DEVELOPMENT OF MOLECULAR TOOLS TO ASSESS WHETHER *ARCOBACTER BUTZLERI* IS AN ENTERIC PATHOGEN OF HUMAN BEINGS

ANDREW L WEBB
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ANDREW LAWRENCE WEBB

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G. Douglas Inglis Thesis Co-Supervisor	Research Scientist	Ph.D.
L. Brent Selinger Thesis Co-Supervisor	Professor	Ph.D.
Eduardo N. Taboada Thesis Examination Committee Member	Research Scientist	Ph.D.
Robert A. Laird Thesis Examination Committee Member	Associate Professor	Ph.D.
Sylvia Checkley External Examiner University of Calgary Calgary, Alberta, Canada	Associate Professor	Ph.D., DVM
Tony Russell Chair, Thesis Examination Committee	Assistant Professor	Ph.D.

DEDICATION

This thesis is dedicated to my partner Jen, who has been a source of endless patience and

support. Furthermore, I dedicate this thesis to my parents, for their unwavering confidence in

me and their desire to help me do what I love.

ABSTRACT

The pathogenicity of *Arcobacter butzleri* remains enigmatic, in part due to a lack of genomic data and tools for comprehensive detection and genotyping of this bacterium. Comparative whole genome sequence analysis was employed to develop a high throughput and high resolution subtyping method representative of whole genome phylogeny. In addition, primers targeting a taxon-specific gene (quinohemoprotein amine dehydrogenase) were designed to detect and quantitate *A. butzleri*. The application of these methods showed that *A. butzleri* is present at high frequencies but low densities in diarrheic and healthy people, and specific strains are associated with human enteritis. The developed tools were also used to determine that *A. butzleri* is common in wastewater, survives tertiary wastewater treatment, and may be transmitted to people via ingestion of contaminated surface water. Diverse subtypes of *A. butzleri* occur in the environment, but pathogenicity is likely strain-specific and/or dependent on other factors such as host resistance.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AMA	Antimicrobial Agent
ASIA	*Arcobacter* Selection and Isolation Agar
ASIB	*Arcobacter* Selection and Isolation Broth
AWC	Adjusted Wallace Coefficient
BBS	Bolton Broth (CM0983, Oxoid) with Bolton supplement (SR0183E, Oxoid)
BLAST	Basic Local Alignment Search Tool
CB	Columbia Broth (Difco and BBL Microbiology, Lawrence, KS)
CBA	Columbia Agar (DF0944-17-0, Difco) amended with 10% sheep blood
CFO	Confined Feeding Operation
CGF	Comparative Genomic Fingerprinting
CGFO	Comparative Genomic Fingerprinting Optimizer
CHR	Chinook Health Region
CI	Confidence Interval
CINA	Non-approximated Confidence Interval
CRH	Chinook Regional Hospital
DNA	Deooxyribonucleic Acid
EMA	Ethidium Monoazide
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction
IAC	Internal Amplification Control
ID	Index of Diversity
JMA	Johnson and Murano Agar
JMB	Johnson and Murano Broth
KBA	Karmali Agar (CM0935, Oxoid) with Bolton supplement (SR0183E, Oxoid)
KSA	Karmali Agar (CM0935, Oxoid) with Karmali supplement (SR0167, Oxoid)
MIC	Minimum Inhibitory Concentration
MIST	Microbial In Silico Typer
MLST	Multilocus Sequence Typing
NCBI	National Center for Biotechnology Information
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RAST	Rapid Annotation Using Subsystem Technology
rRNA	Ribosomal Ribonucleic Acid
SNP	Single Nucleotide Polymorphism
SWA	Southwestern Alberta
SymD	Symmetric Distance
UPGMA	Unweighted Pairwise Grouping with Arithmetic Mean
UVB	Ultraviolet B (shortwave)
WGS	Whole Genome Sequencing
WWTP	Wastewater Treatment Plant

CHAPTER ONE

Literature review

"…as we know, there are known knowns; there are things we know we know. We also know there are known unknowns; that is to say we know there are some things we do not know. But there are also unknown unknowns – the ones we don't know we don't know… it is the latter category that tends to be the difficult ones."
Donald Rumsfeld, United States Secretary of Defense, Feb 12, 2002
(*Transcript, Press Operations, United States Department of Defense)*

1.1. INTRODUCTION

In 1992, the *Arcobacter* genus was proposed to separate a number of aerotolerant species from other taxa within the *Campylobacteraceae* family of *Epsilonproteobacteria* (1-3). *Arcobacter* species were first detected in aborted livestock fetuses (4, 5), but since then the *Arcobacter* species *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* have been detected in people reporting diarrheic symptoms (6-9). In particular, *A. butzleri* is the fourth most commonly detected *Campylobacteraceae* in human beings with enteric illness (10). The association between *A. butzleri* and people with watery diarrhea, intestinal cramping, and dehydration (11, 12) has led many to conclude that this bacterium is an emerging pathogen; however, direct evidence of its pathogenicity is lacking at present. A major limitation in ascertaining whether *A. butzleri* is a public health concern is the lack of genomic data and robust tools for the comprehensive detection, isolation, and genotyping of the bacterium.

1.2. GROWTH AND MORPHOLOGY

Arcobacter butzleri is a Gram negative bacterium with a curved shape and a single polar flagellum (Figure 1.1) (1, 13). It can grow at temperatures as low as 4°C (14) and in anoxic atmospheres (15), although optimal conditions are aerobic or reduced-oxygen atmospheres (1, 3) at temperatures between 25°C (10) and 37°C (16). Although data are limited, the metabolism of *A. butzleri* is likely similar to that of *Campylobacter* species, as they are often co-isolated on

Figure 1.1. *Arcobacter butzleri* visualized by scanning electron microscopy.
White bars represent increments of 0.5 μm in length.

media that are semi-selective for campylobacters (1, 3, 13, 17). In addition, arcobacters and

campylobacters share similar cultural morphologies. When grown on media containing blood,

most taxa within the family *Campylobacteraceae* form smooth, round colonies 1-3 mm in

diameter with a greyish color (1). *Arcobacter butzleri* is capable of forming a biofilm (18) in order

to resist temperature extremes, and saline, acidic and biocidal stressors (Table 1.1), and it

possesses strain-specific resistance to a range of antimicrobial agents (AMAs) (19-23) (Table 1.2).

The adaptability of this bacterium to such a broad range of growth conditions may explain its

ubiquity; *A. butzleri* has been detected in all types of livestock (22, 24-26) and their associated

food products (27-32), seafood (33, 34), household pets (35), wastewaters (15, 36),

environmental waters (32, 37) including contaminated groundwater (38, 39), and both diarrheic

(40-42) and non-diarrheic human beings.

Table 1.1. Experimental inhibition of *A. butzleri* growth using environmental stressors.		
Stressor	Viability	Reference
Acidity		
pH 4.0	> 24 hours	(43)
pH 5.0	> 24 hours	(43)
pH 7.0	> 5 days	(44)
pH 8.0	> 5 days	(44)
Chlorine		
1.16 mg/l	5 min	(45)
Starvation		
Filtered (drinking) water	> 35 days[a]	(45)
Salinity		
3.5% NaCl	> 96 hours	(44)
5.0% NaCl	> 96 hours	(44)
Temperature		
5°C	> 77 days	(46)
10°C	> 20 days	(46)
15°C	> 10 days	(46)
48°C	> 24 hours	(43)
52°C	30 min	(47)
56°C	16 min	(47)
60°C	5 min	(47)

[a] *Arcobacter butzleri* remained culturable between 16 and 21 days in filtered water, but membrane permeability stains suggested that cells remained viable but non-culturable for more than 35 days.

1.3. ECOLOGY

1.3.1. *Arcobacter butzleri* in animals and animal products. *Arcobacter butzleri* has been isolated

from clinically-healthy livestock at all levels of the "farm-to-fork" continuum, although

prevalence varies greatly by animal and type of sample (e.g. skin versus feces), as well as the

detection methods applied in each study (Table 1.3). Greater rates of detection of *A. butzleri* are

reported from feces and intestinal contents of pigs and cattle compared to chickens, yet the

rates of detection on carcasses at slaughter are greater for chickens than for other meat animals

(26, 48, 49). Considering the ubiquity of *A. butzleri* in slaughterhouses and processing facilities

(50-53), it is not surprising that this bacterium is also prevalent in retail animal products such as

meat and dairy (52, 54, 55). Studies suggest that contamination of retail meats likely occurs

Table 1.2. Experimental inhibition of *A. butzleri* growth using antimicrobial agents.				
Antimicrobial	Mechanism of action	MIC$_{50/90}$ (mg/l)	Breakpoint (mg/l) [a]	Reference
Aminocoumarin				
Novobiocin	Energy transduction inhibition	Resistant at 30		(56)
Aminoglycoside				
Amikacin	Protein synthesis inhibition	4/64	≤16	(20)
Gentamicin	Protein synthesis inhibition	0.5/1	≥8	(18)
Kantamycin	Protein synthesis inhibition	Susceptible at 30		(56)
Streptomycin	Protein synthesis inhibition	Susceptible at 300		(56)
Tobramycin	Protein synthesis inhibition	2.0/16	≤4	(20)
β-lactam cephalosporin				
Cefaclor	Cell wall synthesis inhibition	8/128	≤8	(20)
Cefazolin	Cell wall synthesis inhibition	128/>128	≤8	(20)
Cefepime	Cell wall synthesis inhibition	4/8	≤8	(20)
Cefixime	Cell wall synthesis inhibition	1/>128	≤1	(20)
Cefoperazone	Cell wall synthesis inhibition	512/512	≥64	(18)
Cefotetan	Cell wall synthesis inhibition	16/128	≤16	(20)
Ceftriaxone	Cell wall synthesis inhibition	4/128	≤8	(20)
Cefuroxime	Cell wall synthesis inhibition	32/128	≤8	(20)
B-lactam penicillin				
Ampicillin	Cell wall synthesis inhibition	128/256	≥32	(18)
Amoxicillin	Cell wall synthesis inhibition	8/>128	≤8	(20)
Piperacillin	Cell wall synthesis inhibition	512/512	≥128	(18)
Macrolides				
Azithromycin	Protein synthesis inhibition	Intermediate at 15		(56)
Clindamycin	Protein synthesis inhibition	64/>128	≤0.5	(20)
Erythromycin	Protein synthesis inhibition	Intermediate at 15		(56)
Rokitamycin	Protein synthesis inhibition	16/>128	-	(20)
No family				
Chloramphenicol	Protein synthesis inhibition	16/64	≤8	(20)
Imipenem	Cell wall synthesis inhibition	1/2	≤4	(20)
Oxytetracycline	Protein synthesis inhibition	Susceptible at 30		(56)
Tetracycline	Protein synthesis inhibition	Susceptible at 30		(56)
Trimethoprim	DNA synthesis inhibition	512/512	≥16	(18)
Vancomycin	Cell wall synthesis inhibition	512/512	≥32	(18)
Quinolones				
Ciprofloxacin	Topoisomerase II/IV ligase inhibition	4/>8	>4	(18)
Difloxacin	Topoisomerase II/IV ligase inhibition	2/16	≤4	(20)
Enrofloxacin	Topoisomerase II/IV ligase inhibition	2/4	≤2	(20)
Levofloxacin	Topoisomerase II/IV ligase inhibition	0.5/4	≤2	(20)
Marbofloxacin	Topoisomerase II/IV ligase inhibition	0.5/16	≤1	(20)
Norfloxacin	Topoisomerase II/IV ligase inhibition	2/8	≤1	(20)

[a] Concentration of AMA below which *A. butzleri* is considered susceptible for a given minimum inhibitory concentration (MIC) level.

Table 1.3. Frequency of detection of *A. butzleri* in livestock and retail meats.

Sample origin	Samples	Prevalence (%)	Reference
Chicken carcass			
Iran	100	26	(57)
Switzerland	248	53.6	(52)
USA [a]	119	78.2	(23)
USA [b]	12	91.7	(23)
Chicken feces			
Chile	20	10.7	(48)
Japan	234	6.8	(22)
Switzerland	1090	1.4	(52)
Chicken meat			
Iran	100	28	(57)
Ireland	94	52.1	(55)
Japan	41	46.3	(32)
Japan	100	15	(54)
Switzerland	238	15.1	(52)
Cow carcass			
Belgium	247	8.1	(51)
Switzerland	208	19.7	(52)
Cow feces			
Belgium	276	1.4	(25)
Chile	75	30.7	(48)
Switzerland	210	0	(52)
Cow meat			
Ireland	108	20.4	(55)
Japan	90	1.1	(54)
Switzerland	150	0	(52)
Pig carcass			
Belgium	169	1.8	(53)
Switzerland	300	19.7	(52)
Pig feces			
Belgium	294	31.6	(26)
Chile	135	49.6	(48)
Japan	250	6	(22)
Switzerland	250	21.6	(52)
Pig meat			
Belgium	47	14.9	(53)
Ireland	101	21.8	(55)
Japan	100	4	(54)
Switzerland	52	0	(52)

[a] Pre-scald
[b] Post-chill

during slaughter and processing, although the mechanism of contamination may vary by livestock animal (23, 53, 58). *Arcobacter butzleri* has also been detected in sea creatures such as fish (59, 60), mussels and clams (61), which has been attributed to contamination of environmental waters with human and animal waste. It is possible that cross-contamination of seafood occurs during processing and at retail in a similar manner to that of domesticated livestock (50-53), although no such studies are currently available.

1.3.2. *Arcobacter butzleri* **in water sources.** *Arcobacter butzleri* is often detected in surface waters (Table 1.4), which tends to coincide with the detection of indicators of fecal contamination (36, 37, 62). The bacterium is also ubiquitous in municipal and animal wastewater (15, 36, 63). A critical feature of wastewater treatment plants (WWTPs) is the removal of organisms that pose a risk to human health prior to wastewater discharge into environmental waters such as rivers or oceans. However, *A. butzleri* has been detected in treated wastewater effluent (37) and in treated solid waste that is spread onto fields as fertilizer (15); it is likely that *A. butzleri* enters surface waters directly via wastewater discharge or indirectly via field runoff.

1.3.3. *Arcobacter butzleri* **associated with plants and plant products**. *Arcobacter butzleri* has been detected in vegetable wash water, and from carrots, lettuce and spinach at processing facilities and at retail (64-66). Outbreaks of bacterial enteritis have been associated with consumption of raw vegetables contaminated with bacterial pathogens such as *Escherichia coli* and *C. jejuni* (67). Considering that *A. butzleri* has been detected in treated wastewater (36, 37) and solid waste (15), it is plausible that contamination of vegetable crops occurs during fertilization or irrigation with contaminated water. Given the propensity for *A. butzleri* to resist temperate extremes (46, 47), antimicrobial agents (19, 20) and lack of nutrients (45), this bacterium may be able to persist during crop harvesting, washing and storage, and therefore to pose a risk to human beings.

Table 1.4. Frequency of detection of *A. butzleri* in municipal sewage and environmental waters.

Source	Samples	Prevalence (%)	Reference
Canals			
Thailand	7	100	(32)
Lakes			
Spain	29	27.6	(37)
Rivers			
Japan	17	23.5	(32)
Spain	29	55.2	(37)
Seawater			
Italy	6	83.3	(62)
Spain	101	35.6	(37)
Sludge			
Italy	22	72.7	(15)
Spain	27	44.4	(37)
Sewage			
Spain	19	26.3	(37)

1.3.4. *Arcobacter butzleri* in human beings. *Arcobacter butzleri* has been isolated from the stools of diarrheic people (39, 68, 69), both in population studies and in clinical cases. Although *A. butzleri* is commonly co-isolated with known enteric pathogens such as *C. jejuni* (10, 69-71), it has also been reported in diarrheic human beings in the absence of established pathogens. However, the majority of enteric infections are not attributed to a source due to the current limitations of pathogen surveillance methods. Thus, the isolation of *A. butzleri* from diarrheic human beings is insufficient to conclude disease incitation by the bacterium. *Arcobacter butzleri* has also been isolated from people with reduced immunity due to underlying diseases in the absence of symptoms of gastrointestinal diseases; recent work shows that a large number of otherwise healthy diabetic patients were positive for *A. butzleri* (72). The bacterium has also been detected in asymptomatic people (71).

1.4. ISOLATION AND DETECTION

1.4.1. Microbiological detection and isolation. There is no standard microbiological method

available for the detection, isolation, and/or identification of *A. butzleri* in complex matrices such as feces or environmental waters, although many combinations of growth conditions, culturing techniques, and antimicrobial agents have been proposed (Table 1.5). This may be due to the complex nature of *A. butzleri*, which is thought to be genetically diverse and to occupy many niches. *Arcobacter butzleri* is difficult to isolate in a comprehensive manner (17, 73), so culture conditions must therefore be general enough to allow growth of all strains of *A. butzleri*, yet selective enough to inhibit non-target growth by the endless variety of organisms that may be present in complex matrices such as feces. As a result, selective media cannot be relied upon to culture *A. butzleri* in a manner that is completely sensitive (i.e. able to select for minimal number of *A. butzleri* cells), specific (i.e. able to inhibit growth by all non-target taxa), and inclusive (i.e. able to select for growth of all strains of *A. butzleri*). The inability to reliably detect *A. butzleri* in complex matrices leads to underestimation of prevalence and is a serious obstacle to studying the epidemiology of arcobacteriosis.

1.4.2. Molecular detection and identification. Comprehensive molecular methods to detect *A. butzleri* in complex matrices such as feces or water samples do not exist, although a number of novel and modified primer sets that target universal genes for multiplex and/or quantitative Polymerase Chain Reaction (PCR) have been proposed (74-78). Universal genes are an excellent PCR primer target for identification of *A. butzleri* deoxyribonucleic acid (DNA) extracted from pure culture, but as discussed in the section 1.4.1 it is not possible to comprehensively isolate this bacterium from complex matrices. Molecular methods must therefore be able to detect *A. butzleri* DNA extracted directly from complex sample such as feces, which by its very nature will contain non-target DNA that may competitively bind PCR primers. Under these conditions universal gene sequences may not be appropriate targets for sensitive and specific detection of *A. butzleri* DNA by PCR amplification because closely related species often differ in universal

Enrichment (AMA)	Solid medium (technique)[b]	Solid medium AMA	Atmosphere[c]	Temperature (°C)	Reference

Table 1.5. Proposed methods microbiological isolation of *A. butzleri* from complex matrices.

Enrichment (AMA)	Solid medium (technique)[b]	Solid medium AMA	Atmosphere[c]	Temperature (°C)	Reference
Arcobacter media	*Arcobacter* media (dp)	5-fu, amp-B, cfp, nvb, tmp	Mcroaerobic	28	(6)
Arcobacter media	*Arcobacter* media (dp)	amp-B, cfp, tcp	Aerobic	30	(79)
Arcobacter media (5-fu, amp-B, cfp, chx, nvb, tmp)	*Arcobacter* media (dp)	5-fu, amp-B, cfp, chx, nvb, tmp	Microaerobic	28	(26)
Brucella (cfp, chx pip, tmp)	Brain-heart infusion	Cefsulodin, irgasan, nvb	Aerobic	25	(32)
Brucella (cfp, chx pip, tmp)	Mueller-Hinton (dp)	Chx, cfp, pip, tmp	Aerobic	24	(29)
Brucella (cfp, chx pip, tmp)	Mueller-Hinton (dp)	Chx, cfp, pip, tmp	Aerobic	25	(32)
CAT (amp-B, cfp, tcp)	Blood agar (mf)	Amp-B, cfp, tcp	Microaerobic	30	(49)
CAT (amp-B, cfp, tcp)	Blood agar (mf), mCCDA (dp)	None	Aerobic	37	(80)
CAT (amp-B, cfp, tcp)	CAT (dp)	Amp-B, cfp, tcp	Aerobic	37	(80)
CAT (amp-B, cfp, tcp)	Karmali (dp)	None	Aerobic	25, 30	(80)
None	CAT (dp)	Amp-B, cfp, tcp	Aerobic	37	(80)
None	Blood agar (mf), mCCDA (dp)	None	Aerobic	37	(80)
None	*Arcobacter* media (dp)	5-fu, amp-B, cfp, chx, nvb, tmp	Microaerobic	28	(26)
None	*Arcobacter* media (dp)	Amp-B, cfp, tcp	Aerobic	30	(79)

[a] AMAs are 5-fluorouracil (5-fu), amphotericin B (amp-B), cefoperazone (cfp), cyclohexamide (chx), novobiocin (nvb), piperacillin (pip), teicoplanin (tcp), and trimethoprim (tmp).
[b] Plating techniques used were direct plating (dp) or membrane filtration (mf).
[c] Incubation of *A. butzleri* cultures occurred at ambient oxygen conditions (aerobic) or at reduced oxygen conditions (microaerobic) consisting of 5-6% O_2, 6-10% CO_2, 0-7% H_2, and 79-85% N_2.

gene sequences by a small number of interspersed single nucleotide polymorphisms (SNPs).

However, the relative lack of genomic data for *A. butzleri* makes it difficult to identify non-universal gene sequences that are conserved within all strains of this bacterium, especially because members of the family *Campylobacteraceae* are known to be highly genetically diverse (81). Molecular detection methods must therefore be validated against a large number of genetically diverse *A. butzleri* strains in ordered to be considered inclusive for the direct

detection of this bacterium in complex matrices.

1.5. ETIOLOGY

1.5.1. Foodborne infection. As discussed in sections 1.3.1 and 1.3.3, *A. butzleri* is likely

transmitted to human beings via ingestion of contaminated and undercooked or improperly

treated animal and plant products. Previous studies concluded that *A. butzleri* heavily

contaminates carcasses during slaughter, and others have shown that *A. butzleri* is ubiquitous on

the machinery of slaughterhouses (26, 49, 52, 82). In addition, *A. butzleri* is often detected on

animal meat products at retail (51-53, 55). Taken together, these findings indicate that carrier

livestock provide initial contamination of the slaughterhouse environment, and that machinery

is passively contaminated with *A. butzleri* cells within digesta/feces during the slaughter and

meat processing process. The presence of *A. butzleri* on vegetable crops is likely the result of

fecal contamination, which may occur during fertilization of plants with treated or untreated

municipal waste or via irrigation water contaminated with fecal material. Elucidation of 'farm-to-

fork' transmission is a focus for many foodborne pathogens (83, 84), with a primary objective of

mitigating of these pathogens during processing (85-87). However, the ubiquity and biological

characteristics of *A. butzleri* make mitigation of *A. butzleri* in processing plants a challenge; Houf

et al. (50, 88) and Van Driessche et al. (47) showed that the bacterium is resistant to heat and

chemical-based decontamination procedures, and is capable of surviving in chiller and freezer

conditions (46, 89) suggesting that the bacterium remains viable for prolonged periods on retail

meats and vegetables.

1.5.2. Waterborne infection. Studies indicate that *A. butzleri* infection may occur by ingesting

water contaminated with feces; *A. butzleri* has been associated with two disease outbreaks

where groundwater wells were contaminated with fecal material (38, 39). *Arcobacter butzleri* is

present in treated wastewater effluent that is discharged into environmental waters, and it

remains viable in both sterile and contaminated drinking water for extended periods of time

(45). It is therefore possible that *A. butzleri* infection can occur as a result of aquatic recreational

activities, or via improperly treated and/or contaminated drinking water.

1.5.3. Mechanism of infection. As with other taxa within the family *Campylobacteraceae*, *A.*

butzleri likely colonizes the intestines (i.e. in close association with the epithelium) (90, 91), but

evidence for the site and mechanism of infection in human beings is lacking. *Arcobacter butzleri*

has genes that are homologous with those coding for factors associated with survival, adhesion,

and invasion of host epithelial cells in known pathogens such as *C. jejuni* (Table 1.6). In addition,

A. butzleri cells have been shown to adhere to and invade human enterocytes *in vitro* (92, 93),

and there is evidence that *A. butzleri* induces epithelial barrier dysfunction at tight junction

proteins (94).

1.6. GENOMICS

1.6.1. Data availability. The first *A. butzleri* draft genome is approximately 2.3 Mbp in length,

with approximately 2300 coding sequences (56, 95, 96). At present, comprehensive whole

genome sequence data for *A. butzleri* is lacking. Select coding regions such as the 16S ribosomal

ribonucleic acid (rRNA) and 23S rRNA genes have been characterized (76, 78), but whole genome

sequence data are only available for four strains (56, 95-97). In addition, few other *Arcobacter*

species have been sequenced, and a lack of *A. butzleri* gene expression studies prevents

traditional gene identification. As a result, genomic analysis of *A. butzleri* relies heavily on

comparison to species such as *C. jejuni*, which has been better documented and is closely related

by DNA hybridization (1) and 16S rRNA (56) sequence analysis.

1.6.2 Genome annotation and variation. Similar to other *Campylobacteraceae*, *A. butzleri*

strains are genetically diverse in terms of variability within conserved genes and variable

presence/absence of accessory genes (16, 18, 98-100). Variation within conserved genes may be

Table 1.6. Putative *A. butzleri* virulence genes.

Gene	Putative protein group	Putative protein activity	Reference
CadF	Fibronectin binding	Adhesion	(101)
CiaB	Secretion protein	Invasion	(102)
Cj1349	Fibronectin binding	Adhesion	(93)
IrgA	Iron acquisition	Survival, metabolism, competitive exclusion	(103)
HecA	Filamentous hemagglutininin	Adhesion	(104)
HecB	Hemolysin activation	Damage erythrocytes	(56)
MviN	Peptidoglycan biosynthesis	Cell replication	(105)
PldA	Phospholipase	Damage erythrocytes, metabolism, competitive exclusion	(106)
TlyA	Hemolysin	Damage erythrocytes, survival within macrophages	(107)
IroE	Iron acquisition	Survival, metabolism, competitive exclusion	(103)

explained by the presence of a putative mutator bacteriophage within the *A. butzleri* genome

(56, 108), especially because most *Campylobacteraceae* lack functional mismatch repair systems

(56). The variable presence/absence of *A. butzleri* accessory genes has been attributed to the

promiscuous nature of *Campylobacteraceae* (109, 110). This ability to readily integrate foreign

DNA may also account for the accumulation of *A. butzleri* genes homologous to survival and

growth factors in free-living taxa outside of *Campylobacteraceae* (56, 111). However the rate of

promiscuity is strain-dependent for *C. jejuni* (110, 112), so a similar phenomenon may exist for *A.

butzleri*. The existence of both promiscuous and discriminatory strains of *A. butzleri* would

suggest that each survival strategy has competitive advantages and fitness costs. It is possible

that generalist *A. butzleri* strains adapt to inhospitable environments by maintaining accessory

survival factors or obtaining them from native taxa, which would explain increased adhesion

rates of *A. butzleri* strains to intraperitoneal tissues in mice after repeat passage (113). In

comparison, specialist *A. butzleri* strains adapted to a specific host species would not be exposed

to the same variety of selective stressors.

1.7. PATHOGENICITY

1.7.1. Ascertaining pathogenicity. A pathogen is most conveniently defined as an infectious

agent if it causes negative change in biological function (i.e. disease). In the 1890's, Robert Koch

presented four criteria to ascertain the pathogenicity of microorganisms (114). First, the

microorganism should be present in individuals suffering from disease (i.e. those with symptoms

of illness) and absent from healthy individuals (i.e. asymptomatic individuals). Second, the

microorganism should be isolated from the diseased individuals. Third, the isolated

microorganism should induce the expected disease when introduced into a previously healthy

individual. Finally, the introduced microorganism should be isolated from the individual

inoculated with the pathogen. Although Koch's postulates provide a valuable starting point for

ascertaining whether a microorganism incites disease, they do not hold up to the modern

understanding of bacterial pathogenicity and virulence (115). Advances in the detection and

sequencing of genetic material have led to the identification of an increasing number of

unequivocal pathogens that do not adhere to one or more of Koch's postulates. As a result,

Koch's postulates have been amended to reflect the complex relationship that exists between a

pathogen and its host in time and space; that is, pathogenicity must be defined within the

context of both scientific inference of disease causation as presented by Austin Bradford Hill in

1965 (116), and of the sum of host-, pathogen-, and environment-specific factors that influence

the manifestation of disease (117).

