Assessment of Methods for the Proactive Control of *Escherichia coli* O157:H7 in Ruminants

By

Susan Joan Bach

A Thesis
Submitted to the Faculty of Graduate Studies
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Foods and Nutritional Sciences
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FACULTY OF GRADUATE STUDIES

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Susan Joan Bach

To Don, my true companion

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LIST OF ABBREVIATIONS

BHI	brain heart infusion
CFU	colony forming unit
CT-KASMAC	sorbitol MacConkey agar containing 2.5 mg/L potassium tellurite, 0.05 mg/L cefixime, 100 µg/ml ampicillin and 100 µg/ml kanamycin
CT-SMAC	sorbitol MacConkey agar containing 2.5 mg/L potassium tellurite and 0.05 mg/L cefixime
CT-SMACnal	sorbitol MacConkey agar containing 2.5 mg/L potassium tellurite, 0.05 mg/L cefixime and 40 µg/ml nalidixic acid
DM	dry matter
EHEC	enterohemorrhagic *E. coli*
EPEC	enteropathogenic *E. coli*
GLM	general linear model
HC	hemorrhagic colitis
HUS	hemolytic uremic syndrome
IMS	immunomagnetic separation
LB	Luria-Bertani
LAB	lactic acid bacteria
LMG	lactose monensin glucuronate agar
LPS	lipopolysaccharide
LSD	least significant difference
MAC	MacConkey agar
MOI	multiplicity of infection

mTSB	modified tryptic soy broth containing 20.0 mg/L novobiocin, 1.5 g/L bile salts 3
NFM	nonfat milk medium
NLB	sodium lactate broth
NZCYM	broth containing per liter: 10 g NZ amine, 5 g NaCl, 5 g yeast extract, 1 g casamino acids, 2 g $MgSO_4 \cdot 7H_20$
OD	optical density
PBS	phosphate-buffered saline, pH 7.4, containing 15 mM KH_2PO_4, 8 mM Na_2HPO_4, 137 mM NaCl, 2.6 mM KCl
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFU	plaque forming units
pHi	cytoplasmic pH
pHo	environmental pH
ΔpH	transmembrane pH gradient
PMK	plate count-monensin-KCl agar
PT	phage type
SM	salt-magnesium buffer
SMAC	sorbitol MacConkey agar
SLT	Shiga-like toxin
Stx	Shiga toxin
TSA	tryptic soy agar
TSA-AK	tryptic soy agar containing 100 μg/ml ampicillin and 100 μg/ml kanamycin

TSA-NAL	tryptic soy agar supplemented with 200 µg/ml nalidixic acid
TSB	tryptic soy broth
TMR	total mixed ration
VFA	volatile fatty acid
VT1	Vero toxin 1
VT2	Vero toxin 2

ABSTRACT

There was no difference in the fecal shedding of *E. coli* O157:H7 in three groups of yearling steers (three rumen fistulated plus three nonfistulated), inoculated with 10^{10} CFU of *E. coli* O157:H7 and fed one of three different grain diets (85% cracked corn, 15% whole cottonseed and 70% barley, or 85% barley)($P>0.05$). *E. coli* O157:H7 was rapidly eliminated from the rumen of the animals on all three diets, but persisted in the feces of some animals up to 67 days post-inoculation, suggesting that the hindgut is the site of *E. coli* O157:H7 proliferation and a source for fecal shedding. Steers fed the corn diet had a lower fecal pH as compared to the steers fed the cottonseed and barley diets, and significantly fewer animals fed the corn diet shed *E. coli* O157:H7 in their feces ($P<0.005$). *E. coli* O157:H7 was isolated from the water, water trough biofilm, feed and manure associated with the experimentally inoculated steers. Recovery of *E. coli* O157:H7 from the mouth swabs of the steers suggests that the organism can survive in the alkaline saliva of cattle long enough to be transferred to feed, water and other cattle.

In a group of 40 heifers and steers naturally infected with *E. coli* O157:H7, there were no differences in the numbers of animals positive for *E. coli* O157:H7 when fed an 80% concentrate (80% barley and 20% alfalfa silage) or 100% forage (alfalfa silage) diet ($P>0.05$), or during the 48 h fasting periods following each diet ($P>0.05$). The organism was not recovered from the feces of any animals following 12 h of feed withdrawal of the 80% concentrate diet, suggesting that fasting animals on finishing diets for up to 12 h may reduce the numbers of *E. coli* O157:H7 positive animals going to slaughter. There was an increase in the number of *E. coli* O157:H7 positive animals ($P<0.05$) upon re-feeding 100%

forage with 43% of animals being culture positive for *E. coli* O157:H7.

The Rusitec was used to assess the effects of barley, barley silage, timothy and clover diets on the growth and/or survival of 10^7 CFU/ml of *E. coli* O157:H7 in a 20 day study. *E. coli* O157:H7 was eliminated from the barley silage, barley, clover and timothy diets at 60, 72, 120 and 144 h post-inoculation, respectively. The organism was recovered from the feed residues of the fermenters fed all four diets but persisted in the feed of the barley silage fed fermenter 60 h after it was last detected in the rumen fluid. This suggests association of *E. coli* O157:H7 with feed particles may allow it to survive in the rumen.

In the evaluation of the proprietary substances for their effects on *E. coli* O157:H7, the ionophore, monensin sodium, had no effect on the growth and/or survival of *E. coli* O157:H7 in pure culture, at levels which would be typically attained in the rumen (5 to 10 mg/L). Neither *P. freudenreichii* nor *P. acidipropionici* produced inhibitory metabolites against *E. coli* O157:H7 when grown in nonfat milk medium at 32 °C for 4 days and assayed using the direct supernatant spot assay.

Levucell SB20, a feed supplement containing *S. cerevisiae* subspecies *boulardii*, was shown to produce inhibitory metabolites against *E. coli* O157:H7 in the spot agar assay and eradication of 10^4 CFU/ml of *E. coli* O157:H7 was observed in clarified rumen fluid by 1.5 and 2.0 g of Levucell SB20 after 48 h of incubation at 39 °C. No difference in the levels of *E. coli* O157:H7 were observed, however, when 0, 0.5 and 2.0% Levucell SB20 was administered to Rusitec fermentation vessels 8 h following inoculation with 10^4 CFU/ml of *E. coli* O157:H7 ($P>0.05$). This suggests that incorporation of *S. cerevisiae* into feedlot diets at recommended levels (<30 g/head/day) is unlikely to affect the growth and/or

survival of *E. coli* O157:H7 in cattle.

The bacteriophage DC22, was found to lyse *E. coli* O157:H7 when tested on 40 strains of enterohemorrhagic and enteropathogenic *E. coli* using the plaque titration assay. In studies using clarified rumen fluid, DC22 was found to have a high multiplicity of infection (MOI) (> 10^4 plaque forming units (PFU)/CFU) for *E. coli* O157:H7. In the Rusitec, 10^4 CFU/ml of *E. coli* O157:H7 was eliminated from fermenters (n=4) treated with 10^5 PFU/CFU DC22 within 4 h. In wethers, experimentally inoculated with 10^8 CFU *E. coli* O157:H7, DC22 at a level of 10^5 PFU/CFU had no effect on the fecal shedding of the organism over a 30 day period ($P>0.05$). The large MOI of DC22 suggests that DC22 possessed low antibacterial activity against *E. coli* O157:H7 and sufficient numbers of the bacteriophage to cause bacterial infection were probably not achieved in the gastrointestinal tract of the wethers, resulting in a lack of inhibition.

At 22 and 30 °C, acidification of water samples by either 0.5% acetic or 0.5% propionic acid eliminated 10^5 CFU/ml of *E. coli* O157:H7 within 3 and 1 day, respectively, but at 4 °C the organism was still detectable 14 days later. Results indicate that the potential exists for the use of 0.5% acetic or 0.5% propionic acid in water troughs as a means of controlling this possible source of *E. coli* O157:H7.

The effect of a lactic acid bacterial (LAB) inoculant containing 10^5 CFU/g *Pediococcus pentosaceus* and *Propionibacterium jensenii* (P2), on the elimination of 10^5 CFU/g of *E. coli* O157:H7 and 10^5 CFU/g *E. coli* Biotype 1 (EC) inoculated onto barley forage and ensiled for a 42 d period was determined. *E. coli* O157:H7 was not detected in P2 and P2+EC 7 and 15 days post-ensiling, respectively. Bacteriocins of P2 were not

inhibitory to *E. coli* O157:H7 and its inhibition in the silage was likely due to low pH as a result of lactate production. Following exposure of the 42 d silage to air for 28 d, *E. coli* O157:H7 was not detected. Results underlined the importance of adequate ensiling to minimize the spread of *E. coli* O157:H7 among cattle through feed.

Intervention strategies which reduce the fecal shedding of *E. coli* O157:H7 by ruminants and target environmental sources of the organism may reduce numbers of *E. coli* O157:H7 positive cattle.

INTRODUCTION

Since 1982, when it was first identified as a human pathogen, enterohemorrhagic *Escherichia coli* O157:H7 has been implicated in numerous outbreaks of hemorrhagic colitis and the life-threatening hemolytic uremic syndrome (Riley et al., 1983). Although most illness caused by *E. coli* O157:H7 has been largely associated with the consumption of undercooked ground beef (Griffin and Tauxe, 1991) or unpasteurized milk (Chapman et al., 1993; Mechie et al., 1997), outbreaks of infection have also been linked to foods such as radish sprouts (Itoh et al., 1998), apple cider (Besser et al., 1993), mayonnaise (Raghubeer et al., 1995), yogurt (Morgan et al., 1993), venison jerky (Keene et al., 1997), cantaloupe (Beuchat, 1996), lettuce (Ackers et al., 1998), dry-cured salami (CDC, 1995), and water (Ackman et al., 1997; Swerdlow et al., 1992).

Epidemiological investigations have provided evidence that cattle, both dairy and beef, are primary reservoirs of *E. coli* O157:H7 (Hancock et al., 1994; Wells et al., 1991; Zhao et al., 1995). Fecal shedding of *E. coli* O157:H7 is reported to be intermittent in nature and the organism does not cause disease in cattle (Besser et al., 1997). Fecal contamination of carcasses during slaughter and processing is likely the manner in which beef becomes contaminated and the organism is transferred to humans (Besser et al., 1997; Buchanan and Doyle, 1997; Cassin et al., 1998; Chapman et al., 1993b; Elder et al., 2000). Transmission of the organism to other food products is thought to be the result of contact with contaminated bovine products (Griffin and Tauxe, 1991).

E. coli O157:H7 has been isolated from the animal environment and the seasonal pattern of *E. coli* O157:H7 shedding by cattle, with increased prevalence during the summer

months, suggests that environmental replication plays a key role in the ecology of the organism (Hancock et al., 1997; MacDonald et al., 1996; Shere et al., 1998; Wells et al., 1991). The survival of *E. coli* O157:H7 in feed, water, manure and soil indicate that the organism can survive outside the animal host for extended periods of time (Hancock et al., 1998; Kudva et al., 1998; Lynn et al., 1998; Maule, 2000; Rice and Johnson, 2000; Wang et al., 1996).

The maintenance and spread of *E. coli* O157:H7 throughout the animal environment and intermittent shedding of *E. coli* O157:H7 by cattle, suggest a cycle of infection exists in which cattle may be constantly re-infected through environmental sources. The control of *E. coli* O157:H7 fecal shedding by cattle, therefore, requires a multi-faceted approach. Intervention strategies aimed at reducing fecal shedding of *E. coli* O157:H7 in cattle, in addition to targeting environmental sources of the organism, may result in a reduction of this deadly pathogen on the farm.

The objectives of this research were to assess certain dietary components as a means of control of *E. coli* O157:H7 in ruminants, in addition to assessing aspects of the animal environment involved in the spread of the organism. First, to determine the effects of three types of grain diets (85% corn, 15% whole cottonseed and 70% barley, and 85% barley) on the fecal shedding of *E. coli* O157:H7 in experimentally inoculated steers. Second, to determine if fasting and the manner of feeding prior to fasting, had an effect on *E. coli* O157:H7 shedding by cattle, in a herd naturally infected with the organism. Third, to assess the effects of four proprietary agents on the growth and survival of *E. coli* O157:H7, in order to establish their potential as control measures for the shedding of *E. coli* O157:H7

by cattle. This was achieved through *in vitro* studies and the use of the Rumen Simulation Technique (Rusitec). The agents were; (1) Rumensin (monensin sodium) (Elanco Animal Health); (2) *P. freudenrichii* and *P. acidipropionici* (T. Rehberger, Waukesha, WI); (3) Levucell SB20 (*S. cerevisiae* subspecies *boulardii*) (L'Allemand Biochemicals); (4) DC22 (anonymous collaborator). Agents which showed potential in *in vitro* studies were subsequently assessed in experimentally inoculated wethers as a ruminant model. Fourth, to determine the environmental presence of the organism through the sampling of feed, water, manure and mouth swabs of experimentally inoculated steers and their environment. In addition, to assess the survival of *E. coli* O157:H7 in experimentally inoculated barley forage, and to determine the potential for the use of acetic and propionic acids in the control of *E. coli* O157:H7 in animal drinking water, in order to prevent the spread of *E. coli* O157:H7 to the environment and back to cattle.

LITERATURE REVIEW

History and Origins of *E. coli* O157:H7

Two outbreaks of an unusual gastrointestinal illness affecting at least 47 people occurred in the states of Oregon and Michigan in early 1982 (Riley et al., 1983; Wells et al., 1983). The illness was characterized by severe abdominal cramps, watery diarrhea, followed by grossly bloody diarrhea with little or no fever. Conventional examination of stool specimens did not demonstrate the presence of a recognized enteric pathogen such as *Campylobacter, Salmonella, Shigella* or *Yersinia* (Neill, 1989). A rare serotype of *E. coli coli*, O157:H7, was recovered from the stool samples of patients and it was at this time *E. coli* O157:H7 was first identified as a human pathogen (Neill, 1989; Riley et al., 1983; Tarr, 1995).

