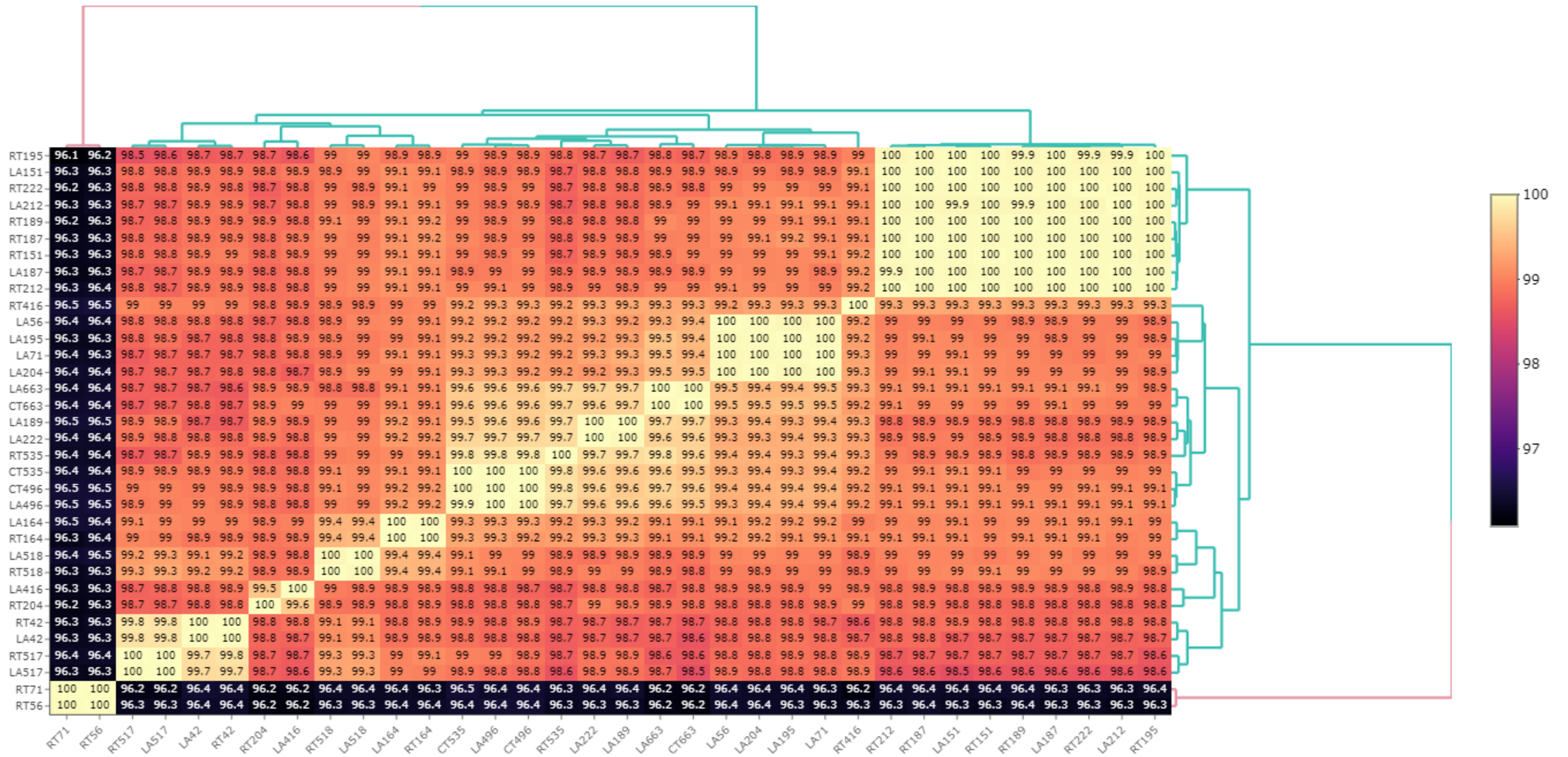

Figure 3-4 Heatmap and dendrogram constructed using pairwise ANI percentages for *F. necrophorum* subsp. *necrophorum* isolates from liver abscesses (LA), rumen tissue (RT), and colonic tissue (CT) from the 2023-3 feedlot study.

	2022-1-CT23	2022-1-LA81	2022-1-LA82	2022-1-LA85	2022-1-LA87	2022-1-RT48	2022-1-CT31	2022-1-CT38	2022-1-CT39	2022-1-CT48	2022-1-CT49	2022-1-CT54	2022-1-CT61	2022-1-CT66	2022-1-CT81	2022-1-CT82	2022-1-CT85	2022-1-CT87	2022-1-LA23	2022-1-LA31	2022-1-LA38	2022-1-LA39	2022-1-LA48	2022-1-LA49	2022-1-LA54	2022-1-LA59	2022-1-LA61	2022-1-LA66
2022-1-CT23	0	7591	7589	7612	8938	55982	7790	7607	7609	55	55915	7245	7521	7773	7596	7590	7587	7478	25	7795	7673	8935	38	7474	7242	7931	7509	7711
2022-1-CT31	7790	6774	6788	6793	7785	55635	0	1590	1590	7765	55985	795	2368	1072	6779	6773	6776	1867	7781	13	1658	7782	7790	1857	788	1101	2384	1002
2022-1-CT38	7607	6950	6948	6967	8168	55388	1590	0	6	7590	55790	1531	3094	1794	6955	6947	6942	2529	7596	1589	75	8169	7607	2529	1530	1839	3108	1725
2022-1-CT39	7609	6950	6950	6967	8172	55390	1590	6	0	7590	55789	1537	3098	1794	6955	6947	6942	2533	7598	1593	77	8173	7609	2530	1532	1843	3114	1727
2022-1-CT48	55	7558	7572	7579	8915	55971	7765	7590	7590	0	55930	7216	7492	7750	7563	7557	7564	7449	42	7770	7658	8910	45	7447	7211	7896	7480	7684
2022-1-CT49	55915	56291	56290	56274	56583	20613	55985	55790	55789	55930	0	55921	56003	56072	56291	56284	56286	56038	55926	55986	55845	56588	55928	56025	55920	55987	55997	56009
2022-1-CT54	7245	6426	6430	6439	7384	55724	795	1531	1537	7216	55921	0	2311	903	6427	6423	6428	1729	7232	786	1606	7383	7239	1721	13	804	2323	832
2022-1-CT61	7521	6264	6270	6279	7145	55729	2368	3094	3098	7492	56003	2311	0	2302	6265	6261	6266	2091	7518	2357	3161	7136	7523	2083	2312	2621	26	2227
2022-1-CT66	7773	6474	6486	6495	7649	55733	1072	1794	1794	7750	56072	903	2302	0	6479	6469	6478	1680	7766	1071	1735	7652	7775	1677	900	1203	2316	79
2022-1-CT81	7596	21	19	36	7228	56087	6779	6955	6955	7563	56291	6427	6265	6479	0	22	29	6289	7593	6782	7021	7231	7594	6281	6426	7052	6247	6409
2022-1-CT82	7590	11	23	32	7224	56081	6773	6947	6947	7557	56284	6423	6261	6469	22	0	15	6283	7587	6780	7015	7223	7588	6276	6420	7044	6243	6401
2022-1-CT85	7587	10	20	31	7235	56081	6776	6942	6942	7564	56286	6428	6266	6478	29	15	0	6288	7578	6783	7010	7230	7583	6281	6425	7051	6248	6410
2022-1-CT87	7478	6286	6294	6307	7466	55786	1867	2529	2533	7449	56038	1729	2091	1680	6289	6283	6288	0	7465	1860	2596	7467	7476	50	1724	2035	2069	1609
2022-1-LA23	25	7582	7586	7603	8941	55987	7781	7596	7598	42	55926	7232	7518	7766	7593	7587	7578	7465	0	7786	7660	8938	23	7465	7227	7922	7504	7704
2022-1-LA31	7795	6781	6791	6796	7780	55634	13	1589	1593	7770	55986	786	2357	1071	6782	6780	6783	1860	7786	0	1655	7775	7791	1850	789	1096	2375	1003
2022-1-LA38	7673	7018	7016	7035	8234	55445	1658	75	77	7658	55845	1606	3161	1735	7021	7015	7010	2596	7660	1655	0	8233	7673	2594	1601	1912	3179	1794
2022-1-LA39	8935	7230	7238	7205	19	56551	7782	8169	8173	8910	56588	7383	7136	7652	7231	7223	7230	7467	8938	7775	8233	0	8933	7457	7388	7983	7128	7584
2022-1-LA48	38	7591	7587	7602	8936	55989	7790	7607	7609	45	55928	7239	7523	7775	7594	7588	7583	7476	23	7791	7673	8933	0	7468	7240	7931	7511	7713
2022-1-LA49	7474	6279	6285	6296	7460	55780	1857	2529	2530	7447	56025	1721	2083	1677	6281	6276	6281	50	7465	1850	2594	7457	7468	0	1720	2033	2063	1609
2022-1-LA54	7242	6423	6431	6444	7389	55725	788	1530	1532	7211	55920	13	2312	900	6426	6420	6425	1724	7227	789	1601	7388	7240	1720	0	803	2326	829
2022-1-LA59	7931	7049	7061	7070	7980	55716	1101	1839	1843	7896	55987	804	2621	1203	7052	7044	7051	2035	7922	1096	1912	7983	7931	2033	803	0	2641	1132
2022-1-LA61	7509	6246	6250	6261	7135	55713	2384	3108	3114	7480	55997	2323	26	2316	6247	6243	6248	2069	7504	2375	3179	7128	7511	2063	2326	2641	0	2241
2022-1-LA66	7711	6406	6418	6427	7583	55669	1002	1725	1727	7684	56009	832	2227	79	6409	6401	6410	1609	7704	1003	1794	7584	7713	1609	829	1132	2241	0
2022-1-LA81	7591	0	22	27	7233	56086	6774	6950	6950	7558	56291	6426	6264	6474	21	11	10	6286	7582	6781	7018	7230	7591	6279	6423	7049	6246	6406
2022-1-LA82	7589	22	0	39	7235	56083	6788	6948	6950	7572	56290	6430	6270	6486	19	23	20	6294	7586	6791	7016	7238	7587	6285	6431	7061	6250	6418
2022-1-LA85	7612	27	39	0	7208	56071	6793	6967	6967	7579	56274	6439	6279	6495	36	32	31	6307	7603	6796	7035	7205	7602	6296	6444	7070	6261	6427
2022-1-LA87	8938	7233	7235	7208	0	56546	7785	8168	8172	8915	56583	7384	7145	7649	7228	7224	7235	7466	8941	7780	8234	19	8936	7460	7389	7980	7135	7583
2022-1-RT48	55982	56086	56083	56071	56546	0	55635	55388	55390	55971	20613	55724	55729	55733	56087	56081	56081	55786	55987	55634	55445	56551	55989	55780	55725	55716	55713	55669

Figure 3-5. Pairwise distance matrix and heatmap displaying the number of core-genome SNPs between *F. necrophorum* subsp. *necrophorum* strains from liver abscesses, rumen tissues, and colonic tissues from the 2022-1 feedlot study

	2023-3-CT496	2023-3-RT518	2023-3-RT535	2023-3-RT151	2023-3-RT164	2023-3-RT42	2023-3-CT535	2023-3-CT663	2023-3-LA187	2023-3-LA189	2023-3-LA204	2023-3-LA222	2023-3-LA56	2023-3-LA496	2023-3-LA518	2023-3-RT187	2023-3-RT56	2023-3-RT71	2023-3-LA535	2023-3-LA663	2023-3-LA71	2023-3-LA164	2023-3-RT204	2023-3-RT222	2023-3-RT517	2023-3-LA195	2023-3-LA212	2023-3-LA42	2023-3-LA416	2023-3-LA517	2023-3-LA151	2023-3-RT189	2023-3-RT212	2023-3-RT416
2023-3-CT496	0	11364	3135	9390	9477	12161	7	4326	9399	4474	8127	4469	8109	10	11361	9386	56997	56970	9314	4326	8130	9477	12550	9383	11274	8123	9387	12159	11458	11277	9383	9386	9380	7009
2023-3-RT518	11364	0	11178	10847	8324	8869	11359	11394	10836	11347	11774	11346	11778	11360	11	10829	56610	56583	8793	11402	11775	8324	11968	10820	8066	11770	10824	8871	10769	8061	10842	10833	10825	11837
2023-3-RT535	3135	11178	0	8888	9112	11562	3142	2781	8897	3221	7582	3216	7564	3143	11175	8886	56640	56613	8726	2783	7585	9112	11509	8883	10688	7578	8883	11560	10783	10691	8883	8886	8876	5790
2023-3-RT151	9390	10847	8888	0	8758	11966	9385	9370	23	9587	9821	9588	9819	9386	10846	36	56205	56176	8368	9368	9824	8758	11277	45	11338	9819	37	11966	9846	11345	23	34	40	8448
2023-3-RT164	9477	8324	9112	8758	0	12086	9476	9138	8757	9164	9832	9163	9834	9479	8313	8752	56633	56606	2053	9140	9835	0	11032	8759	11261	9826	8741	12086	10445	11268	8763	8756	8734	9875
2023-3-RT42	12161	8869	11562	11966	12086	0	12162	12011	11969	12229	12637	12226	12633	12161	8866	11964	56237	56210	11434	12015	12638	12086	13010	11961	1987	12647	11959	2	10834	1992	11969	11964	11948	12310
2023-3-CT535	7	11359	3142	9385	9476	12162	0	4321	9394	4479	8128	4476	8108	11	11356	9381	56998	56971	9313	4329	8131	9476	12551	9378	11275	8122	9384	12162	11459	11280	9378	9383	9377	7012
2023-3-CT663	4326	11394	2781	9370	9138	12011	4321	0	9367	3404	5849	3399	5849	4324	11393	9366	56952	56925	8841	10	5852	9138	11709	9363	11155	5843	9357	12011	10812	11160	9363	9368	9350	5832
2023-3-LA187	9399	10836	8897	23	8757	11969	9394	9367	0	9592	9820	9591	9826	9395	10829	37	56201	56172	8363	9367	9823	8757	11272	44	11341	9818	28	11969	9833	11336	40	33	35	8449
2023-3-LA189	4474	11347	3221	9587	9164	12229	4479	3404	9592	0	6605	7	6583	4480	11338	9583	56975	56946	8750	3400	6606	9164	11768	9586	11371	6599	9586	12227	10833	11376	9582	9583	9579	5214
2023-3-LA204	8127	11774	7582	9821	9832	12637	8128	5849	9820	6605	0	6600	34	8129	11769	9817	57620	57591	9579	5853	5	9832	12213	9822	11755	14	9810	12637	10614	11760	9828	9823	9801	6885
2023-3-LA222	4469	11346	3216	9588	9163	12226	4476	3399	9591	7	6600	0	6582	4477	11335	9582	56972	56943	8749	3401	6603	9163	11761	9585	11368	6596	9581	12224	10828	11371	9583	9582	9572	5211
2023-3-LA56	8109	11778	7564	9819	9834	12633	8108	5849	9826	6583	34	6582	0	8109	11771	9815	57631	57602	9579	5853	35	9834	12199	9818	11751	24	9818	12633	10606	11758	9822	9821	9809	6879
2023-3-LA496	10	11360	3143	9386	9479	12161	11	4324	9395	4480	8129	4477	8109	0	11359	9382	57003	56976	9312	4330	8130	9479	12554	9379	11276	8125	9383	12159	11462	11279	9379	9382	9376	7009
2023-3-LA518	11361	11	11175	10846	8313	8866	11356	11393	10829	11338	11769	11335	11771	11359	0	10822	56609	56582	8784	11401	11772	8313	11959	10819	8061	11761	10817	8868	10758	8056	10841	10826	10818	11832
2023-3-RT187	9386	10829	8886	36	8752	11964	9381	9366	37	9583	9817	9582	9815	9382	10822	0	56193	56164	8358	9366	9820	8752	11255	29	11336	9815	23	11964	9830	11331	39	20	34	8444
2023-3-RT56	56997	56610	56640	56205	56633	56237	56998	56952	56201	56975	57620	56972	57631	57003	56609	56193	0	39	56716	56950	57621	56633	57300	56191	56271	57620	56192	56235	57087	56264	56199	56182	56203	57216
2023-3-RT71	56970	56583	56613	56176	56606	56210	56971	56925	56172	56946	57591	56943	57602	56976	56582	56164	39	0	56689	56923	57592	56606	57273	56164	56244	57591	56165	56208	57060	56237	56170	56153	56174	57187
2023-3-LA535	9314	8793	8726	8368	2053	11434	9313	8841	8363	8750	9579	8749	9579	9312	8784	8358	56716	56689	0	8845	9580	2053	10614	8365	10842	9573	8351	11434	9677	10849	8373	8366	8340	9329
2023-3-LA663	4326	11402	2783	9368	9140	12015	4329	10	9367	3400	5853	3401	5853	4330	11401	9366	56950	56923	8845	0	5856	9140	11715	9363	11157	5847	9359	12013	10816	11162	9367	9366	9360	5832
2023-3-LA71	8130	11775	7585	9824	9835	12638	8131	5852	9823	6606	5	6603	35	8130	11772	9820	57621	57592	9580	5856	0	9835	12216	9825	11758	19	9813	12638	10615	11763	9831	9826	9804	6886
2023-3-LA164	9477	8324	9112	8758	0	12086	9476	9138	8757	9164	9832	9163	9834	9479	8313	8752	56633	56606	2053	9140	9835	0	11032	8759	11261	9826	8741	12086	10445	11268	8763	8756	8734	9875
2023-3-RT204	12550	11968	11509	11277	11032	13010	12551	11709	11272	11768	12213	11761	12199	12554	11959	11255	57300	57273	10614	11715	12216	11032	0	11258	12146	12207	11254	13012	7701	12139	11276	11255	11257	11943
2023-3-RT222	9383	10820	8883	45	8759	11961	9378	9363	44	9586	9822	9585	9818	9379	10819	29	56191	56164	8365	9363	9825	8759	11258	0	11333	9820	38	11961	9837	11328	48	33	43	8443
2023-3-RT517	11274	8066	10688	11338	11261	1987	11275	11155	11341	11371	11755	11368	11751	11276	8061	11336	56271	56244	10842	11157	11758	11261	12146	11333	0	11765	11333	1985	9936	7	11341	11338	11322	11708
2023-3-LA195	8123	11770	7578	9819	9826	12647	8122	5843	9818	6599	14	6596	24	8125	11761	9815	57620	57591	9573	5847	19	9826	12207	9820	11765	0	9810	12649	10608	11772	9826	9823	9801	6879
2023-3-LA212	9387	10824	8883	37	8741	11959	9384	9357	28	9586	9810	9581	9818	9383	10817	23	56192	56165	8351	9359	9813	8741	11254	38	11333	9810	0	11959	9815	11326	56	33	19	8441
2023-3-LA42	12159	8871	11560	11966	12086	2	12162	12011	11969	12227	12637	12224	12633	12159	8868	11964	56235	56208	11434	12013	12638	12086	13012	11961	1985	12649	11959	0	10834	1990	11969	11964	11948	12308
2023-3-LA416	11458	10769	10783	9846	10445	10834	11459	10812	9833	10833	10614	10828	10606	11462	10758	9830	57087	57060	9677	10816	10615	10445	7701	9837	9936	10608	9815	10834	0	9931	9855	9836	9818	10976
2023-3-LA517	11277	8061	10691	11345	11268	1992	11280	11160	11336	11376	11760	11371	11758	11279	8056	11331	56264	56237	10849	11162	11763	11268	12139	11328	7	11772	11326	1990	9931	0	11348	11331	11327	11713
2023-3-LA151	9383	10842	8883	23	8763	11969	9378	9363	40	9582	9828	9583	9822	9379	10841	39	56199	56170	8373	9367	9831	8763	11276	48	11341	9826	56	11969	9855	11348	0	33	45	8447
2023-3-RT189	9386	10833	8886	34	8756	11964	9383	9368	33	9583	9823	9582	9821	9382	10826	20	56182	56153	8366	9366	9826	8756	11255	33	11338	9823	33	11964	9836	11331	33	0	46	8444
2023-3-RT212	9380	10825	8876	40	8734	11948	9377	9350	35	9579	9801	9572	9809	9376	10818	34	56203	56174	8340	9360	9804	8734	11257	43	11322	9801	19	11948	9818	11327	45	46	0	8432
2023-3-RT416	7009	11837	5790	8448	9875	12310	7012	5832	8449	5214	6885	5211	6879	7009	11832	8444	57216	57187	9329	5832	6886	9875	11943	8443	11708	6879	8441	12308	10976	11713	8447	8444	8432	0