In 1996, Fredericks and Relman advocated a new set of guidelines to define disease

causation (115). They proposed the following postulates, for which the wording was designed to

avoid absolute statements regarding forms of proof and strict adherence to every postulate: (i)

nucleic acid sequences belonging to a putative pathogen should be present (preferentially in

diseased organs or anatomic sites) in most cases of an infectious disease; (ii) fewer copies of

nucleic acid sequences belonging to a putative pathogen should occur in hosts or tissues free of

disease; (iii) with resolution of disease, the number of pathogen-associated nucleic acid

sequences should decrease; (iv) a causal relationship is more likely when pathogen-associated nucleic acid sequence copy number correlates with onset and/or severity of disease; (v) the nucleic acid sequences should be consistent with the biological characteristics of that group of organisms; (vi) at the cellular level, nucleic acid sequences should be correlated to areas of tissue pathology; and (vii) sequence-based forms of evidence for causation should be reproducible .

1.7.2. *Arcobacter butzleri* as a potential pathogen. The occurrence of *A. butzleri* in healthy people (72) suggests that this bacterium is not a pathogen. However, incitation of disease requires an infectious agent and a susceptible host in the same time and space (118, 119). Due to the complexity of host immune systems and the protective nature of the microbiota (e.g. colonization resistance), it is possible that A. *butzleri* is able to survive within healthy individuals, and only incites disease under certain environmental conditions (e.g. compromised immune system). Another explanation for the presence of *A. butzleri* in healthy individuals is that *A. butzleri* pathogenicity is strain-specific. By its very nature, an enteric pathogen must be able to survive in the host intestine, adhere to and/or invade host epithelial tissues, and incite disease as a result of cytotoxicity and/or interference with the host immune response (118, 119). Although little is known about the mechanisms of *A. butzleri* infection, the presence/absence of putative virulence factors has been shown to vary by *A. butzleri* strain (93, 111, 120), so it is likely that only certain *A. butzleri* strains possess the full complement of genes necessary to incite disease in its host.

1.7.3. Animal models of infection. Due to the ethical constraints of testing potential pathogens on human beings, animal models are often applied to study the interaction between an infectious agent and its host. Appropriate animal models of infection can be used to study the interactions that occur between a pathogenic bacterium and its host during infection. Wesley et

al. (121) showed that *A. butzleri* colonize neonatal pigs, but the experimental sample size was small and severe gross pathologic changes were not observed. Wesley and Baetz (122) tested strain-dependent *A. butzleri* pathogenicity in chicken and turkey poults; *A. butzleri* strains colonized inbred poults, and in some cases led to mortality. Most recently, IL-10$^{-/-}$ mice have been proposed as a useful model of infection because the subsequent loss of inflammation regulation facilitates the study of *C. jejuni* colonization and pathogenicity (123, 124). Mice are a common model for studying bacterial pathogenicity in human beings because human and murine immune systems are similar (125). Golz et al. (126) tested the pathogenicity of two *A. butzleri* strains, isolated from a human patient and from chicken meat, against IL-10$^{-/-}$ mice that had been subjected to broad-spectrum antibiotics (e.g. to create a dysbiosis in the intestinal microbiota). *Arcobacter butzleri* was able to colonize the antibiotic-modulated microbiome of IL-10$^{-/-}$ mice at population densities that remained stable throughout the sample period, but no histopathological change or overt symptoms of illness (e.g. diarrhea, weight loss) were noted (126). Although intestinal cell apoptosis and compensatory cell proliferation accompanied by upregulation of some pro-inflammatory and inflammation-mediating cytokine production (e.g. TNF, IFN-γ, IL-6, and IL-12p70) was observed in mice inoculated with *A. butzleri*, an increase in inflammation-mediating cytokine FOXP3+ was also noted and most pro-inflammatory cytokines decreased to control levels by day 6 (126). In a companion article (127), the authors reported further strain-dependent upregulation of IL-17A and IL-18, as well as downregulation of production of protective Mucin-2 proteins. These studies (126, 127) provide support that *A. butzleri* induces strain-dependent immune response in mice, but the reported lack of histopathological change and/or overt symptoms of illness in this immunocompromised animal model require further study.

1.8. MOLECULAR EPIDEMIOLOGY, POPULATION STRUCTURE, AND SPECIES SUBTYPING

1.8.1. Molecular epidemiology. As the interplay between *A. butzleri* and human illness is poorly understood, it is useful to employ an epidemiological approach to elucidate its pathogenicity and virulence. Epidemiology is the study of disease in time and space, and more specifically the interaction between a pathogen and human beings at the population level. Thus, molecular epidemiology is the application of DNA-based methods to study the interaction between an infectious agent and its host in time and space. The goal of molecular epidemiology is to elucidate the risk that a pathogen poses to the health of a host species; it entails the identification of potential hosts, reservoirs, environmental factors and transmission pathways by which a pathogen comes into contact with people. In practice, molecular epidemiology is used to attribute human illness to a source of contact with an infectious organism in order to facilitate the development of mitigation strategies. As discussed previously, *A. butzleri* is ubiquitous in livestock (22, 48, 52) and in surface waters contaminated with fecal material (36, 37). It is likely that confined feeding operations (CFOs) function as an important reservoir of *A. butzleri* (58, 128), and its ability to survive for extended periods of time in water containing organic matter (45, 47) suggests that contaminated environmental waters may act as another reservoir for this bacterium.

1.8.2. Population structure. Individuals within a species that possess genotypic variations can be divided into subspecies groups (i.e. strains) based on those variations, which is useful because pathogenicity can be strain-dependent. That is, only certain strains of a species possess the complement of functional genomic machinery to allow for survival, infection, and disease incitation in a host species (129, 130). Previous work has indicated that the same bacterial strain can be non-virulent in one host species, yet highly virulent in another host species. For example, *E. coli* O157:H7 naturally colonizes asymptomatic ruminants, but it is associated with

hemorrhagic diarrhea in infected human beings (131). Thus, the ability to differentiate strains within a species is critical to identify and track those that may be associated with disease.

1.8.3. Species subtyping. Species subtyping is the differentiation of clonal isolates from non-clonal isolates based on shared characteristics (132) towards the study of transmission, survival, colonization and/or incitation of disease in a host species by pathogenic subtypes (133, 134). Genotyping is the subtyping of individuals within a species by comparative analysis of loci within the genome (132). It is noteworthy that genotyping methods tend to be more discriminatory and reliable than phenotype-based methods (112, 135). Whole genome sequencing provides the greatest possible resolution for discriminating isolates based on genotype, because every nucleotide base pair of each isolate can be compared. However, whole genome comparison is too costly and analyses of whole genome data too bioinformatically intensive at present to justify its use to genotype the large number of individuals that are required for epidemiological comparison. Sequencing, assembling and bioinformatics analysis of whole genomes requires specialized and dedicated machinery and computers (136). Alternatively, whole genome analysis can be applied to develop reproducible genotyping techniques that mimic whole genome comparison at a resolution that balances cost and discriminatory power (137).

Multilocus sequence typing (MLST) is the current gold standard for genotyping *A. butzleri*; a portion of six or seven highly conserved housekeeping genes (i.e. core genes) are sequenced, specific alleles are determined based on the SNPs within each gene sequence, and a subtype is assigned based on the allelic pattern (138, 139). However, MLST is time-consuming and it requires specialized equipment (e.g. to ensure the accuracy of SNPs, genes must be sequenced a minimum of two times using a sequencing technology with low error rates), which is problematic because epidemiological studies often require the characterization of a large number of isolates (140). In order to elucidate the pathogenicity of *A. butzleri*, an ideal

genotyping technique should provide highly discriminatory and transferable identification of

strains at a cost and throughput that is accessible to most research groups. Recently Taboada et

al. (141) developed a comparative genomic fingerprinting (CGF) assay to type *C. jejuni* isolates

based on variably present/absent regions of the bacterial genome (i.e. accessory genes). As a

PCR-based method, CGF is more easily deployed by researchers lacking the specialized

equipment and funding required for sequence-based methods such as MLST. Moreover, the CGF

assay may provide greater discrimination between closely related strains (142), which is critical

for surveillance of genetically diverse species such as *C. jejuni* (143) and *A. butzleri (138)*.

1.9. SOUTHWESTERN ALBERTA AS A MODEL AGROECOSYSTEM

Southwestern Alberta, Canada is a large geographical area that possesses a high rate of

enteritis among its human inhabitants (144, 145), but the majority of cases of enteritis are not

linked to an etiological agent and *A. butzleri* is not examined as a pathogen (e.g. VITEK®

Automated Microbial Identification System used at the Chinook Regional Hospital (CRH) does

not include *Arcobacter* species within its database of pathogens). Reasons for the high rate of

enteric disease in this region are currently unknown, but it may be linked to higher densities of

CFOs, higher rates of contact with livestock, and the potential for transfer of fecal material from

CFOs to environmental waters (Figure 1.2). Southwestern Alberta is an ideal model

agroecosystem for the study of waterborne pathogens because it consists of a single primary

water basin, the Oldman River Watershed, which begins relatively pristine in the Rocky

Mountains and becomes progressively contaminated with biological agents as it flows eastwards

and encounters increasingly dense human populations and agricultural activity (146, 147).

Animal feces from pastures and CFOs enters the Oldman River and its tributaries directly or as a

result of runoff from rainwater and crop irrigation (148, 149). In addition, waste produced at

livestock slaughterhouses and processing facilities contributes to municipal WWTPs, the effluent
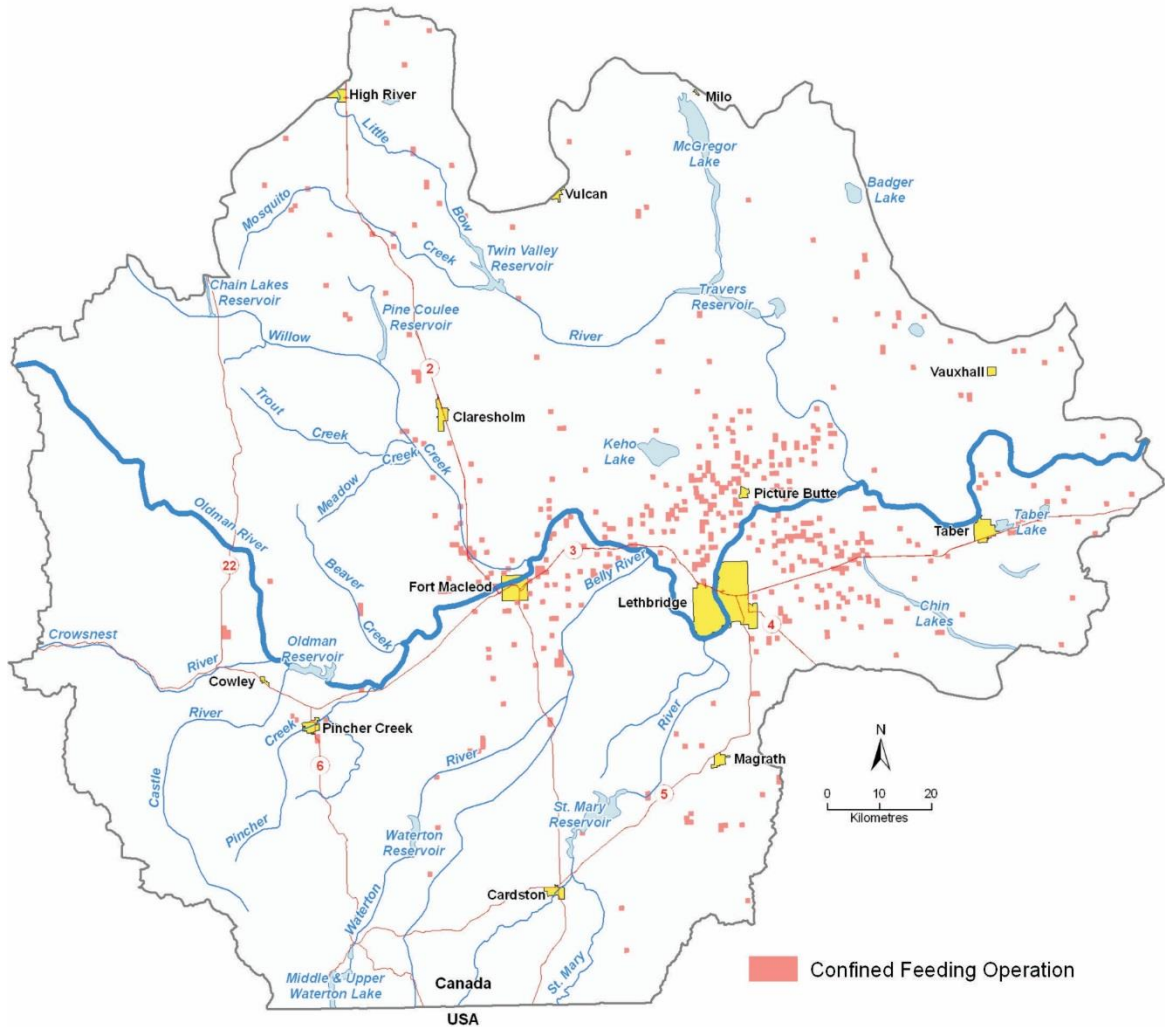
Figure 1.2. Oldman River basin in Southwestern Alberta (SWA) showing the location of confined feeding operations. Source: image is modified from Figure 7.4 in the Oldman River State of the Watershed Report 2010 (146) with permission from the authors.

of which flows into the Oldman River (146, 150).

1.10. KNOWLEDGE GAPS

1.10.1. Colonization versus infection. Elucidation of the pathogenicity of *A. butzleri* is hindered by a lack of understanding of its ecology in people, both diarrheic and non-diarrheic. As discussed in section 1.7.1, a pathogen should be detected more frequently, or in greater densities in diseased individuals. Although experimental evidence to prove incitation of disease in healthy individuals by *A. butzleri* is not currently possible due to the lack of appropriate animal

models, it is possible to compare the frequency of detection and density of *A. butzleri* in cohorts

of diseased and healthy human cohorts. However, few previous studies have compared the

frequency of detection of *A. butzleri* in diseased and healthy cohorts, and no studies have

quantified the densities of *A. butzleri*. This is due in part to the difficulty of obtaining cohorts of

diseased and healthy samples (i.e. stools) in the same time and space, and also to the lack of

standardized tools for comprehensive detection and quantitation of *A. butzleri* in complex

matrices such as feces.

1.10.2. Strain-based pathogenicity. As discussed in section 1.7.2, the presence of *A. butzleri* in

asymptomatic people may indicate that some strains of this bacterium are not pathogenic.

Although *A. butzleri* isolated from diarrheic human beings may be the best candidate strains for

testing strain-specific pathogenicity, experimental testing of strain pathogenicity is not possible

due to the lack of an appropriate animal model of infection. Instead, *A. butzleri* isolated from

diseased and healthy people can be genotyped, which may facilitate identification of candidate

pathogenic strains based on their presence in diseased individuals and absence in healthy

individuals. However, no studies have compared the genotypes of *A. butzleri* isolated from

diseased and healthy human cohorts, which is likely due to a lack of access to appropriate

sample groups and/or genotyping methods.

1.10.3. Population structure. Most cases of *A. butzleri* infection likely result from the ingestion

of contaminated and improperly treated food or water, and less commonly from direct contact

with infected livestock, people, or pets (17). However, the ubiquity of this bacterium in many

possible host species and environmental reservoirs, combined with the number of potential

mechanisms of infection may hinder identification of the original source of contamination. A

number of genotyping techniques have been applied for surveillance of *A. butzleri*, and while the

majority of these studies identified high genotype diversity, they failed to draw further

conclusions about *A. butzleri* population structure in any of the sample sets (17, 99, 100, 151). It

is plausible that the limited throughput of current genotyping methods has hindered the

elucidation of *A. butzleri* population structure because its highly diversity necessitates that a

large number of isolates be genotyped to achieve comprehensive comparison.

1.11. STUDY GOAL AND HYPOTHESES

The overarching goal of my thesis research was to develop and utilize novel molecular

tools to assess whether *A. butzleri* is an enteric pathogen of human beings. The hypotheses

erected were: (1) *A. butzleri* is detected more frequently, and in greater densities in diarrheic

people than in non-diarrheic people; (2) *A. butzleri* pathogenicity is strain-specific, and that non-

pathogenic *A. butzleri* strains are able to colonize human beings as a commensal bacterium; and

(3) *A. butzleri* infection of people in SWA is part of a complex web of transmission pathways

between human beings and their environment (e.g. surface waters, ready-to-eat vegetables,

animals and animal products).

1.12. OBJECTIVES

To test the erected hypotheses, the following three overarching and interrelated project

objectives were established: (i) develop a comprehensive PCR method for detection and

quantitation of *A. butzleri* in complex matrices, and apply this method to diarrheic and non-

diarrheic human stools from SWA; (ii) develop and apply a CGF assay to subtype and compare *A.*

butzleri isolates from diarrheic and non-diarrheic human beings; and (iii) apply my quantitative

detection method and my CGF assay to characterize the population structure of *A. butzleri* in

SWA.

1.13. INTRODUCTION TO CHAPTERS

The research reported herein has resulted in the publication of two peer-reviewed

scientific journal articles, and the submission of two additional manuscripts for publication.

These manuscripts serve as the body of the thesis as follows: (i) whole core genome sequence comparison was employed to develop and validate comprehensive PCR primers for direct quantitative detection of *A. butzleri* in complex matrices, and the method was applied to diarrheic and non-diarrheic stools from people living in SWA (chapter 2); (ii) accessory genome sequence comparison was applied to develop a CGF method for the high-throughput and discriminatory genotyping of *A. butzleri* isolates (chapter 3); the developed quantitative detection method and CGF assay were used to determine the efficacy of wastewater treatment on *A. butzleri* density and strain diversity, and its relation to the *A. butzleri* population structure in human beings (chapter 4); and the developed CGF assay was applied to ascertain the prevalence and population structure of *A. butzleri* in environmental waters as compared to that of municipal wastewater discharge into the Oldman River in SWA (chapter 5). Chapter 6 presents an overview of the results and conclusions drawn from chapters 2-5, and proposes topics for future research.

CHAPTER TWO

Comparative detection and quantification of *Arcobacter butzleri* in stools from diarrheic and non-diarrheic human beings in Southwestern Alberta, Canada[1]

2.1. ABSTRACT

Arcobacter butzleri has been linked to enteric disease in human beings, but its pathogenicity and epidemiology remain poorly understood. The lack of suitable detection methods is a major limitation. Using comparative genome analysis, PCR primers for direct detection and quantification of *A. butzleri* DNA in microbiologically-complex matrices were developed. These primers, along with existing molecular and culture-based methods, were used to detect *A. butzleri* and enteric pathogens in stools of diarrheic and non-diarrheic people (n=1596) living in SWA from May to November 2008. In addition, quantitative PCR was used to compare *A. butzleri* densities in diarrheic and non-diarrheic stools. *Arcobacter butzleri* was detected more often by PCR (59.6%) than by isolation methods (0.8%). Comparison by PCR-based detection found no difference in prevalence of *A. butzleri* between diarrheic (56.7%) and non-diarrheic (45.5%) individuals. Rates of detection in diarrheic stools peaked in June (71.1%) and October (68.7%), but there was no statistically significant correlation between the presence of *A. butzleri* and patient age, sex, or place of habitation. Densities of *A. butzleri* DNA in diarrheic stools (1.6 $\log_{10} \pm 0.59$ copies mg^{-1}) were higher (P=0.007) than in non-diarrheic stools (1.3 $\log_{10} \pm 0.63$ copies mg^{-1}). Of the 892 diarrheic samples that were positive for *A. butzleri*, 74.1% were not positive for other bacterial and/or viral pathogens. The current study supports previous work suggesting that *A. butzleri* pathogenicity is strain-specific, and/or dependent on other factors such as the level of host resistance.

2.2. INTRODUCTION

Nearly 1.7 billion cases of diarrheal disease are reported globally each year (152),

although this is an underestimation of true rates of enteritis as many afflicted individuals do not

have access to or choose not to pursue medical assistance (153). For those seeking diagnosis,

the majority of cases of acute enteritis are not linked to an identified etiological agent (154,

155). Ascertaining the etiology of enteric disease is essential for the development of effective

therapeutics and preventative mitigation strategies. Direct contact with animals and ingestion of

untreated water and/or undercooked animal products are recognized risk factors for acute

enteritis (154), which suggests that a significant number of cases of enteritis are incited by

unidentified biotic pathogens of human or zoonotic origin. Critical components of the

epidemiology of arcobacteriosis and the population structure of *A. butzleri* have yet to be

resolved, in large part because effective culture and/or molecular-based detection methods for

this bacterium have yet to be developed.

Arcobacter butzleri is ubiquitous in the environment (e.g. river water contaminated with

human and/or non-human animal feces) (17, 81, 156). That the bacterium is detected in such a

variety of sources suggests that pathways for transmission among animals and environmental

sources exist, but accurate source tracking of *A. butzleri* is hampered by a lack of standard

detection and isolation methods. Most methods for the isolation of *A. butzleri* from

microbiologically-complex matrices rely on selective enrichments and/or antibiotics to inhibit

the growth of non-target microorganisms (40, 157). In addition, the incubation temperature and

atmosphere utilized for isolation have been inconsistent; temperatures vary from $25^{o}C$ (10) to

$37^{o}C$ (16), and atmospheres range from aerobic (40, 72) to microaerobic (5-6% O_2, 6-10% CO_2, 0-

7% H_2, and 79-85% N_2) and anaerobic (10, 59, 158, 159). Accumulated evidence indicates that no

single medium, temperature, or atmosphere will isolate all strains of *A. butzleri*. For example,

Merga et al. (73) recently compared five media and plating techniques and found that the most effective strategy only detected *A. butzleri* in 70.7% of positive samples.

A number of researchers have utilized primers to detect *A. butzleri* in non-selective enrichment (74, 75). However, no primers have been specifically designed to detect and quantify *A. butzleri* DNA extracted directly from complex matrices without an intermediate enrichment step. Primer development for the detection of microorganisms can be divided into two broad steps: (i) the *in silico* design of primers targeting taxon-specific gene sequences ascertained from comparative analysis of genome data; and (ii) the *in vitro* validation of primer sensitivity (i.e. the minimum detectable amount of target DNA), specificity (i.e. the lack of detection of non-target taxa), and inclusivity (i.e. the detection of all subtypes within a target taxon). During primer design, potential gene targets must be identified and compared to a sequence database to identify marker sites that have conserved nucleotide length, composition, and presence within the target species while being absent from non-target species. As genomic databases cannot contain the entirety of genetic diversity of bacteria, and data are particularly lacking for the genetically diverse *A. butzleri*, developed primers must also be carefully evaluated to ensure sensitivity, specificity, and inclusivity. This is especially true for development of primers to detect DNA in complex matrices such as feces.

I hypothesized that *A. butzleri* is a significant enteric pathogen that is underdiagnosed because of the limitations of culture-based detection. Thus, *A. butzleri* DNA will be more prevalent in stools from diarrheic than from non-diarrheic individuals (i.e. cohorts in the same space and time). Furthermore, *A. butzleri* loads will be higher in diarrheic stools, and the bacterium will be present in diarrheic stools in the absence of other recognized bacterial and viral pathogens. To test these hypotheses, the following objectives were established: (i) use comparative whole genome sequence analysis to select unique, highly conserved, non-variable

loci to develop direct detection and quantification primers for *A. butzleri*; (ii) evaluate the

sensitivity, specificity, and inclusivity of the developed primers; (iii) contrast isolation and PCR

detection frequency of *A. butzleri* in stools of diarrheic and non-diarrheic people (n≈1600) living

in SWA as a model health region; (iv) use quantitative PCR to contrast *A. butzleri* DNA load in

stools from diarrheic and non-diarrheic people; and (v) determine the frequency to which *A.*

butzleri occurs with other recognized bacterial and viral pathogens.

2.3. MATERIALS AND METHODS

2.3.1. Primer design and *in silico* evaluation. The online tool Rapid Annotation Using Subsystem

Technology (RAST) (160) was used to identify open reading frames (ORFs) for genomic

sequences from 12 *A. butzleri* strains available in the National Center for Biotechnology

Information (NCBI) database (PRJNA233527, PRJNA58557, PRJNA158699, PRJNA61483,

PRJNA200766), including eight sequenced by my research group (81), along with whole genomes

from ten additional *A. butzleri* strains (PRJNA309088) provided by Catherine Carrillo (Canadian

Food Inspection Agency). The Basic Local Alignment Search Tool (BLAST) (161) and a program

developed in-house (Concatenator) were used to compare ORFs between *A. butzleri* strains;

those that were redundant or missing from any strains, or that varied in length or sequence

were removed from consideration. The RAST (160) and BLAST (161) tools were also used to

compare the *A. butzleri* genomic sequences to those of four *Arcobacter skirrowii* (PRJNA307998)

and six *Arcobacter cryaerophilus* (PRJNA307600) strains that were sequenced as part of the

current project; any *A. butzleri* ORFs that were detected in *A. skirrowii* or *A. cryaerophilus* were

removed from consideration. The program Geneious (version 5.3.6, Biomatters Ltd, Auckland

NZ) was used to concatenate and align the remaining sequences, and to identify sites for PCR

primer design. Primers for endpoint and qPCR were designed for optimal use with HotStar Taq

Plus DNA polymerase (Qiagen Inc., Toronto ON) and QuantiTect SYBR® Green (Qiagen Inc.).

2.3.2. Primer evaluation.

(i) Primer specificity. Selected PCR primers were tested for specificity against genomic DNA from 22 type strain taxa within the order *Campylobacterales*, including *Arcobacter* spp. (i.e. *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*), *Campylobacter* spp. (i.e. *C. coli*, *C. concisus*, *C. curvus*, *C. fetus* subspecies *fetus*, *C. hominis, C. hyointestinalis* subspecies *hyointestinalis*, *C. insulaenigrae*, *C. jejuni*, *C. jejuni* subspecies *doylei*, *C. lanienae*, *C. lari*, *C. mucosalis*, *C. showae*, *C. sputorum* subspecies *sputorum*, and *C. upsaliensis*), and *Helicobacter* spp. (i.e. *H. canadensis*, *H. pullorum*, *H. pylori*). Amplification reactions consisted of 2.0 μl 10X PCR Buffer containing 15 mM $MgCl_2$ (Qiagen Inc.), 2.0 μl UltraPure BSA (1.0 mg ml^{-1}; Ambion, Life Technologies Inc., Burlington ON), 0.4 μl dNTP mix (10 mM; Bio Basic Canada Inc., Markham, ON), 0.1 μl HotStar Taq Plus DNA Polymerase (5.0 U μl^{-1}; Qiagen Inc.), 1.0 μl ddAbutzF (10 μM; Integrated DNA Technologies, Coralville, IA), 1.0 μl ddAbutzR (10 μM; Integrated DNA Technologies), 2.0 μl DNA template, and 11.5 μl Nuclease-Free Water (Qiagen Inc.). The PCR reaction consisted of activation at 95°C for 5.0 m, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 90 s and elongation at 72°C for 60 s, followed by a final elongation at 72°C for 5 m and storage at 4°C. Amplicons were visualized on a QIAxcel capillary electrophoresis machine (Qiagen Inc.) using the AM320 separation and resolution method, with 15-3000 bp alignment marker and 100-2500 bp size marker.

(ii) Primer inclusivity. Primers were evaluated for their ability to amplify DNA from 130 *A. butzleri* isolates representing 92 different subtypes. The PCR reagents and conditions used for primer evaluation were the same as described for primer specificity. The identity of isolates was confirmed by sequencing the near complete 16S rRNA gene (162). Isolate subtypes were identified using a CGF$_{40}$ method specific to *A. butzleri* (81).

(iii) Primer sensitivity. To determine the limit of detection of developed primers, DNA extracted

from porcine feces seeded with *A. butzleri* was tested; pigs were selected as a monogastric

model for human beings. Multiple fresh samples of feces were collected from three pigs

obtained from the University of Alberta Swine Unit (Edmonton, AB), and were stored at -20°C.