Exposure histories of the patients involved in the outbreaks suggested that illness was associated with consumption of fast-food from the same fast-food restaurant chain in both Oregon and Michigan. *E. coli* O157:H7 was isolated from a raw hamburger patty from the same lot that was used in the Michigan restaurant during the period of the outbreak at a level of 50 organisms per g (Wells et al., 1983). It was hypothesized that *E. coli* O157:H7 contaminated the meat prior to cooking and resulted in illness among some people who ingested the hamburger patties, following its survival of the cooking process. The source of contamination of the meat by *E. coli* O157:H7 was unknown (Riley et al., 1983; Wells et al., 1983).

It is thought that most *E. coli* exist as a benign inhabitant of the gastrointestinal tract of humans and other warm-blooded animals. However, pathogenic *E. coli* strains

exist which are associated with distinct syndromes of diarrheal disease (Levine, 1987). *E. coli* strains which are associated with hemorrhagic colitis and the hemolytic uremic syndrome in humans and produce Shiga-like toxins (SLTs) are referred to as enterohemorrhagic *E. coli* (EHEC) (Kaper, 1994; Whipp et al., 1994). *E. coli* O157:H7 is the predominant EHEC serotype associated with food-borne illness, particularly in North America and the United Kingdom. However, non-O157 EHEC have also been implicated in sporadic enteric infections and possess outbreak potential (Barrett et al., 1992; Boyce et al., 1995; Coia, 1998; Levine, 1987; Karmali, 1989; Rowe et al., 1993).

Five other classes of *E. coli* in addition to EHEC are currently recognized as causes of human gastrointestinal disease and have been categorized on the basis of their pathogenic mechanisms. These five classes are: Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAggEC), and Diffusely Adherent *E. coli* (DAEC) (Buchanan and Doyle, 1997; Kaper, 1994).

It has been proposed that *E. coli* O157:H7 evolved from *E. coli* O55:H7, an EPEC progenitor, that has long been associated with diarrhea in infants worldwide (Armstrong et al., 1996; Mead and Griffin, 1998; Wachsmuth et al., 1997; Whittam et al., 1993). It is thought that *E. coli* O55:H7, which already possessed a mechanism for adherence to intestinal cells, acquired additional virulence traits through horizontal gene transfer and recombination (Mead and Griffin, 1998; Whittam et al., 1993). It has been postulated that following this acquisition of virulence traits, *E. coli* O157:H7 lost its ability to ferment sorbitol and to produce β - glucuronidase (Armstrong et al., 1996).

Clinical Manifestations of *E. coli* O157:H7 Infection

Infection with *E. coli* O157:H7 causes three principal manifestations of illness: hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP). The average time between exposure and illness is 3 to5 days with a range of between 1 to 9 days (Boyce et al., 1995; Neill, 1989; Padhye and Doyle, 1992; Riley, 1987).

HC usually begins with the sudden onset of abdominal cramps followed by non-bloody diarrhea. Vomiting may occur and there is usually little or no fever. The non-bloody diarrhea progresses to grossly bloody diarrhea within 1-3 days in approximately 90% of the cases of diagnosed *E. coli* O157:H7 infection (Chapman, 1995; Tarr, 1995). At this point the patients are rarely febrile and may be experiencing abdominal tenderness (Mead and Griffin, 1998; Tarr, 1995). The duration of bloody diarrhea usually ranges from 2-10 days (Lior, 1994; Mead and Griffin, 1998; Padhye and Doyle, 1992; Tarr, 1995). Approximately 95% of the cases of HC are self limiting and are resolved within 7 days with no obvious sequelae (Griffin and Tauxe, 1991; Mead and Griffin, 1998; Riley, 1987). However, HUS develops in 2 to10% of cases of HC and is commonly diagnosed 2 to14 days after the onset of diarrhea (Boyce et al., 1995; Coia, 1998; Mead and Griffin, 1998; Molenda, 1994; Neill, 1989).

The clinical signs of HUS are the development of acute hemolytic anemia (destruction of red blood cells), thrombocytopenia (low circulating platelets), and acute renal failure (Neill, 1989; Padhye and Doyle, 1992). In HUS, hemoglobin and platelet counts are depressed, while the level of blood urea nitrogen is elevated as compared to

patients without HUS (Gransden et al., 1986).

HUS can occur in people of all ages but is most frequently diagnosed in children less than 10 years of age and in the elderly (Boyce et al., 1995; Buchanan and Doyle, 1997; Griffin and Tauxe, 1991;Tarr, 1995). HUS is probably the most common cause of acute renal failure in children (Boyce et al., 1995). Approximately 50% of the patients with HUS require dialysis and 75% receive blood transfusions. Cardiovascular and acute neurological complications such as heart failure, seizures, stroke, and coma develop in 25% of patients (Mead and Griffin, 1998; Padhye and Doyle, 1992). Approximately 3 to5% of HUS patients die in the acute phase of the disease. Among surviving patients, 5% develop end stage renal disease or suffer from other major sequelae including cholelithiasis (the presence of gallstones in the gall bladder), colonic stricture, chronic pancreatitis, glucose intolerance, and cognitive impairment (Boyce et al., 1995; Coia, 1998; Mead and Griffin, 1998).

TTP is a condition that resembles HUS but with more prominent neurological abnormalities and less renal involvement (Coia, 1998; Mead and Griffin, 1998). It is a syndrome which is usually diagnosed in adults and consists of microangiopathic hemolytic anemia, profound thrombocytopenia and fluctuating neurologic signs. Death frequently is the end result as patients often develop blood clots in the brain (Boyce et al., 1995; Mead and Griffin, 1998).

Biochemical Characteristics

The majority of the biochemical reactions of *E. coli* O157:H7 strains are typical of those of other *E. coli* (Doyle, 1991; Padhye and Doyle, 1992; Ratnam et al., 1988). However, *E. coli* serotype O157:H7 does possess a few distinct biochemical differences.

These include the lack of sorbitol fermentation, the lack of β - glucuronidase activity and enterohemolysin production (Doyle, 1991; Ratnam et al., 1988).

Approximately 96% of *E. coli* strains produce the enzyme β - glucuronidase which is the basis of the development of a rapid fluorogenic assay for the detection of *E. coli* (Feng and Hartman, 1982; Krishnan et al., 1987; Thompson et al., 1990). The indicator 4-methylumbelliferyl β - D glucuronide (MUG) used in this assay is cleaved by β - glucuronidase to yield a fluorogenic end product, methylumbelliferone, which is detectable using ultraviolet light (Ratnam et al., 1988; Thompson et al., 1990). *E. coli* O157:H7 does not produce β - glucuronidase and is negative in the MUG assay.

Approximately 93 - 95% of *E. coli* isolates of human origin ferment sorbitol within 24 h, however, *E. coli* O157:H7 does not (Ratnam et al., 1988; Wells et al., 1983). This particular characteristic has been exploited in the use of sorbitol MacConkey agar which allows for the differentiation between sorbitol-positive *E. coli* (pink colonies) and sorbitol-negative *E. coli* O157:H7 (clear colonies)(Armstrong et al., 1996; March and Ratnam, 1986).

E. coli O157:H7 exhibits other differential sugar fermentation patterns in comparison to non-O157 *E. coli* strains. Sucrose was fermented by 87% of *E. coli* O157:H7 strains in contrast to 42-54% of other *E. coli* strains. Dulcitol and raffinose were fermented by 100% of *E. coli* serotype O157:H7. Approximately 50% of *E. coli* other than serotype O157:H7 fermented dulcitol while 20% of *E. coli* other than serotype O157:H7 fermented raffinose (Krishnan et al., 1987; Ratnam et al., 1988).

Growth and Survival Characteristics

Temperature

E. coli O157:H7 grows rapidly between the temperatures of 30 and 42 °C, with optimal growth occurring at 37 °C. Generation times range from 0.49 h at 37 °C to 0.64 h at 42 ° C (Doyle and Schoeni, 1984). The temperature range for growth and gas production of *E. coli* O157:H7 in *E. coli* medium (EC) within 48 h is 19.3 to 41.0 °C (Raghubeer and Matches, 1990). The organism grows poorly at 44 - 45.5 °C (Doyle and Schoeni, 1984; Gubash et al., 1988). Hence, *E. coli* O157:H7 in food would not be detected using standard enumeration procedures routinely used for detecting *E. coli* in foods and water (Doyle, 1991; Doyle and Schoeni, 1984; Raghubeer and Matches, 1990).

In contrast, in a study of 23 strains of *E. coli* O157:H7 Palumbo et al. (1995) reported that all strains grew from between 10 to 45 °C in brain heart infusion (BHI) broth, with some strains growing at 8 °C and one strain with growth at 49 °C. Growth at elevated temperatures was found to be a function of growth medium, with BHI broth being more permissive than EC broth.

The ability of 18 strains of *E. coli* O157:H7 to grow in EC broth at 42.2, 43.5, 44.5 and 45.5 °C was examined. Ferenc et al. (2000) indicated that the ability of most strains to grow in EC broth at 45.5 °C was dependent on the initial density of CFU. At low densities of CFU the ability to initiate growth was dependent on either low numbers of phenotypic variants tolerant to bile salts in the EC broth at 45.5 °C or to conditioning of the medium with prior elevated numbers of cells. An unknown chemical substance produced in EC broth at 45.5 °C during growth with CFU densities above 2 x 10^{7}/ml was capable of

stimulating the initiation of growth in EC broth at 45.5 °C with an initial CFU density of 2 x 10^{6}/ml exhibiting a phenomenon resembling a quorum response (Ferenc et al., 2000).

Doyle and Schoeni (1984) reported that *E. coli* O157:H7 survives in frozen storage extremely well. No significant difference in number of *E. coli* O157:H7 recovered from ground beef was reported after being frozen at - 80 ° C or being held at -20 °C for 9 months. Duncan and Hackney (1994) reported that *E. coli* O157:H7 can survive refrigeration temperatures and slow growth of the organism occurs at approximately 7 °C. Growth of *E. coli* O157:H7 in BHI broth under optimal conditions (0.5% NaCl; pH 6.5 -7.5) was observed both aerobically and anaerobically at 10 °C, but not at 8 °C (Buchanan and Klawitter, 1992).

pH

The minimum pH for growth of *E. coli* O157:H7 has been reported to be between 4 and 4.5 (Buchanan and Bagi, 1994). The growth rates of *E. coli* O157:H7 were found to be similar between the pH values of 5.5 and 7.5 but declined rapidly below pH 5.5 (Buchanan and Klawitter, 1992). Survival of *E. coli* O157:H7 in acid conditions is affected by type of acidulant and temperature. Mandelic acid has been reported to have the greatest inhibitory effect on *E. coli* O157:H7, however, this acid is not regarded as safe for use as a food preservative. Acetic acid was found to be inhibitory to *E. coli* O157:H7 at pH 4.5 -5.0 and at a concentration of 0.007 M of the undissociated form (Conner and Kotrola, 1995).

It has been reported that *E. coli* O157:H7 strains survived at pH 2 for 24 h in trypticase soy broth (TSB) with a minimal reduction in numbers, while no survivors were

detected after 24 h at pH 12. At extremes in pH, survival of *E. coli* O157:H7 was found to be greater at 4° C than at 25° C (Miller and Kaspar, 1994). A study by Zhao et al. (1993) found that *E. coli* O157:H7 survived in apple cider (pH 3.6 to 4.0) for only 2-3 days at 25 ° C, compared to 10-31 days at 8 °C (Zhao et al., 1993). Although strain differences do occur, *E. coli* O157:H7 has been found to be exceptionally tolerant of acid pH (Conner and Kotrola, 1995; Garren et al., 1997; Miller and Kaspar, 1994).

Acid Resistance

An important property of *E. coli* O157:H7 is the ability to overcome the stress of an extremely acidic environment in addition to moderately acidic environments composed of weak acids. The overall acid survival response in *E. coli* O157:H7 is affected by overlapping systems of acid protection. While acid inducible systems for acid tolerance are present in *E. coli* O157:H7, sustained acid tolerance is not dependent on adaptation but is induced by stationary phase or starvation.

The acid survival systems which have been identified in order to explain the acid tolerance of *E. coli* O157:H7 include an acid-induced oxidative system, an acid-induced arginine-dependent system and a glutamate-dependent system (Lin et al., 1996; Sheridan and McDowell, 1998). The acid- induced oxidative system is a system which is expressed in oxidatively metabolizing bacteria and is repressed by glucose. It is less effective in protecting *E. coli* O157:H7 against weak acids than the arginine and glutamate dependent systems. The functioning of this system requires the gene *rpoS* and it is induced by low pH (Lin et al., 1996). The acid-induced arginine dependent system is active in fermentatively metabolizing cells and offers protection against weak acids. Induction at low

pH requires anaerobisis for its expression (Bearson et al., 1997). The glutamate- dependent system is active in fermentatively metabolizing cells and offers protection against weak acids. In contrast to the arginine and oxidative systems, the glutamate- dependent system is not acid inducible (Bearson et al., 1997; Lin et al., 1996).