Figure 3-6 Pairwise distance matrix and heatmap displaying the number of core-genome SNPs between *F. necrophorum* subsp. *necrophorum* strains from liver abscesses, rumen tissues, and colonic tissues from the 2023-3 feedlot study

Table 3-1 Gene matrix indicating the presence or absence of AMR genes, as detected using StarAMR, in *F. necrophorum* subsp. *necrophorum* strains recovered from liver abscesses, ruminal and colonic tissues from the 2022-1 and 2023-3 feedlot studies. Black indicates presence and white indicates absence.

	Antimicrobial resistance gene					
Strain ID	ant(6)-Ia	aph(3')-III	erm(B)	tet(40)	tet(M)	tet(O)
2022-1 CT23						
2022-1 CT31						
2022-1 CT38						
2022-1 CT39						
2022-1 CT48						
2022-1 CT49				■		■
2022-1 CT54						
2022-1 CT59						
2022-1 CT61						
2022-1 CT66						
2022-1 CT81						
2022-1 CT82						
2022-1 CT85						
2022-1 CT87						■
2022-1 LA31						
2022-1 LA38						
2022-1 LA39	■		■			■
2022-1 LA48						
2022-1 LA49						
2022-1 LA54						
2022-1 LA59						
2022-1 LA61						
2022-1 LA66						
2022-1 LA81						
2022-1 LA82						
2022-1 LA85						
2022-1 LA87	■		■			■
2022-1 RT 48						
2023-3CT496						
2023-3CT535						
2023-3CT663						
2023-3LA187		■			■	■
2023-3LA189						
2023-3LA195						
2023-3LA204						
2023-3LA212		■			■	■
2023-3LA222						
2023-3LA42						
2023-3LA56						

2023-3LA416						
2023-3LA496						
2023-3LA517						
2023-3LA518						
2023-3LA663						
2023-3LA71						
2023-3LA151						
2023-3LA164						
2023-3RT 187						
2023-3RT 189						
2023-3RT 195						
2023-3RT 204						
2023-3RT 56						
2023-3RT 212						
2023-3RT 222						
2023-3RT 71						
2023-3RT 416						
2023-3RT 517						
2023-3RT 518						
2023-3RT 535						
2023-3RT 151						
2023-3RT 164						
2023-3RT 42						

Chapter 4 – First Report of Isolation of *Fusobacterium varium* from Liver Abscesses and Ruminal and Colonic Epithelial Tissues of Feedlot Cattle

Abstract

Objective: Our objective was to isolate and determine prevalence of *Fusobacterium varium* in liver abscesses and the corresponding ruminal and colonic epithelial tissues and ruminal and colonic contents of feedlot cattle.

Materials and Methods: A total of 96 intact liver abscess samples and matched ruminal and colonic tissues and contents from cattle, originating from feedlots that did not receive in-feed tylosin, were collected at slaughter. Liver abscesses, ruminal and colonic tissue were homogenized and then plated, before and after enrichment in lactate or lysine medium with selective antibiotics, onto blood agar and selective lactate or lysine agar for isolation of *Fusobacterium* and to determine prevalence and concentration. Putative colonies were tested by a qPCR assay targeting the *hgdA* gene for species confirmation.

Results and Discussion: None of the liver abscess samples yielded *F. varium* by direct plating, however, *F. varium* was isolated from 3 of 96 (3.1%) following-enrichment of the homogenate in lactate or lysine medium. In contrast to liver abscesses, *F. varium* was isolated by direct plating from 27.1% (26/96) of ruminal epithelial and 3.1% (3/96) of colonic epithelial tissue homogenates. Overall, 10.1%, 77.1%, 44.8%, 86.5%, and 70.1% of liver abscess, ruminal and colonic epithelial tissues and ruminal and colonic contents were positive for *F. varium,* respectively.

Implications and Applications: The increased frequency of isolation and high prevalence of *F. varium* in ruminal tissue and, to a lesser extent, in the colonic tissue confirms its ability to invade tissues and possibly cause bacterial ruminitis. However, the relatively low frequency of *F. varium* isolation and low prevalence in liver abscesses suggest that it is unlikely to be an etiologic agent. Interestingly, there is some evidence that ruminal strains of *F. varium* were resistant to tylosin, therefore, it would be of interest to determine the prevalence in cattle receiving in-feed tylosin.

Key words: Cattle, liver abscesses, *Fusobacterium varium*, rumen, colon

Introduction

Liver abscesses are of economic concern in beef cattle production systems, with negative impacts on live cattle growth performance, carcass yields and quality, along with being the number one cause of liver condemnations at slaughter (Brown and Lawrence, 2010; Reinhardt and Hubbert, 2015; Harris et al., 2018). Much research has been done to elucidate their etiology, pathogenesis, and to explore and evaluate mitigation strategies to minimize the economic burden (Jensen et al., 1954; Reinhardt and Hubbert, 2015; Amachawadi and Nagaraja, 2016, 2022; Pinnell and Morley, 2022).

Fusobacterium necrophorum, a normal inhabitant of the bovine rumen, has been identified as the primary etiologic agent in the development of liver abscesses (Scanlan and Hathcock, 1983; Nagaraja and Chengappa, 1998). However, research has established that liver abscesses are polymicrobial infections, with a multitude of bacterial genera and species identified based on culture-dependent (Scanlan and Hathcock, 1983; Nagaraja and Chengappa, 1998) and -independent methods (Weinroth et al., 2017; Amachawadi et al., 2021; Pinnell et al., 2022). The generally accepted etiology and pathogenesis for liver abscess formation was described by Jensen et al. (1954), detailing the interplay between ruminal acidosis and the subsequent damage to ruminal epithelial tissue allowing the migration of bacteria through the ruminal wall and into portal vein circulation, and then reach the liver and colonize to result in liver abscess formation. Recently, Schwarz et al. (2023) reported that *F. varium*, and not *F. necrophorum,* was the dominant *Fusobacterium* in the bovine rumen. *Fusobacterium varium* and *F. necrophorum* share morphological and biochemical similarities, including colony morphology, ability to utilize lactate and lysine, and production of indole (Schwarz et al., 2023; Deters et al., forthcoming). Additionally, *Fusobacterium varium* is a known human and animal pathogen, and is implicated

in a variety of infections, including abscesses and necrotic infections (Ohkusa et al., 2002; Legaria et al., 2005; Foster et al., 2009; Pett et al., 2014; Rachana et al., 2019). In light of the recent and unexpected finding that *F. varium* is the most dominant *Fusobacterium* species in the bovine rumen, and because the organism has invasive and pathogenic potential, we hypothesized that *F. varium* may be present in liver abscesses. Therefore, the goal of this study was to investigate whether *F. varium* was present in liver abscesses and determine its prevalence and concentration in the corresponding ruminal and colonic tissues and contents, collected at slaughter, of feedlot cattle.

Materials and Methods

Sample Collection

Intact liver abscess samples and matched ruminal epithelial and colonic epithelial tissue and content samples were collected from 96 cattle originating from feedlots that did not feed tylosin phosphate during the finishing period. Cattle originated from 15 feedlots across the midwestern United States, with 1 to 19 cattle sampled from each feedlot. Samples were collected at a commercial slaughter facility in the southern United States, over a 7-month period, between June and December 2022. Slices of livers with intact abscesses were placed into plastic freezer bags, whereas contents and tissue sections of rumen and colon were placed into sterile 50-ml centrifuge tubes, stored on wet ice, and shipped overnight to the laboratory at Kansas State University.

Sample Processing

Liver abscess samples were surface sterilized by searing the surface of the liver with a flame-heated metal spatula prior to excising approximately 5 g of sample consisting of capsular wall and purulent materials, which were suspended in 45 ml of sterile phosphate buffered saline

(PBS; Sigma, St. Louis, MO). Ruminal tissues and colonic tissues were rinsed with sterile double distilled H_2O. Ruminal papillae were cut with sterilized scissors and the mucosal layer of the colonic tissue was removed with a sterile scalpel and approximately 5 g of tissues were weighed and suspended in 45 ml of sterile PBS. The liver and epithelial tissue suspensions were homogenized using a Nutribullet Pro Blender (Nutribullet, Los Angeles, CA) and homogenized samples were used for bacterial isolation and qPCR analysis. Ruminal and colonic contents were strained through 3 layers of cheesecloth to remove large particles. Colonic contents were often pasty and required a 1:1 (weight/volume) dilution in sterile PBS before straining. Strained ruminal and colonic contents were used for qPCR analysis for *F. varium* detection and quantification, before and after enrichment.

Enrichment media

A pre-reduced, anaerobically sterilized peptone yeast extract medium (PY) with 100 m*M* of lactate (PY-La) or lysine (PY-Ly) as the major carbon source, and each supplemented with josamycin (3 µg/ml), vancomycin (4 µg/ml) and norfloxacin (1 µg/ml) (PY-La JVN or PY-Ly JVN) was used for the enrichment of homogenized tissue samples and strained ruminal and colonic content samples (Holdeman et al., 1977; Schwarz et al., 2023; Deters et al., forthcoming).

F. varium isolation

A sterile cotton swab was used to spot inoculate each sample homogenate onto a plate each of blood agar (Remel Inc., Lenexa, KS), PY-La JVN agar, and PY-Ly JVN agar, and then an inoculating loop was used to streak from the spot for isolation of single bacterial colonies. Inoculated plates were incubated in an anaerobic glove box for 48 h at 37° C. Presumptive *F. varium* colonies (small [2 to 4 mm], greyish yellow, round and flat) were picked and streaked

onto blood agar plates, and incubated anaerobically for 48 h at 37°C. The colony morphology of *F. varium* is distinctly different from *F. necrophorum* subsp. *necrophorum* but requires some experience to differentiate from subsp. *funduliforme* (Figure 4-1). The species confirmation was by quantitative PCR (qPCR) assay targeting the *hgdA* gene (Deters et al., 2024). Additionally, 1 ml of sample homogenate was inoculated into 9 ml each of PY-La JVN and PY-Ly JVN broths and incubated at 37°C for 24 h. Enriched tissue homogenate samples were streaked onto blood agar for isolation.

F. varium qPCR detection and quantification

Tissue homogenates were analyzed by qPCR assay before and after enrichment to determine concentration and overall prevalence of *F. varium*. Strained ruminal and colonic contents were subjected to qPCR assay to determine concentration of *F. varium* before enrichment. Strained content samples were additionally inoculated into PY-La JVN and PY-Ly JVN broth (1 ml into 9 ml broth), incubated for 24 hours at 37° C, and *F. varium* prevalence in enriched samples was determined by qPCR assay.

DNA extraction

DNA was extracted from colonies of pure cultures grown on blood agar for qPCR analysis to confirm species. Briefly, a well-isolated, single colony was suspended into 50 µl of sterile, ddH_2O and mixed thoroughly. The DNA suspension was boiled for 10 min, centrifuged at 9,300 RCF for 5 min at room temperature, and the supernatant was used in the qPCR assay for species confirmation. The MagMax-96 DNA Multi-sample Kit (Applied Biosystems, Foster City, CA) was used according to the manufacturer's protocol for the isolation of DNA from liver abscess, ruminal and colonic epithelial tissue homogenates for use in the qPCR assay. DNA from enriched liver abscess, ruminal epithelial, and colonic epithelial samples, and from strained

ruminal and colonic content samples, were isolated and purified using the GeneClean Turbo Kit (MP Biomedicals, Solon, OH), following the manufacturer's protocol for qPCR analysis.

Quantitative PCR Assay

A qPCR assay was performed that targeted the *hgdA* gene, which encodes for 2-hydroxyglutaryl dehydratase, for identification of *F. varium* and to differentiate from *F. necrophorum,* and to quantify and determine overall prevalence in liver abscesses, ruminal and colonic epithelial tissues and ruminal and colonic contents (Deters et al., 2024). Assay running conditions were as follows: 95°C for 5 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 40 sec, using the BioRad CFX96 Real-Time System (BioRad, Hercules, CA).

Results and Discussion

F. varium in Liver Abscesses

None of the liver abscess samples yielded *F. varium* by direct plating of the homogenate onto the blood agar, PY-La JVN or PY-Ly JVN agar. However, the plating of homogenized samples after-enrichment yielded *F. varium* from three of the 96 (3.1%) liver abscess samples (Table 4-1). Although this is the first report of isolation of *F. varium* from liver abscesses of feedlot cattle, there is a report on the isolation from multifocal hepatic abscesses in a concentrate-fed lamb (Foster et al., 2009). *Fusobacterium varium* is a human and animal pathogen, implicated in a variety of anaerobic infections, including necrotic infections (Legaria et al., 2005; Manson McGuire et al., 2014; Rachana et al., 2019; Lee et al., 2022). In addition, there are reports of *F. varium* isolation from suppurative infections of gastrointestinal tract, oral cavity, respiratory tract and feet of animals (Chirino-Trejo et al., 2003; Hattel et al., 2004; Brooks et al., 2014).