No antibiotics were administered to the pigs. To produce cells for incorporation into feces, *A.*

butzleri ATCC49616 was cultured in triplicate on Columbia Agar (DF0944-17-0, Difco) amended

with 10% sheep blood (CBA) in microaerobic atmosphere (i.e. 5% O_2, 3% H_2, 10% CO_2, and 82%

N_2) at 37°C for 48 h. Biomass from the three cultures was removed from the surface of the

medium and combined in Columbia Broth (CB). The absorbance (A_{600}) was adjusted to 0.5, which

contained approximately 2.0×10^9 cells ml^{-1}. The suspension was diluted with CB in a ten-fold

dilution series. Feces were thawed and 1.0 ml from each dilution of *A. butzleri* cells was

thoroughly mixed into 10 g of the feces. The control treatment consisted of 10 g of feces mixed

with 1.0 ml of sterile CB. Three 0.2 ± 0.02 g subsamples were removed from the seeded feces

and stored at -20°C for later DNA extraction. To enumerate *A. butzleri* cells by culture, 1.0 g of

the seeded feces was suspended in 9.0 ml of CB and diluted in a ten-fold dilution series, and 100

μl of each dilution was spread on CBA in duplicate, cultures were incubated in a microaerobic

atmosphere (i.e. 5% O_2, 3% H_2, 10% CO_2, and 82% N_2) at 37°C, and colonies were enumerated at

the dilution yielding 30 to 300 CFU after 48 and 96 h. The experiment was conducted two times

on separate occasions.

DNA was extracted from the frozen feces subsamples using a QIAamp DNA Stool Mini Kit

(Qiagen Inc.) according to the manufacturer's specifications for pathogen detection. As an

internal amplification control (IAC), 2 μl of DNA (1×10^6 copies μl^{-1}) from a synthesized gene

designed using the *Pyrococcus yayanosii* genome (163) was added to the feces subsamples prior

to extraction; this bacterium is an obligate piezophilic hyperthermophilic archaeon isolated from

deep-sea hydrothermal sites (164). The IAC targets a 268 bp sequence in a putative

carbohydrate kinase (PfkB family; AEH23732.1) using the primers IAC-f (3'-

GGTATGCTAGCCCCGCTTAGGGT-5') and IAC-r (3'-TGCTCCAGAAAAGATGTCCAGCGG-5', and was

synthesized by Integrated DNA Technologies. The presence and quantities of the IAC was

confirmed by real-time PCR amplification on a Stratagene Mx3005P qPCR System (Agilent

Technologies, Santa Clara CA) using the following reagents: 10 µl 2X Quantitect SYBR Green

(Qiagen Inc.), 2.0 µl UltraPure BSA (1.0 mg ml^{-1}; Ambion), 1.0 µl primer IAC-f (10 µM; Integrated

DNA Technologies), 1.0 µl primer IAC-r (10 µM; Integrated DNA Technologies), 2.0 µl DNA

template, and 4.0 µl Nuclease-Free Water (Qiagen Inc.). Samples were quantified in duplicate

reactions. The amplification conditions were one cycle at 95°C for 15 m, followed by 40 cycles of

15 s at 94°C, 30 s at 64°C, and 30 s at 72°C for data acquisition. Direct endpoint detection of *A.*

butzleri DNA was carried out as described above for primer specificity. Quantitative PCR

detection of *A. butzleri* was carried out on a Stratagene Mx3005P qPCR System (Agilent

Technologies) using the following reagents: 10 µl 2X Quantitect SYBR Green mastermix (Qiagen

Inc.), 2.0 µl UltraPure BSA (1.0 mg ml^{-1}; Ambion), 1.0 µl ddAbutzF (10 µM; Integrated DNA

Technologies), 1.0 µl ddAbutzR (10 µM; Integrated DNA Technologies), 2.0 µl DNA template, and

4.0 µl Nuclease-Free Water (Qiagen Inc.). Samples were quantified in duplicate reactions. The

amplification conditions were one cycle at 95°C for 15 m, followed by 40 cycles of 30 s at 94°C,

90 s at 65°C, and 60 s at 72°C for data acquisition. At the end of amplification, melt curve

analysis was conducted. The quantitative PCR data were analysed using MxPro (Version 4.10,

Agilent Technologies Inc.).

2.3.3. Detection and quantification of *A. butzleri* in diarrheic and non-diarrheic stools.

(i) Ethics approval. Scientific and ethics approval to isolate, detect, and quantify *A. butzleri* from

diarrheic and non-diarrheic human beings (i.e. healthy volunteers) was obtained from the

Regional Ethics Committee of the former Chinook Health Region (CHR) and from the University

of Lethbridge Human Subject Research Committee.

(ii) Acquisition of stool samples. A total of 1506 stool samples were obtained from diarrheic individuals submitting samples to the CRH between May 1 and November 25, 2008. Stool samples from diarrheic people were suspended in Cary-Blair medium (165) for transportation to the CRH in Lethbridge, AB. In addition, stool samples were obtained from 90 non-diarrheic volunteers from October 27, 2008 to November 12, 2008. Samples were kept at 4°C for no longer than 24 h. Information provided with the samples included stool collection date, along with the age, sex, and place of habitation (i.e. postal code) of the submitting individual. Using the same method as described for seeded porcine feces, 0.2 ± 0.02 g subsamples were taken from stools and stored at -20°C for later DNA extraction.

(iii) Isolation of *A. butzleri*. Media for isolation of *A. butzleri* were CBA, Karmali Agar (CM0935, Oxoid) with Karmali supplement (KSA; SR0167, Oxoid), Karmali Agar (CM0935, Oxoid) with Bolton supplement (KBA; SR0183E, Oxoid), *Arcobacter* Selection and Isolation Agar (ASIA) (166), and Johnson and Murano Agar (JMA) (167). The isolation method varied by medium: membrane filtration (158) was used for CBA; direct plating of 100 μl of the processed sample was used for KS, KB, and ASIA; and Bolton Broth (CM0983, Oxoid) with Bolton supplement (BBS; SR0183E, Oxoid) was used for enrichment culture with subsequent isolation on KS, KB, ASIA, and JMA. The CBA cultures were incubated at 37°C for up to ten days, and all other agar media were incubated at both 30°C and 37°C for 72 h. All cultures were maintained in a high hydrogen atmosphere (i.e. 5% O_2, 30% H_2, 10% CO_2, and 55% N_2). For enrichment cultures, 25 μl of each sample was added to 2.0 ml of BBS and incubated at both 30°C and 37°C. At 24 and 48 h, 10 μl of the enrichment was streaked on the KS, KB, ASIA, and JMA.

Two colonies per morphology per medium per sample were collected and streaked for purity on CBA, and examined microscopically for cell size, shape, and motility. Genomic DNA was

extracted from isolates using the DNeasy Blood and Tissue Kit (Qiagen Inc.) according to

manufacturer specifications and an automated system (Model 740, Autogen, Holliston, MA).

Arcobacter butzleri DNA was identified by taxon-specific PCR using the same reagents and

conditions as specified for primer specificity, and sequencing of the near complete 16S rRNA

gene (162). All recovered *A. butzleri* isolates were subtyped using CGF_{40} (81).

(iv) Extraction of total DNA from feces and direct detection of *A. butzleri* DNA. The IAC was

added to all stool subsamples, and genomic DNA was extracted using the QIAamp DNA Stool

Mini Kit (Qiagen Inc.). Quantitative PCR for the IAC and endpoint PCR for *A. butzleri* were

conducted as described for seeded porcine feces. Amplifications were scored as positive or

negative, and only samples that were positive for the IAC in the absence of *A. butzleri*

amplification were considered to be true negatives.

(v) Specificity of primers in stools by sequencing of direct PCR amplicons. To confirm the

specificity of amplification, 90 arbitrarily-selected amplicons were sequenced. In order generate

enough product for sequence analysis, the *A. butzleri* PCR reaction volume was doubled to 40 µl,

containing 4.0 µl 10X PCR Buffer with 15 mM $MgCl_2$ (Qiagen Inc.), 4.0 µl UltraPure BSA (1.0 mg

ml^{-1}; Ambion), 0.8 µl dNTP mix (10 mM; Bio Basic), 0.2 µl HotStar Taq Plus (5.0 U $µl^{-1}$; Qiagen

Inc.), 2.0 µl ddAbutzF (10 µM; Integrated DNA Technologies), 2.0 µl ddAbutzR (10 µM; Integrated

DNA Technologies), 4.0 µl DNA template, and 23 µl Nuclease-Free Water (Qiagen Inc.). The PCR

reaction mix was activated at 95°C for 5 m, followed by 35 cycles of denaturation at 94°C for 30

s, annealing at 65°C for 60 s, and elongation at 72°C for 30 s, a final elongation at 72°C for 5 m,

and storage at 4°C. Products were purified with a MinElute 96 UF Purification Kit (Qiagen Inc.),

and rehydrated to 20.0 µl. Sequencing was conducted by Eurofins MWG Operon, and sequences

were aligned in Geneious (Version 5.3.6, Biomatters) and identified using the BLAST program in

NCBI.

(vi) Quantification of *A. butzleri* DNA extracted from stools. DNA from human diarrheic (n=69)

and non-diarrheic (n=50) stools collected during the same time period (i.e. October 27 to

November 11, 2008) that tested positive for *A. butzleri* by direct detection PCR was quantified by

qPCR using the same conditions as for seeded porcine feces.

 (vii) Comparison of *A. butzleri* prevalence to known pathogens. The current study was part of a

larger study examining the prevalence of bacterial and viral pathogens in stools from diarrheic

and non-diarrheic people living in SWA. All samples were processed by staff at the CRH for

Aeromonas spp. (i.e. *A. caviae*, *A. hydrophilia*, *A. salmonicida*, *A. sobria*, and *A. veronii*) (168),

Edwardsiella spp. (*E. hoshinae* and *E. tarda*) (169), *Campylobacter* spp. (*C. coli, C. fetus, C. lari, C.

jejuni*) (170), *E. coli* 0157:H7 (171), *Plesiomonas shigelloides* (169), *Salmonella enterica enterica*

(171), *Shigella spp.* (*S. boydii, S. dysenteriae*, *S. flexneri*, *S. sonnei*) (171), *Staphylococcus aureus*

(172), *Vibrio* spp. (*V. alginolyticus*, *V. cholerae*, *V. fluvialis*, *V. metschnikovii*, *V. mimicus*, *V.

parahemolyticus*, and *V. vulnificus*) (173), and *Yersinia* spp. (Y. *enterocolitica*, *Y. pestis*, *Y.

pseudotuberculosis*, and *Y. ruckeri*) (174). In addition, RNA viruses (Norovirus GI, GII, GIII, GIV,

Sapovirus, Rotavirus, Astrovirus) were detected using Taqman PCR (175) (D. Leblanc, G. D. Inglis,

V. F. Boras, J. Brassard, and A. Houde, submitted for publication).

(viii) Data analysis. All statistical analyses were carried out using SigmaPlot (version 12.0, Systat

Software, San Jose CA). The chi-square test of independence was used to calculate significant

differences in prevalence of *A. butzleri* between diarrheic and non-diarrheic people by culture-

based isolation and by PCR-based detection, as well as for calculating significant differences in

prevalence of *A. butzleri* in diarrheic humans by age, sex and location. The chi-square test of

independence was also used to ascertain possible difference in rates of coinfection of *A. butzleri*

with known pathogens in diarrheic human beings. In order to determine if significant differences

existed in the rate of coinfection of *A. butzleri* with more than two tested pathogens, the rate of

coinfection for each pathogen was compared to the mean coinfection of all other pathogens.

The Mann-Whitney Rank Sum test was used to calculate significant difference between

abundance of *A. butzleri* in stools from diarrheic and non-diarrheic human beings

2.4. RESULTS

2.4.1. Primer design and *in silico* evaluation. Comparative whole genome sequence analysis of

Arcobacter species revealed 1906 conserved ORFs. Of the 66 ORFs that were not present in *A.*

skirrowii or *A. cryaerophilus*, 48 did not contain sufficient length or sequence variation, and 42

were also longer than 300 bp. These 42 ORFs were concatenated for further analysis. The gene

sequence for PCR amplification was required to be no more than 200 bp long, with a primer

length between 19 and 23 nucleotides, a GC content of 35% to 65%, a melting temperature of

60°C to 68°C, and self-annealing or cross-annealing stretches less than four bp in length. The

designed primers (ddAbutzF: 5'-AGTGATGGTGGAGTTGCTAGTC-3'; ddAbutzR: 5'-

GTTGCAGGAGCTTTTTCACTCC-3') targeted a sequence that was identified as part of a putative

gene encoding the gamma subunit of quinohemoprotein amine dehydrogenase

(WP_004510536.1). *In silico* analysis of 22 *A. butzleri* strains (PRJNA233527, PRJNA58557,

PRJNA158699, PRJNA61483, PRJNA200766, and PRJNA309088) identified a single copy of the

target sequence per genome. The predicted PCR product was 137 bp, and was unique to *A.*

butzleri by BLAST analysis (176)*.* In addition, the primer target sequences were identical to all

available *A. butzleri* genomes, and the closest non-target match possessed 79% query coverage.

2.4.2. Primer evaluation.

(i) Primer specificity. Of the 22 taxa within *Campylobacterales* that were evaluated, only *A.*

butzleri produced a detectable PCR amplification product when tested with the ddAbutz

primers.

(ii) Primer inclusivity. All 130 isolates (100%) were amplified by PCR using the ddAbutz primers.

(iii) Primer sensitivity. The ddAbutz primers amplified *A. butzleri* DNA at concentrations as low as 0.6 Log_{10} copies mg^{-1} by endpoint PCR and qPCR, which equated to a minimum detection limit of 1.1 copies per reaction (Figure 2.1).

2.4.3. Detection and quantification of *A. butzleri* in diarrheic and non-diarrheic stools.

(i) Isolation of *A. butzleri*. The overall rate of detection of *A. butzleri* by culture-based isolation using a variety of media and plating methods was low (0.8%), and there was no difference (P=0.81) in detection between diarrheic and non-diarrheic individuals (Table 2.1). For culture positive samples, 8 of 13 were positive by a single method, and membrane filtration on CBA was the most inclusive (46%). No *A. butzleri* isolates were obtained by direct plating of processed stools onto KS. No medium and plating technique was specific to *A. butzleri*; each selected for at least one non-target bacterium (Table 2.2). There were too few *A. butzleri* positive stools to compare the effectiveness of direct plating compared to enrichment techniques.

(ii) Total DNA extraction and detection of *A. butzleri* DNA. Of the 1596 human stool samples tested, an IAC and/or *A. butzleri* amplicon were not observed in extracted DNA from 26 samples (1.6%). Of the remaining 1570 stools, 1482 samples were obtained from diarrheic people and 88 were obtained from non-diarrheic people. The overall prevalence of *A. butzleri* was 60%, and there was no difference (P=0.13) in prevalence of *A. butzleri* DNA between diarrheic (57%) and non-diarrheic (46%) stools. The rate of detection of *A. butzleri* in diarrheic individuals varied throughout the sample period with peaks at the beginning and the end of the summer (Figure 2.2). No correlation was observed between *A. butzleri* prevalence in diarrheic stools with sex (P=0.37), age (P≥0.26), or place of habitation (P=0.15) (Table 2.3).

(iii) Specificity of PCR primers in diarrheic stools by PCR amplification. All 90 (100%) of the amplicons from human stools that were sequenced were identified as *A. butzleri* by BLAST analysis. Trimmed sequences were 93 bp to 95 bp in length. All trimmed sequences were
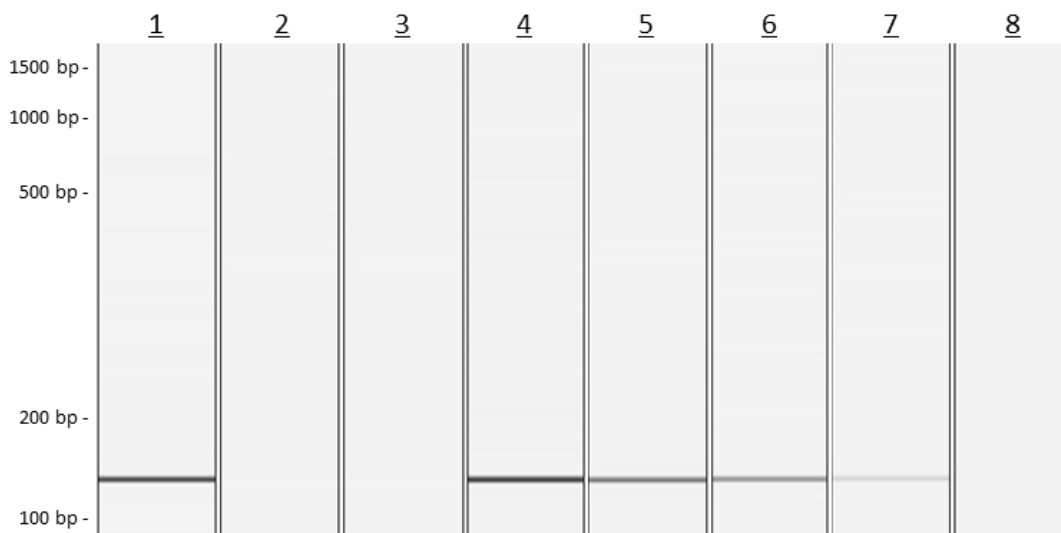
Figure 2.1. Direct PCR detection of *A. butzleri* L130 extracted from inoculated pig feces by targeting the single-copy quinohemoprotein amine dehydrogenase gene with novel ddAbutz primers. Amplicons were visualized on a QIAxcel capillary electrophoresis machine (Qiagen Inc.) using the AM320 separation and resolution method with 15-3000 bp alignment marker and 100-2500 bp size marker. Lane 1, *A. butzleri* L130 positive PCR controls; lane 2, H_2O (Optima) negative PCR control; lane 3, total DNA extracted from feces inoculated with sterile Columbia broth (Difco) as a negative control; lane 4, DNA from feces inoculated with *A. butzleri* L130 at a density of 3.6 Log_{10} copies/mg; lane 5, DNA from feces inoculated with *A. butzleri* L130 at a density of 2.6 Log_{10} copies/mg; lane 6, DNA from feces inoculated with *A. butzleri* L130 at a density of 1.6 Log_{10} copies/mg; lane 7, DNA from feces inoculated with *A. butzleri* L130 at a density of 0.6 Log_{10} copies/mg; lane 8, DNA from feces inoculated with *A. butzleri* L130 at a density of 0.06 Log_{10} copies/mg.

identical, so a single consensus sequence was compared to the NCBI database.

(iv) Quantification of *A. butzleri*. Overall cell density in human stool samples was 1.4 ± 0.62 log_{10} cells mg^{-1}, but quantities of DNA were higher (P=0.007) in stools of diarrheic (1.6 $log_{10} \pm 0.59$ copies mg^{-1}) than non-diarrheic (1.3 $log_{10} \pm 0.63$ copies mg^{-1}) people.

(v) Comparison of *A. butzleri* prevalence to known pathogens. Of the 1482 diarrheic samples examined, 390 (26%) were positive for recognized bacterial and/or viral pathogens. Of the samples positive for *A. butzleri*, 661 (74%) were not positive for other bacterial and/or viral pathogens. None of the recognized pathogens were more likely to be co-detected with *A. butzleri* (P≥0.26) (Table 2.4).

Stool	CBA (mf)[b]	KSA (dp)[c]	KSA (en)[d]	KBA (dp)[e]	KBA (en)[f]	ASIA (dp)[g]	ASIA (en)[h]	JMA (en)[i]
1					+[a]			
2	+		+	+	+			+
3	+		+	+	+			
4								+[a]
5				+[a]				
6						+		+
7				+[a]				
8	+				+	+	+	
9	+[a]							
10	+[a]							
11	+[a]							
12						+	+	
13				+[a]				
Total	6	0	2	5	4	3	2	3

[a] Unique isolation
[b] Membrane filtration (158) onto CBA
[c] Direct plating of 100 µl of processed stool sample onto KSA
[d] Enrichment culture in BBS followed by subsequent isolation on KSA
[e] Direct plating of 100 µl of processed stool sample onto KBA
[f] Enrichment culture in BBS followed by subsequent isolation on KBA
[g] Direct plating of 100 µl of processed stool sample onto ASIA (166)
[h] Enrichment culture in BBS followed by subsequent isolation on ASIA (166)
[i] Enrichment culture in BBS followed by subsequent isolation on JMA (167)

Table 2.1. Detection of *A. butzleri* by isolation.

2.5. DISCUSSION

2.5.1. Efficiency of *A. butzleri* detection methods. In the current study, the detection of *A. butzleri* by isolation was compared to detection by PCR amplification. The rate of detection of *A. butzleri* in human stools by isolation was low (0.8%) compared to PCR-based detection (60%).

Others have found that PCR was more effective than culturing for detection of *A. butzleri* in human stools (72), seawater (62), and wastewater and chicken carcasses (177). Fera et al. (72) suggested that the decreased rate of detection observed in selective and enrichment media may be the result of competition by non-target members of the source microbiota, along with difficulty replicating source conditions for growth in a controlled system. In addition, the use of

Table 2.2. Isolated bacterial taxa.

Isolated taxa	CBA (mf)[a]	KSA (dp)[b]	KSA (en)[c]	KBA (dp)[d]	KBA (en)[e]	ASIA (dp)[f]	ASIA (en)[g]	JMA (en)[h]
Acinetobacter sp.	+			+				
Actinomyces sp.	+							
Alistipes sp.	+			+	+			+
Anaerobiospirillium sp.				+				
Arcobacter butzleri	+		+	+	+	+	+	+
Arcobacter cryaerophilus	+			+				+
Arcobacter skirrowii				+				+
Bacillus sp.	+							
Bacteroides sp.	+			+				+
Bifidobacterium sp.	+		+	+				
Campylobacter coli	+			+				
Campylobacter concisus	+	+	+	+	+			+
Campylobacter curvus	+	+	+	+	+			
Campylobacter gracilis	+			+				
Campylobacter hyointestinalis				+				
Campylobacter jejuni	+	+	+	+	+	+	+	+
Campylobacter lanienae	+	+	+	+	+			
Campylobacter showae	+	+	+	+	+			
Campylobacter upsaliensis	+						+	+
Catabacter sp.	+					+		+
Christensella sp.					+			
Citrobacter sp.		+		+				+
Desulfovibrio sp.	+				+			
Eggerthella sp.	+			+				+
Enterobacter sp.								+
Enterococcus sp.	+	+	+	+				+
Escherichia sp.		+		+				+
Facklamia sp.	+							
Fastidiosipila sp.				+				
Gordonibacter sp.					+			
Halomonas sp.								+
Helicobacter sp.	+	+		+				
Klebsiella sp.			+					
Lactobacillus sp.		+		+	+			+
Micrococcus sp.		+			+			
Mobiluncus sp.	+							
Ochrobactrum sp.				+				
Parabacteroides sp.				+				
Parasutterella sp.	+							
Pediococcys sp.				+				
Phascolarctobacterium sp.	+	+	+		+			+
Propionibacterium sp.	+			+				
Pseudomonas sp.		+	+	+	+			+
Staphylococcus sp.	+							
Sutterella sp.	+	+	+	+	+			+
Veillonella sp.	+	+		+	+			
Total	29	15	12	30	16	3	3	19

[a] Unique isolation
[b] Membrane filtration (158) onto CBA
[c] Direct plating of 100 µl of processed stool sample onto KSA
[d] Enrichment culture in BBS followed by subsequent isolation on KSA
[e] Direct plating of 100 µl of processed stool sample onto KBA
[f] Enrichment culture in BBS followed by subsequent isolation on KBA
[g] Direct plating of 100 µl of processed stool sample onto ASIA (166)
[h] Enrichment culture in BBS followed by subsequent isolation on ASIA (166)
[i] Enrichment culture in BBS followed by subsequent isolation on JMA (167)

Figure 2.2. Rate of detection (%) of *A. butzleri* in stools from diarrheic humans, as determined by direct endpoint PCR targeting the single-copy quinohemoprotein amine dehydrogenase gene with novel ddAbutz primers. The total number of human stools processed by month were 209 (May), 232 (June), 199 (July), 228 (August), 225 (September), 198 (October), and 191 (November).

enrichment culture has been shown to reduce the diversity of other enteric pathogens (178, 179), and antimicrobial agents in *A. butzleri* selective media may also reduce diversity (19). This is problematic because antimicrobial agents are often required to inhibit growth of non-target taxa that could exclude *A. butzleri*. Frequently, presumptive *A. butzleri* (i.e. based on colony morphology) turned out to be *Alistipes spp.*, *Bacteroides spp.*, *Catabacter spp., Citrobacter spp., Helicobacter* spp., and *Campylobacter* spp. in particular. Previous studies have noted a similar lack of specificity for culture isolation of *A. butzleri* from feces (10, 73).

Table 2.3. Direct PCR detection of *A. butzleri* in diarrheic stools.

Category	Samples (n)	Rate of infection (%)	P value
Sex			
Male	599	61.8	0.37
Female	873	59.5	
Age (years)			
0-4	215	62.3	0.53
5-18	112	55.4	0.26
19-64	747	61.3	0.46
65+	398	59.0	0.52
Habitation [a]			
Rural	560	57.7	0.15
Urban	887	61.4	

[a] Rural or urban location of habitation was ascertained from postal codes submitted by diarrheic individuals.

2.5.2. Prevalence of *A. butzleri* in human stools. The overall prevalence of *A. butzleri* in human stools was 60%, which is much higher than rates of 25% or less reported by others (40-42, 72). The high rate of detection of *A. butzleri* observed in the current study may be attributed to the use of primers designed and validated for maximum efficiency in complex matrices. While previous studies evaluated primer sensitivity and/or specificity, they typically did not examine inclusivity. In contrast, the primers used in the current study were designed and evaluated with an emphasis on inclusivity. PCR inclusivity is the ability of primers to amplify all subtypes of the target taxon, and it is reduced as a result of poor binding efficiency at the primer binding site. It is therefore important to select a target site that lacks sequence variation within the targeted bacterium so that it is not susceptible to competitive binding by non-target taxa. The PCR primers used in previous studies target universal gene sequences such as 16S rRNA (42), 23S rRNA (40), *hsp*60 (74), and *gyr*A (41). In the current study, non-universal gene sequences that were conserved within *A. butzleri* were identified, thereby circumventing the potential pitfalls of PCR amplification of universal genes. To validate primer inclusivity, 130 *A. butzleri* isolates representing 92 different CGF subtypes were tested, and the primers successfully amplified the

Table 2.4. Detection of *A. butzleri* and recognized enteric pathogens in diarrheic stools

Pathogen	Positive samples (n)	Coinfections with *A. butzleri* (n)	Rate of coinfection (%)	P value
Aeromonas spp. [a]	9	6	66.7	---
C. coli	16	9	56.3	0.94
C. difficile [a]	7	5	71.4	---
C. jejuni	183	103	56.3	0.68
E. coli O157:H7	17	11	64.7	0.54
Salmonella spp.	25	15	60.0	0.79
Astrovirus	20	10	50.0	0.49
Norovirus GI	16	7	43.8	0.26
Norovirus GII	110	66	60.0	0.53
Norovirus GIII [a]	0	0	---	---
Norovirus GIV [a]	1	1	100	---
Rotavirus	14	6	42.9	0.26
Sapovirus	26	16	61.5	0.66
Total	444	255	57.4	---

[a] Pathogen was not detected in enough samples to be statistically viable.

gamma subunit of the quinohemoprotein amine dehydrogenase gene (WP_004510536.1) for all 130 isolates. In comparison, previous studies have evaluated inclusivity of their primers against a relatively small number (one to seven) of *A. butzleri* isolates (74, 77, 78, 180).