The acid tolerance response has been defined as a response resulting from a two-stage process which involves an initial adaptation to a pH range between 5 and 6 followed by an acid challenge at a pH below 4 (pH 2.5 -3.5) (Goodson and Rowbury, 1989; Sheridan and McDowell, 1998). The acid challenge selects for acid resistant cells as a result of the previous acid exposure. Acid-adapted cells can survive pH conditions of 2.5-3.5 while non- adapted cells cannot survive below pH 4 (Sheridan and McDowell, 1998). *E. coli* O157:H7 has been shown to modulate acid tolerance levels in response to changes in extracellular pH, increasing tolerance when the external pH is mildly acidic (Jordan et al., 1999).

The sustained acid tolerance of *E. coli* O157:H7 is not dependent on adaptation but is induced by stationary phase or starvation. This system is referred to as the pH independent acid survival system and is part of a general protective mechanism which is exhibited by stationary phase cells. As bacteria enter the stationary phase and finally cease to grow, morphological and genetic changes occur in order to prolong its survival and increase its resistance to numerous stresses. Entrance into stationary phase by *E. coli* results in the production of 30 - 50 proteins which are regulated by the transcription factor δ^S, which is encoded for by *rpoS*. Sustained acid tolerance in *E. coli* O157:H7 is therefore part of a general stress protection system triggered by its entrance into stationary phase

(Cheville et al., 1996). It is responsible for sustained acid resistance (AR) in *E. coli* O157:H7, which is defined as the survival of stationary phase cells at extremely low pH (Small et al., 1994). Stationary phase bacteria are 1,000 to 10,000 times more resistant to acid than exponentially growing cells and acid resistance does not require prior exposure to a low pH (Waterman and Small, 1996). The interruption of *rpoS* expression has been shown to significantly reduce the acid resistance of *E. coli* O157:H7 (Arnold and Kaspar, 1995; Cheville et al., 1996; Waterman and Small, 1996).

Outbreaks of *E. coli* O157:H7 infection involving acidic foods have drawn attention to the acid tolerance properties of the organism (Morgan et al., 1993; Besser et al., 1993; Zhao et al., 1993; Raghubeer et al., 1995). In addition to resulting in enhanced survival in food, acid tolerance may play an important role in the pathogenesis of *E. coli* O157:H7 by promoting its survival during passage through the stomach (Cheville et al., 1996).

Outbreaks of *E. coli* O157:H7 Infection

Following the identification of *E. coli* O157:H7 as the causative agent of human illness in 1982 in two separate outbreaks in Oregon and Michigan (Riley et al., 1983), numerous outbreaks associated with *E. coli* O157:H7 have been reported in Canada, the United States, Japan and the United Kingdom (Ackman et al., 1997; Besser et al., 1993; Cody et al., 1999; Griffin and Tauxe, 1991; Keene et al., 1997; Michino et al., 1999; Morgan et al., 1988; Ostroff et al., 1990; Ryan et al., 1986; Swerdlow et al., 1992; Swerdlow and Griffin, 1997; Thomas et al., 1996). In 1993 a multi-state outbreak in the United States which involved 700 people and 4 states resulted in a dramatic increase in

awareness and concern about food borne illness associated with *E. coli* O157:H7 (Bell et al., 1994). The largest reported outbreak occurred in 1996 in Sakai city, Japan with a total of 9578 cases and 11 deaths (Bettelheim, 1997; Michino et al., 1999; Watanabe et al., 1996). An outbreak in Lanarkshire, Scotland in the same year resulted in 20 deaths and 496 cases of food poisoning (Liddell, 1997). An outbreak in Walkerton, Ontario in 2000 associated with contaminated drinking water resulted in 167 confirmed cases of *E. coli* O157:H7 infection and 6 deaths (Bruce-Grey-Owen Sound Health Unit, 2000). Increased recognition of *E. coli* O157:H7 as a cause of human illness has resulted in increased surveillance for *E. coli* O157:H7 and identification of the organism worldwide.

Geographic Distribution

E. coli O157:H7 infections have been reported most frequently in Canada, the United States and the United Kingdom, although illnesses due to *E. coli* O157:H7 have been reported from over 30 countries on six continents (Boyce et al., 1995; Chapman, 1995; Doyle, 1991; Griffin and Tauxe, 1991; Mead and Griffin, 1998; Nataro and Kaper, 1998; Waters et al., 1994). *E. coli* O157:H7 is an important pathogen in Japan and Europe while in countries such as Australia, Argentina, Chile and South Africa, non-O157 serotypes of EHEC are a more frequent cause of HC and HUS (Bettelheim, 1998a; Nataro and Kaper, 1998).

Infections due to *E. coli* O157:H7 appear to be more common in Canada than in the United States (Boyce et al., 1995; Griffin and Tauxe, 1991). In the United States outbreaks appear to be more common in the northern states than in the southern states (Chapman, 2000; Griffin and Tauxe, 1991; Nataro and Kaper, 1998; Slutsker et al., 1997).

In Canada, *E. coli* O157:H7 infection has been reported more frequently from the western provinces (Griffin and Tauxe, 1991; Nataro and Kaper, 1998). The highest annual provincial rates of verocytotoxin-producing *E. coli* (VTEC) infection for the period between 1992 and 1995 were noted for Prince Edward Island (10.2 per 100,000), Manitoba (7.6 per 100,000) and Alberta (7.4 per 100,000) (Spika et al., 1998).

Rural versus Urban Environments

Michel et al. (1999) investigated the risk of VTEC infection associated with living in a rural as opposed to an urban area, by examining the geographic distribution of VTEC infections in Ontario, Canada, between the years of 1990 and 1995. Reported annual rates of VTEC infection were consistently higher in rural areas (5.4 per 100,000) as compared to urban areas (4.4 per 100,000). Spatial analyses revealed that cattle density was a significant indicator of VTEC infection in many regions of Ontario and it was suggested that an increased risk for VTEC infection existed among rural residents. The association of increased cattle density with increased risk of VTEC infection was also a likely explanation for the high incidence of VTEC infections in Prince Edward Island, where the cattle density (0.17 cattle ha $^{-1}$) approximates that of the regions of Ontario with high incidences of VTEC infection (0.2 cattle ha $^{-1}$) (Michel et al., 1999).

Seasonal Influence

Infections associated with *E. coli* O157:H7 appear to be more common during the summer months, with the majority of cases occurring during the months of May through September (Boyce et al., 1995; Chapman, 2000; Coia, 1998; Mead and Griffin, 1998;

Ostroff et al., 1989a; Pai et al., 1988; Slutsker et al., 1997; Waters et al., 1994). This seasonality parallels the increased prevalence of *E. coli* O157:H7 in cattle during the summer months (Boyce et al., 1995; Chapman et al., 1997b; Hancock, 1997; Hancock et al., 1994; Hancock et al., 1997a; Lynn et al., 1998; Mechie et al., 1997).

Incidence

In the United States it has been estimated that *E. coli* O157:H7 infection accounts for more than 20,000 cases of illness and result in as many as 250 deaths per year (Boyce et al., 1995; Coia, 1998). Results of the recently initiated Foodborne Disease Active Surveillance Network (Foodnet) by the CDC reported the incidence *E. coli* O157:H7 in the United States for 1996 to be 3 per 100,000 population (CDC, 1996). In 1999, a total of 38 outbreaks of *E. coli* O157:H7 infection were reported to the CDC. The outbreaks were reported from 80 states and affected 1897 people (CDC, 2001b). The Laboratory Center for Disease Control in Canada reported 1,334 cases associated with *E. coli* O157:H7 in 1995, which represents approximately 5 per 100,000 population (LCDC,1995). In the United Kingdom the highest rates of infection were reported in Scotland with 9.8 cases per 100,000 reported in 1996 (Coia, 1998). Lower rates of infection of 1.29 per 100,000 population were reported in England and Wales in 1996 (Coia, 1998).

Modes of Transmission

Food vehicles

The majority of outbreaks of *E. coli* O157:H7 infection have been traced to foods derived from cattle, with undercooked ground beef and raw milk frequently implicated (Bell

et al., 1994; Borczyk et al., 1987; Martin et al., 1986; Ostroff et al., 1990; Padhye and Doyle, 1992; Riley et al., 1983; Wells et al., 1983, and Willshaw et al., 1994). Investigations of the outbreaks in Oregon and Michigan in 1982, as well as the multi-state outbreak in the western United States in 1993, implicated contaminated ground beef as the vehicle of transmission (Bell et al., 1994; Riley et al., 1983; Wells et al., 1983). Wachsmuth et al. (1997) reported that ground beef has been implicated in more than 50% of the 75 outbreaks in the United States between 1982 and 1995 in which the vehicle of transmission was identified.

The fecal contamination of carcasses during slaughter and processing is likely the manner in which *E. coli* O157:H7 is transferred to beef (Cassin et al., 1998; Chapman et al., 1993b; Elder et al., 2000). The process of grinding the beef may further compound the problem by introducing the pathogen into the interior of ground meat patties where it is more likely to survive inadequate cooking (Boyce et al., 1995; Mead and Griffin, 1998). Ground beef often includes the meat from many carcasses and a few infected animals could potentially contaminate a large quantity of ground beef (Boyce et al., 1995).

Although contaminated and improperly cooked ground beef is the principle vehicle implicated in *E. coli* O157:H7 infections, numerous other foods have been linked to *E. coli* O157:H7 outbreaks. Among these are fresh-pressed apple cider (Besser et al., 1993), yoghurt (Morgan et al., 1993), jerky made from deer meat (Keene et al., 1997), potatoes (Chapman et al., 1997a; Morgan et al., 1988), unpasteurized milk (Borczyk et al., 1987; Chapman et al., 1993b ; Hancock et al., 1994; Wells et al., 1991), unpasteurized goat's milk (Bielaszewska et al., 1997), mayonnaise (Raghubeer et al., 1995; Weagent et al., 1994),

alfalfa sprouts (CDC, 1997), radish sprouts (Hara-Kudo et al., 1997; Itoh et al., 1998), lettuce (Ackers et al., 1998), turkey roll (Salmon et al., 1989), dry-cured salami (CDC, 1995), dry fermented sausage (Paton et al., 1996), and cantaloupe (Beuchat, 1996). In cases where non-bovine foods have been linked to *E. coli* O157:H7 outbreaks, cross-contamination as a result of contact with bovine products or contamination with feces of wild or domestic animals has often been suspected (Armstrong et al., 1996; Besser et al., 1993; Borczyk et al., 1987; Chapman et al., 1993a; Chapman et al., 1997b; Doyle, 1991; Hancock et al., 1994; Mead and Griffin, 1998).

The low concentrations of organisms found in foods responsible for *E. coli* O157:H7 associated outbreaks suggests that the infectious dose is low (Armstrong et al., 1996). In the 1982 outbreaks in Oregon and Michigan, fewer than 50 organisms per g were isolated from the frozen hamburger patty in the suspect lot in Michigan (Wells et al., 1983). In the large multi-state outbreak in the western United States in 1993, microbiological testing of ground beef from lots consumed by people who became ill suggested an infective dose of *E. coli* O157:H7 of fewer than 700 organisms and possibly as low as 100 organisms (Armstrong et al., 1996; Tuttle et al., 1999; Wachsmuth et al., 1997). Investigations of an outbreak associated with dry-cured salami provided an estimate of the infectious dose to be fewer than 50 organisms (CDC, 1997). In an outbreak in Connecticut in 1993 between 40 and 930 organisms per g were isolated from the implicated hamburgers (Roberts et al., 1995). A community outbreak in Wales in 1993 associated with beefburgers was used to estimate the presence of *E. coli* O157:H7 in the burgers. Less than 2 organisms per 25 g sample of meat caused illness, implying that the infectious dose of *E. coli* O157:H7 could

be as low as 10 organisms (Willshaw et al., 1994). The levels of contamination in the food samples incriminated in *E. coli* O157:H7 outbreaks support the hypothesis of a low infectious dose (Armstrong et al., 1996; Neill, 1989). Although the infectious dose is unknown it has been estimated to be 10 or less organisms, similar to the infectious dose of *Shigella* (Duncan and Hackney, 1994). The infectious dose of *E. coli* O157:H7 is also affected by variables such as stomach pH, food composition and host susceptibility (Cassin et al., 1998; Gannon, 1999).

Water

Outbreaks of *E. coli* O157:H7 infection have been associated with drinking water and swimming (Ackman et al., 1997; Brewster et al., 1994; Keene et al., 1994 Paunio et al., 1999; Swerdlow et al., 1992). In 1989 a large outbreak of *E. coli* O157:H7 infection occurred in Cabool, Missouri. This outbreak was associated with drinking water from an unchlorinated water supply (Swerdlow et al., 1992). The outbreak resulted in 243 cases of illness and 4 deaths. In three swimming- associated outbreaks epidemiological studies suggest that fresh-water lakes were the source of *E. coli* O157:H7 infection. In these outbreaks, illness was presumably due to the bather swallowing small amounts of contaminated lake water (Keene et al., 1994; Paunio et al., 1999; Swerdlow et al., 1992). Person-to-person transmission and contaminated water in a children's wading pool were linked to an outbreak in Scotland in 1994 (Brewster et al., 1994). A recent outbreak of *E. coli* O157:H7 infection was associated with contaminated municipal water in Walkerton, Ontario, and resulted in 167 confirmed cases of *E. coli* O157:H7 infection and 6 deaths (Bruce-Grey-Owen Sound Health Unit, 2000).

Person-to-person Transmission

During the large outbreak in 1993 in the United States due to beef burgers, approximately 11% of the cases were identified as secondary (Bell et al., 1994). In Wales, 17% of the identified cases of *E. coli* O157:H7 infection were the result of contact with an index case. The household transmission rate of *E. coli* O157:H7 infection in Wales between 1994 and 1996 was estimated to be 7%, with household contacts of children aged 1-4, and adults aged 15-34 years, most likely to contract infection from an index case patient (Parry and Palmer, 2000). This pattern of transmission suggests that person-to-person spread by the fecal-oral route is likely, as opposed to food borne transmission.