The need for enrichment in lactate or lysine medium prior to isolation of *F. varium* from liver abscess samples suggests that the concentration of *F. varium* in the purulent material/capsule was

too low for isolation by direct plating. The three liver abscess samples that yielded *F. varium* also yielded isolates of *F. necrophorum* subsp. *necrophorum*, but not *F. necrophorum* subsp. *funduliforme* by direct plating of the sample homogenate. Based on the qPCR assay, one of the 96 liver abscess samples was positive for *F. varium* before enrichment and the concentration was 4.0 x 10^4 per g of purulent material (Table 4-2). Of the 96 samples, 8 (8.4%) and 9 (9.5%) liver abscess samples were positive for *F. varium* after enrichment in PY-La JVN and PY-Ly JVN broth, respectively. Overall, the qPCR assay revealed that a total of 10 (10.4%) of 96 liver abscess samples contained *F. varium.*

F. varium in Ruminal and Colonic Epithelial Tissues

In contrast to liver abscesses, *F. varium* was isolated from 27.1% (26/96) of ruminal epithelial and 3.1% (3/96) of colonic epithelial tissue homogenates by direct plating (Table 4-1). In four of the 26 ruminal epithelial tissues and 1 of the 3 colonic epithelial tissues, the species was quantifiable, and the mean concentration ranged from 10^4 to 10^5 CFU per g of tissue (Table 4-2). After enrichment of the tissue homogenate in PY-La JVN or PY-Ly JVN broth, 31 of 70 (44.3%) and 27 of 93 (29%) remaining ruminal and colonic tissues, respectively, yielded *F. varium* isolates. In total, 57 (59.4%) *F. varium* isolates from ruminal tissues and 30 isolates (31.3%) from colonic tissues were obtained. Interestingly, ruminal or colonic epithelial tissue samples of three cattle that yielded *F. varium* from liver abscesses did not yield *F. varium*, either by direct plating or plating after enrichment. Ruminal and colonic tissue homogenates that were not quantifiable by qPCR prior to enrichment were subjected to enrichment in PY-La JVN and PY-Ly JVN broth to detect prevalence. A majority of the ruminal tissues enriched in either PY-La JVN (73.9%) or PY-Ly JVN (76.1%) broth were positive for *F. varium.* In contrast, the prevalence of *F. varium* in colonic tissues was lower (44.2% in PY-La JVN and 43.2% in PY-Ly

JVN broth) than that of the ruminal tissue. Overall, 77.1% and 44.8% of ruminal and colonic tissues, respectively, were positive for *F. varium* by qPCR assay (Table 4-2). The increased frequency of isolation (59.4%) and prevalence (77.1%) from ruminal tissues indicates the ability of *F. varium* to invade the tissue and potentially colonize, which was likely the source of *F. varium* entrance into portal circulation to reach the liver. Similar to *F. necrophorum,* the acidosis-induced ruminal damage may facilitate entry and proliferation of *F. varium* in the ruminal epithelium (Jensen et al., 1954; Amachawadi and Nagaraja, 2022). This is also the first report of isolation of *F. varium* from ruminal and colonic tissues. *Fusobacterium varium* is considered as an active tissue invader among the known virulent *Fusobacterium* species, such as *nucleatum, necrophorum,* etc. (Manson McGuire et al., 2014; Umaña et al., 2019). In the whole genome analyses of two novel *F. varium* isolates from ruminal contents of beef cattle, Schwarz et al. (2023) identified virulence genes associated with the invasion of mammalian epithelial cells. A key virulence factor that permits *F. necrophorum* to evade the host defense mechanisms to survive and colonize ruminal epithelial tissue and liver tissue is leukotoxin (Narayanan et al., 2001). The two ruminal *F. varium* isolates that were whole genome sequenced did not contain the leukotoxin gene. However, both strains contained genes for cellular invasion and pathogenesis similar to those of human pathogenic strains of *F. varium* (Schwarz et al., 2023). It is possible that invasion of colonic tissue could also be the source of *F. varium* in liver abscesses, although this seems unlikely due to the lower frequency of isolation and prevalence. Even for *F. necrophorum,* the role of colon as a possible source of bacterial entry into portal circulation has not been determined (Amachawadi and Nagaraja, 2022). Interestingly, the two ruminal isolates from Schwarz et al. (2023) exhibited marked resistance to tylosin phosphate and monensin compared to *F. necrophorum*. In our study, liver abscess samples were collected from cattle that

did not receive in-feed tylosin, therefore, it would be of interest to determine the prevalence in cattle receiving in-feed tylosin.

F. varium in Ruminal and Colonic Contents

The qPCR assay detected *F. varium* in 19 of 96 ruminal contents (19.8%) in quantifiable concentrations, with a mean concentration of 1 x 10^4 CFU per g. In contrast, only 2 of 96 (2.1%) colonic content samples had quantifiable concentration, with a mean concentration similar to that of the ruminal contents (1.7 x 10^4 CFU/g). However, a majority of the remaining ruminal and colonic content samples were positive for *F. varium* after enrichment in either PY-La JVN or PY-Ly JVN broth. There was no difference in the detection level between the two enrichment media. Overall, 86.5% and 70.1% of ruminal and colonic contents were positive for *F. varium,* respectively, (Table 4-2). Concentrations of *F. varium* did not differ between epithelial tissues and their contents, though more *F. varium* was detected in contents without the need for enrichment than in epithelial tissues, suggesting *F. varium* is a normal member of the bacterial community of the rumen, and possibly of the colon. This observation confirms the evidence provided by Schwarz et al. (2023), based on near-full length 16 S rRNA sequencing, that *F. varium* was prevalent in all the ruminal content samples of cattle examined. In a previous study, we reported prevalence of *F. varium* in almost all the ruminal content samples (98%), collected at slaughter, tested by qPCR assay (Deters et al., 2024). Previous reports of isolation of *F. varium* from ruminal contents and feces of cattle included strains capable of degrading phenolic acids, such as ferulic acid, dihydrovanillin and vanillin, and amino acids (Chen et al., 1988; Shinjo et al., 1990; Bailey and Love, 1993). Schwarz et al. (2023) also reported that *F. varium* grew well in culture media developed for the enrichment of *F. necrophorum*. Because *F. varium* is able to grow in a medium with lactate as the major carbon source, much of the work done in

the past to quantify ruminal *F. necrophorum* populations that relied on culture-based methods, which depended on lactate utilization and indole production from tryptophan, may have unknowingly included *F. varium* (Tan et al., 1994; Nagaraja et al., 1999). As such, ruminal *F. necrophorum* concentration may have been unintentionally inflated, thus overestimating the abundance of *F. necrophorum* in the rumen.

Implications and Applications

The study to determine whether *F. varium* is involved in liver abscesses of cattle was deemed needed because of the recent finding that *F. varium* is more dominant than *F. necrophorum* in ruminal contents of cattle, and because it is a known human and animal pathogen. Based on culture-based and PCR-based analyses, 10 of 98 liver abscesses tested contained *F. varium* and *F. varium* was isolated in pure culture from three abscesses. The increased frequency of isolation and high prevalence of *F. varium* in ruminal tissue and, to some extent, in the colonic tissue confirms its ability to invade tissues and possibly be associated with bacterial ruminitis. However, the relatively low prevalence and low concentrations of *F. varium* in liver abscesses sampled in this study suggests that it is likely not an etiologic agent.

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Table 4-1. Frequency of Isolation of *Fusobacterium varium* from liver abscesses, ruminal epithelial and colonic epithelial tissues collected from feedlot cattle at slaughter

Sample type	No. of samples	No. of isolates/total (%)		
		Isolation by direct plating	Isolation after enrichment[1]	Total
Liver abscesses	96	0/96 (0)	3/96 (3.1)	3/96 (3.1)
Ruminal epithelium	96	26/96 (27.1)	31/70 (44.3)	57/96 (59.4)
Colonic epithelium	96	3/96 (3.1)	27/93 (29)	30/96 (31.3)

[1]Enriched for 24 hours in peptone-yeast extract medium containing lactate (100 m*M*) or lysine (100 m*M*) as the major energy source and supplemented with josamycin, vancomycin, and norfloxacin at 3 µg/mL, 4 µg/mL, and 1 µg/mL, respectively.

Table 4-2 Prevalence and concentration of *Fusobacterium varium*, based on quantitative PCR assay, before and after enrichment, in liver abscesses, ruminal, and colonic epithelial tissues and contents, collected from feedlot cattle at slaughter

		Before enrichment		Prevalence after enrichment, No. positive/total (%):		
Sample type	No. of samples	No. positive/total (%)	Mean concentration (CFU/g)	Peptone yeast extract-lactate plus JVN[1]	Peptone yeast extract-lysine plus JVN[1]	Total, No. positive/total (%)
Liver abscess	96	1/96 (1)	4.0×10^4	8/95 (8.4)	9/95 (9.5)	10/96 (10.4)
Ruminal epithelium	96	4/96 (4.2)	1.1×10^5	68/92 (73.9)	70/92 (76.1)	74/96 (77.1)
Colonic epithelium	96	1/96 (1)	3.5×10^4	42/95 (44.2)	41/95 (43.2)	43/96 (44.8)
Ruminal contents	96	19/96 (19.8)	1.06×10^4	57/77 (74)	64/77 (83.1)	83/96 (86.5)
Colonic contents	96	2/96 (2.1)	1.68×10^4	59/94 (62.8)	66/94 (70.2)	68/96 (70.1)

[1]JVN = Josamycin (3 µg/mL), Vancomycin (4 µg/mL), Norfloxacin (1 µg/mL)

Figure 4-1 Colony morphology of *Fusobacterium varium*, *F. necrophorum* subsp. *necrophorum* and subsp. *funduliforme* on blood agar plate

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Chapter 5 *Fusobacterium varium* in liver abscesses, rumen, and colon of cattle: Prevalence, isolation, and characterization

Abstract

Fusobacterium varium, previously referred to as biotype C of *F. necrophorum* or *F. pseudonecrophorum*, has recently been revealed to be the predominant *Fusobacterium* species in the bovine rumen. *Fusobacterium varium* has been implicated in a wide range of infections in humans, and is considered to be an active tissue invader. Additionally, *F. varium* has recently been isolated from liver abscesses, ruminal epithelial tissues, and colonic epithelial tissues from feedlot cattle. Thus, the objective of this study was to investigate the effects of various dietary and management strategies on the prevalence of *F. varium* in liver abscesses, and ruminal and colonic tissues and contents using culture-based and qPCR-based methods. Our second objective was to conduct whole genome sequencing (WGS) analysis to determine the genetic relatedness of strains recovered from different locations, and to evaluate the virulence and antimicrobial resistance genes in the genomes. *Fusobacterium varium* was isolated from liver abscesses, liver tissues, ruminal epithelial tissues, and colonic epithelial tissues using culture-based methods, before and after enrichment, from two feedlot studies and one experimental study. Additionally, *F. varium* was detected and quantified in liver abscess, liver tissue, and ruminal and colonic tissue and contents using a qPCR assay targeting the *hgdA* gene. Twenty-two isolates from liver abscesses (n = 9), liver tissue (n = 1), ruminal epithelial tissues (n = 9) and colonic epithelial tissues (n = 4) were selected from the two feedlot studies for use in the WGS-based analyses. The presence of *F. varium* in samples did not differ between cattle fed tylosin compared to cattle not fed tylosin, nor did it differ between cattle with ruminal acidosis and cattle without ruminal acidosis. Overall, high dietary starch or erratic feed delivery did not impact the prevalence of *F.*

varium in samples either. Whole genome sequencing revealed high levels of genetic relatedness between one set of strains recovered from matched ruminal and colonic tissues and the liver abscess from one animal. The *tet(O)* gene, encoding resistance to tetracycline antibiotics was observed in 18% of the strains. Multiple virulence genes, including *FomA*, *DnaK,* and *groEL* were found within the genomes. These findings highlight the pathogenic potential of *F. varium* and emphasize the need for further research investigating its role in liver abscess development in feedlot cattle.

Key Words: *Fusobacterium varium,* liver abscesses, rumen, colon, virulence, whole genome sequencing

Introduction

Fusobacterium varium, previously referred to as *F. necrophorum* biotype C and later as *F. pseudonecrophorum*, is a Gram-negative, rod-shaped, anaerobic bacterium that is frequently found in the gastrointestinal tract of animals and humans (Bailey and Love, 1993; Schwarz et al., 2023). Among the known pathogenic species of *Fusobacterium*, *F. varium* is recognized as an active tissue invader (Umaña et al., 2019) and has been implicated in a multitude of infections in humans and animals (Shinjo et al., 1990; Ohkusa et al., 2002; Legaria et al., 2005; Minami et al., 2009; Manson McGuire et al., 2014; Pett et al., 2014; Shamriz et al., 2015; Rachana et al., 2019; Lee et al., 2022). While infections with *F. varium* are less common than those caused by other species of *Fusobacterium*, their frequency has been increasing in recent years, often resulting in the need for extended antibiotic treatment durations and higher mortality rates and is considered, particularly in Asian countries, as an emerging pathogen in humans (Lee et al., 2022).

Fusobacterium varium has been isolated from bovine rumen fluid and feces (Chen et al., 1988; Shinjo et al., 1990). A recent study, based on 16S ribosomal RNA sequencing, has revealed *F. varium* as the predominant *Fusobacterium* species in the bovine rumen, which accounted for 46% of the total *Fusobacterium* species compared to 31% of *F. necrophorum* (Schwarz et al., 2023). *Fusobacterium necrophorum* is a well-known member of the ruminal microbial community, and of the two subspecies, *necrophorum* and *funduliforme,* the subsp. *necrophorum* has been identified as the primary causative agent in liver abscesses (Scanlan and Hathcock, 1983; Nagaraja and Chengappa, 1998). *Fusobacterium necrophorum* and *F. varium* share many growth and biochemical similarities, including colony morphology, energy substrates utilization (lactate and lysine), and ability to produce indole from tryptophan (Schwarz et al., 2023; Deters et al., 2024a)

The finding that *F. varium* is the dominant species of *Fusobacterium* in the rumen led to the development of a three-plex real time, quantitative PCR (qPCR) assay to differentiate and quantify *F. varium* and the two subspecies of *F. necrophorum* in ruminal contents of cattle (Deters et al., 2024a). In ruminal content samples collected from feedlot cattle (n= 345) at the time of slaughter, the subsp. *funduliforme* and *F. varium* were present in nearly all samples (98 to 100%), and surprisingly, the subsp. *necrophorum* was prevalent in 29% of samples at quantifiable concentrations (10^3 to 10^5 CFU per ml) and about 15% at below the detection limit (< 10^3 CFU per ml; Deters et al. 2024a). The preliminary whole genomic analysis of two *F. varium* strains isolated from the rumen revealed the presence of virulence genes implicated in the invasion of mammalian epithelial cells (Schwarz et al., 2023). Interestingly, the two strains were highly resistant to tylosin. In a preliminary study, Deters et al (2024b) reported that *F. varium* was prevalent in 3% and 9% of liver abscesses samples collected from cattle that originated from feed yards, which did not include tylosin in the finishing diet, based on culture and the qPCR assay, respectively. In the same study, the *F. varium* was also detected and isolated from ruminal and colonic epithelial tissues of the liver abscessed cattle. In light of the recent findings of prevalence of *F. varium* in liver abscesses, ruminal epithelial and colonic tissues, the identification of virulence genes implicated in tissue invasion, and resistance to tylosin, the current work aimed to investigate the effects of in-feed tylosin administration, feeding and management strategies, and experimentally-induced ruminal acidosis on prevalence and concentrations of *F. varium* in liver abscesses and corresponding ruminal and colonic epithelial tissues and contents. In addition, a subset of strains were whole genome sequenced to genetically compare strains from liver abscesses, ruminal and colonic tissues, and to identify genes associated with virulence and antimicrobial resistance.

Materials and Methods

Cattle from two feedlot studies and one experimental study were utilized to collect healthy livers, abscessed livers and matched ruminal and colonic contents and tissues to determine prevalence, frequency of isolation and concentrations of *F. varium* by culture- and qPCR-based analyses.

Effects of in-feed tylosin administration.

Samples were collected from beef-on dairy heifers that were part of a feedlot study conducted in 2022, designed to evaluate the effects of an antibiotic alternative (probiotic plus zeolite; *Saccharomyces cerevisiae* CNCM I-1077 fed at 0.5 g per heifer per day to provide 1 x 10^{10} CFU and calcium clinoptilolite zeolite fed at 1.2% on a DM basis) on the performance, carcass characteristics, and incidence of liver abscesses. The study included a negative control (no tylosin or antibiotic alternative treatment) and a positive control that received in-feed tylosin phosphate (Tylan 100, Elanco Animal Health, Greenfield, IN) at a target rate of 68 mg/head/day. The heifers were in 27 separate lots harvested across seven dates from the fall of 2022 to spring of 2023. The study population, arrival processing, number of pens, treatment allocation, diet composition, feeding and management procedures, harvest and liver abscess incidence have been previously described (Theurer et al., 2024; Wilson et al., 2024). Heifers averaged 228 days (range: 205-262) on feed and were harvested at a commercial packing plant. Subsets of 94 heifers with apparently healthy, non-abscessed livers (33 from the negative control, 32 from the tylosin-fed group and 29 from the probiotic plus zeolite group) and 96 heifers with abscessed livers (27 from the negative control, 32 from the tylosin-fed, and 29 from the probiotic plus zeolite group) were utilized in the study. At harvest, slices of liver tissues, healthy or with an intact abscess, and the corresponding matched ruminal epithelial tissues (5 to 10 cm piece from

the ventral sac) were collected, stored on ice and shipped overnight to the Anaerobe Laboratory in the College of Veterinary Medicine at Kansas State University for processing.

Effect of experimentally-induced ruminal acidosis.

Steers that were sampled at necropsy to determine the effects of experimentally-induced ruminal acidosis on prevalence and concentrations of *F. varium* in liver, ruminal and colonic epithelial contents and tissues were part of a study designed to develop a nutritional model to experimentally induce liver abscesses (McDaniel et al., 2024). Steers in two treatment groups (n=20), which included a control group fed a low-starch diet and an acidotic group fed a high-starch diet formulated to induce ruminal acidosis (acidotic group) were utilized in the study. Each group had 10 Holstein steers (initial BW = 84.9 ± 7.1 kg), which were housed in individual pens in an environmentally controlled, biosafety level-2 barn. The low-starch control diet fed to the control group was based on dry-rolled corn and wet corn gluten feed and the high-starch diet fed to the acidotic group was based on steam-flaked corn. The steam-flaked corn -based diet was fed for 3 d and then switched to the low-starch control diet for 2 d. The 5-d acidotic cycle was repeated three more times. The details of the study including IACUC approval, diet composition, feeding and management procedures, duration of the study, euthanasia and necropsy have been described (McDaniel et al., 2024). Liver tissues and ruminal and colonic contents and epithelial tissues were collected at necropsy, stored on ice and shipped to the Anaerobe Laboratory in the College of Veterinary Medicine at Kansas State University.

Effect of dietary starch concentration and feeding management regimen.

Liver abscesses, ruminal and colonic tissues and content samples were collected at slaughter from steers which were part of a feedlot study conducted in 2023, designed to determine the effects dietary starch concentration and feeding management regimen on growth performance,

carcass characteristics and prevalence of liver abscesses. Steers were assigned to 1 of 4 dietary and feeding management strategies in a 2 x 2 factorial arrangement. One factor was a dietary treatment of low starch (49.1%; **CON**) or high starch concentration (64.4%; **HOT**). The second factor was a feeding management strategy designed for consistent feed delivery (**REG**) or randomized variations (**ERR)** in both quantity (85% followed by 115% of the previous 4-d average randomly once per week) and delivery time (randomly delayed for 1, 2, 3, or 4 h twice per week)**.** None of the study diets included tylosin. Steers were shipped to a commercial beef processing facility for slaughter. The details of the study design, number of steers, number of pens, dietary composition, overall feeding and management strategies, and incidence of liver abscesses have been described by (Schneid et al., 2024). At harvest, matched liver abscess, ruminal epithelial, colonic epithelial, ruminal content and colonic content samples were collected from cattle with liver abscesses and shipped on ice overnight to the Anaerobic Laboratory in the College of Veterinary Medicine at Kansas State University for processing.