2.5.3. Comparative detection of *A. butzleri* in diarrheic and non-diarrheic stools. *Arcobacter butzleri* is the fourth most commonly isolated *Campylobacter*-like organism from diarrheic humans (10), but few studies have compared the prevalence of *A. butzleri* in diarrheic and non-diarrheic humans. I hypothesized that if *A. butzleri* is an emerging pathogen, it would be significantly more prevalent in stools from diarrheic than non-diarrheic people. Even though a much higher prevalence of *A. butzleri* was detected in stools compared to previous studies, there was no significant difference between diarrheic and non-diarrheic groups. Collado et al. (40) also found no difference in prevalence between stools from diarrheic and non-diarrheic people in Chile, although there were too few *A. butzleri* positive stools for statistical comparison. In South Africa, Samie et al. (71) used PCR to compare prevalence of *A. butzleri* in stools from diarrheic and non-diarrheic individuals and found no significant difference. These findings

contrast with those of recognized enteric pathogens, which are more prevalent in diarrheic than non-diarrheic individuals (181).

2.5.4. Comparative quantification of *A. butzleri* in diarrheic and non-diarrheic stools. In situations where the pathogenicity of enteric bacteria is uncertain (182, 183), quantification of microorganism density can provide evidence in support of pathogenicity (i.e. an increase in density of a microorganism in diseased individuals). For example, Phillips et al. (184) observed that viral loads of the recognized pathogen, Norovirus GII were much greater in diarrheic than non-diarrheic individuals, and Brassard et al. (185) observed that viral loads of the emerging pathogen, Torque teno virus were much greater in diarrheic than non-diarrheic people. To my knowledge, the current study is the first to compare densities of *A. butzleri* in diarrheic and non-diarrheic people. Although *A. butzleri* DNA loads were low in both diarrheic and non-diarrheic individuals, the density of *A. butzleri* DNA in stools from diarrheic people was slightly higher than in stools from non-diarrheic individuals. It is uncertain whether the difference in DNA loads between the two groups is biologically relevant (i.e. that pathogenic subtypes exist and contribute to the differential density), or is confounded by the diseased status of the diarrheic group. This warrants further investigation.

2.5.5. Epidemiology of diarrheic individuals infected with *A. butzleri*. The prevalence of *A. butzleri* in diarrheic human stools increased with the onset of summer, and it remained relatively high throughout the sample period, but there was no correlation between rate of detection of *A. butzleri* and patient age or sex. Previous studies also found no correlation between *A. butzleri* infection and patient age or sex (41, 71). In comparison, host infection by pathogenic campylobacters is influenced by both age and sex (71, 186, 187), as is infection by other emerging pathogens such as *H. pylori* (71) and Torque teno virus (185). There was no correlation between *A. butzleri* infection and place of habitation (i.e. whether patients lived in an urban or

rural area). However, it was not possible to ascertain the degree to which people living in urban versus rural locations interacted with livestock (e.g. through occupational exposure). Thus, it was not possible to determine whether there was a correlation between direct contact of people with livestock and infection by *A. butzleri*.

2.5.6. Co-isolation of *A. butzleri* with recognized pathogens. In the current study, 74% of *A. butzleri* positive diarrheic human stool samples were not positive for recognized pathogens. The most commonly detected bacterial pathogen was *C. jejuni*, but the rate of co-infection with *A. butzleri* was not significantly greater than with other pathogens. Although it is difficult to directly compare my results with previous studies (i.e. because the pathogens detected varied, as did the methods of detection), others reported that significant numbers of samples ranging from 16% (71) to 60% (41) were positive for *A. butzleri* and not for recognized pathogens. Considering that most cases of enteritis are not attributed to a single pathogenic species (154, 155), and that the majority of cases of enteritis are not linked to an etiological agent (153), the isolation of *A. butzleri* in the absence of other pathogens does not necessarily indicate that *A. butzleri* incites disease. Furthermore, my observation that *A. butzleri* is equally and highly prevalent in diarrheic and non-diarrheic individuals supports the conclusion that *A. butzleri* does not possess species-wide pathogenicity.

2.6. CONCLUSIONS

The current study examined the prevalence and abundance of *A. butzleri* in stools from diarrheic and non-diarrheic people living in SWA. It was hypothesized that, as an emerging enteric pathogen, the prevalence and abundance of *A. butzleri* will be greater in diarrheic than in non-diarrheic people. Culture-based isolation and novel direct detection PCR primers were used to detect *A. butzleri* in 1596 human stools. The vast majority of *A. butzleri* infections were not detected by culture-based isolation, that there was no difference in prevalence of *A. butzleri*

between diarrheic and non-diarrheic cohorts, and that *A. butzleri* DNA loads were only slightly

greater in diarrheic stools. Thus, it was concluded that either *A. butzleri* is not a pathogen, or the

strain of *A. butzleri* and/or the status of the host regulates pathogenicity (e.g. *A. butzleri* is an

opportunistic pathogen in a similar manner to *H. pylori* (188)). The application of high-

throughput subtyping methods such as CGF_{40} (81) is necessary to ascertain whether specific

strains of *A. butzleri* are associated with disease in human beings, with confirmation using

models of pathogenicity/virulence.

CHAPTER THREE

Development of a Comparative Genomic Fingerprinting assay for rapid and high resolution genotyping of *Arcobacter butzleri*[2]

3.1. ABSTRACT

Molecular typing methods are critical for epidemiological investigations, facilitating disease outbreak detection and source identification. Studies on the epidemiology of the emerging human pathogen *Arcobacter butzleri* is currently hampered by the lack of a subtyping method that is easily deployable in the context of routine epidemiological surveillance. The purpose of this study was to design and validate a CGF method for high-resolution and high-throughput subtyping of the *A. butzleri*. Comparative analysis of the genome sequences of eleven *A. butzleri* strains, including eight strains newly sequenced as part of this project, was employed to identify accessory genes suitable for generating unique genetic fingerprints for high-resolution subtyping based on gene presence or absence within a strain. A set of eighty-three accessory genes was used to examine the population structure of a dataset comprised of isolates from various sources, including human and non-human animals, sewage and water (n=156). A streamlined assay (CGF_{40}) based on a subset of 40 genes was subsequently developed through marker optimization. High levels of profile diversity, 121 distinct profiles were observed among the 156 isolates in the dataset, and a high Simpson's index of diversity (ID) observed (ID>0.969) indicate that the CGF_{40} assay possesses high discriminatory power. At the same time, my observation that 115 isolates in this dataset could be assigned to 29 clades with a profile similarity of 90% or greater indicates that the method can be used to identify clades comprised of genetically similar isolates. The CGF_{40} assay described herein combines high resolution and

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repeatability with high throughput for the rapid characterization of *A. butzleri* strains. This assay will facilitate the study of the population structure and epidemiology of *A. butzleri*.

3.2. INTRODUCTION

Arcobacter butzleri is closely related to the pathogen *Campylobacter jejuni* (189), and it has been isolated from surface waters, livestock, and animal products (99, 190-192). The pathogenicity of *A. butzleri* has yet to be resolved (99, 138); although *A. butzleri* has been isolated from the stools of diarrheic human beings, which is highly suggestive of pathogenicity (56, 74, 76), it has also been obtained from non-diarrheic individuals (71, 156) suggesting that it is a commensal or that non-pathogenic strains or subtypes exist within the species.

An important facet in the study of pathogens is epidemiology-based analysis of their incidence and distribution. Molecular subtyping or genotyping, which allows the classification of a bacterial species into distinct strains or subtypes based on genetic variation (40, 193), forms one of the pillars of molecular epidemiology, through which the identification of etiological agents, patterns of transmission, and potential outbreaks can be carried out with enhanced precision (194). Until recently, the study of *A. butzleri* has been hampered by the lack of advanced methods for subtyping. A recently developed MLST scheme (195) provides excellent identification of subtypes and has been utilized to examine genetic diversity in *A. butzleri* isolated from people, livestock and animal products (99, 138). However, this method remains a resource-intensive and relatively low-throughput means of subtyping, which limits the number of isolates that can be analyzed by most research groups (196, 197), as evidenced by the relatively small number of isolates that have been contributed to the MLST database for *A. butzleri* by the global research community (*n*=683, PubMLST accessed on October 21, 2014). More importantly, the lack of a highly deployable subtyping method suitable for use in routine surveillance has precluded the large-scale epidemiological surveys required to fully assess the

potential role of *A. butzleri* as an emerging pathogen of humans.

Recent advances in sequencing technologies (i.e. next generation sequencing) and bioinformatics have made it possible to rapidly obtain draft whole genome sequencing (WGS) data (198) and it is likely that methods based on WGS analysis, including whole genome MLST, will eventually become the new standard for microbial subtyping in an epidemiological context (199, 200). However, until the resources required for WGS-based subtyping allow it to become practical enough to be deployed in large-scale epidemiological surveillance, there is a continuing need for methods that fulfill performance criteria such as discriminatory power and repeatability, and convenience criteria such as throughput, cost and ease of use (194). Recently, Taboada et al. (141) employed whole genome analysis to develop a CGF method for high-resolution subtyping of *C. jejuni* that was highly concordant with MLST but better suited to large-scale surveillance due to improved throughput and cost relative to MLST. Moreover, by targeting a large number of accessory genes (e.g. 40 loci), the CGF method showed improved discriminatory power compared to MLST, allowing the differentiation of closely related strains with distinct epidemiology (141, 142).

The overall goal of the current study was to develop a highly discriminatory CGF assay for *A. butzleri* by employing the strategy described by Taboada et al. (141) for *C. jejuni*. Objectives were to: (i) select *A. butzleri* isolates for WGS; (ii) utilize whole genome sequence data to identify candidate CGF target genes in the accessory genome; (iii) screen CGF targets against a panel of *A. butzleri* isolates to determine accessory gene frequency and assess accessory genome variability; (iv) select a subset of CGF targets for development of a CGF_{40} assay; and (v) evaluate the ability of the CGF_{40} assay to reliably reproducibly discriminate *A. butzleri* strains. The development of highly deployable genotyping techniques that are suitable for use in routine surveillance will improve my ability to distinguish strains of *A. butzleri* and

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facilitate the study of its epidemiology.

3.3. MATERIALS AND METHODS

3.3.1. Ethics statement. Scientific and ethics approval to isolate *A. butzleri* from diarrheic and non-diarrheic human beings (i.e. healthy volunteers) was obtained by GDI from the Regional Ethics Committee of the former CHR and from the University of Lethbridge Human Subject Research Committee. The requirement for informed written consent was waived by the CHR Regional Ethics Committee and the U of L Human Subject Research Committee for subsamples of stools submitted by diarrheic people as the samples were submitted for the detection of enteric pathogens at the CRH and the identities of patients was not disclosed. Informed written consent as mandated by the U of L Human Subject Research Committee was obtained from all healthy volunteers in advance of the submission of stool samples for the isolation of *A. butzleri* and other enteric bacteria.

3.3.2. *Arcobacter butzleri* isolation and DNA extraction. *Arcobacter butzleri* were isolated from a stool sample obtained from eleven diarrheic humans, and from two stools obtained from one non-diarrheic human, as well as from non-human animal feces, sewage, and river water collected in SWA during 2008 and 2009. Isolates were streaked for purity and stored at -80°C in CB with 30% glycerol. Isolates from glycerol stocks were grown on CBA in a microaerobic atmosphere (5% O_2, 3% H_2, 10% CO_2, and 82% N_2) at 37°C for 24-48 hr, and biomass was collected from the surface of the agar medium. An automated system (Model 740, Autogen, Holliston, MA) was used to extract genomic DNA. Putative *A. butzleri* isolates were identified by PCR amplification using an *Arcobacter* PCR-multiplex assay (76).

3.3.3. Whole genome sequencing and assembly. In order to design a CGF assay for *A. butzleri* it was necessary to perform a comparative whole genomic analysis of strains representing diverse sources and genetic backgrounds. To minimize possible genetic bias amongst strains selected for

WGS, *A. butzleri* isolates from diverse sources were genotyped using Amplified Fragment Length

Polymorphism (AFLP) analysis as described previously (201, 202), and eight strains representing

highly diverse AFLP profiles were chosen for sequencing (Figure 3.1). For WGS analysis, DNA was

extracted using a DNEasy Blood and Tissue Kit (Qiagen Inc, Toronto, ON). The identity of isolate

DNA was tested by sequencing approximately 1000 bp of the 16S rRNA gene and by comparing

the results with *A. butzleri* sequences within the NCBI genetic database (161, 176). The DNA for

isolates to be sequenced was quantified by spectrophotometry (A_{600}) (Ultrospec 3100 pro, GE

Healthcare Life Sciences, Baie d'Urfe, QC). Isolates were sequenced as paired-end, 100 bp reads

on a HiSeq platform (Illumina Inc., San Diego, CA) with Phred30 (99.9%) base-calling accuracy

(203), and reads were *de novo* assembled into contigs using ABySS (204) with specifications for

short paired-end reads. Sequencing data for the *A. butzleri* isolates were accessioned in the NCBI

genetic sequence database as a single bioproject (PRJNA233527).

3.3.4. Detection and identification of coding sequences. The RAST tool (160) was used to

identify ORFs for the eight sequenced *A. butzleri* genomes, as well as three previously available

genome assemblies (RM4018 - PRJNA58557, ED1 - PRJNA158699, JV22 - PRJNA61483). The

genome assembly for a fourth strain, 7h1h (PRJNA200766), was not available at the time that

the comparative genomic analysis was performed, however the four published WGS strains were

included in all subsequent *in silico* CGF analyses.

To identify core and accessory genes, the ORFs from each genome were searched against the

eleven genome assemblies using BLAST (161, 176), with filtering to remove redundant results

from likely orthologous genes. ORFs present in all assemblies were identified as core, and all

non-redundant ORFs absent from one or more strains were designated as accessory.

 3.3.5. Identification of candidate accessory genes for CGF assay development. To simplify CGF

 assay design, accessory genes with limited genotypic potential due to a highly biased population

Clade	Isolate ID	Source	Location	Collection Date
1	121	municipal wastewater	Fort Macleod, AB, CA	05/10/2008
1	17	horse feces	Diamond City, AB, CA	02/07/2009
2	151	non-diarrheic human stool	Lethbridge, AB, CA	30/09/2008
2	150	non-diarrheic human stool	Lethbridge, AB, CA	30/09/2008
2	149	Non-diarrheic human stool	Lethbridge, AB, CA	01/04/2009
3	56	municipal wastewater	Fort Macleod, AB, CA	29/06/2008
3	82	pig feces	Lethbridge, AB, CA	12/08/2009
3	B0113	municipal wastewater	Lethbridge, AB, CA	07/05/2008
3	B0132	municipal wastewater	Lethbridge, AB, CA	07/05/2008
3	88	municipal wastewater	Fort Macleod, AB, CA	10/06/2008
3	100	diarrheic human stool	Blairmore, AB, CA	30/07/2008
4	147	diarrheic human stool	Lethbridge, AB, CA	01/04/2009
5	B1140	diarrheic human stool	Blairmore, AB, CA	31/07/2008
5	51	diarrheic human stool	Lethbridge, AB, CA	08/05/2008
6	25	diarrheic human stool	Lethbridge, AB, CA	26/10/2008
7	40	municipal wastewater	Lethbridge, AB, CA	08/03/2009
8	64	surface water	Oldman River	07/05/2008
9	69	diarrheic human stool	Lethbridge, AB, CA	27/04/2009
10	123	municipal wastewater	Fort Macleod, AB, CA	14/05/2008
11	30	diarrheic human stool	Magrath, AB, CA	28/10/2008
12	141	municipal wastewater	Lethbridge, AB, CA	12/08/2008
13	B0117	municipal wastewater	Fort Macleod, AB, CA	07/05/2008

Figure 3.1. Isolates of *A. butzleri* from diverse sources selected for whole genome sequence analysis based on AFLP profile comparison. AFLP clades were defined using an 85% similarity threshold (dashed line). A total of eight strains (green) were selected for WGS, an additional ten strains (orange) were included in the dataset for assessment of CGF discrimination and concordance (Figure 3.3), and four strains (red) were not included as part of the CGF dataset.

distribution (i.e. present in greater than 80% of strains or present in fewer than 20% of strains) were eliminated from further consideration as candidate markers. Moreover, for groups of accessory genes that presented redundant patterns of presence and absence in the dataset (i.e. genes that are typically linked and provide limited additional discrimination), only one representative gene from each unique pattern was considered as a candidate marker for CGF development. Short genes (i.e. <300 bp) and/or those containing gaps or polymorphisms that might affect PCR primer design were also discarded. Accessory genes meeting the above criteria were identified and used to design an expanded CGF assay (i.e. the reference assay) to examine the population structure of a diverse collection of *A. butzleri* isolates (n=152) based on accessory genome variability. Data from these isolates, which were recovered from river water, raw and treated sewage, diarrheic and non-diarrheic human beings, and non-human animals in SWA was used in conjunction with *in silico*-derived (205) CGF data from four published genome-

sequenced strains (RM4018 - PRJNA58557, ED1 - PRJNA158699, JV22 - PRJNA61483, 7h1h - PRJNA200766). CGF profiles were also generated *in silico* using the program Microbial *In Silico* Typer (MIST) (205) for the eight isolates sequenced *de novo* to allow for comparison with PCR-derived CGF data, thus facilitating assessment of marker performance. A dendrogram representing an estimate for a 'reference phylogeny' was constructed from the binary (i.e. presence and absence) data for those genes that generated data fully concordant with *in silico*-predicted CGF profiles (n=72). Hierarchical clustering was performed by Unweighted Pairwise Grouping with Arithmetic Mean (UPGMA) using the *hclust* function in R (206) and the simple matching coefficient of genetic similarity.

3.3.6. Optimization of markers for development of final CGF assay. The program CGF Optimizer (CGFO) (207), which calculates the Adjusted Wallace Coefficient (AWC) and the symmetric distance (SymD) (208-211) to assess the concordance between clustering results from sets of prospective CGF markers and a reference phylogeny, was used to identify a subset of accessory genes yielding high concordance to the reference phylogeny generated using the expanded CGF assay. Briefly, CGFO was used to subsample sets of candidate accessory genes and to compute the AWC of each set to the reference phylogeny; the 40 loci that were most concordant with the reference phylogeny (i.e. the set with the highest AWC) were selected for the final CGF_{40} assay.

3.3.7. CGF assay development. Primer3 (212) was employed to design PCR primers for genes selected for CGF assays. The programs MultiPLX (213) and CGF Multiplexer (207) were used to arrange primers with compatible thermodynamic properties into multiplex pools that would generate amplicons differing by at least 100 bp to facilitate unambiguous scoring of marker presence or absence. The CGF profiles obtained *in silico* (205) and by multiplex PCR amplification for the sequenced strains were compared to ascertain primer sensitivity and specificity, and primer pair concentrations within each multiplex were adjusted to optimize product

amplification (Table 3.1). In addition, the reproducibility of the final CGF_{40} assay was tested by running duplicate PCR reactions for a set of 24 *A. butzleri* isolates (23 test isolates plus 1 control). To generate a CGF profile, eight PCR reactions targeting five loci per reaction were performed for each *A. butzleri* isolate. Individual PCR reactions (25 μl) contained 2.0 μl of genomic DNA, 2.5 μl of 10X incubation mix without $MgCl_2$ (MP Biomedicals, Solon, OH; 1X), 2.5 μl of $MgCl_2$ (MP Biomedicals; 2.5 mM), 0.5 μl of a deoxynucleoside triphosphate pool (0.2 mM), 1.0 μl of the multiplex primer pool (0.4 μM), 0.2 μl Taq DNA Polymerase (MP Biomedicals; 1 U μl^{-1}), and 16.3 μl Optima water (Fisher Scientific, Ottawa, ON). PCR conditions consisted of 32 cycles of denaturation at 93°C for 30 s, annealing at 60°C for 90 s, and extension at 72°C for 60 s. After a final extension step at 72°C for 5 min, PCR products were stored at 4°C, and visualized using a QIAxcel automated capillary electrophoresis system (Qiagen Inc.) with a QIAxcel 2400 Sample DNA Screening Kit (Qiagen Inc.), QX 15-1000 bp alignment marker (Qiagen Inc.), and 30 ng μL^{-1} QX 50-800 bp Size Marker (Qiagen Inc.). Capillary electrophoresis lanes were scored for amplification of the five loci targeted (i.e. scored as present or absent) in each multiplex PCR, resulting in a 40-digit binary profile for each isolate. Isolate profiles were clustered using the simple matching coefficient in BioNumerics (version 6.6, Applied Maths, Austin, TX), and isolate similarity was visualized as an UPGMA dendrogram.

3.3.8. Assessment of CGF discrimination and concordance. PCR data for the reference and CGF_{40} assays was generated for the 152 *A. butzleri* isolates. The CGF profiles of four previously published genome-sequenced strains (RM4018, ED1, JV22, and 7h1h) were also obtained *in silico* (205). To verify concordance between the expanded CGF and CGF_{40} assays, binary data from each assay was subjected to hierarchical clustering by UPGMA using the *hclust* function in R (206) and the simple matching coefficient of genetic similarity. The online 'Comparing Partitions' tool (208) was used to calculate the discriminatory power of each assay and the concordance

	Product Size (bp)	Primer Forward (5' to 3')	Primer Reverse (5' to 3')	Concentration[b] (µM)
Multiplex 1	150	GCATCCTCTTCCTCCATCAT	TCGAATAAATCCCCTACCCTT	12
	250	ATACACCACCAGATGAGCTG	TAACGTACCGCATCCATTGA	10
	400	AGTGCCCGTTCTATTGGTAT	GCATAAAGAGCTTCTCCTCC	8
	500	ACTCTTCCCGAATCTGCAAT	TCTCCAATTCCTTGTCCTATTGT	10
	600	AGTCATGCAATCCTAACGAGA	AGGAGCCTACTATGTACCTCT	10
Multiplex 2	150	TTTTCATTGGGAAGAAGAATTTAGT	TCCAATTCATAAATATCTCTTGGTGA	12
	250	TCTTTTAAAGAAGACAGCTGTAGT	TTTTGCAACACCTAATCTTGC	18
	350	TGATACAGGAATTATAAGAAGTGTTCC	GCATGAACTTCAACTCCAGG	5
	450	TGGAAATGACAGAGGATGGT	AGTAACGGATGAGCTTTTAAATTT	8
	600	TTGGGCTATTATGTCCCCAG	TCGTACAACTGGCATAGCTT	7
Multiplex 3	200	CCTCAACTTCTAACAGCAGG	CTCACATCACCCAATCCACT	8
	300	TGGAATATCATAAACCAAAAATTGTTT	TTCATTGCAAATCCGCCTTT	10
	450	ACAGCATCCTTGATTCTAGCA	GTGTAATCATAGCCCAAATCCA	12
	550	TGAAATAATGAATGAACACAATAGCA	GTGCACAACCTAAAACCTCA	10
	700	GACAGGAACAGAGGGAAGTC	AGCATCTTTATTTGTCGCACT	10
Multiplex 4	200	TGATGAAACACTAGAAAATAAGGCT	CCAGTAAAACCTCTGTCAGC	11
	350	TCACTTTTAGGTACTCACGACT	GCTATAAAACTTGCACCTTTATCG	9
	450	CAAAGATTTCTACGGGAAATTTGT	ACATCCTTTGCCTCTTTAAAAGA	9
	550	TCGAGGACAAGCAGATTCAA	GCCATTTCTACTTCCATTGTGT	7
	700	ACAGCAGTAACATTACAGGG	TCAAAAGCAATTCCACCACT	11
Multiplex 5	150	TCTATAGGTGCTGACCCACT	GCCGCAATACTTCCAAAACT	9
	250	TTTACAGGAGCTTGGACATCA	TTTTACCATCATCTTCAACCCA	9
	400	CATCGTCCTTCAGTCGAATAT	GGAAACCATTTTCTTTTGCCA	9
	550	GTCATTTTTACACCACCTGCA	TCAAAACGCTTAGCCAAATCT	12
	700	ACTTTTTGCTTCTCAAAGTAGAAC	CCTCTGAAAAATTGAAATAATATACCC	10
Multiplex 6	150	GGTTGGGGAAAACTGCTTTT	TCTCTTGATTTTTAGTTTCAATCTCT	10
	250	TGCTATGGGTGCAATGGTTA	AAGATTCTAGCAACACCCGA	8
	400	TGGGGACATGAAAACTGGAA	TTCACATACTTTCTCAGGCATT	10
	550	ACTATGGCTATATATGCGAAGAAA	TCCATAAATGTTTCAACTCAGGA	10
	650	GGAATTGCCGAGTTTACACG	TGAGCTCCATGTTGTATTGGA	10
Multiplex 7	200	ACTCCATTTGTGCTTATTGGA	TCTTGAACTAGCCAAAAGTGC	10
	350	TCGAAATATCTTTTAGCTTCAAGAA	AAAACATCATTTTCTTTTGCCCA	10
	450	AGAGTTTGGATGGAAAACTGT	TGCAACTATTCCATCAAAACCA	10
	550	GGTTCAACACCAGGAACAAA	TGCAACACCTATCATCTCATTT	10
	700	GGAAAAGGCAAAGAATCCTCA	ACCATCGCCAGACTTCATTA	10
Multiplex 8	150	TGCAAGAAATGGTGGAACAA	CCTGTTGCAATAGTTGGTGT	10
	250	TGGTAGAAGAAACAATAAAAAGATTTG	AGTCTTGATTTATCGACAGTTCT	10
	350	TTTTGTTTGAAGCTTATTCGTGA	AGTCCATATCCTTTCTCTCTCA	8
	450	AGGAGCTGTTGAGATTTTCAA	GTCGTTGCTCATCTGCTTTT	7
	550	GATGCTGGATTTTGTATGGCT	AGCCAAGAAACTTTCAATATCTCT	10

Table 3.1. Primers for PCR amplification of CGF$_{40}$ markers[a].

[a] Primer pairs were selected and grouped into multiplexes using Primer3 (212), multiPLX (213), and CGF Multiplexer (207).
[b] Multiplex primer pair concentrations were optimised for T$_a$=60°C.

between assays. The discriminatory power of each CGF assay was calculated using Simpson's ID (214), and the concordance was calculated as the AWC value between the CGF_{40} assay and the reference phylogeny. A "tanglegram" was generated using a custom R script to compare dendrograms for CGF_{40} and the reference phylogeny. This script is available online at https://gist.github.com/peterk87/d92f81ae475063792f49. Briefly, the script generates the dendrograms from binary CGF_{40} and reference phylogeny data and rearranges the CGF_{40} dendrogram with respect to the reference phylogeny in order to maximize structural concordance or minimize entanglement of branches using the "untangle_step_rotate_1side" function from the R package *dendextend* (https://github.com/talgalili/dendextend). It then uses the reference phylogeny to create color-coded linkage groups at a 90% cluster similarity level and plots the color-coded tanglegram.

3.4. RESULTS

3.4.1. Whole genome sequence assembly and comparison. Illumina 100 bp read paired-end sequencing of *A. butzleri* isolates (*n*=8) produced an average of 132 ± 37.0 times coverage based on an assembly size of 2.27 Mbp ± 0.09, with a GC content of 27.3% ± 0.90 and 2.10 ± 1.70 ambiguous bases per 100 kbp. The *de novo* assemblies contained 444 ± 146 contigs and 2.28 x 10^3 ± 129 predicted ORFs. In total, 2.47 x 10^4 coding sequences were identified from the assembled contigs, and 1.42 x 10^3 core and 1.63 x 10^3 unique accessory genes were identified by comparative genomic analysis of the eleven strains included in this study. After removing genes with biased population distribution, those with redundant patterns of presence and absence, or those presenting problems for subsequent PCR primer design, a set of eighty-three candidate accessory genes was identified and used to design an expanded CGF assay aimed at examining the population structure of a large set of *A. butzleri* isolates (n=156) based on shared accessory genome content. Data from eleven accessory genes was discarded due to discordance between

in silico-predicted CGF profiles and laboratory results on eight isolates sequenced *de novo* as part of this project. The reference CGF-based phylogeny was established from the remaining seventy-two accessory genes.