Person-to-person spread of *E. coli* O157:H7 has also been identified in day care centers as the primary means of transmission of infection (Swerdlow and Griffin, 1997). Person-to-person transmission has been identified in secondary cases of illness in a nursing home outbreak and has been implicated in an outbreak involving venison jerky (Keene et al., 1997; Ryan et al., 1986). It has been suggested that the spread of *E. coli* within families and in institutionalized settings may occur through means other than food borne transmission (Doyle, 1991; MacDonald et al., 1996).

Bovine-to-human Transmission

Three incidences of apparent direct transmission of *E. coli* O157:H7 from bovines to humans have been reported (Renwick et al., 1993; Rice et al., 1996; Synge et al., 1993). In 1992, in Ontario, Canada, a 13-month-old boy was hospitalized with hemorrhagic colitis following prolonged contact with calves on a dairy farm. The identical phage type of *E. coli* O157:H7 was isolated from the calf and the child, providing strong evidence that the calves

were the source of infection (Renwick et al., 1993).

In Scotland, in 1993, a 15-month-old child who lived adjacent to a farm became ill with *E. coli* O157:H7 (Synge et al., 1993). One of the 84 samples collected from dung pats of cattle as well as from dogs that roamed the farm yielded an isolate of the same phage type and unusual plasmid profile as the isolate from the child. It was suggested that the dogs may have carried the organism into the household, resulting in the infection of the child.

An additional case of *E. coli* O157:H7 infection acquired from livestock occurred in a 10-year-old boy who was actively involved in raising livestock (Rice et al., 1996). Upon comparison of the isolates from the boy, one of the cattle and one of the sheep, all had indistinguishable pulsed field gel electrophoresis (PFGE) patterns. Given the large diversity of subtypes of *E. coli* O157:H7 distinguishable by PFGE it has been suggested that infection due to direct livestock exposure was likely (Rice et al., 1996).

In Ontario, Canada in 1999, an outbreak of *E. coli* O157:H7 infection among visitors to a petting zoo resulted in 159 illnesses (Warshawsky et al., 1999). Direct contact with farm animals as a result of school visits to farms resulted in an outbreak of *E. coli* O157:H7 infection in Pennsylvania and Washington in the spring and fall of 2000 (CDC, 2001a). Children were allowed to touch cattle, calves, sheep, goats, llamas, chickens and pigs. Lack of appropriate handwashing prior to eating and eating while petting the animals may have been associated with infection (CDC, 2001a; Warshawsky et al., 1999). Contact with livestock should be considered when infections due to *E. coli* O157:H7 occur in humans.

Virulence Factors

Verocytotoxins

In 1977 Konowalchuk et al. reported the presence of a potent cytotoxin in the culture filtrates of a number of strains of *E. coli*. This cytotoxin was referred to as Vero toxin (VT). It was that was lethal to cultured Vero (African green monkey kidney) cells and was found to be quite distinct from the heat-labile and heat-stable enterotoxins of *E. coli* (Konowalchuk et al., 1977). Additional studies revealed a VT produced by *E. coli* O157:H7 was neutralized by antiserum against a very potent cytotoxin produced by *Shigella dysenteriae* type 1 (O'Brien et al., 1982; 1983). It was subsequently shown that VT represents a group of toxins which are structurally and functionally related to Shiga toxin of *S. dysenteriae* type 1. As a result, VT has also been called SLT and Shiga toxin (stx).

Two VTs produced by *E. coli* O157:H7 were subsequently reported by Scotland et al. (1985). The first toxin, which was neutralized by antiserum against Shiga toxin was named SLT-I or VT1. The second toxin which was not neutralized by antiserum against Shiga toxin was named SLT-II or VT2. It has been reported that VT2 is found predominantly in culture filtrates, while VT1 is found mainly in cell lysates (O'Brien and Holmes, 1987; Scotland et al., 1988). Isolates of *E. coli* O157:H7 may produce VT1, VT2 or both VT1 and VT2 (Nataro and Kaper, 1998; O'Brien and Holmes, 1987; Strockbine et al., 1986). It has been shown that most strains of *E. coli* O157:H7 produce VT2, which has been shown to be 1000 times more cytotoxic to human renal microvascular endothelial cells than VT1 (Law, 2000; Mead and Griffin, 1998; Ostroff et al., 1989b).

In addition to VT1 and VT2, four variants of VT2 have been identified. A variant

of VT2 (VT2v) has been isolated from verocytotoxin producing *E. coli* (VTEC) from edema disease in pigs (Marques et al., 1987; Weinstein et al., 1988). It is unusual in that it is cytotoxic to Vero cells but not HeLa cells, and the genes encoding the toxin are not carried by a bacteriophage (Marques et al., 1987). MacLeod et al. (1991) later reclassified this toxin a VT2e. Another VT2-variant was found to be expressed by *E. coli* O91:H21 isolated from a human patient with HUS (Oku et al., 1989). Like VT2e (previously VT2v), this toxin is not cytotoxic to HeLa cells and the terms VT2-variant-human (VT2vh) and VT2-variant-porcine (VT2vp) were introduced to differentiate between these two variant forms of VT2. The variants VT2vh and VT2vp have been shown to be antigenically different from VT2 and have a low level of homology with the VT2 B subunit gene. The genes that encode VT2vp were shown to have 91% overall nucleotide homology with VT2 and shared 93% and 84% amino acid homology in the A- and B- subunits, respectively (Itoh et al., 1991; Weinstein et al., 1988). Two other VT2 variants have been identified, VT2c and VT2d (Chart, 2000). They resemble VT2 more closely than VT2vp, sharing 97% and 99% amino acid homology in the A- and B- subunits, respectively.

All members of the Shiga-toxin family are compound toxins possessing the basic A-B subunit structure (Nataro and Kaper, 1998; O'Brien and Holmes, 1987). The A subunit contains the enzymatically active molecule and the B subunit binds the entire toxin to the target eucaryotic cell (Tarr, 1995). The A subunit is cleaved to A_1 and A_2 fragments which remain linked by a disulfide bond. The A_1 fragment contains the enzymatic activity while the A_2 subunit links the A subunit to the 5 B subunits (Nataro and Kaper, 1998). Both VT1 and VT2 are composed of a single A subunit and 5 B subunits and are encoded on

bacteriophage genomes located on the chromosome of *E. coli* O157:H7 (Mead and Griffin, 1998; Strockbine et al., 1986).

The molecular weights of the A and B subunits in VT1 and VT2 have been reported as being 31,000 - 33,135 and 5,000 - 8,000 Daltons, respectively (Coia, 1998; Doyle, 1991; Nataro and Kaper, 1998; O'Brien et al., 1983; O'Brien and Holmes, 1987). VT1 is structurally and biochemically similar to the Shiga toxin of *S. dysenteriae*, from which it differs by one amino acid. While the amino acid sequence of VT1 is highly conserved, sequence variation occurs in VT2 variants (Nataro and Kaper, 1998). VT1 and VT2 share approximately 56% amino acid homology and 58% nucleotide homology (Tarr, 1995).

The proposed mode of action of the toxin commences with the B subunit of the toxin binding to a specific cell surface receptor, globotriaosylceramide (Gb_3) allowing for its internalization via endocytosis (Doyle, 1991;Nataro and Kaper, 1998; Neill, 1989). The A subunit is translocated to the cytoplasm where the A_1 fragment catalyzes the enzyme inactivation of 28S rRNA of the 60S ribosomal subunit resulting in inhibition of protein synthesis and cell death (Doyle, 1991; Nataro and Kaper, 1998; Tarr, 1995).

The receptor for VT1 and VT2, Gb3-expressing endothelial cells, are present in the colon, the central nervous system, the pancreas, renal glomerular microvasculature, and renal tissue cells (Schmidt et al., 1995). The resultant disruption of protein synthesis leads to the death of any cell which possesses the Gb3 receptor (Nataro and Kaper, 1998). Damage to renal endothelial cells by this process is probably the primary phenomenon resulting in HUS (Coia, 1998; Nataro and Kaper, 1998; Padhye and Doyle, 1992). Cattle

do not have vascular receptors for Gb_3 (Pruimboon-Brees et al., 2000) and are not affected clinically by the presence of *E. coli* O157:H7.

A 60 MDa Plasmid (pO157)

An additional factor which is thought to contribute to the virulence of *E. coli* O157:H7 is a 60-MDa plasmid. This large plasmid of approximately 90 kb is referred to as pO157and has been reported to be present in the majority of EHEC (Levine et al., 1987). Several conflicting observations have been made in regards to the adherence properties of *E. coli* O157:H7 and the role of the 60-MDa plasmid in imparting an adhesin to the bacterium (Junkins and Doyle, 1992; Karch et al., 1987; Sherman et al., 1987; Tzipori et al., 1989). The recent sequencing of the 60-MDa pO157 plasmid did not identify *bfp* and *AAFI* gene clusters which are encoded on the large plasmids of EPEC implying that a fimbrial adhesin is likely chromosomally encoded as opposed to being encoded on pO157 (Paton and Paton, 1998; Wieler et al., 1996).

The 60-MDa plasmid also contains the gene *ehxA* encoding for enterohemolysin (Nataro and Kaper, 1998). Enterohemolysin (Ehx) is a newly discovered *E. coli* hemolysin which is genetically related but not identical to *E. coli* α-hemolysin (*HlyA*) (Bauer and Welch, 1996; Schmidt et al., 1995). *E. coli* expresses two main types of hemolysin, α-hemolysin which is synthesized and exported from the cell during bacterial growth , and β-hemolysin which is released upon cell death (Smith, 1963). In contrast to α-hemolysin, enterohemolysin can only be detected only on blood plates containing washed erythrocytes (Beutin et al., 1989).

Enterohemolysin (Ehx) has been characterized as an RTX (repeats in toxin) toxin

due to its demonstrated similarities to the RTX family of toxins. These similarities include conserved sequence features in proteins (13 tandem repeats at the C-terminus), the presence of a stretch of approximately 200 hydrophobic amino acids in the N-terminal region, and activity which is calcium dependent and heat labile (Bauer and Welch, 1996; Nataro and Kaper, 1998; Schmidt et al., 1995). Members of this family of secreted bacterial cytotoxins are produced from a wide variety of gram-negative human and animal pathogens and include broadly acting hemolysins and narrowly acting leukotoxins, which lyse leucocytes from only certain species (Bauer and Welch, 1996).

There seems to be an association between verotoxin production and the production of enterohemolysin, since enterohemolysin has been found in 93.8% of verotoxin producing strains of *E. coli* (Beutin et al., 1989). Approximately 94% - 100% of *E. coli* strains of serotype O157 tested were found to produce an enterohemolysin (Beutin et al., 1989; Nataro and Kaper, 1998; Schmidt et al., 1995). It has been reported that the incidence of enterohemolysin in non-O157 groups, although present, is significantly lower than that in O157 isolates (Beutin et al., 1989; Law and Kelly, 1995; Nataro and Kaper, 1998).

The significance of enterohemolysin as a true virulence factor is not known. It has been suggested that since the growth of *E. coli* O157:H7 is enhanced by the presence of heme and hemoglobin, lysis of erythrocytes by the enterohemolysin would release these sources of iron and aid in infection (Law and Kelly, 1995; Nataro and Kaper, 1998). It has been suggested that the virulence of *E. coli* O157:H7 is a multifactorial phenomenon and it is likely that verocytotoxins, intimin and enterohemolysin act synergistically to disturb important cell functions and subsequently cause infection (Meng et al., 1998;Schmidt et al.,

1995). The frequent association of enterohemolysin with *E. coli* O157:H7 make it useful as a marker for the detection of *E. coli* O157:H7 and is suggestive of the possible role of enterohemolysin in bacterial virulence (Beutin et al., 1989; Law and Kelly, 1995; Schmidt et al., 1995).

Locus of Enterocyte Effacement (LEE)

The LEE is a 35-kbp chromosomal region which encodes the virulence factors for attaching and effacing (AE) lesions (McDaniel et al., 1995). This large chromosomal locus is a feature which is common to both EHEC and EPEC. The LEE locus contains the *eaeA* (*E. coli* attaching and effacing) gene which encodes a 94-kDa outer membrane protein, intimin, which is necessary to produce actin polymerization and localized recruitment of F-actin to the site of bacterial attachment in the formation of AE lesions (Kaper, 1994; Louie et al., 1993). Four different types of intimin, designated α, β, γ and δ have been identified by Adu-Bobie et al., 1998. The gene coding for intimin γ has been detected in *E. coli* O157:H7 serotypes by PCR (Adu-Bobie et al., 1998).

A 78 kDa receptor for intimin, termed translocated intimin receptor or Tir is also encoded for by LEE (DeVinney et al., 1999). This receptor is synthesized and translocated into the host epithelial cells. The bacteria themselves, therefore, express both the attachment protein, intimin, and the receptor protein, Tir. Tyrosine phosphorylation after insertion of Tir into the epithelial cell membrane increases its apparent molecular mass to 90 kDa in EPEC, but not in EHEC. The interaction of Tir with EPEC intimin triggers actin nucleation beneath the adherent bacteria but the accumulation of tyrosine phosphorylated proteins beneath adherent EHEC does not occur (Kenny, 1999). EHEC

Tir and EPEC Tir both bind intimin and induce extensive cytoskeletal rearrangements within the host membrane, indicating that tyrosine phosphorylation is not required for pedestal formation (DeVinney et al., 1999; Kenny, 1999; Paton et al., 1998).