Processing of healthy and abscessed livers, ruminal contents and epithelial tissues and colonic contents and epithelial tissue samples.

The capsule of an intact liver abscess was seared with a hot spatula and then opened with a sterile scalpel. Similarly, healthy liver slices were seared and a sterile scalpel was used to slice them open for collection of inner parenchymal tissue samples. Five grams of healthy liver tissue pieces, purulent material and pieces of the inner wall of liver abscesses combined, excised ruminal and colonic epithelial tissues were suspended in 45 ml of sterile phosphate buffered saline, and homogenized in a blender (Nutribullet Pro Blender, Los Angeles, CA) for approximately 1 min. Because of the thick consistency, the colonic contents were diluted at a 1:1 ratio with sterile phosphate buffered saline and vortexed. The ruminal contents and diluted

colonic contents were strained through four layers of cheesecloth and the strained samples were used to determine concentrations of *F. varium*. One milliliter of strained ruminal and colonic contents was inoculated into 9 ml of peptone-yeast extract lactate or lysine broth with selective antibiotics, josamycin (3 mg/L), vancomycin (4 mg/L) and norfloxacin (1 mg/L) (PY-La JVN and PY-Ly JVN, respectively) and incubated at 37° C for 24 hours. If the pre-enrichment sample was initially negative by qPCR assay (below the detection limit), then the enriched sample was used for detection of the absence or presence of *F. varium.*

Species confirmation and quantification of *F. varium*.

A real-time quantitative PCR assay targeting the *hgdA* gene, which encodes for 2-hydroxyglutaryl dehydratase, was used for species confirmation of putative colonies and for the quantification of *F. varium* in liver, ruminal and colonic samples (Deters et al., 2024a).

F. varium isolation.

Sterile cotton swabs were used to spot inoculate healthy liver, liver abscess, ruminal, and colonic sample homogenates onto blood agar (Remel Inc., Lenexa, KS) and PY-La JVN and PY-Ly JVN agar. Inoculating loops were then used to streak from the spots and inoculated plates were incubated anaerobically for 48 h at 37°C to obtain isolated single colonies. Presumptive *F. varium* colonies (greyish yellow, round, smooth and flat; Deters et al., 2024a) were picked and streaked onto blood agar plates, and incubated anaerobically for 48 h at 37°C. Species identification was by the qPCR assay described above. If the direct inoculation of sample homogenate onto blood agar did not yield *F. varium*, the enriched tissue homogenates were streaked onto blood agar for isolation of *F. varium*.

DNA extraction.

Ruminal and colonic epithelial tissues, healthy liver and liver abscess tissue samples were subjected to DNA extraction using the MagMax-96 DNA Multi-Sample Kit (Applied Biosystems, Waltham, MA) according to the manufacturer's protocol for isolation of genomic DNA. Briefly, 100 µL of tissue homogenates or contents were combined with 100 µL of Multi-Sample DNA Lysis Buffer and homogenized. Homogenates were then added to the microtiter processing plate with 100% isopropanol, sealed, and shaken for 3 min on a microtiter plate shaker. DNA Binding Bead Mix was added and pelleted against a magnetic stand, beads were washed, dried, and DNA eluted. Eluted DNA was stored at -20°C until further use. DNA extraction and purification from ruminal and colonic content samples, and all samples enriched in PY-La JVN or PY-Ly JVN broths were performed using the GeneClean Turbo Kit (MP Biomedicals, Solon, OH) following the manufacturer's protocol. Briefly, 1 ml aliquots of the samples were boiled for 10 min then centrifuged for 5 min at 9,300 RCF, and the supernatant used for DNA isolation using the GeneClean Turbo Kit.

Whole genome sequencing

Bacterial strains

A total of 14 *F. varium* strains isolated from liver abscesses (n= 5), ruminal epithelial tissues (n= 5), and colonic tissues (n= 4) were whole-genome sequenced. Strains were chosen as matched sets originating from at least two of the three tissues from the same animal. The strains were grown in pre-reduced, anaerobically sterilized Brain Heart Infusion broth (PRAS-BHI; Difco™) to a late-log phase (absorbance 0.6 to 0.65 at 600 nm) for DNA extraction.

DNA extraction

Bacterial DNA was extracted from the 14 *F. varium* strains using a phenol-chloroform extraction protocol described by Green and Sambrook (Green and Sambrook, 2017) with modifications. Briefly, cultures were spun down at 5,000 RCF for 10 min to pellet bacterial cells, and the pellet was suspended in 1 ml buffer B1 (Qiagen) and 2 µl RNAseA (100mg/ml). Twenty microliters of lysozyme solution (100 mg/ml) was added to the suspension and incubated at room temperature for 30 minutes. Following incubation, 45 µl of Proteinase K (100 mg/ml) was added and samples were incubated at 50°C for 30 minutes. Each sample was then split into two microcentrifuge tubes for the remainder of the procedure. One volume (approx. 500 µl) phenol/chloroform/isoamyl alcohol (25:24:1) solution was added to each tube, mixed thoroughly by inversion and centrifuged at 3,000 RCF for 5 min with the resulting supernatant carefully transferred to a new tube using a micropipette. This extraction step was performed a total of 3 times, at which point samples were combined with 1 volume (approx. 250 µl) of chloroform: isoamyl alcohol (24:1) and centrifuged for 5 min at 3,000 RCF. The supernatant was transferred to a new tube for overnight DNA precipitation in 2.5 volume (approx. 750 µl) ice cold 100% ethanol and 0.1 volume (approx. 25 µl) NaOAc (pH 7.5) at -20°C. The following day, DNA pellets were washed 3 times with ice cold 70% alcohol, dried completely under laminar flow, and then re-dissolved in 100 µl nuclease-free DI water. Extracted DNA was stored at -20°C until further use.

DNA quality control, library preparation, and sequencing.

Extracted DNA was assessed for quality by spectrophotometry using a Biotek Take3 microvolume plate (Bioteck Synergy H1 reader, Agilent). If necessary, DNA was further purified using Mag-bind Total Pure magnetic beads (Omega Bio-tek) according to the manufacturer’s instructions. High molecular-weight DNA libraries were prepared using the

Rapid Sequencing Kit and sequencers on a MinION sequencer (Oxford Nanopor Technologies, Oxford, United Kingdom) using a Flongle adapter and flow cell (R10.4.1. chemistry) according to manufacturer instructions.

Genome Assembly

After sequencing, raw reads were base called in MiniKNOW using the Super-accurate model, and trimmed and filtered by size with Porechop (v0.2.4) prior to assembly. Genome assemblies were generated using Flye de novo assembler (v2.9.3), and annotated using Bakta (Schwengers et al., 2021).

Bioinformatic analyses.

The presence of antimicrobial resistance genes and plasmids in each genome was analyzed using StarAMR (v0.10.0). Relatedness between strains was determined using FastANI (v1.33), and reported as a heatmap created with the Heatmaply package in R. Mashtree was used to construct phylogenetic trees of all strains. The Virulence Factor Database (VFDB) was used to scan genome sequences for the presence of virulence genes (Chen et al., 2005). Additionally, a blast search was performed on all of the strains using a custom database created from previously identified potential virulence genes in *Fusobacterium* species (Bista et al., 2022).

Statistical analysis

All analyses were done using the GLIMMIX procedure in SAS v9.4, and significance for all analyses was reported as a $p < 0.05$, with tendencies reported at $p < 0.1$.

Total isolation-based *F. varium* prevalence was analyzed using a binary response distribution (1= observed, 0= absent) and the Residual PL estimation technique with the logit link. Where appropriate, fixed effects in the models included treatment, animal status, sample

type, and all possible interactions of the three; while random effects included animal ID, sampling date/processing date, and pen.

Total qPCR-based *F. varium* prevalence was analyzed using the same binary response distribution using the maximum likelihood estimation technique with the Gauss-Hermite Quadrature likelihood approximation, also with the logit link. The same fixed and random effects were used for total isolation-based prevalence and total qPCR-based prevalence analyses. The model for the analysis of ruminal epithelial *F. varium* concentrations utilized the restricted maximum likelihood estimation technique for Gaussian response distribution. The fixed effect included in the model was treatment, with pen and sampling date included as random effects where appropriate.

Results

Effects of in-feed tylosin administration on *F. varium* prevalence in liver tissues, ruminal epithelial tissues, and liver abscesses.

At harvest, the total liver abscess (LA) incidences were 37.8%, 35.4%, and 41.9% in the control, probiotic plus zeolite, and tylosin groups, respectively. Neither tylosin nor probiotic plus zeolite had any effect of the LA incidence. Heifers in the probiotic plus zeolite group tended to have fewer liver abscesses compared to the tylosin-fed group (Theurer et al., 2024). Matched healthy liver, abscessed liver, and ruminal epithelial tissue samples were collected randomly from a subset of 190 heifers. The heifers sampled included 60 from the control group with 27 abscessed liver and 33 healthy livers, 62 from the probiotic plus zeolite group with 30 abscessed livers and 32 healthy livers, and 68 from the tylosin group with 39 abscessed livers and 29 healthy livers. The frequency of *F. varium* isolations from healthy livers, abscessed livers, and matched ruminal epithelial tissues by direct plating of the tissue homogenate or after enrichment

in peptone yeast extract broth supplemented with lactate or lysine and selective antibiotics are shown in table 5-1. Fewer samples yielded *F. varium* by direct isolation compared to the number of samples that yielded strains after enrichment.

There were no treatment effects and no significant interaction between treatment, sample type, or liver health status regarding the total isolation-based prevalence of *F. varium.* The frequency of total *F. varium* isolation (direct plating plus enrichment) differed between healthy and abscessed livers ($p = 0.0145$) and between liver samples and ruminal tissues ($p < 0.0001$). The total isolation-based prevalence of *F. varium* was significantly higher in ruminal epithelial tissues than in liver tissues (80% vs. 47.9%, $p < 0.0001$), regardless of treatment group or liver health status. Across both sample types (liver and ruminal) and all three treatments, heifers with abscessed livers had a higher isolation-based prevalence of *F. varium* than heifers with healthy livers ($p = 0.0145$).

The prevalence, before and after enrichment, and concentrations of *F. varium* based on qPCR assay are shown in table 5-2. There were no significant interactions in the qPCR-based total *F. varium* prevalence between treatment, sample type (liver vs. ruminal tissues), or liver status (healthy vs. liver abscess). Neither healthy liver tissue nor abscessed liver tissues were positive for *F. varium* via qPCR prior to enrichment. However, a significant proportion (range 37.5 to 62.1%) of ruminal epithelial tissues from heifers with healthy livers or abscessed livers were positive for *F. varium* prior to enrichment, with concentrations averaging 10^5 CFU per g of tissue. The total qPCR-based prevalence of *F. varium* did not differ by treatment group, regardless of sample type, nor were there any statistically significant difference between heifers with abscessed or healthy livers, aside from in ruminal epithelial tissues. Liver abscesses were found to be significantly more likely to contain *F. varium* detected by qPCR assay after

enrichment than healthy liver tissues (p= 0.0196). Additionally, there appeared to be a numerical difference in overall qPCR-based *F. varium* by sample type, with apparently higher prevalence in ruminal epithelial tissues than in liver tissues, although these differences were not statistically significant. A trend was observed for higher *F. varium* prevalence in liver tissues from non-abscessed heifers in the control treatment compared to liver tissues from non-abscessed heifers in the probiotic treatment (p = 0.0631).

Due to the lack of pre-enriched qPCR liver tissue and liver abscess samples containing quantifiable concentrations of *F. varium*, only ruminal epithelial tissue *F. varium* concentrations were statistically compared. There was no interaction between liver health status and treatment, and no significant differences in *F. varium* concentrations were evident between liver health status or treatment groups.

Effect of experimentally induced ruminal acidosis on *F. varium* prevalence

Matched ruminal epithelial tissue, colonic tissue, and liver tissue samples were collected from 20 Holstein steers at necropsy. Ten steers were from the control treatment, and ten were from the experimentally induced acidosis treatment. None of the steers had liver abscesses at necropsy. Numerically, it appeared that steers in the induced ruminal acidosis treatment group exhibited a higher prevalence of *F. varium* in ruminal epithelial tissues than steers in the control group, at 60% vs. 20%, respectively, when prevalence was determined using isolation-based methods, however, this difference was not statistically significant (Table 5-3). When qPCR was used for the determination of *F. varium* prevalence, there was no difference between ruminal epithelial tissues in the control or ruminally acidotic groups. Ruminal and colonic content samples were also subjected to qPCR for detection and quantification of *F. varium*, with ruminal

contents seeming to have a higher prevalence than colonic contents, although this difference was not statistically significant (Table 5-4).

Effect of dietary starch concentration and feeding management regimen.

This study was designed to determine the impact of dietary starch concentration, 49.1% (CON) vs. 64.4% (HOT) and feeding management regiment, consistent feed quantity and delivery (REG) and randomized variation in feed quantity and delivery time (ERR) on the incidence of liver abscesses. At harvest, steers fed the HOT diet had a higher incidence of liver abscesses compared to the control (55.1% vs. 33.4%, respectively). Feeding management regimen, regular vs. erratic, did not affect the liver abscess incidence (Schneid et al., 2024). To determine the frequency of *F. varium* isolation via culture method, and prevalence and concentrations by qPCR, we randomly collected liver abscess samples and matched ruminal and colonic epithelial tissues from 159 steers. Of the steers sampled, 26 were from the CON REG treatment, 23 from the CON ERR treatment, 55 from the HOT REG treatment, and 55 from the HOT ERR treatment. No interaction between treatment and sample type was observed in the study. The total isolation-based prevalence of *F. varium*, which included strains obtained before and after enrichment of the samples, varied significantly between the three sample types (liver abscess, ruminal and colonic epithelial tissues) collected, regardless of the treatment group ($P <$ 0.001). The total isolation-prevalence was significantly higher in ruminal epithelial tissues (75.5%) than in colonic epithelial tissues (30.2%, $P <$0.001) or liver abscesses (17%, $P < 0.001$) (Table 5-5). The total isolation-based prevalence of *F. varium* was higher in colonic epithelial tissues than in liver abscesses ($P = 0.0022$).

While numerical differences were observed, no statistical difference in total isolation-based prevalence of *F.* varium was observed between treatment groups for either liver abscesses or

ruminal epithelial tissues. However, total isolation-based prevalence was significantly higher in colonic epithelial tissues from the CON ERR treatment than the HOT ERR treatment (P = 0.0098), with total isolation-based *F. varium* prevalence of 56.5% and 18.2%, respectively. There were no significant differences observed between any of the other treatments for isolation-based prevalence of *F. varium* within colonic epithelial tissue samples (Table 5-5).

There was no significant difference into total PCR-based *F. varium* prevalence (presence before or after enrichment) between ruminal epithelial tissue and liver abscess samples, nor was there a significant difference in the total qPCR-based prevalence between colonic epithelial and ruminal epithelial tissues. However, the *F. varium* prevalence was significantly higher in colonic epithelial tissues than liver abscesses (62.3% vs. 37.7%, $P < 0.0001$). Numerically, both the HOT REG and HOT ERR treatments resulted in lower total qPCR-based prevalence compared to the CON REG and CON ERR treatments, but these differences were no significant. The total qPCR-based prevalence was numerically lower in samples from the HOT REG treatment than in the HOT ERR treatment, but again, these observed differences were not found to be statistically significant. There were no significant differences observed in qPCR-based total prevalence between treatments within any of the three tissue types (Table 5-6).

No liver abscess samples had quantifiable *F. varium*, and only one colonic epithelial tissue sample had a quantifiable concentration of *F. varium* (Table 5-6). Therefore, only ruminal epithelial tissues were included for statistical analysis. There were no significant differences in *F. varium* concentrations between the treatment groups, with all treatment groups averaging 10^5 CFU/g of ruminal epithelial tissue.

Whole genome sequencing of 14 F. varium strains

A total of 14 *F. varium* strains were selected for whole genome sequencing and subsequent bioinformatic analyses. Strains were selected as matched sets isolated from the liver abscess, ruminal epithelial tissue, and colonic epithelial tissue from 4 animals, and from the matched liver abscess and ruminal epithelial tissue from a fifth animal. The assembled *F. varium* genomes ranged in size from 3.39 Mb to 3.61 Mb in length, with the average assembly length being 3.48 Mb. The GC content of the genomes ranged from 29.21% to 29.72%, with an average of 29.4%. These genome lengths and GC content were consistent with those reported by Schwarz et al. for their novel *F. varium* strains of bovine origin.

Average nucleotide identity Analysis.

Average nucleotide identity (ANI) analysis was used to generate pairwise comparison scores for all 14 strains. The resultant pairwise comparison values were plotted as a heat map and dendrogram in R studio. The heatmap is shown in figure 5-1, where ANI percentages were rounded to the nearest 0.001%. The generated ANI pairwise comparison values ranged from 98.677% to 99.996%. A cluster of highly genetically similar strains was observed between the strains isolated from the liver abscess, colonic epithelial tissue, and ruminal epithelial tissue from animal 323, along with the strains isolated from the colonic epithelial tissue and ruminal epithelial tissue from animals 309 and 460, with $\geq$ 99.9% ANI observed between all strains within the cluster. Previously published work has suggested using an ANI pairwise comparison value of >99.99% to establish clonality between strains for the purpose of outbreak tracing (Rodriguez et al., 2024). Using these guidelines, clonality could be inferred between the liver abscess and colonic epithelial strains from animal 323, along with the colonic epithelial and ruminal epithelial strains from animals 309 and 323. None of the other matched sets of strains

met the previously suggested ANI criteria for establishing clonality, nor was any apparent clustering by tissue type observed in the generated ANI heat map (figure 5-1).