3.4.2. A 'reference phylogeny' for a sample population of *A. butzleri* isolates. A reference phylogeny for a comprehensive set of *A. butzleri* isolates (n=156) recovered from river water, raw and treated sewage, diarrheic and non-diarrheic people, and non-human animals was derived from the binary (i.e. presence and absence) data for the expanded CGF assay. The phylogenetic distribution of twelve genome-sequenced strains, which includes four previously sequenced strains and eight strains sequenced as part of this study, shows that all but two sequence type while the remaining strains are from diverse sequence types. An average of ten distinct alleles was observed at each of the seven MLST loci, and the lack of shared alleles suggests significant genetic diversity among the twelve WGS strains. Although this dataset does not represent a comprehensive sampling of the *A. butzleri* population, a comparative genomic analysis of these isolates would be expected to capture significant accessory genome diversity. The reference phylogeny contained a total of 31 multi-isolate clades when a ≥90% isolate similarity threshold was applied (Figure 3.2). The largest clade (Clade 5) comprised 12 isolates strains (149 and 151) belong to distinct CGF clades. Moreover, the *in silico* MLST data (Table 3.2) is consistent with the CGF results since strains 149 and 151 share the same, albeit novel, from four human diarrheic stool samples. Clade 31 contained all of the isolates recovered from two non-diarrheic human stools. Isolates from non-human animals clustered together and distinctly from other isolates. Although human isolates clustered with water isolates (clades 2 and 31, respectively), there were no clades that contained isolates from both diarrheic and non-diarrheic human beings. None of the four previously sequenced strains included in this dataset clustered at the 90% similarity level with the *A. butzleri* isolates from SWA.
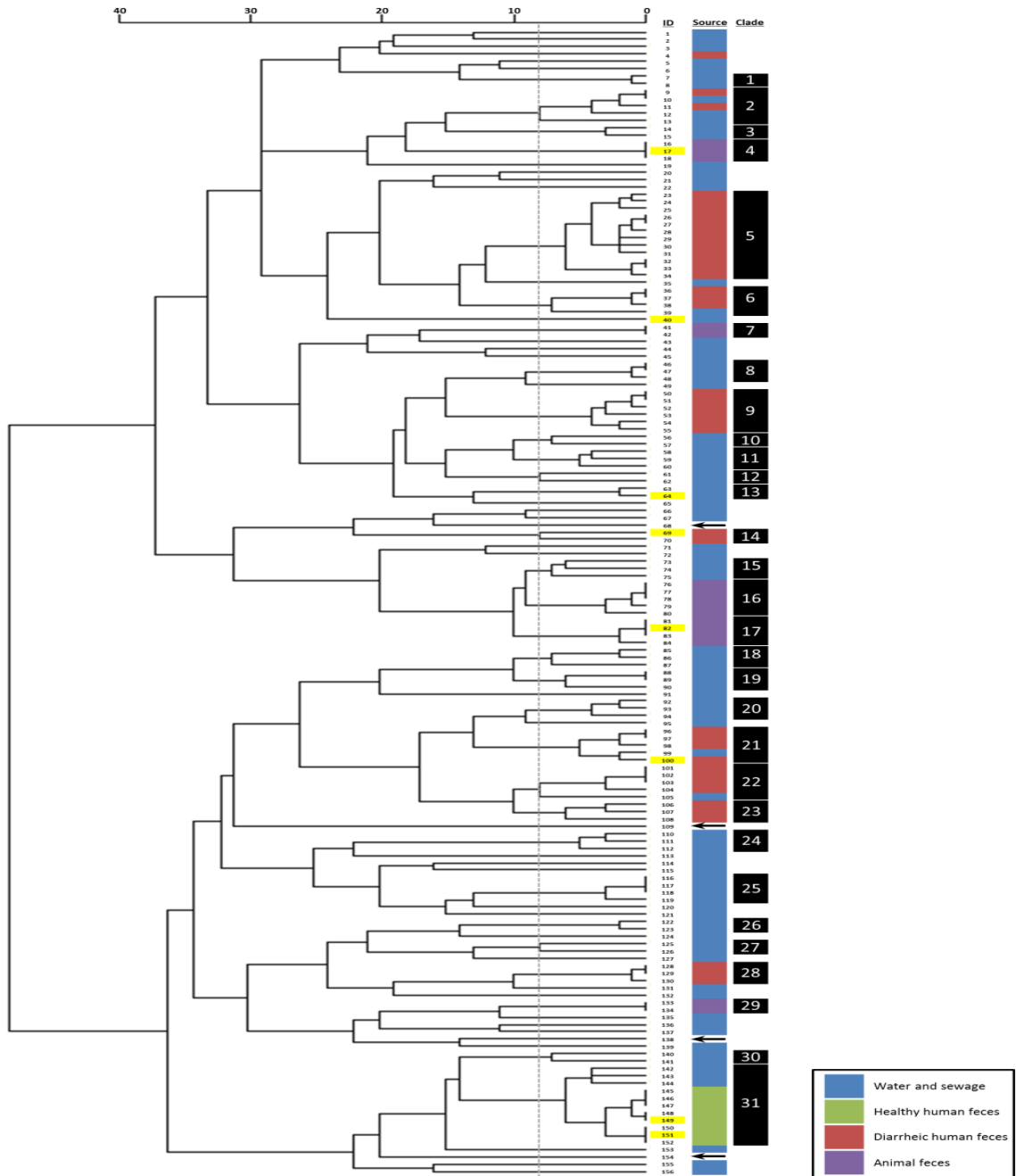
Figure 3.2. Reference genealogy of *A. butzleri* isolates (*n*=156). Clusters were calculated by simple matching comparison of 72 accessory genes using pairwise coefficients and UPGMA analysis. The scale represents fingerprint similarity based on the total number of shared loci between isolate profiles and the total number of loci in the assay. Dashed grey line represents a 90% similarity threshold used for clade definition. Isolates sequenced as part of this study are highlighted in yellow; ID 17 (strain L353, PRJNA233527), ID 40 (strain L355, PRJNA233527), ID 64 (strain L348, PRJNA233527), ID 69 (strain L352, PRJNA233527), ID 82 (strain L354, PRJNA233527), ID 100 (strain L349, PRJNA233527), ID 149 (strain L351, PRJNA233527), ID 151 (strain L350, PRJNA233527). Published reference *A. butzleri* strains are designated with arrows and include ID 68 (strain 7h1h, PRJNA200766), ID 109 (strain JV22, PRJNA61483), ID 138 (strain RM4018, PRJNA58557), ID 154 (strain ED-1, PRJNA158699).

Table 3.2. Identification of *A. butzleri* isolates by Reference CGF and MLST typing.

Isolate ID [a]	CGF Clade [b]	MLST Subtype [c]	AspA	AtpA	GlnA	GltA	GlyA	Pgm	Tkt
17	4	New ST; 4/7 matches with ST387	23	7	11	11	221	87	178
40	n/a	New ST; 2/7 matches with ST27	15	66	124	37	178	2	6
64	13	New ST; 4/7 matches with ST87	23	7	34	19	176	76	51
68	n/a	New ST; 6/7 matches with ST303/ST347	150	4	1	122	220	194	52
69	14	New ST; 3/7 matches with ST177	4	133	1	15	346	102	6
82	17	Existing ST18	4	4	4	4	139	4	89
100	21	New ST; 2/7 matches with ST62	14	45	128	55	47	17	50
109	n/a	New ST; 5/7 matches with ST12	3	17	16	20	new	231	7
138	n/a	Existing ST1	1	1	1	1	1	1	1
149	31	New ST; 4/7 matches with ST170	209	15	15	48	169	74	86
151	31	New ST; 4/7 matches with ST170	209	15	15	48	169	74	86
154	n/a	New ST; 5/6 matches with ST142	55	37	32	40	71	57	32

[a] Only those CGF isolates for which MLST data was available (via *in silico* subtyping) are shown.
[b] Reference CGF clades were defined using a 90% similarity threshold, which corresponds to ~ 7 mismatches, and only those clades corresponding to multiple isolates were provided clade numbers.
[c] Sequence Type (ST) information was unavailable for all but one strain (82), and the remaining strains represent novel sequence types; in each case, the closest ST is described under "Comments".

3.4.3. Analysis of CGF_{40} concordance with reference phylogeny. After 1.0×10^4 iterations, CGFO (207) retrieved 40 accessory genes for CGF_{40} that had an AWC of 1.0 with respect to the reference phylogeny. Analysis of the 156 *A. butzleri* isolates yielded high Simpson's ID (Table 3.3) and AWC (Table 3.4) values for both assays at 90% and 95% similarity thresholds. In addition, direct comparison showed that clusters in the reference and CGF_{40} phylogenies were highly concordant (Figure 3.3). At 90% similarity, isolates from 29 of the 31 clades identified in the reference phylogeny also clustered together when analysed using the CGF_{40} assay. Moreover, of the 54 isolates that shared identical CGF_{40} profiles, 45 also shared identical profiles when

Table 3.3. Simpson's ID [a] for *A. butzleri* isolates (*n*=152) genotyped by CGF$_{40}$.

Partitioning Method	Assay	Partitions [b]	Simpson's ID	CI (95%)	CINA (95%)
Binary Pairwise Similarity (UPGMA)	Reference	87	0.984	0.978-0.991	0.977-0.992
	CGF$_{40}$	86	0.987	0.983-0.992	0.982-0.992

[a] Simpson's ID, confidence interval (CI), and non-approximated confidence interval (CINA) were calculated using the online tool of the Comparing Partitions Website (http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Tool).
[b] Partitions were denoted at the 95% similarity level, which was calculated using the simple matching coefficient in BioNumerics (version 6.6, Applied Maths, Austin, TX).

analysed with the expanded set of 72 markers.

3.4.4. Analysis of CGF$_{40}$ reproducibility. To assess assay reproducibility, the CGF$_{40}$ analysis was repeated for 24 *A. butzleri* isolates on separate occasions. Concordance analysis revealed that 907 of the 920 data points assessed (98.6%) had identical presence/absence patterns in both runs.

3.5. DISCUSSION

Enteritis is inflammation of the alimentary canal (i.e. enteron) that is often characterized by diarrhea, abdominal pain, dehydration, loss of appetite, fever and nausea (215). Southwestern Alberta was selected for the study because this region possesses high rates of enteritis (144), which has been attributed to dense livestock populations in the region (149, 216, 217). *Arcobacter butzleri* is closely related to *C. jejuni* and it is considered to be an emerging pathogen by some (17, 74, 190) because it has been isolated from diarrheic people (111, 156). However, its pathogenicity and reservoirs/pathways of transmission for potentially pathogenic genotypes have yet to be elucidated. In order to understand the relationship between *A. butzleri* and human illness a method is required for the rapid and accurate genotyping of *A. butzleri* strains to facilitate epidemiological studies.

A number of subtyping methods have recently been used to examine genetic diversity of *Arcobacter* and to compare genotypes between sources. Douidah *et al*. proposed a two-stage

Table 3.4. Adjusted Wallace Coefficient values [a] of CGF_{40} compared to the reference phylogeny for *A. butzleri* isolates (*n*=152).

Partitions [b]	Reference (90% Similarity)	Reference (95% Similarity)
CGF_{40} (90% Similarity)	0.88 (0.83-0.93)	0.62 (0.53-0.71)
CGF_{40} (95% Similarity)	0.92 (0.89-0.95)	0.87 (0.83-0.91)

[a] Adjusted Wallace Coefficient values were calculated using the online tool of the Comparing Partitions Website (http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Tool).
[b] Partitions were denoted by 90% accessory gene pairwise similarity, which were calculated using the binary simple matching algorithm in BioNumerics (version 6.6, Applied Maths).

approach using Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) and Pulsed Field Gel Electrophoresis (PFGE) for subtyping of human and animal *Arcobacter* isolates (17, 138, 218). For *A. butzleri* a scheme for MLST, a leading method for related organisms such as *C. jejuni* and *H. pylori*, has recently been developed (17, 138, 218). No *A. butzleri* sequence types have been directly linked to human illness, but given the relative paucity of data both in the literature and within the global MLST database (219) it is difficult to assess whether the *A. butzleri* MLST data generated so far is representative of large-scale population or epidemiological trends. Moreover, despite the demonstrated ability of MLST to accurately distinguish subtypes of *A. butzleri* and other bacteria, the resources required to generate MLST data for the substantial numbers of *A. butzleri* isolates that are necessary for comparative epidemiological investigations may be prohibitive for many research groups. Comparative genomic fingerprinting provides a high-resolution and high-throughput alternative to MLST that is also deployable in the context of large-scale epidemiological surveillance (141, 142). The CGF method identifies intraspecies relationships by targeting accessory loci that are representative of genetic variation throughout the genome. The phylogenetic signal in accessory genome content variation has been examined in several bacterial species and shown to be highly

CGF72 ID Source

CGF40

% CGF$_{72}$ Similarity

50 60 70 80 90 100

Water and
sewage

Healthy
human feces

Diarrheic
human feces

Animal feces

% CGF$_{40}$ Similarity

100 90 80 70 60 50
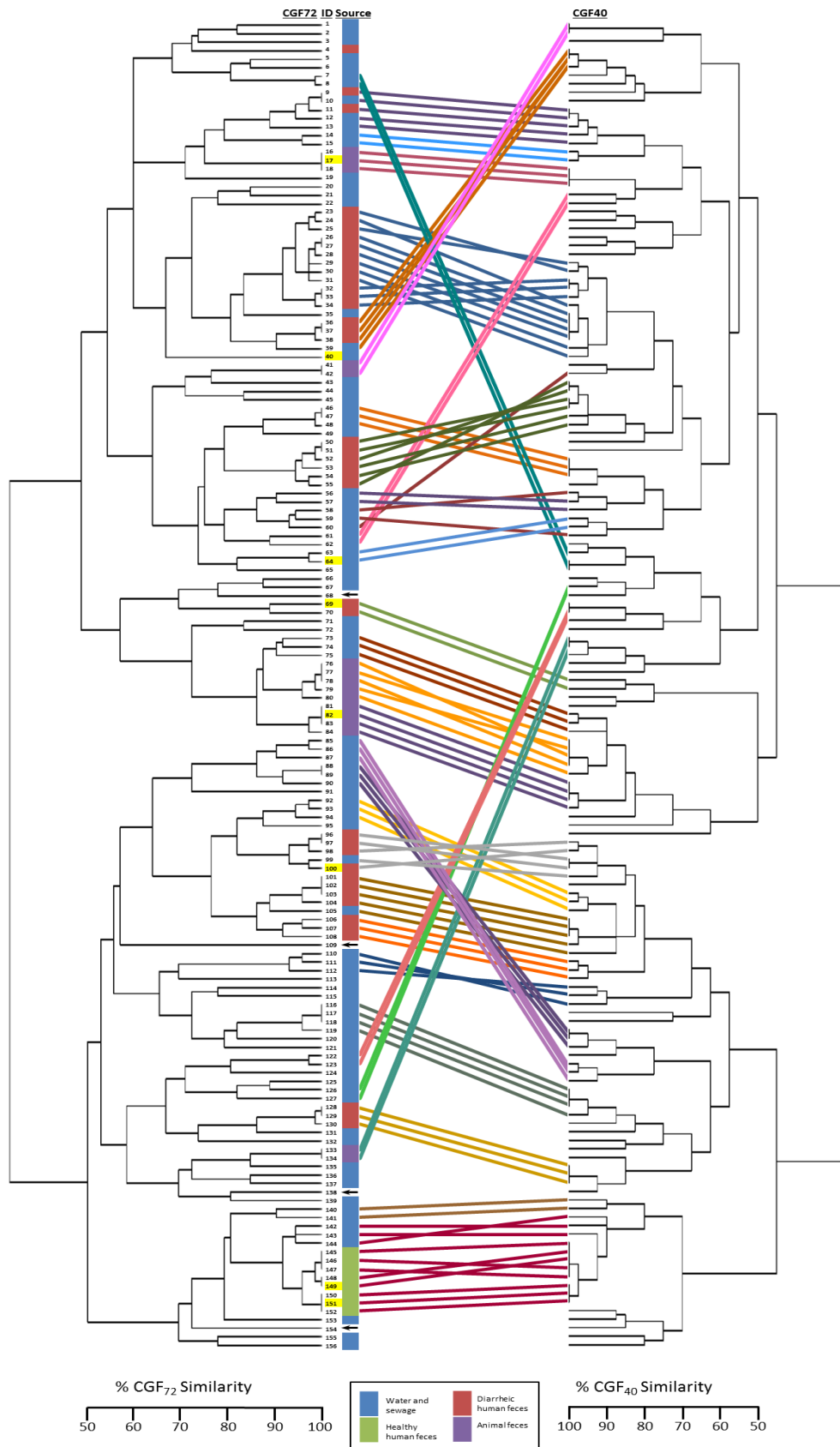
Figure 3.3. Tanglegram of Reference CGF and CGF$_{40}$ genealogies for *A. butzleri* isolates (*n*=156). Coloured lines represent isolates within clusters in the reference cladogram that are ≥90% similar to one or more other isolates. Scales represent fingerprint similarity based on the total number of shared loci between isolate profiles and the total number of loci in the assay. Coloured lines also indicate the location of the same isolate in the CGF$_{72}$ and the CGF$_{40}$ cladograms. Scales represent fingerprint similarity based on the total number of shared loci between isolate profiles and the total number of loci in the assay. Isolates sequenced as part of this study are highlighted in yellow; ID 17 (strain L353, PRJNA233527), ID 40 (strain L355, PRJNA233527), ID 64 (strain L348, PRJNA233527), ID 69 (strain L352, PRJNA233527), ID 82 (strain L354, PRJNA233527), ID 100 (strain L349, PRJNA233527), ID 149 (strain L351, PRJNA233527), ID 151 (strain L350, PRJNA233527). Published reference *A. butzleri* strains are designated with arrows and include ID 68 (strain 7h1h, PRJNA200766), ID 109 (strain JV22, PRJNA61483), ID 138 (strain RM4018, PRJNA58557), ID 154 (strain ED-1, PRJNA158699).

concordant with that contained in other forms of genetic variation (for examples, see (140, 220, 221)). Such loci are binary (i.e. present or absent) and determination of their allelic status does not require sequencing, with assessment possible by PCR amplification. In addition, CGF assays target sufficient loci to distinguish between closely related strains that may be indistinguishable by other methods (141) while generating phylogenetic signal that is consistent with that of MLST (222). Previous work has shown the CGF assay for *C. jejuni* to be highly predictive of MLST, and although each method clustered strains similarly, CGF provided additional discrimination within those groups (141, 142). In Canada, the CGF method is being used to analyse *C. jejuni* isolates generated through several large-scale surveillance networks, which will facilitate the study of campylobacteriosis through the holistic comparison of *C. jejuni* subtypes collected from a diverse range of sources and infection cases (223). In addition to being a close phylogenetic relative of *C. jejuni*, two features of the *A. butzleri* pan-genome identified through my comparative genomic analysis suggested that it would be an excellent species for the development of a CGF-based genotyping assay. The *A. butzleri* strains showed significant variability in accessory genome content, which facilitates a high level of discriminatory power among genotypes. The CGF$_{40}$ assay is based on a marker optimization process that yielded phylogenetic clusters that were highly concordant those observed in the reference phylogeny

and it provided a high discriminatory power for differentiation of isolates from diverse sources.

In addition, the majority of isolates that were identical by CGF_{40} analysis also proved to be

identical or highly similar using the larger number of markers. This suggests that the finalized set

of 40 loci were appropriate for high resolution genotyping of *A. butzleri* strains, and that there

may be an "efficiency plateau" above which additional loci do not sufficiently increase

discriminatory power to justify their inclusion in the assay. The CGF_{40} assay was found to be

easily deployable; 32 isolates could be processed (i.e. from stock to digital phylogeny) in a

typical workday by a single individual using one thermal cycler and capillary electrophoresis

system.

Previous efforts to characterize *A. butzleri* have identified a high degree of genetic

variation but have failed to associate specific genotypes in a geographic or temporal context (73,

100, 128, 138). In the current study, 29 *A. butzleri* clades were identified within the CGF_{40}

phylogeny when compared at a similarity threshold of 90% or greater and 121 distinct (i.e. non-

identical) CGF_{40} profiles were observed among the 156 isolates analysed. Of interest, each of the

four previously genome-sequenced strains in the public databases formed their own clades in

both CGF-based phylogenies. Taken together, these results suggest that the density of marker

sampling targeted by the CGF assay described herein provides sufficient power for discriminating

isolates at a high level of resolution. At the same time, my observation that 115 of the 156

isolates in this dataset could be assigned to clades with a profile similarity of 90% or greater

suggests that this level of discriminatory power does not compromise the ability to identify

clades comprised of genetically similar isolates. It is noteworthy that although the CGF_{40} assay

was developed using isolates primarily obtained from SWA, the dataset used for the

comparative genomic analysis to identify potential CGF markers also included several genome-

sequenced isolates from international sources. Moreover, it is my intention to further validate

the CGF$_{40}$ assay by examining *A. butzleri* populations in a pan-Canadian and an international context.

Arcobacter butzleri were isolated from the stools of diarrheic and non-diarrheic human beings living in SWA, as well as from river and sewage samples throughout SWA during 2008 and 2009. The clustering of isolates from human beings with isolates from river and sewage waters throughout SWA over the same time period suggests that it may be possible for *A. butzleri* strains to be transferred between people and their environment. Although it was not possible to identify clades that included isolates from human beings and non-human animals in the current study, this may be due a lack of overlap between sampling periods for human and non-human animals. Further research may identify a linkage between *A. butzleri* found in human beings and non-human animals through concurrent and comprehensive sampling; the rapid and inexpensive characterization of isolates using the developed CGF$_{40}$ method will be very useful in this regard.

To my knowledge no studies conducted to date have examined the carriage and shedding of *A. butzleri* strains in diarrheic and non-diarrheic human beings, and although *Arcobacter* species have been detected in and occasionally isolated from the stools of non-diarrheic individuals (71, 156), this is the first time that *A. butzleri* has been isolated from stools of a non-diarrheic person sampled on two separate occasions. Individuals were sampled six months apart and periodic shedding of the same *A. butzleri* genotype suggests that strains of this bacterium may chronically colonise people without inciting disease. Colonization of healthy human beings by *A. butzleri* may occur in a similar manner to the closely related pathogen *C. jejuni*, which has been shown to colonise healthy people more frequently in areas with endemic rates of infection (224-226). Thus, it may be possible to relate genotypes to endemic disease rates by characterizing *A. butzleri* isolates from diarrheic and non-diarrheic human beings.

3.6. CONCLUSIONS

Whole genome sequencing and comparative genomic analysis of *A. butzleri* isolated from diverse sources and demonstrated that accessory gene variation among strains can be used for high-throughput, high-resolution, and reproducible subtyping of this bacterium. Although WGS analysis will eventually become the gold standard in epidemiological genotyping of pathogenic bacteria, until WGS data are routinely deployed for surveillance of highly prevalent pathogens, the CGF_{40} assay described herein will allow the scientific community to address key knowledge gaps about the epidemiology of arcobacteriosis toward the prevention and mitigation of enteric disease. Furthermore, the CGF_{40} assay developed is highly deployable and will allow researchers and clinicians to efficiently compare the genetic diversity, persistence, and prevalence of *A. butzleri* subtypes in different sources, and to rapidly and efficiently identify relevant strains as candidates for WGS analysis.

CHAPTER FOUR

Efficacy of wastewater treatment on *Arcobacter butzleri* density and strain diversity[3]

4.1. ABSTRACT

Arcobacter butzleri is a suspected waterborne enteric pathogen that is ubiquitous in the environment, but the degree to which wastewater treatment prevents *A. butzleri* entry into environmental waters and the risks posed are not well established. Untreated and treated wastewater samples (n=260) were collected weekly from the Lethbridge and Fort Macleod wastewater treatment facilities in SWA, Canada from May 2008 to April 2009. Human diarrheic stools (n=2709) from the CRH, which services communities in SWA, were processed daily. *Arcobacter butzleri* was isolated from stools and wastewater, and isolates were genotyped using a novel comparative genomic fingerprinting method. Densities of the bacterium were determined by quantitative PCR. High densities of *A. butzleri* were detected in untreated wastewaters at both Lethbridge and Fort Macleod, locations that use different wastewater treatment processes. At both locations, biological and mechanical wastewater treatment significantly decreased but did not eliminate the number of viable *A. butzleri* and fecal coliforms in effluent, and tertiary ultraviolet B (UVB) irradiation reduced numbers further. Overall genetic diversity of *A. butzleri* was greater in Lethbridge wastewater, but survival during treatment was not strain-dependent. *Arcobacter butzleri* isolated from diarrheic humans shared common subtypes and were most closely related to treated wastewater effluent. The current study demonstrates that wastewater treatment processes differentially affect *A. butzleri* viability, and viable cells enter environmental waters via wastewater effluent discharge and pose a risk of enteric disease in human beings.

4.2. INTRODUCTION

Arcobacter butzleri is the fourth most commonly detected *Campylobacter* species or *Campylobacter*-like organism in human beings with enteric disease (10), but its mechanisms of transmission have yet to be determined. The presence of *A. butzleri* in drinking water has been linked to multiple enteric disease outbreaks (38, 39), and this bacterium possesses many genetic traits characteristic of waterborne free-living pathogens (56). *Arcobacter butzleri* grows in aerobic, anoxic, and anaerobic environments (3, 56), at temperatures as low as 10 °C (46, 227), and in the presence of a wide range of antimicrobial agents (19, 20). *Arcobacter butzleri* has been detected in human stools and livestock waste, and the presence of *A. butzleri* in surface waters has been linked to fecal contamination (36, 37).

Recent studies indicate that *A. butzleri* in urban wastewaters survive treatment and are discharged into environmental waters (36, 37). WWTPs utilize a combination of mechanical (i.e. screens and sedimentation), biological (i.e. activated sludge and bioreactors), and enhanced (i.e. nutrient removal, chlorine, and UVB irradiation) processes (228, 229) to remove enteric pathogens prior to discharge of effluent into environmental waters. These methods limit the number of fecal coliforms that are released into environmental waters, but their effects on *A. butzleri* cell density, viability, and genetic diversity have not been documented. Considering that *A. butzleri* is a potential pathogen that displays greater survival capacity in water containing organic material, it is likely that environmental waters contaminated with *A. butzleri* serve as reservoirs of human infectious cells for this enteric pathogen.

The purpose of the current study was to determine the efficacy of standard wastewater treatments on the viability of *A. butzleri* at two WWTPs that discharge treated municipal wastewater into the Oldman River in SWA, Canada, and to compare the genotypes of *A. butzleri* in wastewater with those from diarrheic people over a 1-year period. I hypothesized that

wastewater treatment would reduce the number of viable *A. butzleri* cells entering the Oldman River as effluent, and genotypes surviving treatment also occur in people with diarrhea. Primary objectives were to: (i) utilize novel quantitative PCR to measure total and viable densities of *A. butzleri* in wastewaters at various stages of the treatment process; (ii) compare the density of *A. butzleri* in Lethbridge and Fort Macleod wastewater (the two major municipal inputs in SWA, that utilize different treatment processes); and (iii) comparatively examine the genetic diversity of *A. butzleri* shed in stools from diarrheic people with those in wastewaters.

4.3. MATERIALS AND METHODS

4.3.1. Ethics Statement. The University of Lethbridge Human Subject Research Committee approved the collection and analysis of stool samples from diarrheic human beings (Internal File 2012-015). In addition, the CHR Research Committee approved the transfer of diarrheic stool samples to Agriculture and Agri-Food Canada for the isolation of *Arcobacter*, *Campylobacter*, and *Helicobacter* species (Research Study Proposal 2012-02).