LEE includes a cluster of genes (*sepA* through *sepI*) which encodes a type III secretion system, with the *sepB* gene being involved in the translocation of at least five proteins involved in AE formation (McDaniel et al.,1995). This system represents a transport apparatus that is responsible for the secretion of other LEE encoded proteins, including EspA, EspB, and EspD, which are required for signal transduction in host cells and for the formation of AE lesions (Jarvis and Kaper, 1996; Perna et al., 1998). EspA forms filamentous structures which act as primary adhesins and form the delivery apparatus for the type III secretion system. By means of the EspA filaments, other secreted proteins, EspB, EspD and Tir may be delivered to the host cell (Chart, 2000; Jarvis and Kaper, 1996; Paton and Paton, 1998).

Although EHEC and EPEC are organisms which cause two distinctly different diseases, they both share the ability to elicit AE lesions on intestinal epithelial cells (Levine, 1987; Moon et al., 1983). All the genes necessary for the formation of AE lesions are present on LEE. Primary adhesion of *E. coli* O157:H7 with the host cell likely occurs by the formation of EspA filaments, which involves the contact-dependent type III secretion system which forms part of the signal-transduction apparatus for AE lesions. The proteins EspB, EspD and Tir are translocated to the surface of the host cell by means of these filaments, where Tir inserts into the host cell membrane. Subsequent attachment of *E. coli* O157:H7 occurs as a result of the binding of the bacterial outer membrane protein, intimin,

a product of the *eaeA* gene, and Tir. This complex and unique mechanism results in intimate attachment of the bacteria to the large intestine (Kenny et al., 1997; Potter and Finlay, 2000). The AE lesions are characterized by localized destruction of brush border microvilli, followed by intimate attachment of the bacteria to apical enterocyte membranes and accumulation of polymerized actin beneath the adherent bacteria (Knutton et al., 1989).

While adhesion of *E. coli* O157:H7 to intestinal cells may be an important aspect of the organism's pathogenic potential, there is no direct evidence from human cases to assess the nature of intestinal colonization by *E. coli* O157:H7 (Doyle, 1991; Karmali, 1989; Ryan et al., 1986). Infection with *E. coli* O157:H7 results in little or no fever which is suggestive of a non-invasive organism which does not enter the circulatory system (Riley et al., 1983). Mathews et al. (1997) reported that *E. coli* O157:H7 was able to invade RPMI-4788, but not HeLa, human cell lines. Colonization of the human large intestine by *E. coli* O157:H7 has not been clearly demonstrated (Heuvelink et al., 1995; Riley, 1987).

Several animal models have been developed to demonstrate the pathological features of *E. coli* O157:H7 infection (Bryant et al., 1989; Dean-Nystrom et al., 1998; Francis et al., 1986; Moon et al., 1983; Pai et al., 1986; Sherman et al., 1988; Tzipori et al., 1986). In animal models *E. coli* O157:H7 has been shown to colonize the terminal ileum, cecum and colon of gnotobiotic piglets and neonatal calves by the AE (Dean-Nystrom et al., 1997; Francis et al., 1986; Tzipori et al., 1986;1987). Although many of these animal models indicate the susceptibility of rabbits, piglet and calves to the effects of verocytotoxin-producing *E. coli*, they do not produce all of the manifestations associated with HC and HUS in humans (Scotland et al., 1990).

Sources of *E. coli* O157:H7

Cattle as a Reservoir of *E. coli* O157:H7.

Since the first two outbreaks of *E. coli* O157:H7 infection in 1982, which were associated with consumption of hamburger patties from a fast-food restaurant, a bovine source of *E. coli* O157:H7 has been implicated (Riley et al., 1983; Wells et al., 1983). Further evidence suggesting cattle as a reservoir resulted following a large outbreak of *E. coli* O157:H7 infection among kindergarten children in south-western Ontario in 1996 (Borczyk et al., 1987; Orskov et al., 1987). An epidemiological investigation following the outbreak revealed a significant association between infection and the consumption of unpasteurized milk. Later, *E. coli* O157:H7 was isolated from seven of the cattle on the dairy farm. These findings provided direct evidence that cattle may be a primary reservoir of *E. coli* O157:H7 involved in human disease.

The association of *E. coli* O157:H7 with undercooked hamburgers and unpasteurized milk led to further investigations as to the role of cattle as a reservoir of *E. coli* O157:H7 (Buchanan and Doyle, 1997; Griffin and Tauxe, 1991). Numerous studies have reported isolation of *E. coli* O157:H7 from healthy beef and dairy cattle, showing that cattle are asymptomatic carriers of the organism (Borczyk et al., 1987; Chapman et al., 1993a; Chapman et al., 1997b; Hancock et al., 1994; Mechie et al., 1997; Montenegro et al.,1990; Wells et al., 1991; Zhao et al., 1995).

Initial studies showed a low prevalence of *E. coli* O157:H7 in the feces of cattle (Faith et al., 1996; Hancock et al., 1994). For example, in one study the prevalence of *E. coli* O157:H7 was reported to be 0.28% in dairy cattle and 0.71% in beef cattle with a herd

prevalence of 8.3% and 16% respectively (Hancock et al., 1994). Recent studies have reported much higher levels. Chapman et al. (1997a) isolated *E. coli* O157:H7 from the feces of 15.7% (752/4800) of cattle over a 1-year period. The monthly prevalence ranged from 4.8 - 36.8%, with the highest prevalence occurring in the spring and late summer (Chapman et al., 1997b). Van Donkersgoed et al. (1999) isolated *E. coli* O157:H7 from 7.5% of fecal samples collected from cattle at slaughter, with the prevalence of *E. coli* O157:H7 in the fecal samples of yearling cattle (12.4%) being higher as compared to cull cows (2.0%). Recently, Elder et al. (2000) reported a prevalence of *E. coli* O157:H7 of 28% (91/327), from fecal samples taken from slaughter cattle during July and August. In surveys of feedlots in the United States, *E. coli* O157:H7 was isolated from 63 to 100 % of feedlots, indicating widespread distribution of *E. coli* O157:H7 in cattle operations (Dargatz et al., 1997; Elder et al., 2000; Hancock et al., 1997b,c).

The incidence of *E. coli* O157:H7 has been reported to be higher in weaned calves (4.8- 5.3%) and heifers (1.8-3.0%) than pre-weaned calves (0.9-2.9%) and adult cattle (0.15 - 0.4%)(Garber et al., 1995; Hancock et al., 1997a; Wells et al., 1991; Zhao et al., 1995). A recent study conducted by Laegreid et al.(1999) on the prevalence of *E. coli* O157:H7 in beef calves before weaning found that 87% of 15 herds and an average of 7.4% of individual animals were culture positive for *E. coli* O157:H7. This suggests that calves are infected with *E. coli* O157:H7 prior to weaning and before their entry into feedlots (Laegreid et al., 1999).

The reason for the differences in the prevalence of *E. coli* O157:H7 in young animals and adults may be attributed to age-related differences in rumen function, the stress

of weaning, dietary changes, transport, sorting and mixing, immune response and management factors (Garber et al., 1995; Heuvelink et al., 1998; Laegreid et al., 1999; Wells et al., 1991). Alternatively, it has been suggested that a large number of calves may be infected prior to weaning through direct or indirect maternal transmission and through contaminated water (Laegreid et al., 1999).

E. coli O157:H7 is not pathogenic in cattle and therefore fecal shedding of *E. coli* O157:H7 occurs in clinically normal cattle (Hancock et al., 1994; Montenegro et al., 1990; Wells et al., 1991). The fecal shedding of *E. coli* O157:H7 in cattle has been reported to be intermittent in nature, characterized by short periods with a relatively high prevalence of excretion separated by longer periods of reduced or undetectable shedding (Besser et al., 1997; Dargatz et al., 1997; Garber et al., 1995; Hancock et al., 1997a; Wells et al., 1991; Zhao et al., 1995).

Levels of *E. coli* O157:H7 shed by cattle have been reported to range from 10^2 to $> 10^6$ CFU/g (Gansheroff and O'Brien, 2000; Zhao et al., 1995). There is a tendency for *E. coli* O157:H7 shedding in cattle to be seasonal in nature, with peak occurrences between the months of May and September (Hancock et al., 1997a; Hancock et al., 1994; Mechie et al., 1997; Van Donkersgoed et al., 1999). This seasonal pattern may contribute to the similar seasonal pattern of *E. coli* O157:H7 associated food borne illness in humans (Ostroff et al., 1989a).

It has been suggested that the hindgut of cattle is the site of *E. coli* O157:H7 proliferation and represents a source for fecal shedding of the organism (Brown et al., 1997; Whipp et al., 1994; Rasmussen et al., 1999). Support for this theory comes from numerous

studies which have found no association between populations of *E. coli* O157:H7 in the rumen and fecal shedding of the organism (Harmon et al., 1999; Tkalcic et al., 2000). Rasmussen et al. (1999) have also reported that conditions in the colon and cecum likely have a more direct influence on the shedding of *E. coli* in the feces than conditions in the rumen.

Potter and Finlay (2000) have reported that *E. coli* O157:H7 strains are able to attach to bovine intestinal epithelial cells of cattle using a unique mechanism which is mediated by a number of proteins, including Tir, intimin, EspA and EspB. Colonic and rectal mucosal explants from steers inoculated with *E. coli* O157:H7 developed AE lesions at the sites of bacterial attachment (Baehler and Moxley, 2000). This provides evidence that the mucosal epithelium of the large intestine may be the site of infection that contributes to the carriage of *E. coli* O157:H7 in cattle. Mathews et al. (1997) have shown that *E. coli* O157:H7 is able to enter bovine mammary epithelial cells (MAC-T) and bovine kidney cells (MDBK), demonstrating localized adherence similar to that observed for EPEC. Phillips et al. (2000) reported that the formation of AE lesions in human and bovine mucosa *in vitro* was restricted to the follicle- associated epithelium of Peyer's patches. This further suggests that attachment may be important in the asymptomatic carriage of *E. coli* O157:H7 in cattle and may provide a reservoir for the fecal shedding of the organism by cattle.

In calves and adult cattle, experimentally inoculated with 10^{10} CFU *E. coli* O157:H7, there was a wide variation among individuals in the magnitude and duration of *E. coli* O157:H7 fecal shedding (Brown et al., 1997; Cray and Moon, 1995). Calves shed *E. coli* O157:H7 in their feces in greater numbers and for a longer period of time than adult

cattle. Shedding of *E. coli* O157:H7 among individual animals was observed for up to 100 days in adult cattle and 144 days in calves (Cray and Moon, 1995). There was no difference observed in the pattern of *E. coli* O157:H7 fecal shedding following re-inoculation indicating that previous infection does not prevent re-infection (Cray and Moon, 1995).

Other Animal Sources of *E. coli* O157:H7

Although epidemiological investigations in animal populations have concentrated on the bovine reservoir of *E. coli* O157:H7, other animal hosts have also been identified (Beutin et al., 1996). Chapman et al. (1997a) reported isolation of *E. coli* O157:H7 from 2.2 % of sheep and 0.4 % of pigs at slaughter, but not from 1000 chickens (Chapman et al., 1997b). Other studies have reported the prevalence of *E. coli* O157:H7 in sheep to vary from between 0 and 31%, indicating that sheep may have a role as important as cattle as reservoirs of *E. coli* O157:H7 (Chapman et al., 1996a, b, c; Fegan and Desmarchelier, 1999; Heuvelink et al., 1998; Kudva et al., 1996).

Trevena et al. (1996) suggested that horses and dogs may be vectors of *E. coli* O157:H7 due to the close contact between farm animals, companion animals and humans. *E. coli* O157:H7 has been detected in 1.1% of horses and 3.1% of dogs (Hancock et al., 1998). *E. coli* O157:H7 has been isolated from wild deer and the feces of wild birds (Hancock et al., 1998; Keene et al., 1997; Rice et al., 1995; Wallace et al., 1997). This suggests that there is the possibility of the transfer of *E. coli* O157:H7 between animals and birds that share the same habitat as cattle.

Prevalence of *E. coli* O157:H7 among cattle is highly variable suggesting the possibility of a reservoir of *E. coli* O157:H7 external to cattle (Hancock et al., 1998). It

has been implied that the intermittent and seasonal nature of *E. coli* O157:H7 shedding in cattle supports the possibility that cattle may be a vehicle for the transmission of the organism as opposed to a reservoir where the organism is maintained for a long period of time (Hancock et al., 1998). Reports on the prevalence of *E. coli* O157:H7 in other animals and birds indicate that animal reservoirs of *E. coli* O157:H7 are not restricted to bovines. It is possible that *E. coli* O157:H7 has not been detected in other animals because they were less likely to be examined for the organism. Sampling of other potential animal sources of *E. coli* O157:H7 more frequently would allow the prevalence of the organism in animals other than cattle to be established. It is likely that *E. coli* O157:H7 has multiple reservoir species, including cattle. Survival of *E. coli* O157:H7 in the environment may also result in the organism being transmitted back to cattle.

The Animal Environment

Johnson et al. (1999) noted that there are many possible habitats for *E. coli* O157:H7 in the farm environment including; manure heaps, ponds, dams and wells, barns calf hutches, straw and other bedding, feed and feed troughs, water and water troughs, farm equipment, ground and pasture, and watercourses. Once present in the environment, the organism can be transferred to other sites by rainwater, wind, removal and spreading of manure, and by animals and humans.

Cattle Feed. Numerous studies have failed to isolate *E. coli* O157:H7 from cattle feed or feed manger swabs (Hancock et al., 1998; Lynn et al., 1998; Rahn et al., 1997). However, Shere et al. (1998) isolated *E. coli* O157:H7 from 3 of 32 (6.3%) feed samples obtained from a dairy farm in Wisconsin. Growth of *E. coli* O157:H7 has been

demonstrated in a variety of cattle feeds *in vitro* at temperatures similar to those which occur on a farm during the summer months (Lynn et al., 1998).