Identification of Antimicrobial Resistance Genes

The *F. varium* strains were screened for the presence of antimicrobial resistance (AMR) genes using StarAMR, which scans bacterial genomes against the ResFinder, PointFinder, and PlasmidFinder databases. None of the strains contained AMR genes within their genomes, nor were any plasmids identified within the genomes.

Virulence factor identification

The Virulence Factor Database was used to conduct a blast search of the *F. varium* genomes for identification of virulence factors (Chen et al., 2005). The percent identity cut off value was set to 70% and the e value cutoff was set to 0.0000001. All of the *F. varium* genomes contained the *groEL* gene, which encodes for the GroEL chaperonin protein involved in *in vivo* protein folding, with approximately 73.9% identity (Yan et al., 2018).

Along with the VFDB, a custom database was used for to search for virulence factors previously identified as having potential relevance to the *Fusobacterium* genus (Bista et al., 2022). Using this database, two additional virulence factors were identified within the *F. varium* genomes: *DnaK* and the acyl-coA dehydrogenase protein sequence. The *DnaK* gene, encoding a molecular chaperone protein, was present in all 14 *F. varium* genomes, with approximately 80% identity. One strain, 2023-3CT458, contained the acyl-CoA dehydrogenase protein sequence within its genome, which encodes for a protein involved in fatty acid metabolism (Fujita et al., 2007).

Discussion

This is a follow-up study to evaluate the involvement of *Fusobacterium varium* in liver abscesses. Recent research has indicated that *F. varium* is the predominant *Fusobacterium* in ruminal contents (Schwarz et al., 2023; Deters et al., 2024a), and our previous study showed that *Fusobacterium varium* was cultured from 3% of liver abscesses sampled, and detected in 10% of those same samples by qPCR (Deters et al., 2024b). The present study aimed to further investigate the prevalence of *F. varium* in liver abscesses, non-abscessed liver tissues, ruminal and colonic epithelial tissues, and ruminal and colonic contents from cattle subjected to varying diets and management strategies. To date, this is only the second study investigating *F. varium* in bovine gastrointestinal tissues and liver abscesses, and the first to report on its prevalence in non-abscessed liver tissues.

Fusobacterium varium is considered to be an active tissue invader among *Fusobacterium* species (Umaña et al., 2019), and shares many morphological and biochemical similarities with *F. necrophorum*, the primary causative agent in liver abscess development in beef cattle, which has been generally accepted to cause liver abscesses through the "ruminitis-liver abscess complex" (Jensen et al., 1954; Nagaraja and Chengappa, 1998). It is hypothesized that high grain finishing diets, resulting in acidosis, lead to damage of the ruminal epithelium, which allows pathogenic bacteria to penetrate the ruminal epithelium, enter into portal vein circulation, and translocate into the liver. Recent studies have called into question the association of ruminal epithelial pathogenesis and liver abscess development (Bester et al., 2016), and have suggested the hindgut as a potential source of liver abscess-causing bacterial pathogens (Bissell and Hall, 2010; Gressley et al., 2011; Li et al., 2012; Sanz-Fernandez et al., 2020). The hindgut has lower buffering capacity than in in the rumen, and is composed of a singular mucosal layer, making it

potentially more susceptible to epithelial damage and increased permeability (Amachawadi and Nagaraja, 2022). Therefore, we wanted to investigate the prevalence of *F. varium* in both the rumen and the colon, and any potential association with its prevalence in liver abscesses.

In the two feedlot studies that included the analysis of colonic epithelial tissue samples, the *F. varium* prevalence ranged from 20% - 78% as determined by qPCR assay, and from 10% - 57% as determined by culture method. In the same studies, ruminal epithelial *F. varium* prevalence ranged from 50% - 95% and 20% -88% by qPCR assay and culture methods, respectively. The findings indicate that *F. varium* can be highly prevalent in the hindgut of grain-fed cattle, with prevalence rates sometimes similar to those seen in ruminal epithelial tissues.

Ribotyping has previously been used to establish clonal connections between *F. necrophorum* strains of ruminal and liver abscess origin (Narayanan et al., 1997). It was found that strains recovered from the ruminal epithelium had identical banding patterns in 8 of 9 cattle sampled. These results support the hypothesis that *F. necrophorum* penetrates the ruminal epithelium to translocate to the liver to establish abscesses. To date, no studies have been published on clonal connections between *Fusobacterium* strains recovered from the hindgut to those isolated from liver abscesses in the same animal.

The genomes of *F. varium* exhibited no apparent clustering by tissue type based on ANI or MinHash analyses for genome similarities. High levels of genetic similarity were observed amongst all 14 *F. varium* strains, with ANI pairwise similarities ranging between 98.67% to 99.99%. When applying the guidelines suggested by Rodriguez et al (2024) for establishing clonality using ANI pairwise comparison values, clonality was inferred between one set of matched liver abscess and colonic epithelium strains from the same animal. Additionally, clonality was inferred between a two more sets of matched ruminal and colonic epithelial strains.

These results give credence to the theory that bacterial pathogens in liver abscesses originate from the rumen and, potentially, the colon. While the results reported here cannot determine whether the bacterial isolate recovered from a liver abscess originated from the rumen or the colon, it does highlight the need for further investigation into the potential for the colon to serve as a source of liver abscess pathogens, especially since it exhibits less robust epithelial integrity than the rumen (Gressley et al., 2011).

The prevalence of *F. varium* was determined before and after selective enrichment, using both isolation- based and qPCR-based methods. Enrichment methods utilized were a combination of supplementation with lactate or lysine, as well as selective antibiotics (josamycin, vancomycin, and norfloxacin) (Deters et al., 2024a). The *F. varium* prevalence in the sampled tissues and contents was not affected by conditions intended to induce ruminal acidosis, nor was it affected by in-feed tylosin administration. Tylosin phosphate, a macrolide antibiotic, is commonly used as a feed additive during the finishing period in beef cattle for liver abscess prevention, and can reduce liver abscess prevalence between 40% - 70% (Nagaraja and Chengappa, 1998; Reinhardt and Hubbert, 2015; Weinroth et al., 2019; Theurer and Amachawadi, 2022). Tylosin is believed to prevent liver abscesses by reducing ruminal concentrations of *F. necrophorum*, which is generally highly susceptible to tylosin administration (Nagaraja et al., 1999). However, a recently published study described marked resistance to tylosin exhibited by two *F. necrophorum* subsp. *necrophorum* strains, and WGS analyses revealed the presence of the *erm(B)* gene, which confers resistance to macrolide antibiotics, within both genomes (Schwarz et al., 2024). Additionally, Schwarz et al.'s characterization of novel *F. varium* strains from ruminal contents indicated marked phenotypical resistance to both tylosin-phosphate and monensin (Schwarz et al., 2023).

In light of these recent findings, we investigated whether any of the *F. varium* genomes from our studies contained antimicrobial resistance (AMR) genes. No AMR genes were found within the genomes based upon genomic analysis. Schwarz et al., 2023 did not report any antimicrobial resistance genes within the genomes of their strains tested, however phenotypic resistance was shown. As such, it would be of interest to examine whether any of our strains exhibited phenotypic resistance to tylosin and/or monensin, even with the absence of AMR genes within the genomes. We also investigated the presence of virulence genes within the genomes of the 14 strains using both the VFDB and a custom database designed for *Fusobacterium* (Bista et al., 2022). All of the strains contained two chaperonins: *GroEL* and *DnaK*. Both genes belong to a highly conserved family of heat shock proteins, and have roles in stress tolerance, and potentially in tolerance to antibiotic stress as well (Singh et al., 2007). Additionally, one strain's genome encoded the acyl-CoA dehydrogenase protein sequence, which encodes a protein involved in fatty acid metabolism and increased bacterial persistence (Beites et al., 2021). Notably, the gene, *lktA*, which codes for leukotoxin, one of the most extensively studied virulence factors in *F. necrophorum* and the major virulence factor thought to be responsible for liver abscess development (Tan et al., 1996; Pillai et al., 2021), was absent in all of the *F. varium* genomes, suggesting a reliance on alternative virulence factors. These results emphasize the need for further research into the mechanisms involved in *F. varium* pathogenesis in infections, including within liver abscesses. Future studies should aim to analyze larger sets of strains recovered from animals within the same cohort to infer more meaningful differences in virulence and AMR genes between strains from various tissue types.

Conclusions

This study reinforced the findings of Schwarz et al. and Deters et al., that *F. varium* is highly abundant in the rumen of grain-fed cattle. We further investigated the prevalence of *F. varium* using culture-dependent and culture-independent methods in colonic epithelial tissues, liver abscesses, and non-abscessed liver tissues, as well as in ruminal and colonic contents, and found that the colon may serve as a source for bacterial translocation into portal circulation. Whole genome sequencing analyses were conducted on 14 *F. varium* strains to assess clonality between the three locations and their virulence potential. The approximate genome sizes and GC content of our novel genomes were similar to those reported previously. While high levels of genetic similarity were observed between strains, a low prevalence of virulence genes, and no AMR genes, were observed. Additionally, these are the first published sequences from strains of bovine liver, colonic epithelial, and ruminal epithelial origin (Genbank records, search last conducted July 16, 2024) (Clark et al., 2016). Given the presence of genes involved in both virulence and antimicrobial resistance, *F. varium* should continue to be investigated as a gut pathogen with potential involvement in liver abscess development in beef cattle.

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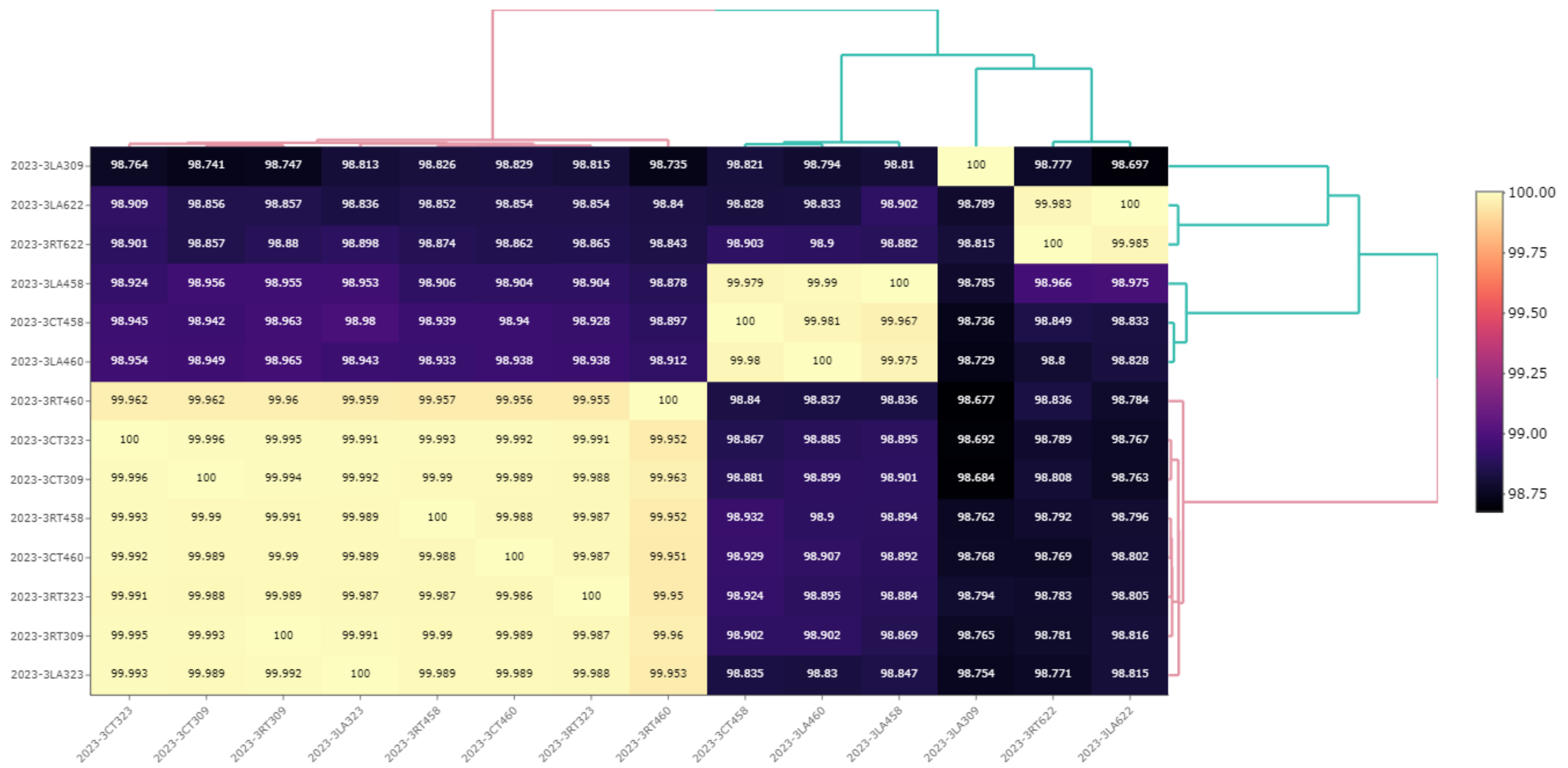

Figure 5-1 Average Nucleotide Index heat map and dendrogram of the 14 matched *Fusobacterium varium* strains isolated from liver, ruminal and colonic tissues of feedlot cattle

Table 5-1 Frequency of isolation of *Fusobacterium varium* from matched liver abscesses, healthy liver tissue, and ruminal epithelial tissues collected at slaughter from beef-on-dairy heifers fed diets with or without probiotic plus zeolite or tylosin

				Isolation, No. positive/total (%)		
Liver abscess status	**Tissues**	**Treatment group**	**No. of samples**	Direct isolation	Isolation after enrichment[2]	**Total**
Healthy (non-abscessed)	Liver tissue	Control (no tylosin)	33	2 (6.1)	17 (54.8)	19/33 (57.6)
		Probiotic (no tylosin)	32	0 (0)	11 (34.4)	11/32 (34.4)
		Tylosin	29	2 (6.9)	8 (29.6)	10/29 (34.5)
	Ruminal epithelial tissue	Control (no tylosin)	33	5 (15.2)	17 (60.7)	23/33 (69.7)
		Probiotic (no tylosin)	32	9 (28.1)	16 (69.6)	25/32 (78.1)
		Tylosin	29	6 (20.7)	17 (73.9)	23/29 (79.3)
Abscessed	Liver abscess	Control (no tylosin)	27	0 (0)	16 (59.3)	16/27 (59.3)
		Probiotic (no tylosin)	30	0 (0)	16 (53.3)	16/30 (53.3)
		Tylosin	39	0 (0)	19 (48.7)	19/39 (48.7)
	Ruminal epithelial tissue	Control (no tylosin)	27	3 (11.1)	21 (87.5)	24/27 (88.9)
		Probiotic (no tylosin)	30	8 (26.7)	17 (77.3)	25/30 (83.3)
		Tylosin	39	5 (12.8)	27 (79.40	32/39 (82.1)

[1]The probiotic product included *Saccharomyces cerevisiae* CNCM I-1077 fed 0.5 g per heifer per day to provide 1 X 10^{10} and calcium clinoptilolite zeolite fed at 1.2% on a DM basis, respectively; Tylosin phosphate was included at a rate of 68 mg per heifer per day.

[2] Homogenized tissue samples were enriched in peptone-yeast extract medium containing lactate (100 m*M*) or lysine (100 m*M*) as the major energy source, and supplemented with josamycin, vancomycin, and norfloxacin at 3 µg/mL, 4 µg/mL, and 1 µg/mL, respectively.

Table 5-2 Prevalence and concentration of *Fusobacterium varium*, based on quantitative PCR assay, in matched liver abscesses, healthy liver tissue, and ruminal epithelial tissues collected from feedlot cattle at slaughter either fed or not-fed tylosin

Liver abscess status	**Tissue Type**	**Treatment group**	**No. of samples**	**Prevalence, no. positive/total (%)**	**Mean concentration, CFU/g**	**Prevalence, no. positive/total after enrichment in (%):** Peptone yeast extract-lactate plus JVN[1]	Peptone yeast extract-lysine plus JVN[1]	**Total**
Healthy (non-abscessed)	Liver tissue	Control	33	0 (0)	N/A	12 (36.4)	14 (42.4)	17/33 (51.5)
		Probiotic (no tylosin)	32	0 (0)	N/A	4 (12.5)	4 (12.5)	6/32 (18.8)
		Tylosin	29	0 (0)	N/A	7 (24.1)	8 (27.6)	8/29 (27.6)
	Ruminal epithelial tissue	Control	33	13 (39.4)	1.93×10^5	20 (100)	20 (100)	33/33 (100)
		Probiotic (no tylosin)	32	12 (37.5)	2.06×10^5	19 (95)	19 (95)	31/32 (96.9)
		Tylosin	29	18 (62.1)	1.48×10^5	8 (72.7)	8 (72.7)	26/29 (89.7)
Abscessed	Liver abscess	Control	27	0 (0)	N/A	18 (66.7)	17 (63)	20/27 (74.1)
		Probiotic (no tylosin)	30	0 (0)	N/A	14 (46.7)	12 (40)	15/30 (50)
		Tylosin	39	0 (0)	N/A	16 (41)	16 (41)	18/39 (46.2)
	Ruminal epithelial tissue	Control	27	16 (59.3)	1.99×10^5	11 (100)	11 (100)	27/27 (100)
		Probiotic (no tylosin)	30	12 (37.5)	2.06×10^5	19 (95)	19 (95)	31/32 (96.9)
		Tylosin	39	22 (56.4)	1.16×10^5	12 (70.6)	12 (70.6)	34/39 (87.2)

[1]Homogenized tissue samples were enriched in peptone-yeast extract medium containing lactate (100 m*M*) or lysine (100 m*M*) as the major energy source, and supplemented with josamycin, vancomycin, and norfloxacin at 3 µg/mL, 4 µg/mL, and 1 µg/mL, respectively.