4.3.2. Diarrheic stool collection and isolation of *A. butzleri*. Human diarrheic fecal samples (n=2709) were obtained daily from the CRH, which services Lethbridge and surrounding communities in the former Chinook Region of SWA from May 2008 to April 2009. Stool samples from diarrheic people were suspended in Cary-Blair medium (165) for transportation to the CRH. Data collected with samples included the date of stool production and patient age, sex, and residence (i.e. postal area). *Arcobacter butzleri* was isolated in a microaerobic environment (i.e. 5% O_2, 3% H_2, 10% CO_2, and 82% N_2) using a combination of membrane filtration, direct plating, and enrichment at 30°C and 37°C (230). Two colonies per morphology per medium per sample were collected, streaked for purity on Columbia agar (DF0944-17-0; Difco) containing 10% sheep blood (CBA) in a microaerobic atmosphere, and examined microscopically for cell size, shape, and motility. Genomic DNA was extracted from isolated *A. butzleri* colonies using the DNeasy

blood and tissue kit (Qiagen Inc.) and an automated system (model 740; Autogen, Holliston, MA) according to the manufacturer's specifications.

4.3.3. Wastewater sample collection and processing. Untreated (n=104) and treated (n=156) wastewater samples were collected weekly from the Fort Macleod and Lethbridge WWTPs in SWA from May 2008 to April 2009. At the time of the study, the wastewater treatment process at the Fort Macleod WWTP consisted of a mechanical bar screen, grit removal, RBC activated sludge contact tank, secondary clarifier, solids removal to a digester and/or recirculated to front of contact tank, and treated effluent release to the Oldman River via a 3 km-long outfall line. At the Lethbridge WWTP, the wastewater treatment process consisted of a mechanical bar screen, grit removal, primary clarifiers, anaerobic, anoxic, and aerobic digesters, secondary clarifiers, removal of activated sludge, UVB irradiation of liquid effluent, and treated effluent release to the Oldman River via a 1 km-long outfall line. Untreated wastewater (i.e. raw sewage) was collected at both sites immediately after mechanical bar screening. Treated liquid effluent was collected at the end of the treatment process (i.e. immediately prior to effluent release into the Oldman River). At the Lethbridge WWTP, treated liquid effluent was collected immediately before and after UVB irradiation.

Samples were collected, maintained on ice, and processed within 6 hr of collection. A total of 100 ml of each sample was filtered through a 150 mm pre-filter (#1001-150, Whatman International Ltd., Maidstone England) and a GMF grade 0.2 μm filter (#1842-090, Whatman). Both filters were vortexed (high setting) in 10 ml of phosphate buffered saline (PBS) (pH 7.X) to release particulates from the filters. The filters were removed, and the suspension was centrifuged at 14 900 X g for 10 minutes. All but 3.0 ml of supernatant was removed by aspiration. The pellet was suspended by vortexing (high setting), and the suspension was used for DNA extraction, quantitation of fecal coliforms, and isolation of *A. butzleri*. For DNA

extraction, aliquots (200 µl) of the pellet were placed in four 2-ml tubes. Ethidium monoazide

(Invitrogen Canada Inc., Burlington, ON, Canada) was added to two tubes (4 µl; final

concentration of 100 µg ml^{-1}), and Optima water alone (4 µl) was added to the other two tubes

under low-light conditions (216). Tubes were placed in the dark for 5 min, lids were opened, and

all tubes were exposed to light emitted from a 500-W halogen light bulb for 1 min on ice; the

light source was situated 10 cm from the samples. An IAC was also added to each sample (230).

Samples were stored at -80°C. The remaining suspension was used to quantify fecal coliforms

and to isolate *A. butzleri*.

4.3.4. Fecal coliform enumeration. To enumerate fecal coliforms, 1.0 ml from each wastewater

suspension was diluted in a ten-fold dilution series in PBS, and 100 µl of each dilution was spread

on mFC Agar (Sigma-Aldrich) in duplicate. Cultures were incubated aerobically for 24 h at 45°C,

and blue colonies were enumerated at the dilution yielding 30-300 CFU per dish. The mean of

the two duplicate cultures was calculated.

4.3.5. Isolation of *A. butzleri* from wastewaters. *Arcobacter butzleri* isolates in wastewater were

recovered and DNA was extracted and identified as described for diarrheic stools.

4.3.6. Quantitative PCR. Extraction of total DNA from wastewater samples was performed using

the Powerlyzer Powersoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad CA) according to the

manufacturer's recommendations. Presence of extracted DNA was confirmed by quantitative

PCR with primers targeting the added IAC, and quantitative PCR with primers targeting a single-

copy gene sequence unique to *A. butzleri* was performed on all successful extractions (230).

Briefly, the IAC was a synthesized gene designed from a 268-bp sequence encoding a putative

carbohydrate kinase (PfkB family; GenBank accession number AEH23732.1) within the genome

of *Pyrococcus yayanosii*, a bacterium that is an obligate piezophilic hyperthermophilic archaeon

isolated from deep-sea hydrothermal sites. The IAC (2 µl at 1 x 10^6 copies µl^{-1}) was added to

concentrated wastewater samples before freezing. Primers to detect and quantify the IAC were

IAC-f (3'-GGTATGCTAGCCCCGCTTAGGGT-5') and IAC-r (3'-TGCTCCAGAAAAGATGTCCAGCGG-5').

The presence and quantities of the IAC were measured by quantitative PCR amplification using a

Stratagene Mx3005P qPCR System (Agilent Technologies, Santa Clara CA). Quantitative primers

for *A. butzleri* (ddAbutzF, 5'-AGTGATGGTGGAGTTGCTAGTC-3', and ddAbutzR, 5'-

GTTGCAGGAGCTTTTTCACTCC-3') were designed using comparative whole genome sequence

analysis; the primers targeted a single copy gene that was identified as part of a putative gene

encoding the gamma subunit of quinohemoprotein amine dehydrogenase of the bacterium

(NCBI reference sequence WP_004510536.1). Quantitative PCR detection of *A. butzleri* was

carried out using a Stratagene Mx3005P qPCR System (Agilent Technologies). At the end of

amplification, melt curve analysis was conducted. Samples were quantified in duplicate

reactions, and the quantitative PCR data were analysed using MxPro (Version 4.10, Agilent

Technologies Inc.)

4.3.7. Subtyping of *A. butzleri* isolates. *Arcobacter butzleri* isolates from wastewater and

diarrheic human stool samples were subtyped using a previously developed high-throughput

and high-resolution CGF method (81) . Briefly, a set of 40 accessory genes representative of

whole genome single nucleotide polymorphism phylogeny were identified via comparative

whole genome sequence analysis, primers were designed and validated, and multiplex end-point

PCR was completed with capillary electrophoresis to generate a 40-digit binary profile for each

isolate. One *A. butzleri* isolate per site per week was arbitrarily selected for CGF characterization.

Isolates were clustered at 95% fingerprint similarity (i.e. less than two locus mismatches) using

simple matching with UPGMA and minimum spanning trees in Bionumerics (version 6.6, Applied

Maths, Austin, TX). Similarity in *A. butzleri* populations between human diarrheic stools and

wastewaters was calculated as the number of shared subtypes multiplied by 2, divided by the

sum of the number of subtypes, multiplied by 100.

4.4. RESULTS AND DISCUSSION

4.4.1. Densities in untreated in wastewater. High densities of *A. butzleri* DNA were consistently

observed in untreated wastewater at the Lethbridge (Figure 4.1A) and Fort Macleod (Figure

4.1B) WWTP. In previous studies Collado et al. (37) detected *A. butzleri* in 100% of sewage

samples and in 96.3% of sludge. In addition, Stampi et al. (15) found *A. butzleri* to be viable at all

stages of solid waste treatment (i.e. primary clarification, activated sludge, thickened sludge, and

anaerobically-digested sludge). At the Lethbridge WWTP, approximately 29.5 ± 20.6% of total *A.*

butzleri cells detected in untreated wastewater were deemed viable by qPCR, and at the Fort

Macleod WWTP 14.4 ± 13.0% of total *A. butzleri* cells were viable. Culture-based quantification

of *A. butzleri* in complex matrices such as wastewater is not accurate because no method of

isolation is comprehensive for all strains of *A. butzleri* (73, 230). PCR methods have been shown

to provide increased detection of *A. butzleri* in complex matrices such as feces (72, 230) and

surface waters (62), but these methods target DNA that has been extracted from lysed cells

regardless of their viability. Thus, the current study utilized qPCR of untreated wastewater

samples to which ethidium monoazide (EMA) had been added prior to DNA extraction, because

EMA is effective for live/dead cell differentiation of *Campylobacter* (216), *Helicobacter* (231,

232), and Salmonella species (233). My results are likely an underestimation of cell viability

because EMA can penetrate cells possessing an intact cell membrane (i.e. viable cells) (234).

4.4.2. Wastewater treatment efficacy. Wastewater treatment greatly decreased densities of

viable *A. butzleri* in Lethbridge (Figure 4.2A) and Fort Macleod (Figure 4.2B) wastewaters. The

overall density of viable *A. butzleri* in treated wastewater at Lethbridge and Fort Macleod was

0.71 $\log_{10}$ ± 0.85 cells ml^{-1} (99.8% reduction) and 1.13 $\log_{10}$ ± 0.77 cells ml^{-1} (81.5% reduction),

respectively. Although qPCR did not show an appreciable reduction in densities of *A. butzleri* as a
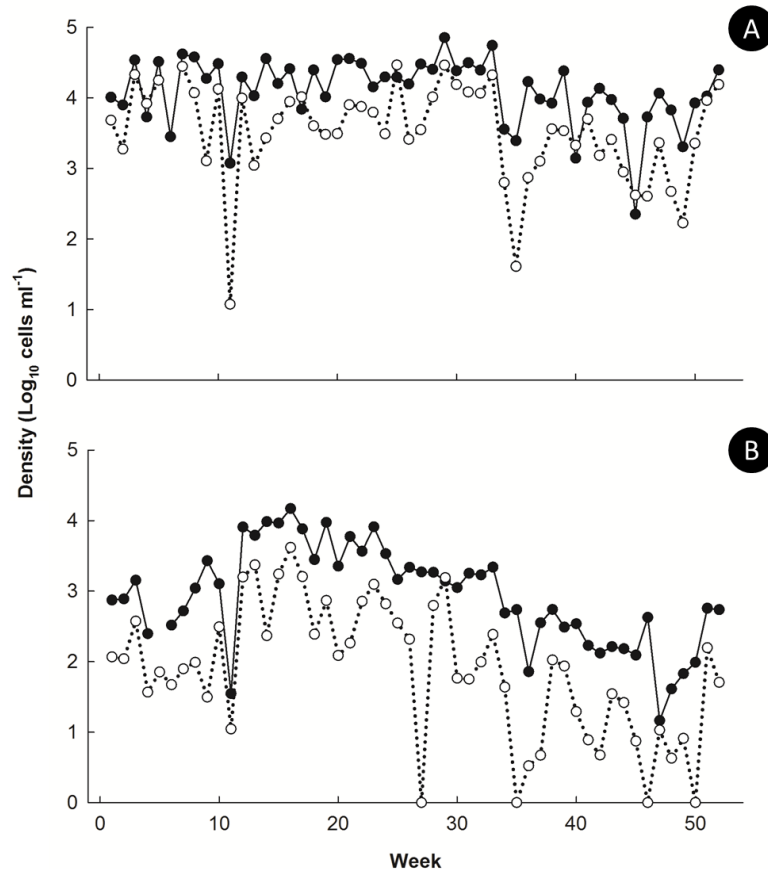
Figure 4.1. Detection of *A. butzleri* DNA by quantitative PCR in untreated wastewater at the Lethbridge WWTP (A) and the Fort Macleod WWTP (B) from May 2008 to April 2009. Solid lines represent total *A. butzleri* DNA and dotted lines represent viable *A. butzleri* DNA. Viable *A. butzleri* cell density was determined by quantitative PCR of samples to which EMA had been added prior to sample storage and subsequent DNA extraction. The quantitative PCR primers target a portion of the putative gene sequence encoding the gamma subunit of quinohemoprotein amine dehydrogenase (WP_004510536.1), for which *A. butzleri* possesses a single gene copy (81).

result of UVB irradiation (Figure 4.2C), the culture-based frequency of detection of *A. butzleri* in treated Lethbridge wastewater was significantly reduced (P<0.01) from 100% before UVB irradiation to 56% after UVB irradiation. This suggests that qPCR of samples treated with EMA may not be a reliable means of determining the effects of UVB irradiation on cell viability; likely because UV radiation disrupts the replicative ability of bacterial cells without compromising
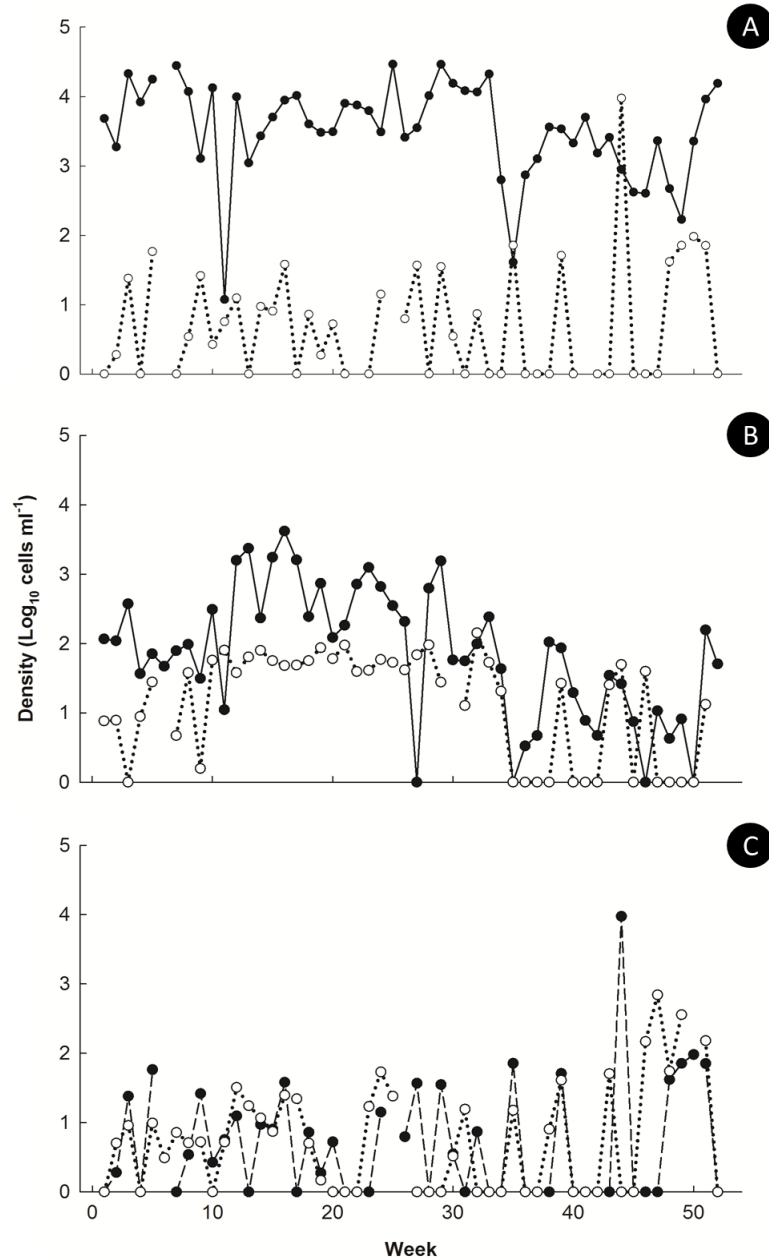
Figure 4.2. Comparison of viable *A. butzleri* DNA by quantitative PCR in untreated wastewater and treated wastewater without UVB irradiation at Lethbridge WWTP (A) and Fort Macleod WWTP (B), and in treated wastewater immediately prior to and after UVB irradiation at the Lethbridge WWTP (C) from May 2008 to April 2009. Solid lines represent untreated wastewater, dotted lines represent treated wastewater effluent prior to discharge into the Oldman River, and dashed lines represent treated wastewater immediately prior to UVB irradiation. The quantitative PCR primers target a portion of the putative gene sequence encoding the gamma subunit of quinohemoprotein amine dehydrogenase (WP_004510536.1), for which *A. butzleri* possesses a single gene copy (81).

membrane integrity (235, 236). This prevented accurate quantification of viable *A. butzleri*

discharged into the Oldman river at the Lethbridge WWTP, although the density of viable *A.*

butzleri in the Oldman River at the Fort Macleod WWTP effluent outfall site increased by 5.0

cells L^{-1}. The density of fecal coliforms in treated wastewater at the Lethbridge WWTP (Figure

4.3A) and the Fort Macleod WWTP (Figure 4.3B) was reduced. In addition, the density of fecal

coliforms in treated wastewater at the Lethbridge WWTP was further reduced by UVB

irradiation. The overall density of fecal coliform indicators in treated wastewater at the

Lethbridge and Fort Macleod WWTPs was 0.5 $\log_{10}$ ± 0.5 cells ml^{-1} (100% reduction) and 2.3 $\log_{10}$

± 0.5 cells ml^{-1} (97.9% reduction), respectively. This equates to an increase in fecal coliform

density in the Oldman River of 27.8 and 72.3 cells L^{-1} at the Lethbridge and Fort Macleod WWTP

effluent outfall sites, respectively. Previous studies demonstrated that wastewater treatment

reduces bacterial pathogens to acceptable densities prior to discharge (237), and that further

treatment with UVB irradiation leads to greater reduction in bacterial pathogen viability (238,

239). At the time of sampling for the current study, the Fort Macleod WWTP handled 1.5 million

liters of wastewater per day using RBC activated sludge removal, secondary clarification, and

biological treatment (i.e. aerobic digestion). At the same time, the Lethbridge WWTP handled

36.0 million liters of wastewater per day using a process comprised of primary clarification,

biological treatment (i.e. anaerobic, anoxic, and aerobic digestion), secondary clarification, and

UVB irradiation. Both the Fort Macleod and Lethbridge processes reduced fecal coliform

indicators to similar levels, although the Lethbridge WWTP handled greater initial densities of

fecal coliforms. In contrast, the Lethbridge wastewater treatment process decreased viable *A.*

butzleri densities to a lower level than the Fort Macleod process, despite greater initial *A.*

butzleri densities in untreated wastewaters at Lethbridge. To my knowledge, my study is the first

to provide a quantitative comparison of the viability and/or density of *A. butzleri* in
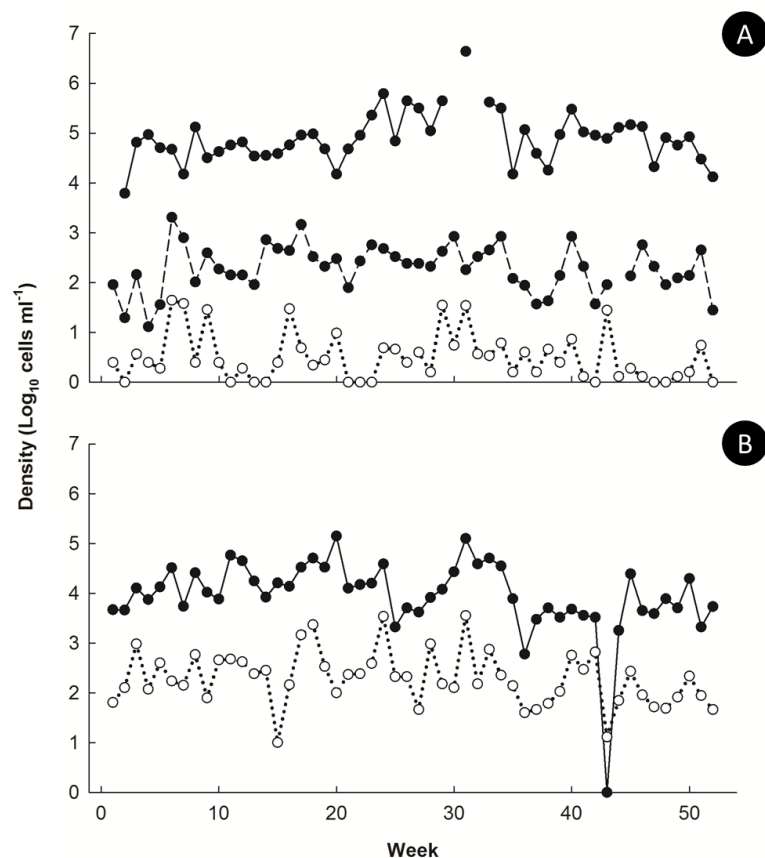
Figure 4.3. Detection of fecal coliform indicators in wastewater from the Lethbridge WWTC (A) and the Fort Macleod WWTP (B) from May 2008 to April 2009. Solid lines represent untreated wastewater, dotted lines represent treated wastewater effluent prior to discharge into the Oldman River, and dashed lines represent treated wastewater immediately prior to UVB irradiation. Fecal coliform indicators were enumerated by spreading 100 µl of ten-fold dilutions of processed wastewater samples on mFC Agar (Sigma-Adrich). Cultures were incubated aerobically for 24 h at 45°C, and blue colonies were enumerated at the dilution yielding 30-300 colony forming units (CFU) per dish.

wastewater by treatment. My results indicate that the effectiveness of wastewater treatment on

viability of *A. butzleri* varies based on the type of process employed (e.g. anaerobic, anoxic, and

aerobic digestion versus aerobic digestion alone), while traditional fecal coliform indicators are

greatly reduced regardless of the specific process. Wery et al. (240) found that the enteric

pathogens *Salmonella spp.* and *C. jejuni* also tended to survive better than fecal indicators during

wastewater treatment, and previous studies showed that *A. butzleri* remains viable during sludge composting (15) and in surface waters contaminated by effluent discharge (36, 37). The high densities of *A. butzleri* that were present in untreated effluent entering WWTPs at the Lethbridge and Fort Macleod WWTPs suggests that this bacterium may be a suitable alternative pathogen indicator in treated solid and liquid waste.

4.4.3. Comparative genomic analysis. Comparative genomic fingerprinting (81) was performed on 688 *A. butzleri* isolates, and 342 subtypes were identified (Figure 4.4). There was no change in *A. butzleri* genetic diversity during treatment at either wastewater facility, but overall genetic diversity was greater at Lethbridge compared to Fort Macleod (Table 4.1). This may be because wastewater input at Lethbridge is more varied than Fort Macleod; although both facilities handle human inputs, waste from pork, and chicken and cheese processing plants are present in Lethbridge (Doug Kaupp, City of Lethbridge, personal communication) but not in Fort Macleod (Dan Segboer, Town of Fort Macleod, personal communication). These additional inputs likely increase the genetic diversity of *A. butzleri* in Lethbridge wastewater because animal holding and processing facilities are suspected reservoirs for *A. butzleri* (50, 58). The lack of a decrease in diversity as a result of wastewater treatment indicates that resistance to deactivation by wastewater treatment is not strain-specific. Finally, *A. butzleri* isolated from untreated wastewaters at Lethbridge and Fort Macleod were most similar to their respective treated wastewaters, and *A. butzleri* from diarrheic samples were most closely related to effluent from both Lethbridge and Fort Macleod (Figure 4.5). These findings provide additional evidence that *A. butzleri* is present in municipal wastewater effluent as a result of surviving the treatment process, and not as a result of post-treatment contamination.

4.5. CONCLUSIONS

The association between *A. butzleri* and human illness is poorly defined. My findings
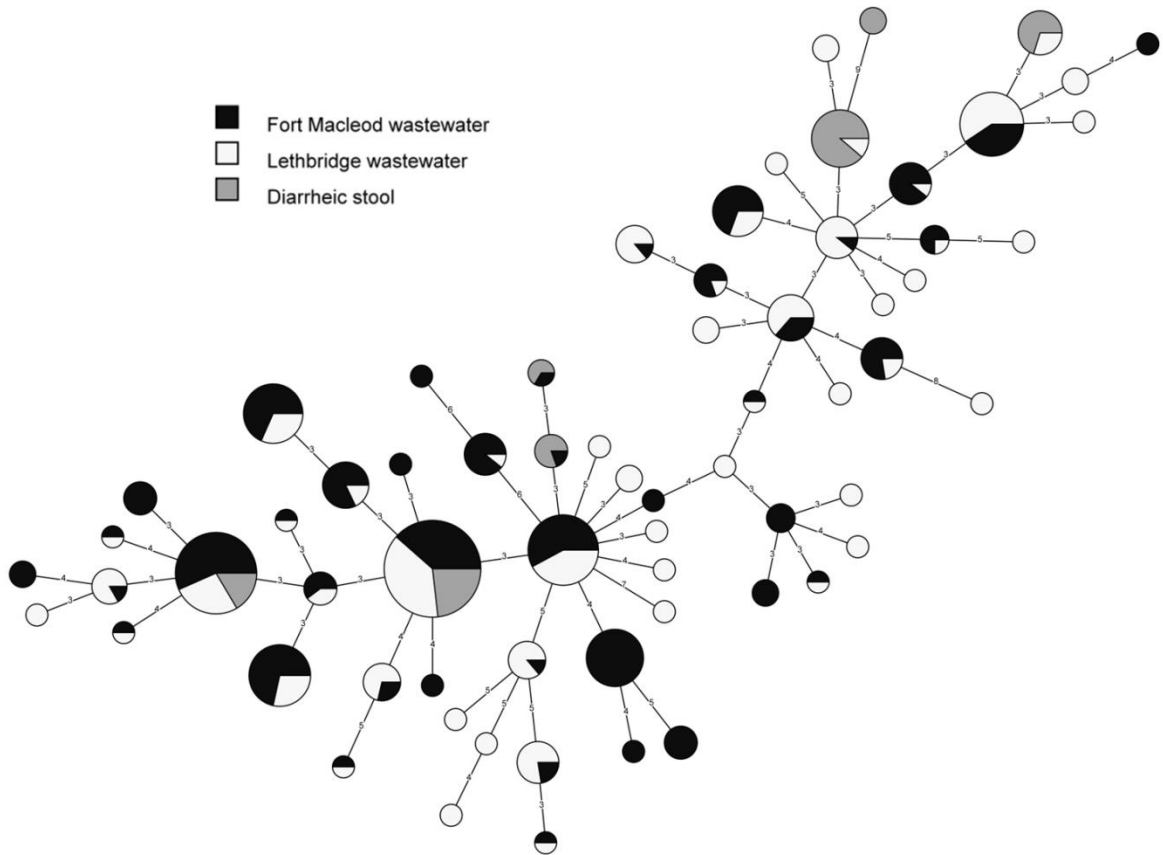
Figure 4.4. Cluster comparison of *A. butzleri* isolated wastewaters from the Fort Macleod and Lethbridge WWTPs, and human diarrheic stools. Clusters represent groups of *A. butzleri* isolates with at least 95% CGF similarity, and isolates that did not cluster are not shown. Lines represent CGF fingerprint mismatches between clusters, where each mismatch is equal to a 2.5% difference in subtype similarity. Minimum spanning tree analysis was conducted using Bionumerics (version 6.6, Applied Maths).

suggest that *A. butzleri* from diarrheic human beings are able to survive the wastewater treatment process, and their presence in environmental waters may pose a risk to human health. In addition, *A. butzleri* isolated from the stools of multiple diarrheic humans often shared the same subtype in time and space, which demonstrates the value of high-throughput genotyping methods such as CGF for identifying potentially pathogenic *A. butzleri* subtypes.

Table 4.1. Genetic diversity of *A. butzleri* in municipal WWTPs and diarrheic human beings.