Growth of *E. coli* O157:H7 was reported in poorly fermented laboratory silage (Fenlon and Wilson, 2000). Two strains of *E. coli* O157:H7, inoculated onto grass and subsequently ensiled under conditions allowing for aerobic spoilage, increased from 10^3 CFU/g to 10^7 CFU/g over a 13 day period at 20 °C. A poor ensiling technique which resulted in delayed fermentation created an environment conducive to the growth of *E. coli* O157:H7. Fecal contamination of grass due to manure application followed by inadequate ensiling may therefore be a significant factor in the persistence of *E. coli* O157:H7 carriage among cattle (Fenlon et al., 2000; Fenlon and Wilson, 2000).

A negative correlation exists between the concentration of acetate and propionate in cattle feeds and the replication of *E. coli* O157:H7. Thus, the routine use of propionic acid as an additive in cattle feeds may reduce exposure of cattle to *E. coli* O157:H7 (Lynn et al., 1998). The seasonal increase in fecal shedding of *E. coli* O157:H7 implies that environmental replication may play a key role in the ecology of *E. coli* O157:H7 in the farm environment. Since it has been shown that *E. coli* O157:H7 can replicate in a variety of feeds, amplification of low levels of *E. coli* O157:H7 may increase the possibility of further dissemination of the organism on the farm.

Animal Drinking Water. Several studies have isolated *E. coli* O157:H7 from water troughs and shallow wells (Faith et al., 1996; Hancock et al., 1998; Jackson et al., 1998; MacDonald et al., 1996; Wilson et al., 1997). The organism has been found to persist in water trough sediments for periods of at least 4 months and may even multiply in this

environment (Hancock, 1997).

A study conducted by Rice and Johnson (2000) used cattle drinking water from two dairy farms in order to assess the survival of *E. coli* O157:H7 and *E. coli* Biotype 1. The survival of *E. coli* Biotype 1, which was present in the manure used as the inoculum, was examined as an indicator of fecal contamination. The pathogen *E. coli* O157:H7 was detected at 5 and 15 °C for 16 days when the water was inoculated with 5×10^3 organisms/ml. In the cattle drinking water inoculated with 1.7×10^2 organisms/ml, *E. coli* O157:H7 persisted for 8 and 4 days at 5 and 15 °C, respectively. The *E. coli* Biotype 1was present in the animal drinking water when *E. coli* O157:H7 was present, indicating that detection of this fecal indicator organism may be of value in monitoring cattle drinking water (Rice and Johnson, 2000).

Under laboratory conditions, *E. coli* O157:H7 has been shown to survive in lake water for up to 35 days at 5 °C and 20 °C (Swerdlow et al., 1992). A study by Wang and Doyle (1998) conducted over a 91 day period found that the survival of *E. coli* in water *in vitro* was greater at 8 °C than at 25 °C. Results of the study also suggest that *E. coli* O157:H7 can enter a viable but nonculturable (VBNC) state in water when encountering environmental and nutritional stresses and thus may be undetected by common laboratory methods but responsible for waterborne outbreaks (McKay, 1992; Wang and Doyle, 1998). This further suggests that contaminated water troughs may be a mechanism by which *E. coli* O157:H7 may be maintained or disseminated within a herd (Faith et al., 1996; Hancock et al., 1998; Shere et al., 1998; Wang and Doyle, 1998)

A recent study has provided evidence that the means by which animal drinking

water becomes contaminated is oral rather than fecal. The presence of *E. coli* O157:H7 was detected in animal drinking water obtained from covered water tanks which had ball-water ports. Since the animal had to physically depress the ball in the port in order to receive water, fecal contamination of this type of water system was unlikely (Shere et al., 1998). *E. coli* O157:H7 has been recovered from the tonsils of cattle which further supports the possibility of oral contamination of animal drinking water (Cray and Moon, 1995). The presence of *E. coli* O157:H7 in the water and its subsequent recovery from fecal samples of cattle suggests cattle may become infected from drinking water (Shere et al., 1998).

Van Donkersgoed (2000) isolated *E. coli* O157:H7 from water troughs in feedlots in addition to incoming water sources. Different subtypes of *E. coli* O157:H7 were isolated from the source water, water troughs and feces of cattle, suggesting a source of *E. coli* O157:H7 other than cattle feces contaminating the water. In addition to this, the isolation of similar subtypes among feed, water and feces implied the transmission of the organism among these sources (Van Donkersgoed, 2000).

Manure. There have been conflicting reports in regards to the association between the application of manure to grazing land and the prevalence of *E. coli* O157:H7 in cattle. A tentative association was found between the application of slurry to grazing land and herd *E. coli* O157:H7 infection status (Hancock et al., 1994). However, subsequent studies reported no association between manure application to pastures or cattle forage crops and *E. coli* O157:H7 fecal shedding by cattle (Garber et al., 1995; Hancock et al., 1997b). A recent study conducted by Bolton et al. (1999) indicated that current waste management practices such as spreading manure on pastureland may increase the carriage rate of *E. coli*

O157:H7 in herds.

The survival of *E. coli* O157:H7 in manure and manure slurry has been observed under various experimental and environmental conditions. Kudva et al. (1998) found that *E. coli* O157:H7 from experimentally inoculated sheep survived for 21 months in manure incubated under environmental conditions. In aerated ovine and bovine manure piles, the organism survived for 4 months and 47 days respectively. In bovine feces with an initial inoculum of 10^5 CFU/g, the survival times of *E. coli* O157:H7 at 5, 22 and 37 °C were 70, 56, and 49 days respectively (Wang et al., 1996). Fukushima et al. (1999) found that *E. coli* O157:H7 survived in bovine feces for 2 to 14 weeks at 5 °C, 1 to 18 weeks at 15 °C, and 3 to 12 weeks at 25 °C, when inoculated with 10^3 CFU/g. Kudva et al. (1998) reported that the survival time of *E. coli* O157:H7 in bovine manure frozen at - 20 °C was at least 100 days (Kudva et al., 1998).

It has been reported that *E. coli* O157:H7 survives for shorter periods of time in manure slurry than in manure. Kudva et al. (1998) showed that levels of *E. coli* O157:H7 in bovine slurry declined from 10^7 CFU/ml to undetectable at 23 and 27 °C within five days. Enhanced survival was found at 4 °C, as levels of 10^5 CFU/ml were present after 28 days. Maule (2000) reported that *E. coli* O157:H7 inoculated into cattle feces and incubated at 18 °C remained detectable for more than 50 days, while in cattle slurry, levels declined from 10^8 CFU/ml to undetectable levels in 10 days.

Contamination with bovine manure has been suggested in several cases of human infection associated with non-bovine food products. Vegetables that were inadequately washed were implicated as the source of *E. coli* O157:H7 infection following isolation of

the pathogen from a garden which was fertilized with manure (Cieslak et al., 1993). The use of manure as fertilizer could explain food borne outbreaks of *E. coli* O157:H7 associated with unpasteurized apple cider, potatoes, radish sprouts and lettuce (Ackers et al., 1998; Besser et al., 1993; Chapman et al., 1997a; Itoh et al., 1998; Morgan et al., 1988). Manure may be a risk factor for transmitting *E. coli* O157:H7 to produce grown on manure-fertilized soil and may serve as a source for the maintenance of the *E. coli* O157:H7 carriage status among cattle herds (Chapman et al., 1997a; Chart, 1998).

Survival of *E. coli* O157:H7 in manure and manure slurry for extended periods of time emphasizes the importance of proper manure management in preventing the spread of the organism to the environment, food and animal crops, and back to cattle.

Soil. Bolton et al. (1999) reported a decline in the numbers of *E. coli* O157:H7 in bovine feces deposited directly on grassland, however, there was a lack of a correlation with counts in bovine feces present in sealed containers placed on the grassland. This may have indicated that a reduction in numbers was due to the organism being washed out of the fecal material as opposed to cell death (Bolton et al., 1999). This was supported by the detection of the organism in the surrounding soil for up to 99 days after the inoculated bovine feces had assimilated into the soil (50 days).

Fenlon et al. (2000) reported that *E. coli* O157:H7 survived in loam and clay soils for a period of 25 weeks and sandy soil for a period of 8 weeks when inoculated with *E. coli* O157:H7 at a level of 10^6 CFU/g of soil. In arable and grass plots inoculated with dairy cattle slurry at an application rate of 660 CFU/m^2, *E. coli* O157:H7 was detected in the top 2.5 cm of the soil and on the grass for the first week after application. Approximately 2%

of the initial inoculum of *E. coli* O157:H7 was transported to the deeper layers of the soil. Transport to drains was mainly associated with rainfall which occurred three and seven days after slurry application, leading to a cumulative loss of 7% of the applied *E. coli* O157:H7 (Fenlon et al., 2000).

Maule (2000) used model systems to determine the persistence of *E. coli* O157:H7 in soil cores. Soil cores containing rooted grass resulted in the greatest survival of the organism, with viable numbers declining from 10^{8} CFU/g soil to between 10^{6} and 10^{7} CFU/g soil after 130 days at 18 °C (Maule, 2000).

Tillage practice, soil type and method of pathogen delivery were found to affect vertical transport of *E. coli* O157:H7 in soil in a study conducted by Gagliardi and Karns (2000). Steady rainfall was applied to soil cores which were treated with manure inoculated with *E. coli* O157:H7. The presence of manure enhanced the survival of *E. coli* O157:H7 in intact (no-till) soils as opposed to disturbed (tilled) soils. High levels of recovery and low leachate levels of *E. coli* O157:H7 from clay loam soils, as opposed to silt loam or sandy loam soils, indicated that growth of *E. coli* O157:H7 could occur if there were minimal leaching losses. Results indicated that if soil pores do not become clogged, the organism could travel vertically through the soil for more than two months after initial application resulting in contamination of food and water (Gagliardi and Karns, 2000).

Barker et al. (1999) found that *E. coli* O157:H7 was able to survive and replicate in *Acanthamoeba polyphaga*, a common environmental protozoa. The authors suggested that a dynamic and mutually beneficial interaction between *E. coli* O157:H7 and *A. polyphaga* existed in laboratory microcosms. In some instances, *E. coli* O157:H7 was

digested as a food source by the protozoa, but in others the bacteria multiplied. Since protozoa are widely distributed in soil, they may represent an important environmental reservoir of *E. coli* O157:H7 (Barker et al., 1999).

Flies. The increased presence of flies around cattle during the summer months represents a potential mechanism for the spread of *E. coli* O157:H7 among animals. A recent study was conducted on the potential of transmission of *E. coli* O157:H7 to fresh-cut apple tissues by fruit flies (Janisiewicz et al., 1999). Following contact with *E. coli*, the fruit flies were contaminated both externally and internally with the bacterium. The flies then transmitted the bacterium to uncontaminated apple wounds and increased the level of contamination (Janisiewicz et al., 1999).

Sasaki et al. (2000) detected *E. coli* O157:H7 in the crop of houseflies (*Musca domestica*) at a level of 5×10^6 CFU, immediately after bacterial feeding. Subsequently, a drop of excretion contained $> 10^4$ bacteria 1 h or 1.8×10^4 bacteria 3 h after feeding, with a decrease in numbers of *E. coli* O157:H7 excreted over the next 24 h. Houseflies appear to be effective vectors of *E. coli* O157:H7 especially immediately after feeding on bovine feces or contaminated water (Sasaki et al., 2000).

E. coli O157:H7 has been isolated from flies collected on dairy farms and feedlots (Hancock et al., 1998; Rahn et al., 1997; Shere et al., 1998). The potential exists for the dispersion of *E. coli* O157:H7 in the environment and to humans by contaminated flies.

Diet and *E. coli* O157:H7 Fecal Shedding by Cattle

High Concentrate versus High Roughage Diets

Several studies have found an association between feed and the fecal shedding of *E. coli* O157:H7 in ruminants (Diez-Gonzalez et al., 1998; Hovde et al., 1999; Kudva et al., 1995; Kudva et al., 1997), however, other studies have found no association (Magnuson et al., 2000; Tkalcic et al, 2000).

The effect of diet quality on the fecal shedding of *E. coli* O157:H7 was investigated using experimentally inoculated sheep as the ruminant model. Differences in diet quality resulted in distinctively different patterns of fecal shedding in sheep (Kudva et al., 1995; Kudva et al., 1997). Sheep that were fed a diet which was high in fiber and low in nutrients (grass hay) shed *E. coli* O157:H7 in larger numbers and for longer periods of time than sheep that were fed a diet low in fiber and high in nutrients (corn and pelleted alfalfa)(Kudva et al., 1997). The average number of days of *E. coli* O157:H7 fecal shedding was 8-15 days and 2-6 days, respectively (Kudva et al., 1997). Results suggested that feed management practices could reduce the frequency of *E. coli* O157:H7 fecal shedding by animals and the duration of time in which animals were positive for the organism.

A study conducted by Diez-Gonzalez et al. (1998) reported that cattle fed concentrate diets shed higher numbers of nonpathogenic *E. coli* in their feces than cattle fed hay diets, and that a higher percentage of the *E. coli* shed by concentrate fed cattle were acid tolerant. Hovde et al. (1999) determined that nonpathogenic coliforms from hay-fed cattle were more sensitive to acid shock than coliforms from grain-fed animals. However, in contrast to the results reported by Diez-Gonzalez (1998), they demonstrated that hay-fed

cattle shed *E. coli* O157:H7 in their feces for a significantly longer period of time than grain-fed animals, 39-42 days and 4 days respectively, and that regardless of diet, the *E. coli* O157:H7 shed were equally acid resistant (Hovde et al., 1999).