Table 5-3 Frequency of isolation of *Fusobacterium varium* from liver tissue and corresponding ruminal and colonic epithelial tissues collected from steers in the control group and in steers with

Treatment group	**Tissue type**	**No. of samples**	**Isolation, No. positive/total (%)**		
			Direct isolation	Isolation after enrichment[1]	Total
Control	Liver tissue	10	0	2 (20)	2/10 (20)
	Ruminal epithelium	10	0	2 (20)	2/10 (20)
	Colonic epithelium	10	0	1 (10)	1/10 (10)
Ruminally acidotic	Liver tissue	10	0	1 (10)	1/10 (10)
	Ruminal epithelium	10	0	6 (60)	6/10 (60)
	Colonic epithelium	10	0	1 (10)	1/10 (10)

[1]Homogenized tissue samples were enriched in peptone-yeast extract medium containing lactate (100 m*M*) or lysine (100 m*M*) as the major energy source, and supplemented with josamycin, vancomycin, and norfloxacin at 3 µg/mL, 4 µg/mL, and 1 µg/mL, respectively.

experimentally induced ruminal acidosis

Table 5-4 Prevalence of *Fusobacterium varium*, based on quantitative PCR assay, in liver tissue and corresponding ruminal, ileal and colonic contents and epithelial tissues collected from steers in the control group and in steers with experimentally induced acidosis

Treatment group	**Sample type**	**No. of samples**	**Prevalence, No. positive/total (%)**	**Mean concentration, CFU/ml or CFU/g**	**Prevalence, no. positive/total after enrichment in (%):**		**Total**
					Peptone yeast extract-lactate plus JVN[1]	Peptone yeast extract-lysine plus JVN[1]	
Control	Liver tissue	10	0 (0)	N/A	1 (10)	0 (0)	1/10 (10)
	Ruminal contents	10	0 (0)	N/A	6 (60)	7 (70)	7/10 (0)
	Ruminal epithelium	10	1 (10)	2.93×10^4	4 (44.4)	3 (33.3)	5/10 (50)
	Colonic contents	10	0 (0)	N/A	2 (20)	0 (0)	2/10 (20)
	Colonic epithelium	10	0 (0)	N/A	2 (20)	2 (20)	2/10 (20)
Ruminally acidotic	Liver tissue	10	0 (0)	N/A	1 (10)	1 (10)	1/10 (10)
	Ruminal contents	10	1 (10)	1.79×10^3	4 (44.4)	7 (77.8)	8/10 (80)
	Ruminal epithelium	10	2 (20)	1.22×10^5	3 (37.5)	3 (37.5)	5/10 (50)
	Colonic contents	10	0 (0)	N/A	0 (0)	0 (0)	0/10 (0)
	Colonic epithelium	10	0 (0)	N/A	0 (0)	2 (20)	2/10 (20)

[1]JVN = Josamycin (3 µg/mL), Vancomycin (4 µg/mL), Norfloxacin (1 µg/mL)

Table 5-5 Frequency of isolation of *Fusobacterium varium* from liver tissue and corresponding ruminal and colonic epithelial tissues collected from cattle fed diets containing low or high starch level under two feeding management regimens[1]

Tissue Type	Dietary starch level	Feeding regimens	Number of samples	Isolation, No. positive/total (%)		
				Direct isolation	Isolation after enrichment[2]	Total
Liver abscess	CON	REG	26	0 (0)	7 (26.9)	7/26 (26.9)^{AB}
	CON	ERR	23	0 (0)	2 (8.7)	2/23 (8.7)^{A}
	HOT	REG	55	0 (0)	9 (16.4)	9/55 (16.4)^{A}
	HOT	ERR	55	0 (0)	9 (16.4)	9/55 (16.4)^{A}
Ruminal epithelium	CON	REG	26	6 (23.1)	17 (85)	23/26 (88.5)^{D}
	CON	ERR	23	5 (21.7)	13 (72.2)	18/23 (78.3)^{CD}
	HOT	REG	55	6 (10.9)	32 (65.3)	38/55 (69.1)^{BCD}
	HOT	ERR	55	1 (1.8)	40 (74.1)	41/55 (74.5)^{CD}
Colonic epithelium	CON	REG	26	0 (0)	9 (34.6)	9/26 (34.6)^{ABC}
	CON	ERR	23	2 (8.7)	11 (52.4)	13/23 (56.5)^{ABCD}
	HOT	REG	55	2 (3.6)	14 (26.4)	16/55 (29.1)^{AB}
	HOT	ERR	55	1 (1.8)	9 (16.7)	10/55 (18.2)^{A}

[1]Two diets included a feedlot finishing diet containing 49.1% (CON) or 64.4% (HOT) starch level, each fed under regular (REG) or erratic (ERR) feeding regimens

[2] Homogenized tissue samples were enriched in peptone-yeast extract medium containing lactate (100 m*M*) or lysine (100 m*M*) as the major energy source, and supplemented with josamycin, vancomycin, and norfloxacin at 3 µg/mL, 4 µg/mL, and 1 µg/mL, respectively.

Differing superscripts indicate significant differences at $p < 0.05$

Table 5-6 Prevalence of *Fusobacterium varium*, based on quantitative PCR assay, in liver tissue and corresponding ruminal and colonic contents and epithelial tissues collected from cattle subjected to 4 different management styles

Tissue Type	Dietary starch level	Feeding regimen	No. of Samples	Prevalence, No. positive/total (%)	Mean concentration, CFU/g	Prevalence, No. positive/total after enrichment in (%):		Total Prevalence
						Peptone yeast extract-lactate plus JVN[1]	Peptone yeast extract-lysine plus JVN[1]	
Liver abscess	CON	REG	26	0 (0)	N/A	8 (30.8)	9 (34.6)	10/26 (38.5)
	CON	ERR	23	0 (0)	N/A	6 (26.1)	9 (39.1)	9/23 (39.1)
	HOT	REG	55	0 (0)	N/A	12 (21.8)	11 (20)	16/55 (29.1)
	HOT	ERR	55	0 (0)	N/A	14 (25.5)	18 (32.7)	24/55 (43.6)
Ruminal epithelium	CON	REG	26	14 (53.8)	8.41×10^4	9 (75)	5 (41.7)	23/26 (88.5)
	CON	ERR	23	12 (52.2)	1×10^5	10 (90.9)	9 (81.8)	22/23 (95.7)
	HOT	REG	55	14 (25.5)	5.64×10^4	24 (58.5)	23 (56.1)	40/55 (72.7)
	HOT	ERR	55	8 (14.5)	7.19×10^4	31 (66)	23 (48.9)	40/55 (72.7)
Colonic epithelium	CON	REG	26	0 (0)	N/A	15 (57.7)	12 (46.2)	17/26 (65.4)
	CON	ERR	23	1 (4.3)	1.81×10^4	14 (60.9)	15 (65.2)	18/23 (78.3)
	HOT	REG	55	0 (0)	N/A	29 (52.7)	28 (50.9)	40/55 (72.7)
	HOT	ERR	55	0 (0)	N/A	20 (36.4)	22 (40)	29/55 (52.7)

[1]JVN = Josamycin (3 µg/mL), Vancomycin (4 µg/mL), Norfloxacin (1 µg/mL)

Chapter 6 Characterization of Five Novel Bacteriophages Lytic to *Fusobacterium necrophorum* subspecies *necrophorum*

Abstract

Fusobacterium necrophorum subsp. *necrophorum*, an anaerobic, Gram- negative bacterium, is a normal inhabitant of the bovine rumen and the primary causative agent of liver abscess formation in feedlot cattle. Liver abscesses are the number one cause of liver condemnation at slaughter, and result in a major economic impact to the beef industry. Currently, the prevention of liver abscesses relies primarily on the in-feed administration of tylosin phosphate, a macrolide antibiotic, but growing concerns over antibiotic usage in food animal production have made the development of antimicrobial alternatives a priority to the beef industry. Bacteriophages, viruses that predate bacteria, are one antimicrobial alternative that has been applied in human and animal medicine with high levels of success. Our objective in this study was to characterize five novel bacteriophages, previously isolated from untreated sewage, for their potential application as an alternative to tylosin for liver abscess prevention. The five bacteriophages were visualized using transmission electron microscopy for morphological characterization, and experiments to determine the host-range, titers, pH stability, and genome composition were performed. A proof of concept study was then conducted using a cocktail of the bacteriophages as a prophylactic for the prevention of liver abscesses caused by *F. necrophorum* in a mouse model. Three bacteriophages were assigned to the family *siphoviridae,* and one bacteriophage was assigned to the *myoviridae* family based on morphology and nucleic acid type. All five bacteriophages were lytic specifically to *F. necrophorum* subsp. *necrophorum*, but exhibited a broad host range within the subspecies, including against strains isolated from

bovine liver abscesses. The proof of concept study showed that prophylactic bacteriophage administration resulted in a 30% reduction in mortality ($P < 0.05$) and a marked reduction in morbidity amongst surviving mice. The results indicated that bacteriophage therapy can be a viable alternative to antibiotics, including tylosin, for the prevention of liver abscesses in feedlot cattle, although more in depth research is required to determine optimum bacteriophage dosages and routes of administration.

Key Words: *F. necrophorum*, bacteriophages, antibiotic alternatives, liver abscesses, cattle

Introduction

Fusobacterium necrophorum is a Gram negative, non-spore-forming, anaerobic bacteria, and is a normal member of the gastrointestinal flora of both humans and animals (Langworth, 1977). In the GI tract, *F. necrophorum* has roles in fermentation of amino acids, lactic acid, and the degradation of proteins. There are two subspecies of *Fusobacterium necrophorum:* subsp. *necrophorum* (biovar/biotype A) and subsp. *funduliforme* (biovar/biotype B), which differ in morphology, biochemical properties, and virulence; specifically, leukotoxin production, which is consistently higher in subsp. *necrophorum* than in subsp. *funduliforme* (Shinjo et al., 1991; Tan et al., 1992, 1996). Aside from being a normal inhabitant of the GI tract, *F. necrophorum* is considered an opportunistic pathogen and is frequently isolated from infections in the oral cavity, genitourinary and respiratory tracts, and other necrotic lesions. In humans, *F. necrophorum* subsp. *funduliforme* is more frequently implicated in disease. It is the causative agent of Lemierre's syndrome and is one of the most common anaerobes isolated from abdominal abscesses and respiratory infections, as well as being associated with septic joint infections, soft tissue abscesses, and infections in the female genitourinary tract (Tan et al., 1996; Brazier, 2006). In animals, *F. necrophorum* subsp. *necrophorum* is more commonly associated with disease, including calf-diphtheria (necrotic laryngitis), foot rot, soft tissue abscessation, and, in cattle, hepatic abscesses (Scanlan and Hathcock, 1983; Nagaraja and Chengappa, 1998; Nagaraja et al., 2005).

Liver abscesses in feedlot cattle are caused by *F. necrophorum* subsp. *necrophorum*, and are the most common cause of liver condemnation at slaughter, resulting in a significant economic impact to the beef cattle industry (Brown and Lawrence, 2010; Reinhardt and Hubbert, 2015). The development of liver abscesses is a consequence of chronic ruminal acidosis and

subsequent damage to the ruminal epithelium as a result of feeding high-grain finishing diets. The compromised ruminal epithelium allows the translocation of *F. necrophorum* into portal vein circulation, where it becomes trapped in, and subsequently colonizes, the liver (Jensen et al., 1954; Nagaraja and Chengappa, 1998). Currently, liver abscess control relies primarily on the use of in-feed antibiotics (Nagaraja and Lechtenberg, 2007). Tylosin phosphate, a macrolide, is the most commonly utilized antibiotic for the prevention of liver abscesses and can result in up to a 90% reduction in ruminal *F. necrophorum* populations, and a 70% reduction in liver abscess prevalence (Nagaraja and Chengappa, 1998; Nagaraja et al., 1999). However, the prevalence of liver abscesses in feedlot cattle has been increasing in recent years and there is increasing scrutiny/restriction over the use of in-feed antibiotics in beef cattle leading to the desire for antimicrobial alternatives for liver abscess prevention in feedlot cattle (Angulo et al., 2009; Harris et al., 2017). Antimicrobial alternatives that have been explored as liver abscess preventatives include essential oils (EO) and *Saccharomyces cerevisiae* fermentation products (SCFP), with varying degrees of efficacy compared to tylosin phosphate (Pukrop et al., 2017; Huebner et al., 2019).

Another antimicrobial alternative, bacteriophages, have recently seen a resurgence of interest in food animal production and food safety (Czaplewski et al., 2016; Fischetti, 2018). Bacteriophages are viruses that infect and kill bacteria, and were first recognized as potential therapeutic agents in the early 20th century, although the widespread adoption of antibiotic therapies stunted bacteriophage research in western medicine for many years. One of the major benefits of bacteriophage therapy over traditional antimicrobial therapies is their narrow spectrum of host-specificity, which reduces the potential for “off-target” effects of treatment and typically results in fewer treatment side effects compared to antibiotics (Veiga-Crespo and Villa,

2009; Loc-Carrillo and Abedon, 2011). Recently, bacteriophages apparently lytic to *Fusobacterium necrophorum* were isolated from ruminal fluid and raw municipal sewage samples (Schnur et al., 2024). In this publication, we seek to further characterize a subset of these bacteriophages, and investigate their potential as an antimicrobial alternative for the prevention of liver abscesses caused by *F. necrophorum*.

Methods

Bacteriophage Propagation:

Bacteriophages SA2, SA3, SA4, SA11, and SA14 were isolated from raw, unfiltered sewage (Schnur, unpublished data). Bacteriophages were propagated on agar plates using a spot-on-lawn technique, and in broth cultures. For propagation on agar media a lawn of late-log growth *F. necrophorum* subsp. *necrophorum* broth culture (approx. 0.8 OD) was spread-plated on a blood agar plate (TSA supplemented with 5% sheep's blood, Remel), onto which 20 µl of bacteriophage lysate was spotted. Plates were incubated anaerobically for 24 h at 37°C, and bacteriophage plaques were harvested as agar plugs using a sterile transfer pipet. Agar plugs were eluted in 1 ml SM buffer (Cold Springs Harbor Protocols, 2006) overnight in 1.5 ml micro-centrifuge tubes. Post-elution, microcentrifuge tubes were centrifuged for 30 s at 12,000 rcf to pellet agar and cellular debris and the supernatant was transferred to a 0.22 µ Spin-X microcentrifuge tube (Corning, NY, USA). Spin-X tubes were centrifuged at 12,000 rcf for 2 min at room temperature with filter-sterilized bacteriophage lysates stored at 4°C until further use.

Propagation of bacteriophages in broth cultures was accomplished by combining 1 ml of overnight broth culture (approx. OD 1.00) of a phage-susceptible *F. necrophorum* strain with 1

ml of bacteriophage suspension into 10 ml of PRAS BHI broth. The mixture was incubated at 37°C until lysis occurred, with optical density (600 nm) readings recorded every hour. Bacterial lysis was indicated by a marked clearing of the inoculum, observed as a decrease in OD to near baseline readings (<0.2, 600 nm). Once lysis occurred, bacteriophage liquid cultures were filter-sterilized twice through a 0.22 µl syringe filter prior to long-term storage at 4°C.

Bacterial Cultures

A total of 60 *F. necrophorum* subsp. *necrophorum*, 31 subsp. *funduliforme*, and 30 miscellaneous *Fusobacterium* sp. strains were used. All *F. necrophorum* strains were originally isolated from bovine liver abscesses. The miscellaneous *Fusobacterium* sp. strains were laboratory isolates of various origin. Additionally, 17 non-*Fusobacterium* strains were used to determine host-range, all of which were laboratory isolates of various clinical origin.

Fusobacterium strains were streaked onto blood agar plates and incubated anaerobically for 48 h at 37°C, sub-cultured in pre-reduced, anaerobically sterilized Brain-Hear Infusion (BHI) broth and their species and subspecies confirmation analyzed via MALDI-TOF mass spectrometry or RAPID-ANAII (Remel, Lenexa, KS). *Bacillus, Enterobacter, Escherichia, Klebsiella, Listeria, Pseudomonas, Salmonella, Serratia, Staphylococcus,* and *Trueperella* strains were streaked onto blood agar plates and incubated aerobically at 37°C for 24 h, and their identities confirmed via MALDI-TOF mass spectrometry.

Media Preparation

Pre-reduced, anaerobically sterilized brain-heart infusion (PRAS BHI, Difco) broth was utilized for all broth-culture experiments. Media was prepared as according to the manufacturer's instruction with the addition of 0.0001% resazurin and 0.05% cysteine HCl, dispensed

anaerobically into hungate tubes, then autoclaved. Prepared BHI was stored at room temperature for up to 1 month until use.

SM buffer for bacteriophage elution was prepared as according to the published protocol by Cold Spring Harbor Laboratory (2006). Prepared SM buffer was autoclaved, filter sterilized through a 0.22 µ filter and aliquoted into 50 ml centrifuge tubes for storage of up to 1 month prior to use.

Bacteriophage Host Range Determination

Host range testing of all 5 bacteriophages was conducted using 60 strains of *F. necrophorum* subsp. *necrophorum*, 31 strains of *F. necrophorum* subsp. *funduliforme*, 27 strains of *F. varium*, and 1 strain each of *F. gastrosuis, F. ulcerans, F. nucleatum* and *F. russii.* Additionally, between 1 to 3 strains each of *Bacillus, Enterobacter, Escherichia*, *Klebsiella, Listeria, Pseudomonas, Salmonella, Serratia, Staphylococcus, Streptococcus,* and *Trueperella*, were utilized in the host range assay. A complete list of the bacterial strains used can be found in Table 6-1. Host range was determined using a modified spot-on-lawn technique (Adams, 1959). Briefly, an overnight liquid culture of each bacterial isolate was plated onto blood agar with a sterile cotton swab to create a bacterial lawn, onto which 10 µl of bacteriophage lysate was spotted. Plates were incubated as per standard growth conditions for the bacterial strain tested, and checked for bacterial lysis after incubation. Lysis was observed as zones of bacterial growth inhibition at the site of the bacteriophage deposit, and scored on a scale with (+++) indicating complete lysis, (++) indicating incomplete lysis, and (-) indicating no observed lysis as according to Litt and Jaroni (2017).