Sample source	Isolates	Subtypes	Simpson's ID	CI (95%)	CINA (95%)
Lethbridge untreated	128	106	0.996	0.994-0.999	0.993-1.000
Lethbridge biological	158	118	0.995	0.992-0.997	0.991-0.998
Lethbridge effluent	58	49	0.993	0.987-0.999	0.984-1.000
Macleod untreated	162	93	0.984	0.978-0.991	0.977-0.991
Macleod effluent	127	72	0.985	0.979-0.991	0.979-0.992
Diarrheic stools	52	9	0.825	0.770-0.880	0.767-0.883
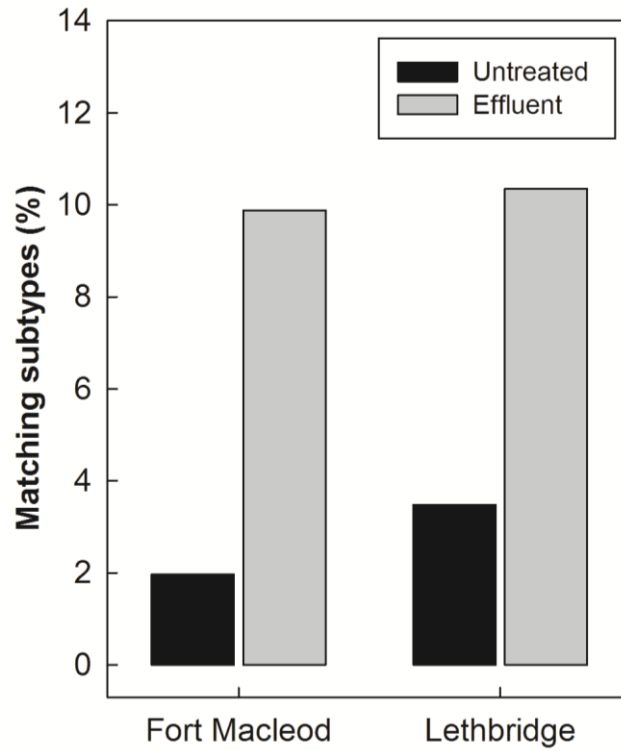
Figure 4.5. Genetic similarity of *A. butzleri* isolated from human diarrheic stools to isolates recovered in wastewater from Fort Macleod and Lethbridge WWTPs in SWA using CGF. Similarity between *A. butzleri* populations was calculated based on the proportion of shared subtypes.

CHAPTER FIVE

Prevalence and Diversity of Waterborne *Arcobacter butzleri* in Southwestern Alberta, Canada[4]

5.1. ABSTRACT

Arcobacter butzleri is a potential enteric pathogen to human beings, but its reservoirs and modes of transmission are largely unverified. Microbiological and molecular detection and subtyping techniques can facilitate surveillance of *A. butzleri* in environmental reservoirs and hosts. *Arcobacter butzleri* were isolated from surface waters (n=676) and treated wastewaters (n=104) in the Oldman River Basin over a 1 year period using eight culture-based techniques. The frequency of detection of *A. butzleri* in surface water was seasonal, peaking during summer months. In the Oldman River mainstem the frequency of detection of *A. butzleri* was greatest at sites directly downstream of outfall sites, and in its tributaries the greatest frequencies were in regions with high densities of confined feedlot operations. *Arcobacter butzleri* isolates (n=500) were subtyped using a CGF method recently developed by my group. *Arcobacter butzleri* isolated from wastewater effluent were most similar to those from the Oldman River directly downstream of the first outfall site (21.8%), while Oldman River tributary isolates were most similar to those from the Oldman River directly downstream of the second outfall site (15.6%). A total of 64 *A. butzleri* subtypes (25.6%) were isolated at more than one sampling period, suggesting that *A. butzleri* persists over time in environmental waters contaminated by fecal material. Evidence indicated that viable *A. butzleri* enters the Oldman River and its tributaries as wastewater from both human and non-human animal populations, which may pose a risk to human health.

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[4] A version of this chapter will be submitted for publication as: **Webb AL, Selinger LB, Boras VF, Taboada EN, Inglis GD.** 2016. Prevalence and diversity of waterborne *Arcobacter butzleri* in southwestern Alberta, Canada.

5.2. INTRODUCTION

Arcobacter butzleri is a Gram negative Epsilonproteobacterium that is considered an

emerging or potential enteric pathogen (17). This bacterium has been detected in diarrheic and

non-diarrheic human beings, farm animals, animal products, wildlife, wastewater, and surface

waters (36, 42, 55, 58, 71, 99). Infection by *A. butzleri* is thought to arise from the ingestion of

food or water that has been contaminated with fecal material (17), but the mechanisms through

which *A. butzleri* is transmitted from the environment to potential hosts are poorly understood,

largely because high-resolution and high-throughput subtyping methods to facilitate

epidemiological investigations of this bacterium have been lacking.

Molecular subtyping is routinely used to study the population structure of

Campylobacter jejuni (223), an enteric pathogen that is closely related to *A. butzleri*. It is now

recognized that individual strains of *C. jejuni* appear to be source-specific 'specialists' (e.g. to

humans or non-human animal), while other 'generalists' can be associated with a variety of

different hosts (241, 242). Comparison of *C. jejuni* strains isolated from host species and their

environment has facilitated the identification of shared transmission pathways between

reservoirs and host species (243). Given that *A. butzleri* and *C. jejuni* share many genetic

characteristics (56) their epidemiology may be similar, so a similar approach may facilitate the

study *A. butzleri* pathogenic genotypes, reservoirs of infectious strains, and modes of

transmission.

Previous studies that utilized MLST found that *A. butzleri* is genetically diverse (99, 138),

but evidence that *A. butzleri* subtypes are unique within or shared between sources (e.g.

humans, non-human animals, surface waters) is lacking. This is likely because the relatively high

cost of MLST hinders the examination of large numbers of isolates, which is often necessary to

resolve cluster patterns in relation to source. Alternate subtyping methods such as AFLP (100)

and PFGE (31) have been applied to *A. butzleri*, but they are labour-intensive and may not

discriminate between closely-related strains (81, 223, 244). The recently developed CGF method

for *A. butzleri* was used to rapidly type a large number of isolates at high resolution (81).

    Arcobacter butzleri has been detected in city wastewater (15, 36), and greater frequency

of *A. butzleri* has been linked to fecal contamination of surface waters (36, 37). In SWA, the

effluent of treated municipal wastewater (i.e. sewage) is discharged into the Oldman River

watershed (146). This region possesses high densities of livestock production and animal waste

that results from intensive agricultural activity enters the Oldman River and its tributaries as

contaminated irrigation water (146, 147, 150). Given the ability of *A. butzleri* to remain viable in

animal feces (48, 166), treated wastewater (15, 36), and surface waters (32, 37), it is likely that

waterborne transmission of this potential pathogen constitutes an enteric disease risk.

    The overall goal of the current study was to apply CGF to identify potential pathways of

waterborne transmission of *A. butzleri* in the Oldman River watershed in SWA, Canada. I

hypothesized that *A. butzleri* enters the Oldman River via its tributaries and city wastewater

effluent. Primary objectives were to: (i) utilize selective media and enhanced plating techniques

to isolate *A. butzleri* from treated effluent at the Fort Macleod and Lethbridge WWTPs (i.e. the

two major municipal inputs), and surface waters in SWA; (ii) conduct CGF profiling to

characterize and subtype a large number of *A. butzleri* isolates; and (iii) conduct comparative

examination of subtype frequency in wastewater and surface waters.

5.3. MATERIALS AND METHODS

5.3.1. Sample collection and processing. Surface water samples (n=676) were collected weekly

from 12 sites along the Oldman River and its tributaries in SWA from May 2008 to April 2009

(Figure 5.1). Treated wastewater effluent samples (n=104) were also collected weekly from the

Fort Macleod and Lethbridge WWTPs. It is noteworthy that at the time of sample collection, the
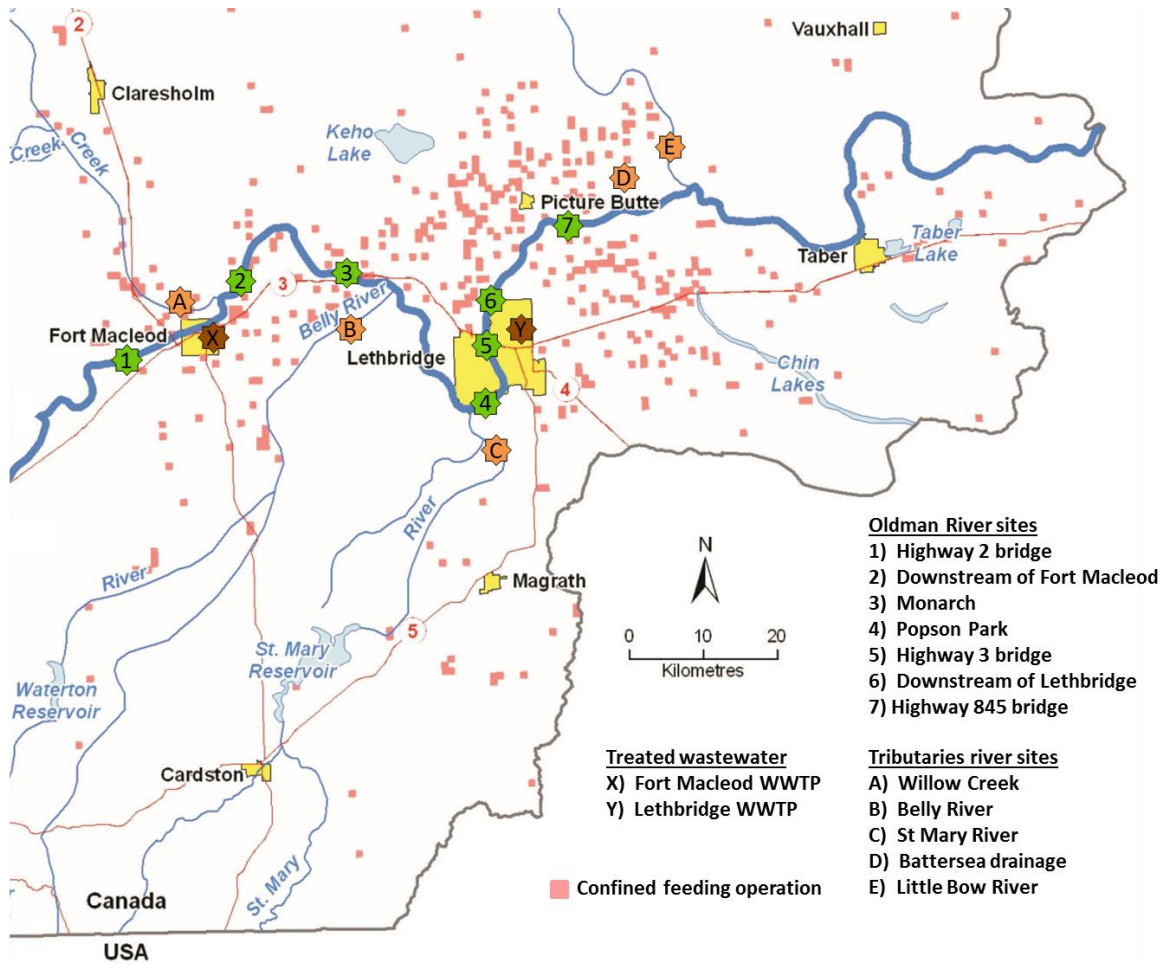
Figure 5.1. Surface water and wastewater sample sites in SWA, and their proximity to confined feeding operations. Water samples were collected weekly from May 2008 to April 2009. Confined feeding operations are defined by the Canadian *Agricultural Operation Practices Act* as "fenced or enclosed land or buildings where livestock are confined for the purpose of growing, sustaining, finishing or breeding by means other than grazing and any other building or structure directly related to that purpose". The Oldman River Basin contains approximately 1.28 million head of cattle, 2.50 million chickens, and 0.40 million hogs (Alberta Agriculture, Food and Rural Development). Source: image is modified from Figure 7.4 in the Oldman River State of the Watershed Report 2010 (146) with permission from the authors.

wastewater treatment process at the Fort Macleod WWTP consisted of a mechanical bar screen, grit removal, RBC activated sludge contact tank, secondary clarifier, solids removal to a digester and/or recirculated to front of contact tank, and treated effluent release to the Oldman River via a 3 km-long outfall line. In comparison, the Lethbridge WWTP used a mechanical activated sludge process comprised of a mechanical bar screen, grit removal, primary clarifiers, anaerobic,

anoxic, and aerobic digesters, secondary clarifiers, removal of activated sludge, UVB irradiation

of liquid effluent, and treated effluent release to the Oldman River via a 1 km-long outfall line.

Surface water and wastewater effluent samples (500 ml) were stored at 4°C for less than 6 hr.

Air temperature data during the sample period were collected daily by the Lethbridge Research

and Development Centre weather station. Subsamples of surface water (250 ml) and

wastewater effluent (100 ml) were filtered through a 150 mm pre-filter (#1001-150, Whatman

International Ltd., Maidstone England) and a GMF grade 0.20 µm filter (#1842-090, Whatman).

Both filters were vortexed vigorously in 10 ml PBS buffer to release particulates. The filters were

removed and the tubes were centrifuged at 14 900 x g for 10 min, and all but 3 ml of

supernatant were removed by aspiration. Pellets were suspended by vortexing and used for

culture-based quantification and/or isolation of fecal coliforms and *A. butzleri*.

5.3.2. Detection and quantification of fecal coliforms. Samples were diluted in a ten-fold

dilution series, and 100 µl from each dilution were spread on mFC Agar (Sigma-Aldrich) in

duplicate. Cultures were incubated for 24 h at 45°C, and CFU were enumerated at the dilution

yielding 30-300 dark blue colonies per dish.

5.3.3. Isolation and identification of *A. butzleri*. Media used to isolate *A. butzleri* from

wastewater and surface waters were CBA, KSA, ASIA (29), and JMA (167). Media for enrichment

of *A. butzleri* were BBS, *Arcobacter* Selection and Isolation Broth (ASIB) (29), and Johnson and

Murano Broth (JMB) (167). The isolation technique varied by medium; membrane filtration (158)

was used for CBA and ASIA, direct plating of 25 µl (river water) and 10 µl (wastewater) of

inoculum was used for KSA, and for enrichments 150 µl of inoculum was incubated in 2 ml of

BBS, ASIB, and JMB before subsequent plating on the respective agar medium. All cultures were

grown at high hydrogen atmosphere conditions (i.e. 5% O_2, 30% H_2, 10% CO_2, and 55% N_2), but

incubation temperature varied by isolation method; KSA and CBA cultures were incubated at

37°C, membrane filtration ASIA cultures were incubated at 30°C, and enriched ASIA and JMA cultures were incubated at 30°C and 37°C. Two colonies per morphology per medium per isolation technique per sample were collected and streaked for purity on CBA, and examined microscopically for cell size, shape, and motility. Genomic DNA was extracted from isolates using the DNeasy Blood and Tissue Kit (Qiagen Inc.) according to manufacturer specifications and an automated system (Model 740, Autogen, Holliston, MA). Extracted DNA was identified as *A. butzleri* by endpoint PCR with ddAbutz primers, which are specific to *A. butzleri* (230).

5.3.4. Subtyping of *A. butzleri* isolates. The CGF method (81) was used to characterize *A. butzleri* isolates recovered from surface water and wastewater effluent. One *A. butzleri* isolate per site per week was arbitrarily selected for CGF characterization. This system was repeated until 500 isolated had been selected.

5.3.5. Data analysis. Comparison of the efficacy of culture-based detection methods was performed using the Chi-square statistic in Sigmaplot (version 12.0, Systat Software Inc., San Jose CA). Comparison of CGF-based *A. butzleri* genotypes was performed using simple matching distance and minimum spanning trees in Bionumerics (version 6.6, Applied Maths, Austin TX).

5.4. RESULTS AND DISCUSSION

5.4.1. Isolation effectiveness by medium and technique. Although methods utilizing KSA were most effective for isolation of *A. butzleri* regardless of the sample source (Figure 5.2), enrichment techniques had greater sensitivity for surface water samples (P=0.016) and direct plating had greater sensitivity for wastewater samples (P=0.001). Each of the eight methods was responsible for solely isolating *A. butzleri* from at least one sample, and non-target bacteria were observed using all methods (data not shown). This was consistent (i.e. lack of specificity and inclusivity) with the previous study that used the same methods to isolate *A. butzleri* from diarrheic stools (230). Others also reported that culture-based methods lack specificity and
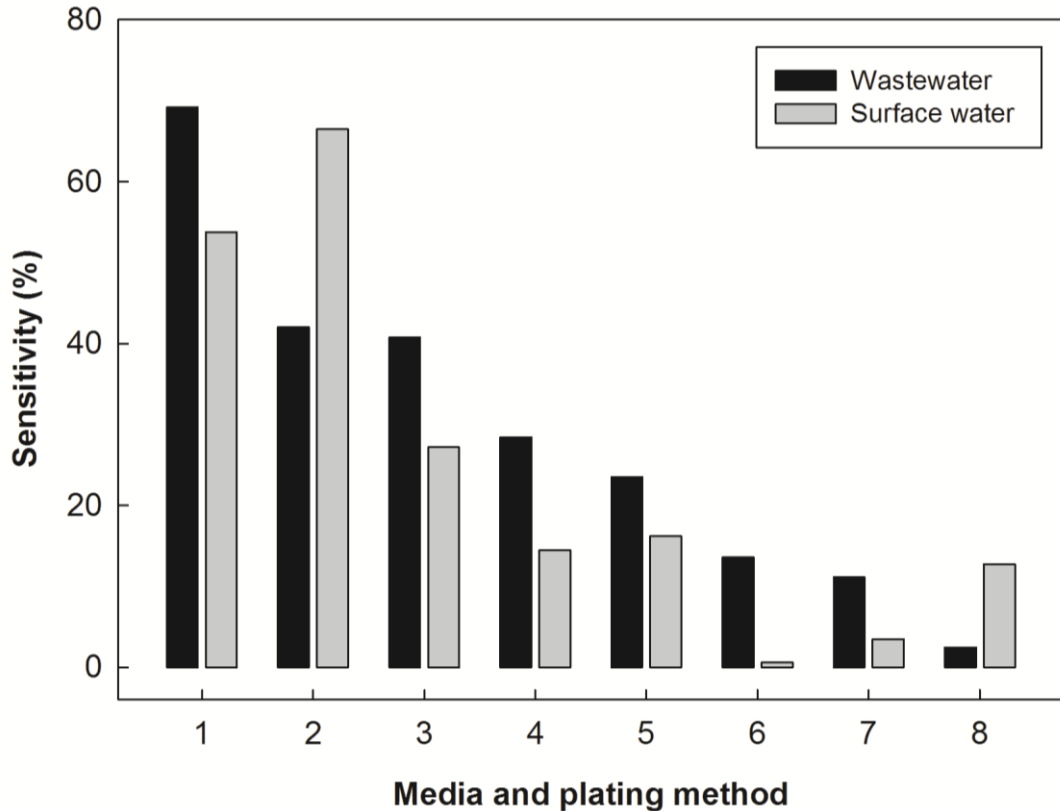
Figure 5.2. Comparative sensitivity of culture methods for selective detection and/or isolation of *A. butzleri*. Sensitivity is defined as the proportion of *A. butzleri* positive surface water (n=173) and wastewater (n=81) samples from which each *A. butzleri* was isolated using each method. Isolation method conditions were as follows: Direct plating onto KSA at 37°C (method 1); enrichment culture in BBS and isolation on KSA at 37°C (method 2); enrichment culture in ASIB (166) and isolation on ASIA (166) at 30°C (method 3); enrichment culture in JMB (167) and isolation on JMA (167) at 30°C (method 4); enrichment culture in JMB (167) and isolation on JMA (167) at 37°C (method 5); membrane filtration (158) on ASIA (166) at 30°C (method 6); membrane filtration (158) on CBA at 37°C (method 7); and enrichment in ASIB (166) and isolation on AISA (166) at 37°C.

inclusivity for the detection of *A. butzleri* in microbiologically-complex matrices (10, 73). It is

therefore important to utilize multiple media and plating techniques to achieve comprehensive

isolation of *A. butzleri*.

5.4.2. Detection of *A. butzleri* in surface waters. The overall frequency of detection of *A. butzleri*

in surface waters peaked during the summer months before decreasing in autumn and winter, a

trend which corresponded with Oldman River flow rates (Figure 5.3). There was no difference
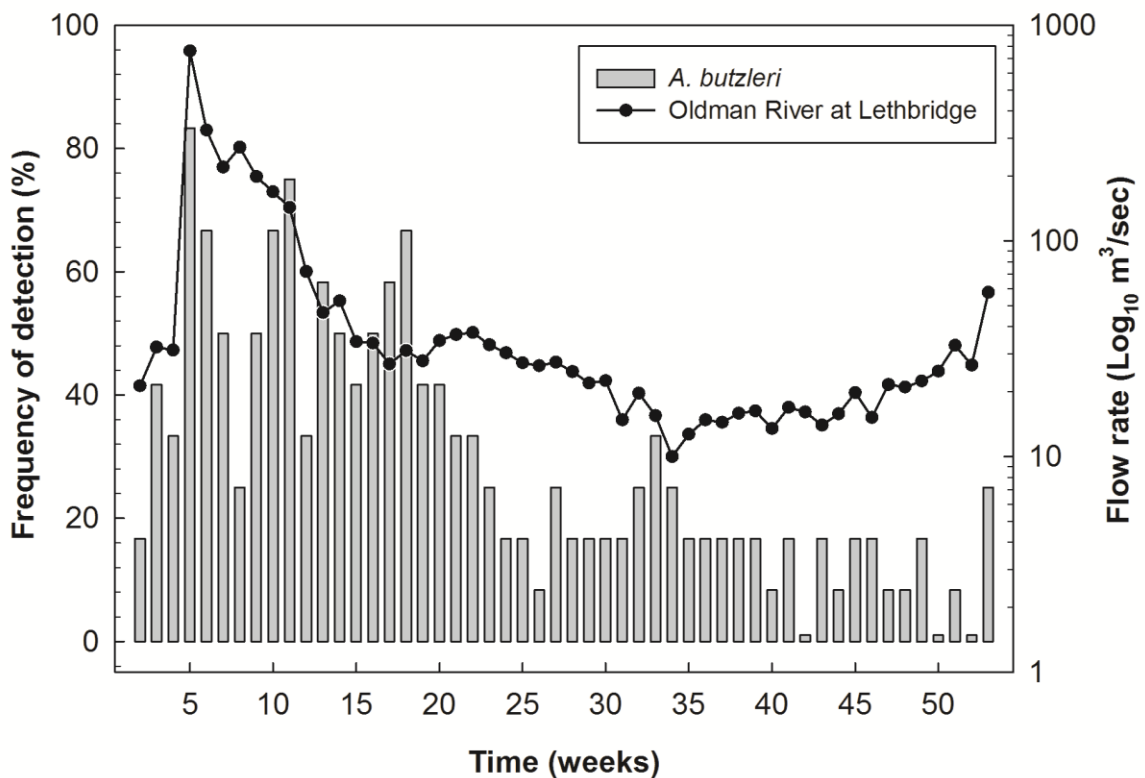
Figure 5.3. Frequency of detection of *A. butzleri* in surface waters in SWA (May 2008 to April 2009). Frequency of detection is defined as the proportion of weekly surface water sample sites (n=12) that were *A. butzleri* positive by at least one culture-based detection method. Oldman River flow rate data was provided by Alberta Environment and Parks.

(P=0.830) between frequency of detection of *A. butzleri* at sites immediately downstream of the

Fort Macleod (71.7%) and Lethbridge (69.8%) outfall sites, and at both sites the frequency of

detection of the bacterium was higher than at any other site along the Oldman River or its

tributaries (P≤0.011). There was no difference (P=0.555) between frequency of detection of *A.*

butzleri at the Battersea drainage (39.6%) and Little Bow River (45.3%), and the bacterium was

more frequently isolated at both of these sites compared to other Oldman River tributaries

(P≤0.019). Densities of fecal coliform indicators corresponded to *A. butzleri* isolation frequency

(Figure 5.4). Others have demonstrated that *A. butzleri* remains viable in feces from cattle (166),

pigs (48), and poultry (245). Aside from sites immediately downstream of wastewater outfalls,

the frequency of detection of *A. butzleri* in the Oldman River and its tributaries was greater at
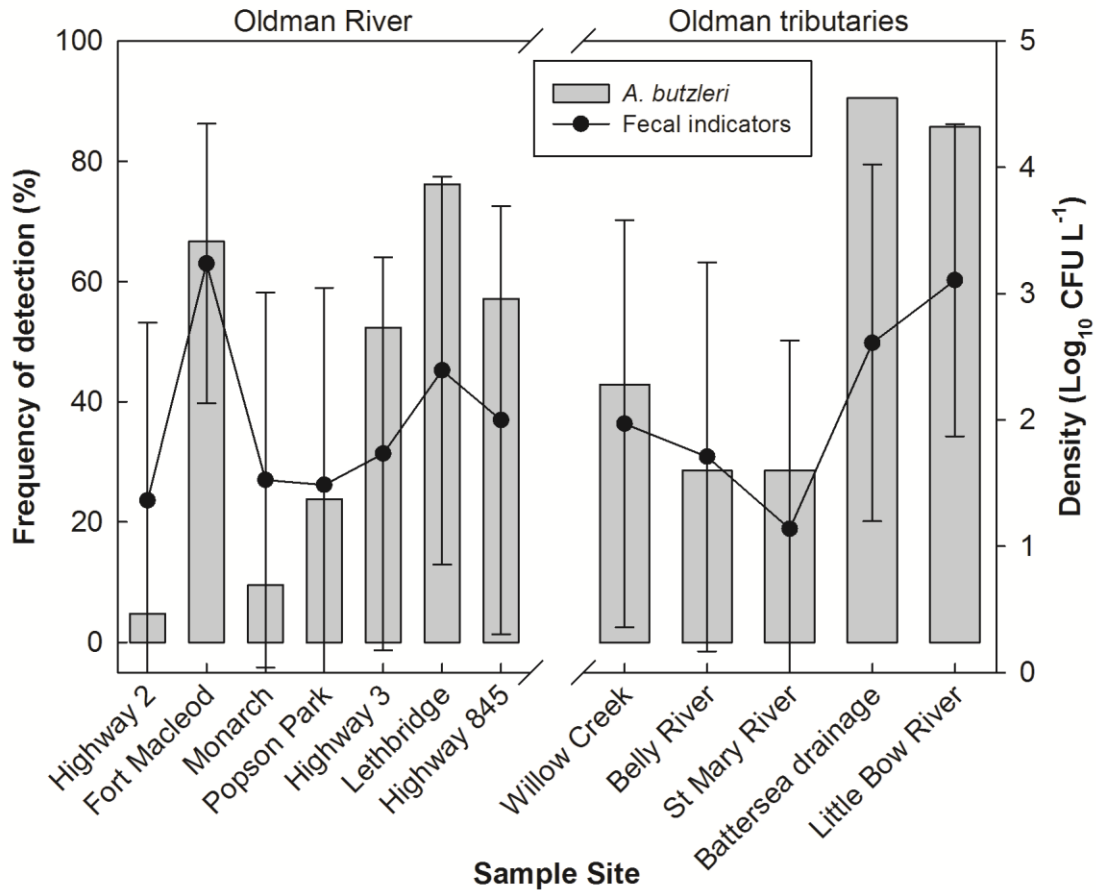
Figure 5.4. Frequency of detection of *A. butzleri* and density of fecal indicators at surface water sample sites in SWA from May to October 2008. Frequency of detection for each sample site is defined as the proportion of weeks (n=26) that were *A. butzleri* positive by at least one culture-based detection method. Vertical lines associated with histogram bars represent standard deviation of the means. Sites are arranged geographically from west to east.

eastern sample sites. As the Oldman River flows west to east from its source in the Rockies into a prairie agroecosystem of increasing agricultural activity, both in terms of contained feedlots (Figure 5.1) and ranging cattle (146). In particular, the Battersea drainage sample site channels waters from a region known as "feedlot alley", which possesses a high density of confined feeding operations. Irrigation canals that divert water from the Oldman River and its tributaries to flow through confined feedlots return to their source river with significantly greater levels of fecal contamination (148), so it is likely that the greater frequency of *A. butzleri* at eastern sampling sites are the direct result of greater livestock inputs.