Conflicting results reported by Tkalcic et al. (2000) found no association between calves fed high concentrate diets or calves fed high roughage diets and the incidence of rumen populations of *E. coli* O157:H7 or fecal shedding of the organism. Although the proliferation of *E. coli* O157:H7 was favored in rumen fluid from animals fed a high roughage diet compared with a high concentrate diet *in vitro*, a similar effect of diet was not demonstrated *in vivo*. Magnuson et al. (2000) reported that grain based finishing diets and forage based growing diets did not affect the duration heifers were culture positive for *E. coli* O157:H7.

Studies have established relationships between rumen volatile fatty acid (VFA) concentrations, rumen pH and feed quality. Roughage diets increase rumen pH and decrease VFA concentrations while high concentrate diets are known to decrease rumen pH and increase VFA concentrations (Magnuson et al., 2000; Owens and Goetsch, 1988). Harmon et al. (1999) found that despite fluctuations in rumen VFAs, there was no correlation between daily rumen VFA concentrations and daily rumen or fecal *E. coli* O157:H7 numbers. Harmon et al. (1999) suggested that rumen populations of *E. coli* O157:H7 remained low and *E. coli* O157:H7 was subsequently introduced into the colon where replication occurred.

The significance of acid resistant *E. coli* O157:H7 in bovine feces remains controversial. Diez-Gonzalez et al. (1998) reported that grain feeding results in a lower

colonic pH than hay feeding resulting in more acid-resistant *E. coli* in grain fed versus hay fed animals. Conflicting results have been reported in regards to the effects of high concentrate and high roughage diets on the shedding of *E. coli* O157:H7 by cattle and the subsequent development of the acid resistance of the organism which need to be resolved (Diez-Gonzalez et al., 1998; Hovde et al., 1999; Russell et al., 2000; Tkalcic et al., 2000). The gastrointestinal tract of cattle is a complex system and many factors likely contribute to the survival of *E. coli* O157:H7 in this system. Further investigations are required to determine the effect of diet on *E. coli* O157:H7 in the gastrointestinal tract of cattle before any recommendations are made in terms of diet as a means of control of *E. coli* O157:H7.

Diet Change, Fasting and *E. coli* O157:H7

In sheep experimentally inoculated with *E. coli* O157:H7, an abrupt change in diet from corn and pelleted alfalfa to grass hay resulted in an increase in *E. coli* O157:H7 fecal shedding while a diet change from grass hay to corn and pelleted alfalfa resulted in decreased levels of O157:H7 (Kudva et al., 1997). In a similar study, a diet change from alfalfa pellets to sagebrush-bunchgrass range (change in location as well) resulted in uniform shedding among lambs and ewes for 15 days, after which all animals were culture negative for *E. coli* O157:H7 and remained culture negative even after periods of fasting (Kudva et al., 1995). A diet change in adult rams from alfalfa pellets to kochia weed followed by 48 h of fasting resulted in animals being consistently culture negative for *E. coli* O157:H7 (Kudva et al., 1995).

Dietary change or diet change in addition to withholding feed appeared to result in increased excretion of *E. coli* O157:H7 in sheep. It has been suggested that the change

in diet induced selective microbial growth in the intestine and levels of *E. coli* O157:H7 shed became detectable. Although diets high in nutrients and low in fibre result in a lower level of *E. coli* O157:H7 fecal shedding, they do not induce the clearance of the organism from the animals. Diets low in nutrients and high in fiber in combination with fasting induced shedding of large numbers of *E. coli* O157:H7 but also caused elimination of the organism (Kudva et al., 1995). The lambs use in the study progressed from pre-ruminant to ruminant metabolism and animals were moved from confinement to graze on an open range as part of a dietary change. These variables may have had an effect on the shedding of *E. coli* O157:H7 in addition to the dietary changes (Kudva et al., 1995).

Studies have shown that dietary stress results in an increase in fecal shedding of *E. coli,* however, feed withdrawal has little effect on the shedding of *E. coli* O157:H7 (Cray et al., 1998; Harmon et al., 1999; Jordan and McEwen, 1998; Kudva et al., 1997). Although fasting resulted in a decrease in VFA concentrations and a resulting increase in the pH of the rumen, there is no correlation between VFA concentrations and rumen and fecal levels of *E. coli* O157:H7 (Harmon et al., 1999). However, diet-stressed calves are more susceptible to *E. coli* O157:H7 infection than well-fed calves (Cray et al., 1998). In cattle shedding *E. coli* O157:H7, feed withdrawal has little effect on *E. coli* O157:H7 fecal shedding or its growth in the rumen. This suggests that fasting prior to slaughter does not increase fecal shedding of *E. coli* O157:H7 and a subsequent increase in the number of *E. coli* O157:H7 positive animals entering the food chain.

Magnuson et al. (2000) conducted a study using sheep as a ruminant model, and developed a fasting and re-feeding regime which resulted in an increased rate of

gastrointestinal tract cell proliferation 24 h after re-feeding following a 24 h period of feed withdrawal. The fasting re-feeding regime was then used in cattle to test the hypothesis that feeding interventions increase the rate of gastrointestinal tract cell proliferation and induce the clearance of *E. coli* O157:H7 from the bovine gastrointestinal tract. A decrease in the number of *E. coli* O157:H7 culture positive animals was observed 72 h after feeding was resumed which coincided with increases in gastrointestinal tract cell proliferation.

Ionophores and *E. coli* O157:H7

Carboxylic polyether ionophore antibiotics, more commonly called ionophores, are a class of compounds produced by various strains of *Streptomyces* (Bergen and Bates, 1984). In 1975 ionophores were approved by the Food and Drug Administration for use in feedlot diets (Russell and Strobel, 1989; USDA:APHIS:VS, 1994). While monensin is the most extensively used ionophore as a feed additive for cattle, others such as salinomycin, lasalocid, narasin, lysocellin, tetronasin and laidlomycin have either been investigated or are used commercially (Russell and Strobel, 1989; Schelling, 1984; Wallace, 1992).

Ionophores are lipophilic substances which have a molecular weight of 500 to 2,000 (Russell and Strobel, 1989). The exterior of the molecule is hydrophobic, while the interior is hydrophilic and able to bind cations. Ionophores are referred to as "antiporters" or "ion bearers" and achieve their effects by modifying the movement of ions across the membranes of rumen microbes (Bergen and Bates, 1984; Russell and Strobel, 1989; Schelling, 1984; Wallace, 1992).

Ionophores differ in their selectivity for cations. Monensin, for instance, has a

higher selectivity for Na^+ while lasalocid has a higher selectivity for K^+, with both being capable of acting as Na^+ or K^+/H^+ antiporters.(Russell, 1987; Russell and Strobel, 1989). Since the composition of feed can affect the concentration and ratio of cations in rumen fluid, the efficiency of ionophores can be dependent on diet (Russell and Strobel, 1989).

Monensin was initially developed as a coccidiostat for poultry however subsequent studies showed that it also has an effect on rumen fermentation (Richardson et al., 1976). The general effect achieved by ionophores such as monensin is improved animal thriftiness as a result of a series of changes in the rumen fermentations and host gastrointestinal physiology (Bergen and Bates, 1984; Schelling, 1984). Effects include a decrease in methane production, an increase in the ratio of propionate to acetate production and a decrease in protein degradation to ammonia (Richardson et al., 1976; Russell and Strobel, 1989; Wallace, 1992). Effects of monensin on rumen fermentation in live animals are similar to those observed in the Rumen Simulation Technique (Rusitec) (Wallace et al., 1980).

The sensitivity of rumen bacteria to ionophores appears to be dependent on the absence of an outer membrane. Gram-positive bacteria, which lack a protective outer membrane are generally sensitive to ionophores while Gram-negative bacteria are generally ionophore resistant (Bergen and Bates, 1984; Russell and Strobel, 1989). It has been suggested that because of this inhibitory effect of ionophores on gram-positive bacteria, the gram-negative population of the rumen is enriched (Bergen and Bates, 1984).

The first outbreaks of *E. coli* O157:H7 occurred shortly after the introduction of ionophores in the mid 1970s. It has been speculated that the physiological changes brought

about in the rumen as a result of ionophore use may have selected for *E. coli* O157:H7 (USDA:APHIS:VS, 1994). However, there is no experimental evidence to support this hypothesis (Herriott et al., 1998). Although ionophores exert an inhibitory effect on Gram-positive bacteria it is not known if the effect of ionophores allows *E. coli* O157:H7 to proliferate in the rumen. Epidemiological studies have reported that the use of ionophores in cattle feed is not related to the fecal shedding of *E. coli* O157:H7 (Dargatz et al., 1997; Garber et al., 1995). Growth of *E. coli* O157:H7 in a variety of cattle feeds *in vitro* occurs whether lasalocid is present in the feed or is not (Lynn et al., 1998). The effect of monensin and other ionophores on the growth and survival *E. coli* O157:H7 has not been thoroughly investigated.

Control Measures

A simulation study preformed by Jordan et al. (1999) has shown that the pre-slaughter interventions which exhibit the most potential in reducing the contamination of beef carcasses with *E. coli* O157:H7 are vaccination and the use of substances which will reduce the shedding of *E. coli* O157:H7 in the feces of cattle. A study conducted by Elder et al. (2000) reported a positive correlation between the fecal and hide prevalence of *E. coli* O157:H7 and subsequent contamination of carcasses with the organism. This implies that a role for the control of *E. coli* O157:H7 exists at the bovine source. There is ample opportunity for cross contamination once the animal enters the packing plant and thus intervention strategies which would reduce the levels of *E. coli* O157:H7 in the feces of cattle may subsequently reduce the cases of foodborne illness associated with this deadly pathogen.

Diet

Epidemiological studies have not confirmed an association between diet and the carriage of *E. coli* O157:H7 by cattle (Dargatz et al., 1997; Hancock et al., 1997b; Hancock et al., 1994; Herriott et al., 1998). Additional studies designed to investigate further the effects of diet on *E. coli* O157:H7 proliferation, acid tolerance and fecal shedding are necessary before any recommendations in changes to dietary strategies as a means of *E. coli* O157:H7 control are made.

Probiotics

The protective flora which establishes itself in the intestines of animals soon after birth is very stable, but can be influenced by certain dietary and environmental conditions. These include antibiotic therapy, overcrowding, feed changes, handling and shipping and new environments. When animals are stressed the balance between beneficial and undesirable organisms may become upset resulting in diarrhea, gastroenteritis and reduced feed intake and growth (Fox, 1988) . The concept of administering probiotics to animals involves the hypothesis that these beneficial microorganisms will combat the effects of stress and prevent establishment of pathogenic microorganisms in the intestine (Fuller, 1989). Probiotics have been defined as "Live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance" (Fuller, 1989).

Probiotics are bacterial (or yeast) preparations which are administered orally or added to the feed. The most commonly used probiotics in animal production are strains of lactic acid bacteria (LAB) *Lactobacillus* spp. and *Streptococcus* spp. Probiotics for

ruminants also include various species of *Bacillus*, *Bifidobacteria* and *Propionibacteria*, *Enterococcus* and fermentation extracts of *Saccharomyces cerevisiae* and *Aspergillus oryzae* (Fox, 1988; Fuller, 1989; Wallace, 1992).

The specific mode of action of probiotics may be mediated by a direct antagonistic effect against a specific group of organisms. The suppression of bacterial numbers may be achieved by the production of antibacterial compounds, competition for nutrients or competition for adhesion sites. Probiotics may also alter microbial metabolism by affecting enzyme activity and stimulate immunity resulting in increased antibody levels and macrophage activity (Fuller, 1989)

Probiotic bacteria have been shown to be effective in reducing the duration of ruminal carriage of *E. coli* O157:H7 in cattle (Zhao et al., 1998). Eighteen bacterial isolates (seventeen *E. coli* strains and *Proteus mirabilis*), which produced metabolites which were inhibitory to *E. coli* O157:H7, were isolated from cattle found not to shed *E. coli* O157:H7. The 18 strains of probiotic bacteria were mixed with 2% sterilized skim milk and orally administered to the calves 2 days prior to inoculation with *E. coli* O157:H7. The administration of the probiotic bacteria reduced the duration of ruminal carriage of *E. coli* O157:H7 from 22-32 days in the control animals to 9-17 days in the animals receiving the probiotics. Fecal shedding was reduced from 25-32 days in the control group to 14-19 days in the probiotic fed group (Zhao et al., 1998).

Vaccination

Vaccination represents an intervention strategy which may be effective in reducing the carriage of *E. coli* O157:H7 by cattle. Studies conducted by Potter and Finlay (2000)

have reported that vaccination of cattle with two recombinant antigens, Tir and EspA, resulted in reduced fecal shedding of *E. coli* O157:H7 when compared with unvaccinated control animals.

Bacteriophage Therapy

Since the discovery of bacteriophages in the early 1900's by Twort and d'Herelle, bacteriophages have been used successfully to control human bacterial pathogens such as *Salmonella*, *Shigella* and *Staphylococcus* (Alsiky et al., 1998; Barrow and Soothill, 1997; Sulakvelidze et al., 2001). Bacteriophages have been used to successfully control enteropathogenic *E. coli* infection in mice (Smith and Huggins, 1982), calves, piglets and lambs (Smith and Huggins, 1983), with phage therapy being more effective when applied before or together with the infective bacteria.