Bacteriophage Morphology:

Morphology of the phages was observed using transmission electron microscopy. Lysates were prepared via the above described agar plate propagation method. Phage lysates were negatively stained using Uranyless (Electron Microscopy Sciences, Hatfield, PA) onto carbon coated copper grids (Electron Microscopy Sciences). The overall morphology, capsid dimensions, and tail length were measured using ImageJ software (Abràmoff et al., 2004).

Bacteriophage Titer Determination:

Bacteriophage titers were determined as plaque forming units (PFU) per ml by serially diluting (1:10) the bacteriophage working stock in SM buffer and performing a modified plaque assay (Adams, 1959). Briefly, serial dilutions were combined with an equal volume of overnight *F. necrophorum* subsp. *necrophorum* broth culture, mixed thoroughly, then spread-plated in triplicate onto blood agar plates. Blood agar plates were incubated anaerobically for 24 h at 37°C and individual bacteriophage plaques were counted.

pH stability of bacteriophages

The pH stability of the bacteriophages was performed using modifications to the protocol described by Litt and Jaroni (Litt and Jaroni, 2017). Briefly, bacteriophages were suspended in sterile PBS adjusted with 1M NaOH or HCl to achieve a pH range of 4-9 (± 0.2). pH adjusted bacteriophage lysates were incubated at room temperature (approx. 22°C) to determine their survival at 1 and 24 h. At the selected time points, surviving bacteriophage concentrations were determined using the bacteriophage titer determination protocol described above.

Bacteriophage survival in cold storage:

Bacteriophage lysates were stored at 4°C, and at -80°C in SM buffer supplemented with 20% glycerol in sterile cryovials. Periodically, the lysates were removed from cold storage to determine survival.

Bacteriophage DNA extraction

For DNA extraction, the Purelink Viral RNA/DNA Mini Kit (Invitrogen, Waltham, MA) was used according to manufacturer instruction. Briefly, 62.5 µl of Proteinase K was added to 500 µl of purified bacteriophage lysate. A lysis buffer and carrier RNA mixture were prepared as according to the formula provided by the manufacturer, and added to each sample. Sample tubes were vortex mixed, incubated at 56° C for 15 minutes then centrifuged for 15 seconds at 6,800 rcf at room temperature. Following centrifugation, 402.8 µl of absolute ethanol was added and each tube was vortexed for 15 seconds. Samples were incubated at room temperature for 5 minutes, at which point the lysates were transferred to viral spin columns, centrifuged at 6,800 x g for 1 min, and the flow-through discarded. This was followed by two washes with 500 µl of wash buffer with the flow-through discarded. Spin columns were dried via centrifuging at 16,000 x g for 1 minute then placed into clean tubes for DNA recovery. DNA recovery consisted of the addition of 50 µl of sterile, RNase-free water to the center of each spin column followed by a 1-minute room temperature incubation period and elution via centrifuging at 16,000 x g for 1 minute. Eluded DNA was stored at -80°C until sequencing.

DNA Analysis and Sequencing

The quantity, concentration, and purity of extracted bacteriophage DNA was analyzed using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). Quality analysis results are shown in table 2. DNA QC, library preparation, and sequencing was performed by

CosmosID (Germantown, MD). Quantification of extracted DNA was performed using a Qubit 4 fluorometer and the Qubit dsDNA HS Assay Kit (Thermofisher Scientific). Libraries were prepared with the Nextara XT DNA Library Preparation Kit (Illumina) and IDT Unique Dual Indexes with a total DNA input of 1 ng, and genomic DNA was fragmented using a proportional amount of Illumina Nextera XT fragmentation enzyme. After the addition of unique dual indexes, samples were subjected to 12 rounds of PCR for library construction. DNA libraries were purified using AMpure magnetic beads (Beckman Coulter) and eluted into Qiagen EB buffer, followed by library quantification using a Qubit 4 fluorometer and the Qubit dsDNA HS Assay Kit. DNA sequencing of the libraries was conducted on the Illumina NovaSeq platform with a 2 x 150 bp read length.

Bioinformatic Analyses

Raw reads were trimmed using Trim galore version 0.6.7 and Flash (version 2.2.00) was used to merge the quality controlled paired-end reads into interleaved outputs to be used for genome assembly. Genomes were assembled using SPAdes (version 3.15.3) with default parameters and the careful flag. Quast was utilized for evaluation of the genome assemblies.

Evaluation of a bacteriophage cocktail for prevention of liver abscesses in a mouse model

Thirty male, 11-week old, BALB-C mice were acquired from Charles River Laboratories for use in the study. Mice were housed in solid-bottomed cages, with each cage containing between 1-5 mice. All mice were maintained on ad libitum water and standard laboratory rat chow. Mice were randomly assigned to one of 3 treatments: *F. necrophorum* control (A; n=10); bacteriophage control (B; n=10), or *Fuso* + phage challenge (C; n=10). Treatment groups B and C received intraperitoneal (IP) inoculation with 0.1 ml of bacteriophage cocktail (suspended in

SM buffer), with the approximate phage concentration of 1 x 10^9 PFU/ml on study day 0. On study day 1, treatment groups A and C received an IP injection of 0.1 ml of *F. necrophorum* strain 8L1 in BHI, at a concentration of 1.65 x 10^8 CFU/ml (final dosage, approx. 1.7 x 10^7 CFU). Mice were monitored at least twice daily for 14 days post-*F. necrophorum* challenge, at which point all surviving mice were humanely euthanized. Necropsies were performed, and both gross and liver-specific pathologies were noted. Additionally, livers were collected from all mice and liver weights were recorded.

Bacteriophage isolation from mouse livers:

Whole mouse livers were homogenized in 5 ml of sterile PBS using a commercial tissue homogenizer immediately after harvesting. One milliliter of homogenate was used for qPCR detection/quantification of *F. necrophorum*, and 1 ml was used for bacteriophage detection/enumeration. Homogenate used for bacteriophage detection was centrifuged at 9,000 x g for 30 sec to pellet debris and the supernatant transferred into a 0.22 μ Spin-X tube, where it was centrifuged at 9,000 x g for 2 min. Spin-X cartridges were discarded and the filtered flow-through was used in 1:10 serial dilutions in SM buffer for enumeration of bacteriophages. Each serial dilution was combined with an equal volume of overnight liquid *F. necrophorum* subsp. *necrophorum* 8L1 culture, mixed by pipetting, then spread-plated in triplicate onto blood agar. Blood agar plates were incubated anaerobically at 37°C for 24 h and assessed for bacteriophage plaque development.

Results

Bacteriophage morphology

Transmission electron microscopy (TEM) was used for visualization and morphological characterization of the bacteriophages. Bacteriophages SA2, SA3, SA11, and SA14 were

assigned to the order Caudovirales based upon morphological characteristics (figure 6-1). Bacteriophages SA3, SA11, and SA14 were further classified based upon morphological characteristics to the family *siphoviridae* due to their long, non-contractile tails and icosahedral capsids, while phage SA2 was classified as belonging to the *myoviridae* family, due to its apparently contractile tail and icosahedral capsid morphology. Capsids ranged in diameter from approximately 73 nm to 85 nm, and tail lengths ranged from approx. 40 nm to 256 nm. Tail widths ranged from approx. 11 nm to 14 nm. Full bacteriophage dimensions are provided in table 6-2.

Numerous attempts were made to visualize bacteriophage SA4 using TEM methods, including the use of positive and negative staining. However, none of these attempts yielded success in visualizing particles resembling typical bacteriophage morphologies. Consistently, a rounded, tailless particle of approximate 64 nm x 51 nm in size was observed under TEM. A photo of this particle is included in figure 6-1c.

Bacteriophage titers:

Bacteriophage titers were determined using a modified plaque assay technique (Adams, 1959), and the quantified bacteriophage stocks solutions were then used for all subsequent experiments to ensure consistency. The bacteriophage stock solutions used for the titer, and all subsequent experiments were propagated in liquid broth culture. The titers for SA3 and SA11 were both 1.2×10^9 PFU/ml, while the concentration of SA2 and SA14 were 7.4×10^8 PFU/ml and 1.5×10^9 PFU/ml, respectively. Bacteriophage SA4 had a titer of approximately 1×10^7 PFU/ml, and was notably more difficult to propagate.

Host Range Determination

Bacteriophage host ranges were determined using a spot-on-lawn technique, with minor modifications (Adams, 1959). The host range assay revealed varied lytic activity against *F. necrophorum* subsp. *necrophorum* strains among the 5 bacteriophages (SA2, SA3, SA4, SA11, and SA14), although lytic activity of all phages was confirmed to be specific only to *F. necrophorum* subsp. *necrophorum*. Over 45 out-group, non-*F. necrophorum* strains were used to determine the specificity of the bacteriophage host range, along with 60 *F. necrophorum* subsp. *necrophorum* strains. Bacteriophages SA2 and SA11 exhibited strong lytic activity against all tested 60 *F. necrophorum* subsp. *necrophorum* strains, and bacteriophages SA3 and SA14 were lytic to all but one of the tested *F. necrophorum* strains (2022-1 LA19). Bacteriophage SA4 was lytic to 36 (60%) of the tested *F. necrophorum* strains. Detailed results of the host-ranging experiment can be found in Table 6-3.

pH and temperature stability

In order to evaluate the stability of the 5 bacteriophages in various pH conditions, phages were incubated in pH-adjusted PBS (pH 4, 5, 6, 7, 8, and 9) for 24 hours. At 1 h and 24 h post-inoculation, aliquots of bacteriophage lysates were spread-plated onto blood agar for plaque assay and subsequent titer determinations.

None of the bacteriophages exhibited any significant reduction in titer concentration after 1 h at any of the tested pH values. Overall, all 5 of the bacteriophages were found to be able to survive in pH values ranging from 4-9 for a duration of 24 hours. However, all phages displayed significantly reduced ($p < 0.02$) titers after 24 hours at pH 4, with these reductions ranging in magnitude from 1 to 4 log-fold. Similarly, it was observed that bacteriophages SA3, SA11, and

SA14 titers were significantly reduced ($p < 0.03$) after 24 h at pH of 9, exhibiting a 1 to 2-log-fold decrease in titer.

All 5 of the bacteriophages survived over 3 years at 4°C with no apparent reduction in titer. Similarly, phages stored in glycerol at -80°C were unaffected by long-term cold storage.

Genome Assemblies and Annotation

It was determined that all 5 bacteriophages were dsDNA viruses, with assembled genome sizes ranging in estimated size from 31,441 bp to 170,234 bp. The GC content of the genomes ranged from 28% - 47%.

Bacteriophage cocktail for the prevention of liver abscesses

A cocktail containing all 6 of the bacteriophages characterized was investigated as a potential antimicrobial alternative for the prevention of liver abscesses caused by *F. necrophorum* in a BALB/C mouse model. Mice were given an IP injection of either *F. necrophorum* subsp. *necrophorum* strain 8L1 (1.7×10^8 CFU/ml "Group A"), the bacteriophage cocktail ($\sim 1 \times 10^9$ PFU/ml, "Group B") or both ("Group C"). None of the mice from group A (n=10) survived more than 72 h post-8L1 challenge, whereas all 10 group B mice survived the full 15-day study period. One mouse from group C died 2 days post-8L1 inoculation, 4 died 4 days post-8L1 challenge, and 2 died 5 days post-challenge. The remaining 3 group C mice survived the full 15-day study period, at which point they were humanely euthanized.

All mice from group A had observable liver abscesses at their time of death, with enumerable amounts of *F. necrophorum* in the liver homogenate (mean 9.23×10^6 CFU/g tissue) and none of the group B mice had observable liver abscesses or detectable amounts of *F. necrophorum* in their livers. The 7 group C mice that died prior to the end of the study period also had observable livers abscesses upon necropsy, with an average liver *F. necrophorum*

concentration of 5.83 x 10^6 CFU/g. The 3 group C mice that survived to the study end had apparently healthy livers, with no observable liver abscesses, though one of the mice had enumerable *F. necrophorum* in its liver (5.15 x 10^5 CFU/g). No bacteriophages were recovered from the livers of any mice in groups A or B, though 4 mice from group C had viable bacteriophages present in the liver at the time of death. The titer of the recovered bacteriophages ranged from 4.89 x 10^4 PFU/g to 8.69 x 10^7 PFU/g, with a mean of 1.14 x 10^6 PFU/g. All 4 of the group C mice with recovered bacteriophages had liver abscesses at the time of death, and enumerable *F. necrophorum* in the liver tissue.

Discussion

The present study was conducted as a follow-up study to Schnur et al., 2024, who reported the isolation of novel bacteriophages lytic to *F. necrophorum* subsp. *necrophorum* from untreated sewage. Additionally, we investigated the potential for these bacteriophages to be used therapeutically for the prevention of liver abscesses caused by *F. necrophorum.*

One of the long-standing challenges with bacteriophage research is the lack of a universally accepted method for bacteriophage classification. Historically, classification was based upon phage morphology observed via electron microscopy (capsid shape, presence or absence of envelope, and number of capsomers) and nucleic acid type (dsDNA, ssDNA, or ssRNA) (Lwoff et al., 1962; Bradley, 1967; Ackermann, 2006). Classification schemes were later expanded over the years to include over 20 families, including the most well studied families *syphoviridae, myoviridae,* and *podoviridae* (Ackermann and Eisenstark, 1974; Ackermann, 2006). Three of the bacteriophages in our study were classified into the *Siphoviridae* family due to their long, noncontractile tails and icosahedral capsid morphology, while one bacteriophage was classified into the *myoviridae* family to the its apparently

contractile tail and icosahedral capsid. One of our novel bacteriophages was unable to be classified using morphology, as no phage-like particles were able to be visualized using TEM. Bacteriophage electron microscopy has been criticized in the past for the high prevalence of poor quality images (Ackermann, 2014). One of the biggest critiques regarding TEM has been the replacement of conventional TEM with digital TEM, the latter of which is known for producing lower quality images that make bacteriophage visualization and measurement more difficult. Additionally, our bacteriophages were difficult to purify and prepare on grids, often exhibiting broken phages and other artifacts. These artifacts and bacteriophage degradation, along with the poorer quality images produced by the use of a digital TEM made identifying, measuring, and classifying our phages more difficult. In the past decade, genomic-based bacteriophage classification schemes have been proposed, both due to the lack of standardization for morphology based classification, and since the dramatic increase in available bacteriophage sequences have revealed much higher levels of genomic diversity than was expected within the existing families and subfamilies (Adriaenssens et al., 2018; Adriaenssens et al., 2020; Barylski et al., 2020; Turner et al., 2021). These new genomic-based classification schemes are still in development, and as such, have yet to become the official standard for classification (Zhu et al., 2022). It would be of great interest to subject the novel bacteriophages characterized in this study to genomic-based classification methods to better elucidate their genetic relationships with one another, as well as with other previously published bacteriophage sequences.

Due to the increasing concern regarding antibiotic resistance, research into antimicrobial alternatives for use in food animal production has been reinvigorated in recent years (Mwangi et al., 2016; Organization, 2017). Among these antimicrobial alternatives, bacteriophages have the advantage of being highly abundant in many environments, easy to isolate, and cost-effective to

produce in large quantities due to their ease of production in large broth cultures (Veiga-Crespo and Villa, 2009; Wittebole et al., 2014). Additionally, bacteriophages are typically much more specific than other antimicrobial alternatives, often times displaying subspecies or even strain-level specificity within a bacterial species (Veiga-Crespo and Villa, 2009). The bacteriophages in our study were found to be specific to *F. necrophorum* subsp. *necrophorum*, with no lytic activity observed against any other *Fusobacterium* species. *Fusobacterium necrophorum* subsp. *necrophorum* is the primary causative agent of liver abscess development in feedlot cattle, and is a normal ruminal inhabitant with roles in lactate and lysine fermentation, and protein degradation (Langworth, 1977; Wada, 1978; Scanlan and Hathcock, 1983). As such, we decided to investigate the pH stability of our bacteriophages to see if they would survive in ruminal conditions. Our pH stability results showed no significant reduction in bacteriophage titers when subjected to the pH range present in the rumen (pH 5-pH 8), and the bacteriophages survived for at least 24 hours at pH values of 4 and 9. These findings support the potential application of our bacteriophages as a feed additive, although further research needs to be undertaken to assess the temperature stability of the phages in ruminal conditions.

To assess the potential of the bacteriophage in liver abscess prevention, we conducted a proof of concept study in a mouse model of liver abscesses induced by pure culture administration of *F. necrophorum*. Liver abscesses were induced via intraperitoneal injection of *F. necrophorum* subsp. *necrophorum*, as according to previously published research (Abe et al., 1976), and bacteriophages were administered intraperitoneally as a cocktail 24 h prior to bacterial infection. We found a significant reduction in mortality and morbidity in the bacteriophage challenge group when compared to the *F. necrophorum* control. However, our model resulted in high levels of mortality amongst mice in the bacterial challenge group, which

was unexpected and created difficulties in assessing the true impact of bacteriophage prophylaxis. Our challenge study utilized a high dose inoculation with an *F. necrophorum* subsp. *necrophorum* strain characterized as expressing high levels of leukotoxin production (Narayanan et al., 1997), which should be adjusted in future studies to achieve liver abscess formation without the high levels of mortality experience in this study. Additionally, alternate strategies for bacteriophage administration, including time of administration relative to bacterial challenge, as well as administration routes, should be explored.