5.4.3. Detection of *A. butzleri* in wastewaters. The frequency of detection of *A. butzleri* in city wastewater effluent was greater (P<0.010) at Fort Macleod (100%) than at Lethbridge (55.8%). Previous studies found viable *A. butzleri* to be present in treated wastewater effluent (36), but to my knowledge my study is the first to compare the frequency of detection of *A. butzleri* in wastewater effluent treated using different treatment processes. At the time of sampling, the Fort Macleod WWTP treated 1.53 million liters of wastewater per day using mechanical bar screen, grit removal, RBC activated sludge contact tank, secondary clarifier, solids removal to a digester and/or recirculated to front of contact tank, and treated effluent release to the Oldman River via a 3 km-long outfall line (Dan Segboer, Town of Fort Macleod, personal communication). The Lethbridge WWTP handled 36.0 million liters of wastewater per day using a mechanical activated sludge process comprised of a mechanical bar screen, grit removal, primary clarifiers, anaerobic, anoxic, and aerobic digesters, secondary clarifiers, removal of activated sludge, UVB irradiation of liquid effluent, and treated effluent release to the Oldman River via a 1 km-long outfall line (Doug Kaupp, City of Lethbridge, personal communication). Although UV irradiation has been shown to reduce the amount of fecal-associated bacteria in wastewater effluent (238, 239), no studies have examined its efficacy against *A. butzleri*. It was concluded that the Lethbridge wastewater treatment process at the time of the study was more effective than the Fort Macleod process for reducing the frequency of *A. butzleri* in effluent discharge, although further study is required to identify the exact cause of this increased effectiveness.

5.4.4. Genetic diversity and persistence. Comparative genomic fingerprinting was conducted on 500 *A. butzleri* isolates, and 250 subtypes were identified (Figure 5.5). Too few *A. butzleri* isolates for statistical comparison were recovered at four sites, but *A. butzleri* isolated from the remaining sites were highly diverse (Table 5.1). A greater proportion of subtypes isolated from
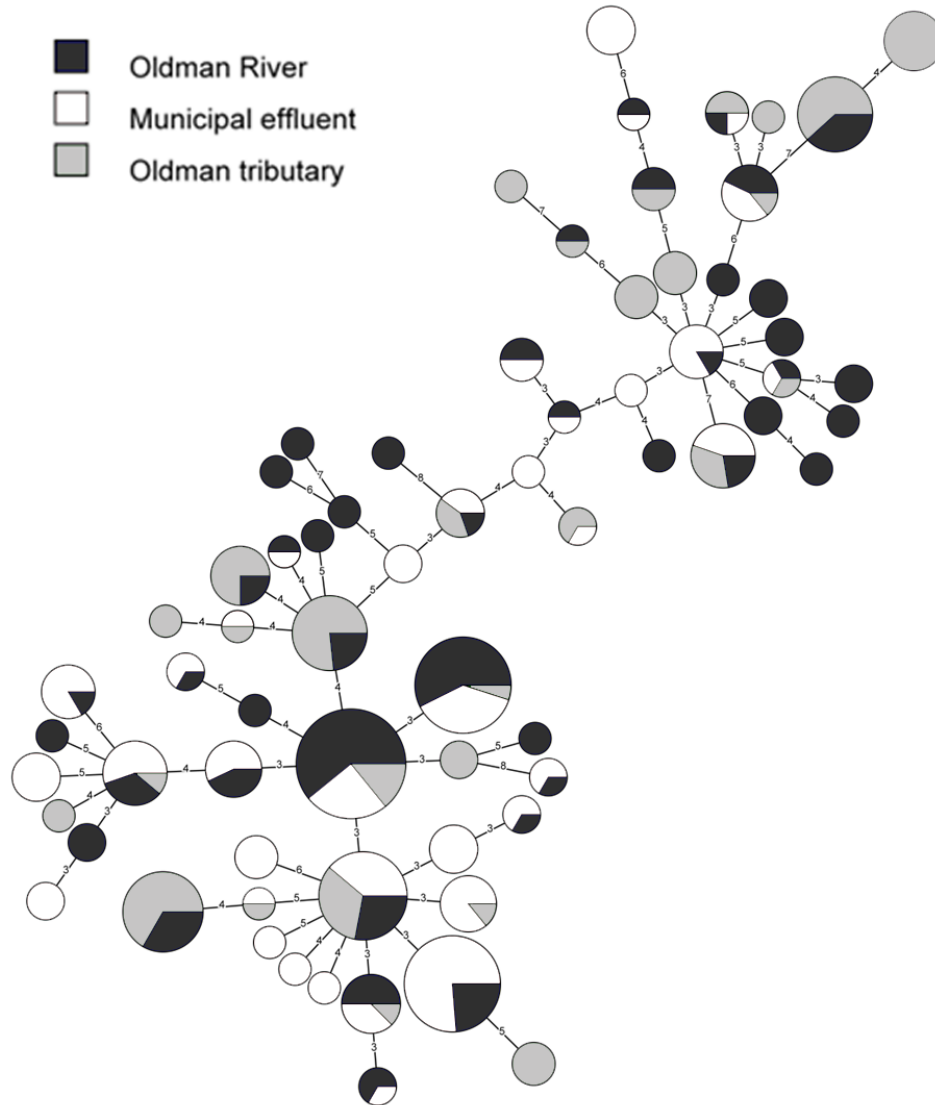
Figure 5.5. Cluster comparison of *A. butzleri* isolated from wastewater and surface waters. Clusters represent groups of *A. butzleri* isolates with at least 95% CGF similarity. Isolates that did not cluster at 95% CGF similarity are not shown. Lines represent CGF fingerprint mismatches between clusters, where each mismatch is equal to a 2.5% difference in subtype similarity. Minimum spanning tree analysis was conducted using Bionumerics (version 6.6, Applied Maths).

Lethbridge effluent were unique compared to Fort Macleod effluent. The sources of waste input at the Lethbridge treatment facility are more varied; large volumes of wastewater from pork, chicken, and cheese processing plants make use of the Lethbridge sewage system, while almost all of the input at Fort Macleod is of human and household origin. Considering that animal

holding and processing facilities are suspected reservoirs for *A. butzleri* (58, 177), the greater

variety of *A. butzleri* inputs likely accounts for the increased genetic diversity in Lethbridge

wastewater. Similarly, the lack of difference in *A. butzleri* genetic diversity (P≥0.077) between

surface water sites may be attributed to the selection of sample sites within a relatively uniform

watershed and agro-ecosystem, which would suggest similar inputs at all surface water sample

sites.

A total of 64 subtypes (25.6%) were identified on more than one occasion (Figure 5.6).

Previously, *A. butzleri* subtypes were found to persist in pig pens (58), poultry slaughterhouses

(192), and dairy facilities (151). Others have suggested that wastewater is a potential reservoir

for *A. butzleri* (177, 246), and my findings indicate that specific *A. butzleri* subtypes in

wastewaters survive treatment to be introduced into and persist in surface waters. *Arcobacter*

butzleri has been shown to remain viable for greater periods of time in water contaminated with

organic material (47). Considering that both the frequency of *A. butzleri* detection and the

number of persistent subtypes declined with increased distance from waste input sites, this

suggests that wastewater acts as a long-term reservoir for *A. butzleri*, with surface waters

contaminated by the bacterium from feces serving as a potential transmission medium.

5.4.5. Isolate similarity. *Arcobacter butzleri* isolated from wastewater effluent was most similar

to isolates from sites along the Oldman River that were immediately downstream of wastewater

outfalls, and similarity decreased with increasing distance (Figure 5.7). In addition, the similarity

between effluent and the Fort Macleod outfall site was significantly greater than that of the

Lethbridge outfall site. These findings provide further evidence that *A. butzleri* enters the

Oldman River as wastewater effluent, and that the Fort Macleod treatment process was less

effective at removing viable *A. butzleri* than the Lethbridge process at the time of the study.

Regardless of their geographical location, Oldman River tributaries were most similar to the

Sample site [a]	Source	Isolates	Simpson's ID[b]	CI (95%)	CINA (95%)
Oldman River					
Highway 2 bridge	Oldman River	1	NC	NC	NC
Downstream of Fort Macleod	Oldman River	59	0.974	0.954-0.995	0.952-0.997
Monarch	Oldman River	1	NC	NC	NC
Popson Park	Oldman River	2	NC	NC	NC
Highway 3 bridge	Oldman River	16	0.958	0.910-1.000	0.886-1.000
Downstream of Lethbridge	Oldman River	83	0.992	0.987-0.996	0.985-0.998
Highway 845 bridge	Oldman River	25	0.973	0.938-1.00	0.929-1.000
Tributaries					
Willow Creek	Tributary	23	0.933	0.871-0.995	0.861-1.000
Belly River	Tributary	4	NC	NC	NC
St Mary River	Tributary	13	0.923	0.805-1.000	0.784-1.000
Battersea drainage	Tributary	41	0.967	0.941-0.993	0.937-0.998
Little Bow River	Tributary	44	0.953	0.919-0.988	0.915-0.992
Wastewater effluent					
Fort Macleod	Wastewater	127	0.986	0.980-0.991	0.979-0.992
Lethbridge	Wastewater	58	0.993	0.987-0.999	0.984-1.000

Table 5.1. Genetic diversity of *A. butzleri* in surface waters and treated wastewater.

[a] Subsites are listed geographically from west to east, and their location with respect to SWA municipalities are indicated in Figure 5.1.
[b] *Arcobacter butzleri* subtype diversity was not calculated (NC) for sample sites that had too few isolates to be statistically relevant.

eastern regions of the Oldman River. Findings by Van Driessche et al. (25) suggest that *A. butzleri* can be transmitted both by direct contact and by sources such as water or feces. As these rivers and tributaries are subject to high densities of livestock production on rangeland and within confined feedlot operations, it is likely that these sites all receive *A. butzleri* via direct deposition of feces in water, and/or via precipitation and irrigation runoff containing animal fecal material.

5.5. CONCLUSIONS

Selective media and enhanced plating techniques were used to detect and isolate *A. butzleri* in surface water and wastewaters, and to link the presence of *A. butzleri* in the Oldman River and its tributaries in SWA with fecal contamination. Conventional treatment decreased, but did not eliminate the amount of viable *A. butzleri* in municipal wastewaters. In addition,
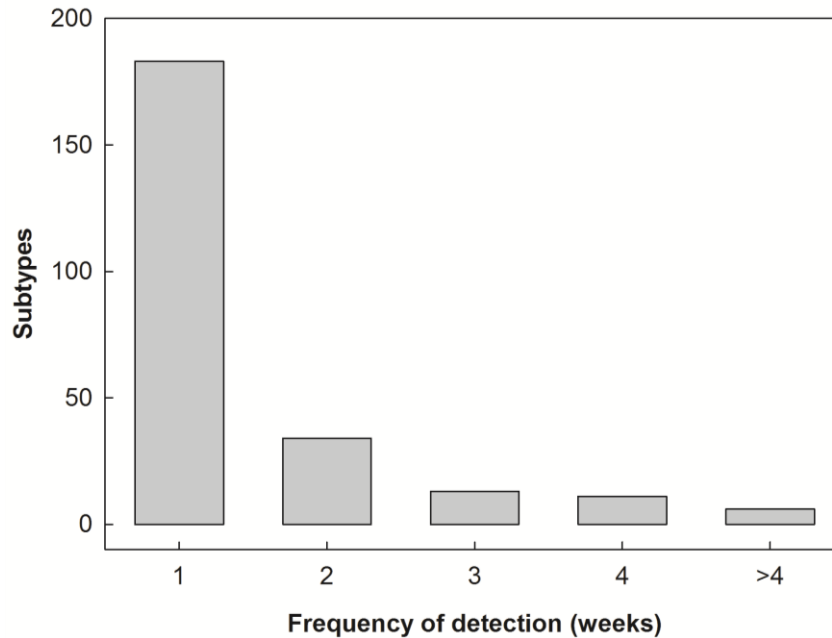
Figure 5.6. Frequency of detection of specific *A. butzleri* subtypes in wastewater and surface waters in SWA, Canada. Frequency of detection refers to the sum of sampling weeks at which any particular subtype was detected, regardless of sample site or weeks elapsed between detection.

subtype analysis indicated that *A. butzleri* is highly genetically diverse, that it remains viable in wastewater and surface waters, and that the bacterium enters surface waters as a result of human and non-human fecal contamination. My findings suggest that the presence of the potential pathogen *A. butzleri* in surface waters may constitute a risk to human health.
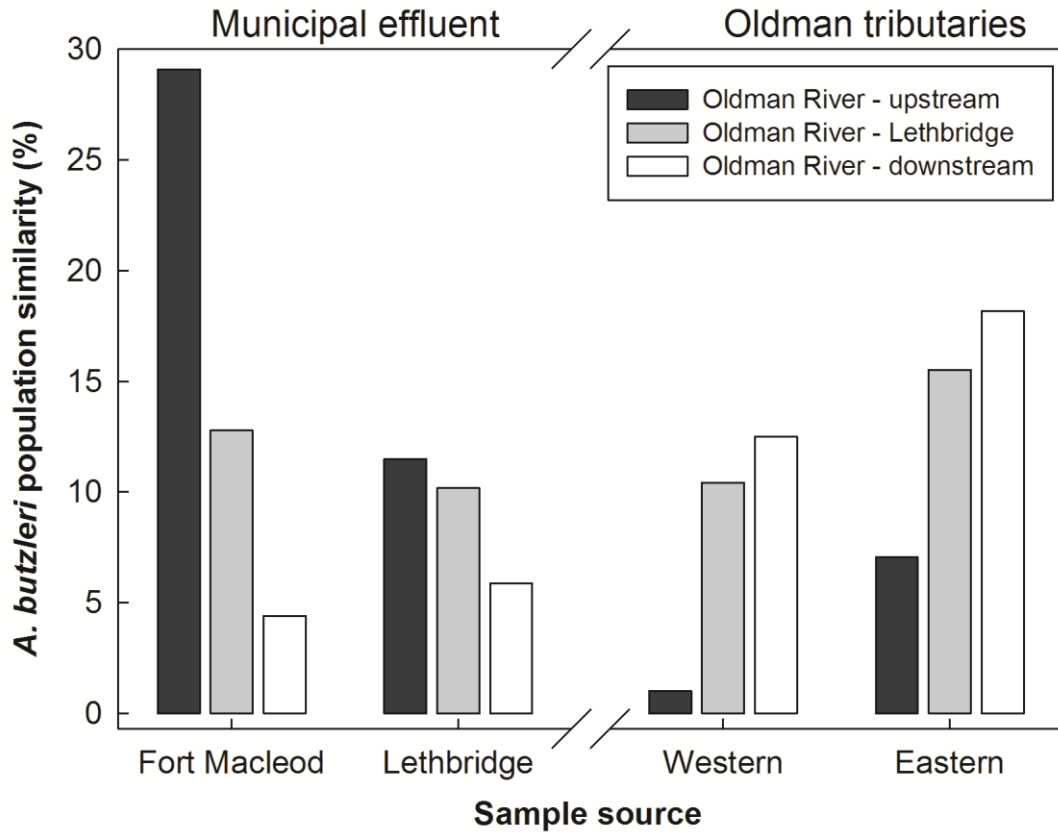
Figure 5.7. Genetic similarity of *A. butzleri* isolated from surface waters and wastewater to the Oldman River by CGF fingerprinting. Similarity in *A. butzleri* populations between each sample source and Oldman River regions is defined as the number of subtypes shared by a pair of sites multiplied by 2, divided by the sum of the number of subtypes at the pair of sites, multiplied by 100. Geolocation of tributaries was determined in relation to Lethbridge; Willow Creek, Belly River and St Mary River are western tributaries, and the Battersea drainage and Little Bow River are eastern tributaries.

CHAPTER SIX

General Discussion

6.1. RESEARCH GOAL

The primary goal of my thesis project was to develop and apply novel tools to ascertain whether *A. butzleri* is a pathogen of human beings in the model agroecosystem of SWA.

6.2. HYPOTHESES, FINDINGS, AND SCIENTIFIC CONTRIBUTIONS

6.2.1. Hypothesis 1. I hypothesized that *A. butzleri* is detected more frequently, and in greater densities in human beings with diarrheic illness. To test this hypothesis, a comprehensive PCR method for detection and quantitation of *A. butzleri* in complex matrices was developed and applied to compare prevalence/densities of the bacterium in diarrheic and non-diarrheic stools obtained from people living in SWA (230). *Arcobacter butzleri* was prevalent in both diarrheic and non-diarrheic individuals, but *A. butzleri* cell densities were greater in diarrheic individuals. Thus, I reject my hypothesis that *A. butzleri* is detected more frequently in diarrheic individuals, although the component of my hypothesis stating that the bacterium will be present in greater densities was supported by my results and warrants further investigation. Aside from the validation of a reliable detection method for *A. butzleri* in complex matrices, the number of *A. butzleri* strains that were whole genome sequenced and the sequence data made available for future studies was tripled. This is important because development of molecular tools for the study of *A. butzleri* pathogenicity has been hindered by the lack of WGS data that is responsible for the poor current understanding of its genomic variability, and specifically, variability among *A. butzleri* strains isolated from different sources. Thus, my sequence data will facilitate the identification of conserved and variable regions within the *A. butzleri* genome, which is a critical component of the molecular biology of this bacterium.

6.2.2. Hypothesis 2. I hypothesized that *A. butzleri* pathogenicity is strain-specific and that non-

pathogenic *A. butzleri* strains are able to colonize human beings as a commensal bacterium. I developed and applied a CGF assay to subtype and compare *A. butzleri* isolates from diarrheic and non-diarrheic human beings (81). *Arcobacter butzleri* subtypes were shared by multiple diarrheic people in SWA during the same week, which suggests that clinically relevant strains of this bacterium exist. In addition, the same *A. butzleri* subtype was isolated from two fecal samples from a healthy individual that were collected 6 months apart; thus, some strains of *A. butzleri* may persistently colonize people as commensal members of the microbiota. These findings support my hypothesis that *A. butzleri* pathogenicity is strain specific and is able to colonize human hosts as a commensal. The putative pathogenic and commensal *A. butzleri* strains identified as part of this study may advance the validation of animal models for *A. butzleri* pathogenicity, and also contribute to future studies to elucidate the pathobiology of the bacterium (e.g. via whole genome comparison, virulence factor expression, and host response).

6.2.3. Hypothesis 3. I hypothesized that *A. butzleri* infection of people in SWA is part of a complex web of transmission pathways between human beings and the environment. The quantitative detection method and CGF assay developed were applied to characterize the population structure of *A. butzleri* in SWA as a model ecosystem (247, 248). Findings provided strong evidence that *A. butzleri* is highly prevalent in environmental waters as a result of contamination from municipal and agricultural wastewaters, and that the presence of *A. butzleri* in environmental waters poses a risk to human health. These findings support my hypothesis that *A. butzleri* transmission occurs among human beings via contamination of the local environment. The comparison of *A. butzleri* prevalence and density in diarrheic and non-diarrheic human cohorts, and wastewaters and surface waters in the same time and space is an unprecedented step in the elucidation of *A. butzleri* epidemiology. This is notable because human stool samples are difficult to obtain, especially in a manner that allows for

epidemiological comparison with healthy cohorts and their environment. For example, studies of

A. butzleri prevalence in diarrheic patients often do not include a healthy control group (6, 41).

This makes it difficult to determine whether the prevalence of *A. butzleri* in diarrheic sample

groups is greater that the prevalence that would be expected in non-diarrheic individuals. The

samples used in my project were obtained from a centralized medical laboratory that process all

clinical samples for SWA, and my healthy cohort treatment group consisted of stools from non-

diarrheic individuals in the same geographical space and time as the diarrheic samples. Results

showed that *A. butzleri* prevalence and densities in both human and environmental sources vary

over time, and that some strains are source-specific while others are present in both human

beings and their environment.

6.3. REMAINING KNOWLEDGE GAPS AND FUTURE RESEARCH

6.3.1. Elucidation of the contribution of livestock to the population dynamics of *A. butzleri*.

During the course of my thesis research it became apparent that environmental waters and

retail meats contaminated with feces from cattle, chicken, and pigs are a likely mechanism of *A.*

butzleri transmission (52, 55). In addition, river waters and irrigation canals downstream from

CFOs have increased levels of fecal contamination. However, samples were only collected at one

location downstream from an area of high CFO irrigation/wastewater input, and in this case, no

samples were obtained upstream of potential input sources. Considering the high density of

CFOs in close proximity to surface waters in SWA, appropriate application of my CGF subtyping

method may demonstrate a relationship between animal-associated *A. butzleri* subtypes with

human diarrheic illness. An ideal scenario would be to temporally collect livestock, surface

water, and human fecal samples (diarrheic and non-diarrheic) during the same time period.

Greater focus should also be placed on contrasting densities and population dynamics of *A.*

butzleri in animal feces at CFO wastewater discharge sites with those in surface waters upstream

and downstream of each site. In the future, my qPCR and CGF methods will be valuable to study the potential transmission of *A. butzleri* from animals and animal products to humans, in order to provide evidence that such a pathway constitutes a risk of disease.

6.3.2. Elucidation of the contribution of crop agriculture to *A. butzleri* population dynamics.

Arcobacter species have been detected on ready-to-eat vegetables including carrots (64), lettuce (66), and spinach (65), but the manner in which they are contaminated and the risks that such contamination poses to human health have not been extensively studied. The results of my thesis research support previous findings that *A. butzleri* remains viable in municipal wastewater and solid waste throughout treatment (15, 36, 37, 247, 248). Considering that municipal solid waste is routinely applied to fields as fertilizer (249) and that treated wastewater is used as irrigation water as part of water reclamation initiatives (250), it is possible that this bacterium is transmitted to vegetable crops prior to harvesting or during processing. Similar to the scenario presented previously for the contamination of livestock slaughterhouses, it is plausible that vegetable crops are contaminated with *A. butzleri* during fertilization and/or irrigation cause widespread contamination of processing facilities (64-66). My direct detection and CGF subtyping methods can be applied to screen ready-to-eat vegetables for *A. butzleri* prevalence and density from "farm to fork", and CGF-based surveillance will facilitate identification the source of contamination.

6.3.3. Coinfection dependent pathogenicity of *A. butzleri*. As previously presented in section 1.3.4, detection of *A. butzleri* in the absence of known pathogens is not sufficient evidence that illness is incited by *A. butzleri*. Despite commonly being co-isolated with known pathogens (10, 69-71, 230), the possibility that *A. butzleri* pathogenicity is dependent upon coinfection with other microorganisms has not been examined to date. Bacterial-viral coinfections have been reported previously (251, 252), but coinfection by two pathogenic bacterial species is poorly

understood. The majority of *A. butzleri* putative pathogenicity factors are genetically similar to those of closely related pathogens like *C. jejuni*, which suggests a common mechanism of infection, yet putative virulence factors critical to *C. jejuni* pathogenicity are missing from the *A. butzleri* genome (56). For example, *C. jejuni* virulence protein secretion is dependent on its flagellar apparatus, which likely functions as a type 3 secretion system (253, 254). However, the flagellar apparatus of *A. butzleri* is evolutionarily distinct from *C. jejuni* (56), and there is no evidence that the *A. butzleri* flagellar apparatus plays a role in pathogenicity. While the association between the presence of these putative virulence genes and *A. butzleri* adhesion and/or invasion is not well characterized (93, 120), *A. butzleri* has been shown to impair epithelial barrier integrity in enterocytes (94, 255). Thus, it is possible that *A. butzleri* facilitates invasion of host cells by a second pathogen such as *C. jejuni*, and that *A. butzleri* benefits from the resulting *C. jejuni* infection. Whether *A. butzleri* instigates, participates in, and/or benefits from the pathogenicity of a second microorganism is not currently known, and my molecular identification and characterization tools can be applied to test the importance of *A. butzleri* as an accessory and/or opportunistic pathogen.

6.3.4. Validation of putative pathogenic *A. butzleri* strains in human beings using enterocyte and animal models. Currently, studies examining the infection of mammals by *A. butzleri* have utilized a limited number of *A. butzleri* strains in cell culture and animal models. However, the strains examined were arbitrarily selected without any direct evidence of pathogenicity. The majority of strains of closely related pathogens such as *C. jejuni* that are isolated from livestock have not been detected in diarrheic humans, and therefore may not represent a risk of infection for human beings (256, 257). In addition, both *C. jejuni* and *A. butzleri* have been detected in asymptomatic people (72, 258), so it is clear that at least some strains of these bacteria are non-virulent under appropriate conditions (e.g. an immunologically competent host). Thus, there is a

significant chance that arbitrarily selected *A. butzleri* strains are non-pathogenic. To guard

against this and to ensure the selection of appropriate strains, it is important to use high-

throughput genotyping methods such as CGF to identify *A. butzleri* strains that are present in

both livestock and diarrheic humans, which either suggests transmission from one host to the

other or a similar point of infection (242). Pathogenic strains of *C. jejuni* have been shown to

possess unique virulence factors (130) and to modify gene expression (259) during infection, and

similar *A. butzleri* studies would serve to elucidate its pathobiology. It would be beneficial to

sequence the genomes of prospective pathogenic and commensal strains of *A. butzleri*, and to

comparatively examine their pathobiology in enterocyte and animal models. Whole genome

comparison of candidate pathogenic and non-pathogenic *A. butzleri* strains would allow for the

identification and quantification of putative virulence factors and their expression during

infection. As animal models of disease do not currently exist for *A. butzleri* (e.g. germ-free and

gnotobiotic ASF mice), research should examine the use of alternative models in combination

with rationale-based strain selection. For example, intestinal xenografts (i.e. an immunologically

naïve model) (260) and intestinal loops (261) may be appropriate. My quantitative direct

detection method will be of value in assessing evaluations of pathogenesis (e.g. measuring

change in *A. butzleri* density over the course of the infection).

6.3.5. Targeted temporal and geographic study design to increase statistical validity. The high

genetic diversity of *A. butzleri* within and between reservoirs and host species necessitates

comparison of a large number of isolates in order to draw conclusions regarding the local and

global population dynamics of this bacterium. In addition, little is known about the changes in *A.*

butzleri population structure that occur over time, so "snapshot" sampling cannot be considered

representative of the diversity of subtypes within an ecosystem. Therefore, future studies should

focus on isolating *A. butzleri* more frequently (i.e. daily) and in greater numbers (i.e. more

colonies per sample). At the same time, isolation methods should be tested for comprehensive

isolation of a large number of diverse *A. butzleri* genotypes in order to mitigate the potential

effects of culture condition bias on population structure analysis (262) .

Although methodological constraints remain a limiting factor because the costs involved

(i.e. money and time) increase with longer sampling period, greater sampling frequency, number

of sites sampled, number of isolation methods applied per sample, and number of isolates

processed per isolation method. Many of these constraints can be mitigated via the

implementation of an appropriate project design. For example, the sample period could be

trimmed by focussing on summer months, during which my research findings indicate *A. butzleri*

prevalence peaks in both environmental waters and diarrheic people. An emphasis on sampling

sites closer to municipal wastewater outfall sites (i.e. directly upstream and downstream) and

CFO irrigation canals should be prioritized because they provide high frequencies of *A. butzleri*

isolation (36, 37). Finally, the lack of an effective standard culture-based isolation procedure may

be circumvented by pre-screening water samples for *A. butzleri* DNA using my direct detection

PCR primers to mitigate false-negative detection results based on the application of a limited

number of isolation methods.

6.3.6. Establishment of an international database for isolated *A. butzleri* CGF profiles. Access to

a large number of subtypes is critical in order to understand and compare *A. butzleri* genetic

diversity within and between populations. During my thesis research, I generated CGF profiles

for 1150 *A. butzleri* isolates from humans, non-human animals, wastewaters and environmental

waters. My findings, and those of others, indicated that *A. butzleri* is extremely genetically

diverse (36, 81, 99). The establishment of an online database for *A. butzleri* CGF subtypes would

facilitate characterization of *A. butzleri* genetic diversity using the largest number of isolates

from many sample sets and sources. Although such a database exists for the MLST subtyping

method, only 598 isolates have been added to the *A. butzleri* pubMLST website (as of 20 March 2016) since it was published in 2009 (138). The disparity between the sum of *A. butzleri* isolates that have been contributed to the MLST database and number of CGF isolates that were profiled as part of this thesis is likely due to a combination of the high-throughput nature of CGF and the relative impracticality of sequence-based methods such as MLST. A Canadian CGF profile database already exists for *C. jejuni* (263), which allows members of the scientific community to contribute to and benefit from this typing method, and a similar database for *A. butzleri* would further efforts to understand the molecular genetics and pathogenicity of this emerging pathogen.

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