Conclusions

This study was the first to characterize 5 novel *F. necrophorum* subsp. *necrophorum* lytic bacteriophages. All 5 bacteriophages were found to be lytic only to subsp. *necrophorum,* but have a wide host range within the subspecies. Morphological characterization was achieved using TEM, which identified 4 of the 5 phages as being tailed phages with icosahedral capsids. Furthermore, genetic sequencing of all 5 bacteriophage confirmed their novelty to the currently deposited bacteriophages accessible in GenBank. Finally, a proof-of-concept study highlighted the potential for the bacteriophages to be used as a cocktail for the successful prevention of liver abscesses caused by *F. necrophorum* subsp. *necrophorum* in a mouse model, with significant reductions in morbidity and mortality observed. Future research should be conducted to further evaluate the potential of these bacteriophages as an antimicrobial alternative for liver abscess prevention in beef cattle.

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Table 6-1. Bacterial strains used in the host ranging experiments for characterization of 5 novel bacteriophages isolated from untreated sewage

Genus	Species	Subspecies	Number of strains	Source
Bacillus	*cereus*		1	Laboratory isolate
Bacillus	*subtiilis*		1	Laboratory isolate
Enterococcus	*casselflavis*		1	Laboratory isolate
Escherichia	*coli*		1	Laboratory isolate
Enterococcus	*faecalis*		2	ATCC
Fusobacterium	*gastrosuis*		1	Laboratory isolate
Fusobacterium	*necrophorum*	*necrophorum*	60	Laboratory isolates
Fusobacterium	*necrophorum*	*funduliforme*	31	Laboratory isolates
Fusobacterium	*russii*		1	Laboratory isolate
Fusobacterium	*ulcerans*		1	Laboratory isolate
Fusobacterium	*varium*		27	Laboratory isolates
Klebsiella	*aerogenes*		1	ATCC
Klebsiella	*pneumoniae*		1	ATCC
Listeria	*monocytogenes*		1	ATCC
Pseudomonas	*aeruginosa*		1	ATCC
Proteus	*hauseri*		1	ATCC
Staphylococcus	*aureus*		1	ATCC
Salmonella	*enterica*	*enterica*	2	ATCC
Serratia	*marcesens*		1	Laboratory isolate
Streptococcus	*pyogenes*		1	ATCC
Trueperella	*pyogenes*		1	Laboratory isolate

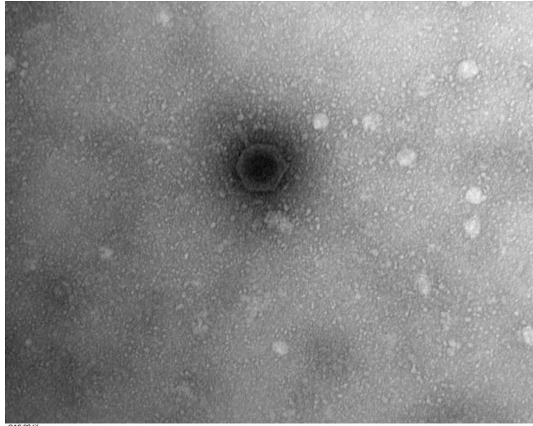
Table 6-2 Measurement of the 5 novel bacteriophages, as observed under transmission electron microscopy

Bacteriophage ID	Capsid		Tail	
	Length, nm	Width, nm	Length, nm	Width, nm
SA2	75.074	78.253	39.218	11.16
SA3	85.798	77.734	218.419	13.226
SA4	64.998	51.133	n/a	n/a
SA11	76.206	73.498	254.045	14.391
SA14	77.695	73.625	256.669	12.82

Table 6-3 Results of the host ranging experiments performed using a modified spot on lawn technique. +++ indicates complete lysis, - indicates no lysis

		Bacteriophage ID				
Bacterial species	**Strain ID**	**SA2**	**SA3**	**SA4**	**SA11**	**SA14**
F. necrophorum subsp. *necrophorum*	2016-13 102A	+++	+++	+++	+++	+++
	2016-13 11A	+++	+++	+++	+++	+++
	2016-13 148A	+++	+++	+++	+++	+++
	2016-13 14A	+++	+++	+++	+++	+++
	2016-13 28A	+++	+++	+++	+++	+++
	2016-13 29A	+++	+++	+++	+++	+++
	2016-13 31A	+++	+++	+++	+++	+++
	2016-13 35A	+++	+++	+++	+++	+++
	2016-13 36A	+++	+++	+++	+++	+++
	2016-13 390A	+++	+++	+++	+++	+++
	2016-13 40A	+++	+++	+++	+++	+++
	2016-13 41A	+++	+++	+++	+++	+++
	2016-13 430A	+++	+++	+++	+++	+++
	2016-13 439A	+++	+++	+++	+++	+++
	2016-13 46A	+++	+++	+++	+++	+++
	2016-13 54A	+++	+++	+++	+++	+++
	2016-13 56A	+++	+++	+++	+++	+++
	2016-13 57A	+++	+++	+++	+++	+++
	2016-13 58A	+++	+++	+++	+++	+++
	2016-13 61A	+++	+++	+++	+++	+++
	2016-13 70A	+++	+++	+++	+++	+++
	2016-13 73A	+++	+++	+++	+++	+++
	2016-13 76A	+++	+++	+++	+++	+++
	2016-13 77A	+++	+++	+++	+++	+++
	2016-13 83A	+++	+++	+++	+++	+++
	2016-13 86A	+++	+++	-	+++	+++
	2016-13 87A	+++	+++	+++	+++	+++
	2016-13 882A	+++	+++	+++	+++	+++
	2016-13 88A	+++	+++	+++	+++	+++
	2016-13 890A	+++	+++	+++	+++	+++
	2016-13 90A	+++	+++	+++	+++	+++
	2016-13 93A	+++	+++	+++	+++	+++
	2016-13 98A	+++	+++	+++	+++	+++
	8LI	+++	+++	+++	+++	+++
	2016-13 9A	+++	+++	+++	+++	+++
	2016-13 10A	+++	+++	+++	+++	+++
	2016-13 17A	+++	+++	+++	+++	+++
	2022-1-CT39	+++	+++	-	+++	+++

2022-1-LA35	+++	+++	-	+++	+++
2022-1-LA31	+++	+++	-	+++	+++
2022-1-LA34	+++	+++	-	+++	+++
2022-1-LA32	+++	+++	-	+++	+++
2022-1-LA41	+++	+++	-	+++	+++
2022-1-LA30	+++	+++	-	+++	+++
2022-1-CT31	+++	+++	-	+++	+++
2022-1-LA33	+++	+++	-	+++	+++
2022-1-LA36	+++	+++	-	+++	+++
2022-1-LA38	+++	+++	-	+++	+++
2022-1-LA20	+++	+++	-	+++	+++
2022-1-LA22	+++	+++	-	+++	+++
2022-1-LA26	+++	+++	-	+++	+++
2022-1-LA19	+++	-	-	+++	-
2022-1-LA21	+++	+++	-	+++	+++
2022-1-LA24	+++	+++	-	+++	+++
2022-1-LA25	+++	+++	-	+++	+++
2022-1-LA17	+++	+++	-	+++	+++
2022-1-LA18	+++	+++	-	+++	+++
2022-1-RT20	+++	+++	-	+++	+++
2022-1-CT38	+++	+++	-	+++	+++
2023-3-374	+++	+++	-	+++	+++



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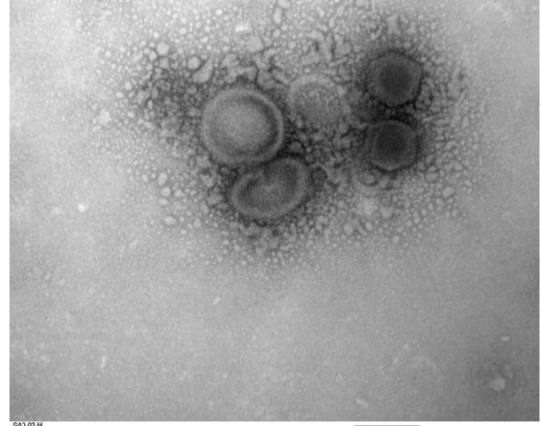

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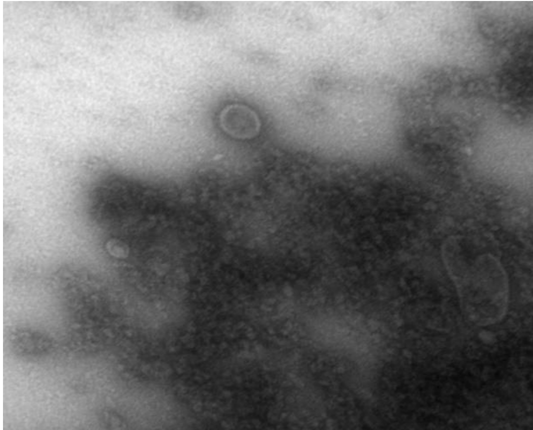

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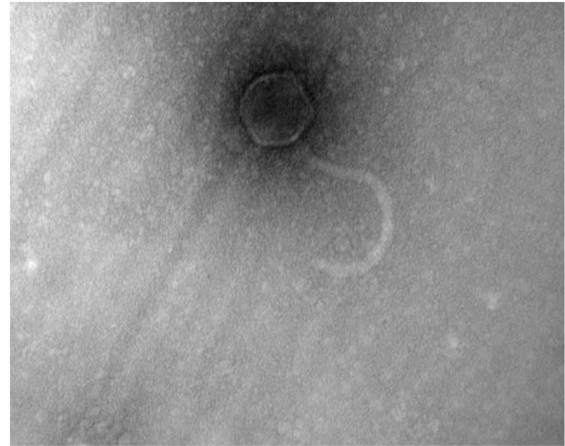

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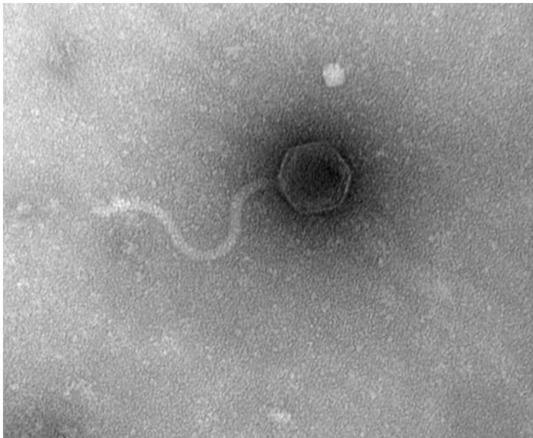

e.

Figure 6-1 Visualization of bacteriophages as observed using transmission electron microscopy. a) bacteriophage SA2. b) bacteriophage SA3. c) bacteriophage SA4. d) bacteriophage SA11. e) bacteriophage SA14

Chapter 7 Conclusions

The objectives of the research described in this dissertation were to reevaluate the etiology and pathogenesis of liver abscesses in feedlot cattle, with a focus on the potential involvement of *Fusobacterium varium*, and the assessment of bacteriophages as an antimicrobial alternative for liver abscess prevention. Specifically, a real-time qPCR assay was developed for the detection and quantification of *F. necrophorum* subspecies and *F. varium* in ruminal contents of feedlot cattle at the time of slaughter, whole genome sequencing (WGS) analyses was conducted on 64 *F. necrophorum* subsp. *necrophorum* strains recovered from matched ruminal epithelial and colonic epithelial tissues and liver abscesses from cattle at the time of slaughter for genomic comparisons between strains from the different locations to provide insight into liver abscesses etiology and strain pathogenicity, *F. varium* was isolated for the first time from liver abscesses, ruminal epithelial tissues, and colonic epithelial tissues collected from cattle at the time of slaughter with a follow-up study was conducted to investigate the impact of various dietary and management factors on the prevalence of *F. varium* within these tissues and conduct WGS analyses on a subset of recovered isolates from matched sample sets for genomic comparisons, and five novel bacteriophages lytic to *F. necrophorum* subsp. *necrophorum* were characterized for application as an antimicrobial alternative to tylosin phosphate for liver abscess prevention in feedlot cattle.

The development of a rt qPCR assay for the identification and quantification of *F. necrophorum* subspecies and *F. varium* in ruminal fluid provides a quick, reliable method for investigating bacterial prevalence amongst beef cattle. Ruminal *F. necrophorum* has been theorized to penetrate damaged epithelial tissue to enter the portal vein and translocate to the liver to cause liver abscesses. Interestingly, the described study revealed that *F. varium* and *F.*

necrophorum subsp. *funduliforme* were highly prevalent in ruminal content samples, while *F. necrophorum* subsp. *necrophorum* was only present in less than one third of samples tested, although there was a significant difference in prevalence in samples from cattle with liver abscesses at slaughter than in cattle with apparently healthy, non-abscessed livers. Since liver abscesses take multiple days to form, the presence of *F. necrophorum* in the rumen at the time of slaughter may not always correlate with liver abscess presence. Future research should investigate the use of this rt qPCR assay for analyzing samples taken from cattle at various timepoints throughout the finishing period to examine whether correlations between *F. necrophorum* presence and liver abscess formation can be revealed.

Fusobacterium necrophorum subsp. *necrophorum* is the primary causative agent in liver abscess formation, therefore whole genome sequencing-based analyses were conducted to investigate genetic relatedness between strains recovered from numerous locations from the same animal, and to investigate the presence of virulence and antimicrobial resistance genes in the strains. High levels of genetic similarity were observed between strains from the same animal, reinforcing the hypothesis that bacterial pathogens originate in the gastrointestinal tract, including the hindgut, and translocate from there into the liver. Numerous conserved virulence genes were identified within the genomes of all strains, including the *lktA* gene, the major virulence factor associated with liver abscess development, and hemagglutinin, another major virulence factor involved in pathogenicity of the organism. Additionally, a surprisingly high prevalence of antimicrobial resistance (AMR) genes was identified within the genomes, including the *ermB* gene, which encodes for resistance to tylosin phosphate. These findings emphasize the need for antimicrobial alternatives to tylosin for liver abscess prevention, and

warrant further research into the potential horizontal gene transfer between ruminal bacteria resulting in the acquisition of AMR genes.

Following the findings of high prevalence of *F. varium* in ruminal contents of cattle, a study was conducted in an effort to isolate *F. varium* from liver abscesses, ruminal epithelial tissues, and colonic epithelial tissues in feedlot cattle. *Fusobacterium varium* is an active tissue invader, and has been implicated in ulcerative colitis in humans, therefore it is of interest to investigate whether it has roles in liver abscess formation. Three of ninety-six liver abscesses sampled (3.1%) had culturable amounts of *F. varium*, while the qPCR assay described previously detected *F. varium* in ten of the ninety-six samples. Additionally, *F. varium* was present in a majority of ruminal epithelial tissue samples, and approximately one half of colonic epithelial samples. These findings are in agreement with recent studies revealing liver abscesses to be highly polymicrobial infections, and raise questions on the potential synergistic role of *F. varium* in liver abscess formation.

A follow-up study was conducted to investigate how the prevalence of *F. varium* in ruminal epithelial, colonic epithelial, liver abscess, and liver tissue samples is affected by various dietary and management factors. Previous research has reported resistance to tylosin in *F. varium* strains recovered from bovine ruminal fluid, and the results of the present study showed no significant differences in *F. varium* in cattle receiving in-feed tylosin administration compared to cattle not receiving tylosin. Similarly, ruminal acidosis, either experimental, or caused by increased dietary starch levels and/or erratic feed bunk management strategies, did not affect the prevalence of *F. varium* in ruminal tissues, liver tissues, or liver abscesses. Interestingly, erratic feeding of a control, moderate starch diet resulted in a significantly higher prevalence of *F. varium* in colonic epithelial tissues than a combination of a high dietary starch level with erratic

feeding. The colon has been hypothesized as a potential source of bacterial liver abscess causing pathogens, so management strategies that promote *F. varium* colonization in the colon may increase their potential to translocate from there to the liver, especially if the colonic epithelium becomes damaged. Whole genome sequencing analysis of a subset of these isolates revealed a high level of genetic relatedness between matched colonic, ruminal, and liver abscess strains, making it of importance to evaluate liver abscess mitigation strategies that also have post-ruminal effects. While the genome analysis did not reveal the presence of macrolide resistance genes, it would be of clinical relevance to test for phenotypic resistance in the recovered isolates, especially due to the lack of effect on *F. varium* prevalence even with in-feed tylosin administration.

Bacteriophages have been used as alternatives to traditional antibiotic therapies for over a century, and have the benefit of exhibiting a very high level of specificity to their host bacterium. Five bacteriophages lytic to *F. necrophorum* subsp. *necrophorum* were characterized and used in a proof of concept mouse study for the prevention of liver abscesses. The phages exhibited a wide host range within subsp. *necrophorum*, including lytic activity against ruminal, colonic, and liver abscess strains. When the phages were combined into a cocktail and used as prophylaxis against *F. necrophorum* infection in a mouse liver abscess model, significant reductions in mortality and morbidity were observed. Future studies should investigate the efficacy of bacteriophages in the rumen and/or colon for reduction in *F. necrophorum* populations for the purposes of liver abscess prevention. It is important to note that the liver abscess model resulted in a high level of mortality in infected mice, which is not typical in cattle with liver abscesses, therefore future models should aim to reduce the mortality of infected animals to create a more typical liver abscess presentation (low mortality, relatively few clinical symptoms).

The research findings in this dissertation highlight the potential use of genomic technologies for understanding the etiology of liver abscesses, including identifying the location of origin for liver abscess-causing pathogens, and identifying novel pathogens associated with liver abscess formation. Additionally, bacteriophages were shown to be a simple, effective method for reducing *F. necrophorum* induced mortality and morbidity in a mouse model. These findings can be applied for the development of more targeted therapeutics for the prevention of liver abscess in beef cattle, including the development of antimicrobial alternatives to tylosin phosphate.

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