A study conducted by Kudva et al. (1999) found that three O157-specific bacteriophages lysed liquid cultures of *E. coli* O157:H7 at 4 and 37 °C *in vitro*. Culture aeration and a high multiplicity of infection was required for complete lysis with no single phage being able to completely lyse the culture. Full lysis of the bacterial culture occurred after 8 h of incubation with aeration at 4 and 37 °C when infected with three phages at a multiplicity of infection (MOI) of 10^3 plaque forming units (PFU)/CFU. In non-aerated cultures complete elimination of the bacteria resulted after 5 days of incubation at 4 °C.

Waddell et al. (2000), treated calves inoculated with 3×10^9 CFU *E. coli* O157:H7, with 10^{11} PFU of each of six O157-specific bacteriophages on days -7, -6, -1, 0 and 1 post-inoculation. The administration of the bacteriophages significantly reduced the duration of fecal shedding of *E. coli* O157:H7 by the calves. Shedding of *E. coli*

O157:H7 was observed in the control group (*E. coli* O157:H7 only) for 6 to 14 days while animals in the bacteriophage-treated group shed the organism for 6 to 8 days.

Studies have suggested that a combination of bacteriophages is more effective than the use of one bacteriophage in eliminating *E. coli* O157:H7. The use bacteriophage therapy is also more effective when applied before or with the infective bacteria, and this may place limitations on their use as a control measure for the fecal shedding of *E. coli* O157:H7 by cattle (Kudva et al., 1999; Smith and Huggins, 1982; Smith and Huggins, 1983; Waddell et al., 2000).

Additional Control Measures

Additional intervention strategies include the use of coumarins, sodium chlorate, prebiotics, synbiotics and colicins (Anderson et al., 2000; Duncan et al., 1996; Gyles, 2000; Murinda et al., 1996). Plant metabolites, such as coumarins, have been found to inhibit *E. coli* O157:H7 *in vitro*. The growth of *E. coli* O157:H7 was inhibited under aerobic and anaerobic conditions by esculetin, umbelliferone and scopoletin. The mechanism of inhibition by coumarins is unknown. However, coumarins are known to be enzyme inhibitors and act as antioxidants in biological systems (Duncan et al., 1998). Coumarins are found in a wide variety of animal forages and in fruits and vegetables consumed by humans.

Anderson et al. (2000) found that chlorate exerted an inhibitory effect on *E. coli* O157:H7 in ruminal contents *in vitro*. *E. coli* O157:H7 and *Salmonella* possess a respiratory nitrate reductase, which reduces chlorate to chlorite intracellularly, resulting in cell death. The antimicrobial properties of chlorate are greater at 5 mM as opposed to 1.25

mM and at pH 6.8 as opposed to pH 5.6 (Anderson et al., 2000).

Colicins (bacteriocins produced by *E. coli)* have been shown to exhibit inhibitory activity against *E. coli* O157:H7 in *in vitro* studies and may have potential for the control of *E. coli* O157:H7 in cattle (Murinda et al., 1996). The use of prebiotics (feed ingredients which stimulate microbial growth, e.g. inulin) and synbiotics (a combination of probiotics and prebiotics), also represent potential intervention strategies for the reduction of *E. coli* O157:H7 fecal shedding by cattle. Few reports have been published on the use of colicins, prebiotics and synbiotics in the control of *E. coli* O157:H7.

Detection of *E. coli* O157:H7 in Bovine Feces

A variety of methods have been developed for the isolation of *E. coli* O157:H7 from foods and clinical samples (Padhye and Doyle, 1992; Vernozy-Rozand, 1997). Culture methods for the isolation of *E. coli* O157:H7 used in prevalence studies in cattle are far from standardized. Different studies use methods of varying sensitivity, making comparisons between studies difficult. To effectively study the epidemiology of *E. coli* O157:H7 in cattle, the organism must be isolated from a large population of other bacteria which are present in the feces of cattle (Sanderson et al., 1995; Wallace and Jones, 1996; Zhao et al., 1995).

Enrichment Media

The use of enrichment media is required to detect the presence of low numbers of *E. coli* O157:H7 from food and feces (DeBoer and Heuvelink, 2000). An enrichment culture step increases the sensitivity of the assay by allowing for the growth of *E. coli*

O157:H7 cells to detectable levels while suppressing high levels of background microflora (Okrend et al., 1990b; Padhye and Doyle, 1992).

Modified trypticase soy broth (mTSB) is an enrichment medium which is commonly used in the isolation of *E. coli* O157:H7. Trypticase soy broth is modified through the addition of bile salts (1.5 g/L) and dipotassium phosphate (1.5 g/L). This medium may be further supplemented with novobiocin (20 µg/ml) (Doyle and Schoeni, 1987) or acriflavine (10 µg/ml)(Kim and Doyle, 1992). Acriflavine and novobiocin have been reported to reduce the growth of gram-positive organisms, naturally present in beef, while bile salts are inhibitory to non-enterics (Bennet et al., 1995; Doyle and Schoeni, 1987; Padhye and Doyle, 1991a; Szabo et al., 1986).

The mTSB may be supplemented with low levels of cefixime (0.05 µg/ml), vancomycin (40 µg/ml) and tellurite (2.5 µg/ml)(Sanderson et al., 1995). A mixture of vancomycin (8 µg/ml), cefixime (0.05 µg/ml) and cefsulodin (10 µg/ml) may be added to either buffered peptone water (BPW), pH 7.2 (10 g/L peptone, 5 g/L sodium chloride, 3.5 g/L sodium phosphate, dibasic, 1.5 g/L potassium phosphate, monobasic) or mTSB. These enrichment broths are referred to as BPW-VCC (Chapman et al., 1994) and EEB (EHEC enrichment broth)(Weagent et al., 1995), respectively. Vancomycin is used to suppress the growth of *Aeromonas* while cefixime and cefsulodin suppress the growth of *Proteus* spp. (Chapman et al.,1991; Vernozy-Rozand, 1997). The incorporation of tellurite into the liquid media serves to inhibit other strains of *E. coli* and non-sorbitol fermenting strains of bacteria such as *Providencia* spp. or *Aeromonas* spp. (Zadik et al., 1993).

Padhye and Doyle (1991a) described an enrichment medium, mTSB, which was

not supplemented with novobiocin (dm TSB), but contained Casamino acids (10g/L) and acriflavine-HCl (10 mg/L) (dmTSB-CA) (Padhye and Doyle, 1991a). This enrichment medium was used in conjunction with a sandwich, enzyme-linked immunosorbent assay (ELISA) for the detection of *E. coli* O157:H7 in foods. This method has been used in surveys of dairy herds to determine the prevalence of *E. coli* O157:H7 from fecal samples (Garber et al., 1995; Zhao et al., 1995).

Another enrichment medium which has been used for the detection of *E. coli* O157:H7 from bovine fecal samples is EC (*Escherichia coli*) broth with novobiocin (20 µg/ml)(Faith et al., 1996; Heuvelink et al., 1998; Shere et al., 1998). This medium contains bile salts at a level of 1.5 g/L as the selective agent against gram positive bacteria. Other enrichment media used in studies to detect the prevalence of *E. coli* O157:H7 fecal shedding in cattle include mTSB (Garber et al., 1995; Wells et al., 1991), BPW-VCC (Mechie et al., 1997), TSBcv (Besser et al., 1997; Hancock et al., 1994; Hancock et al., 1997b; Rice et al., 1997), TSBc (Hancock et al., 1997c) and mTSB-CA (supplemented with both novobiocin and acriflavine) (Zhao et al., 1995).

Sanderson et al. (1995) compared the use of TSBcv and TSBcvt as enrichment media for the detection of *E. coli* O157:H7 from bovine feces. It was found that overnight enrichment of fecal swabs at 37 ° C in TSBcv followed by plating onto cefixime tellurite sorbitol MacConkey agar (CT-SMAC) was slightly more sensitive, though not significantly so, than the use of TSBcvt as an enrichment media for the detection of *E. coli* O157:H7 from bovine fecal samples. The incorporation of tellurite into CT-SMAC increased the recovery of *E. coli* O157:H7 twofold. The use of immunomagnetic separation (IMS) was

slightly more sensitive, however, the difference was not significant (Sanderson et al., 1995).

Szabo et al., (1990), incorporated an enrichment step, in an attempt to increase the sensitivity of a hydrophobic grid membrane filter enzyme-labeled antibody procedure for *E. coli* O157:H7 detection in foods and bovine feces. They reported that while mTSB at 43 °C for 16-20 h with agitation (100rpm) provided optimum recovery of *E. coli* O157:H7 from meats, TSB under the same conditions yielded optimum recovery from fecal samples (Szabo et al., 1990). The validity of this conclusion is questionable since the temperature range for the growth of some strains of *E. coli* O157:H7 is 30 - 42 °C, with poor growth resulting at temperatures above 41 °C (Raghubeer and Matches, 1990).

Stephens and Joynson (1998) assessed the ability of several selective enrichment broths to support recovery and growth of acid/salt stressed *E. coli* O157:H7. The addition of bile salts to mTSB rendered the medium inhibitory to stressed cells of *E. coli* O157:H7 at 37 °C with a 1.5 $\log_{10}$ reduction in *E. coli* O157:H7 cells recovered as compared to TSB. Addition of other selective agents such as novobiocin or acriflavine to mTSB had negligible effects on the numbers recovered at 37 ° C. Vancomycin, cefsulodin and cefixime additions, however, resulted in a 2.5 $\log_{10}$ reduction in numbers of *E. coli* O157:H7 recovered compared to recovery from TSB alone at 37 ° C. In contrast, the addition of novobiocin, acriflavin and bile salts to make mTSB had no effect on the recovery of unstressed cells of *E. coli* O157:H7 (Stephens and Joynson, 1998). Supplementation of TSB with acriflavine or novobiocin did not affect recovery of *E. coli* O157:H7 significantly while the supplementation of BPW with vancomycin, cefsulodin and cefixime resulted in a 0.4 $\log_{10}$ decrease in the recovery of unstressed cells.

It has been suggested that non-selective enrichment media, as is commonly used in *Salmonella* detection procedures, is necessary for the effective recovery of low levels of stressed *E. coli* O157:H7 cells (Stephens and Joynson, 1998). Although enrichment media are used to amplify low levels of *E. coli* O157:H7 to detectable levels, selective agents are often used to suppress the growth of numerous other microorganisms which occur in bovine feces (Chapman et al., 1994; Vernozy-Rozand, 1997).

The use of mTSB with novobiocin or mEC with novobiocin, are the selective enrichment broths which appear to be the most appropriate for the detection of *E. coli* O157:H7 from bovine feces. The enrichment broths are minimally selective and provide limited differential specificity favoring the isolation of *E. coli* O157:H7 as opposed to other Gram-negative bacteria which may be present in the fecal sample (DeBoer and Heuvelink, 2000).

Plating Media

E. coli O157:H7, unlike approximately 95% of other *E. coli* strains, does not ferment sorbitol within a 24 h period (Krishnan et al., 1987; Ratnam et al., 1988). This characteristic has been exploited by the use of MacConkey agar containing sorbitol (SMAC), instead of lactose, as a differential medium for the detection of *E. coli* O157:H7 (March and Ratnam, 1986). The growth of *E. coli* O157:H7 and other non-sorbitol fermenting (NSF) organisms on SMAC medium occur as colorless colonies while sorbitol fermenting (SF) *E. coli* colonies appear bright pink.

The frequency of NSF organisms other than *E. coli* O157:H7 in feces has been reported to be 10-20%. The breakdown of the frequency of NSF organisms in 240 stool

samples processed was: *E. coli* O157:H7, 0%; non O157:H7 *E. coli*, 6%; *Proteus* spp., 4%; *Morganella* spp., 2%; other coliform bacteria and *Pseudomonas*, 3% (March and Ratnam, 1986). The use of SMAC medium provides a simple means of distinguishing *E. coli* O157:H7 from other fecal microflora. It has been reported that in addition to a sensitivity of 100%, SMAC medium had a specificity of 85% and an accuracy of 86% (March and Ratnam, 1986).

Many modifications to SMAC have been described, all aiming at improving the selectivity of the media for the isolation of *E. coli* O157:H7. Ratnam et al. (1988) found that rhamnose was not fermented within 24 h by approximately 89% of 174 *E. coli* O157:H7 strains tested, but was fermented by 84-91 % of 11, 601 other *E. coli* tested. Rhamnose fermentation reactions, however, were found to be inconsistent and variable within individual strains (Ratnam et al., 1988). Cefixime is an antibiotic which suppresses the growth of *Proteus* spp. Biochemical tests on human and animal feces during 1987-1990 revealed that 15% of the NSF organisms isolated were *Proteus* spp (Chapman et al., 1991). The addition of rhamnose (0.5%) and cefixime (0.05 ug/ml) to SMAC agar (CR-SMAC) was reported to improve selectivity for the isolation of *E. coli* O157:H7 as compared to SMAC (Chapman et al., 1991; Zadik et al., 1993).

The incorporation of 4-methylumbelliferyl-β-D-glucuronide (MUG) (0.1 g/L) into MacConkey sorbitol agar (MSA) (MSA-MUG) allows for the differentiation of microorganisms based on the production of the enzyme β-glucuronidase. Approximately 96% of *E. coli* other than serotype O157:H7 are β-glucuronidase positive, while *E. coli* O157:H7 is β-glucuronidase negative (Ratnam et al., 1988) The MUG substrate is

hydrolyzed by β-glucuronidase to yield a fluorogenic end product, methylumbelliferone, that is detectable under a UV light source. *E. coli* O157:H7 may be differentiated on MSA-MUG by the lack of fluorescence when colonies are exposed to UV light (Padhye and Doyle, 1991a).

Okrend et al. (1990c) added 5-bromo-4-chloro-3-indoxyl-β-glucuronide (BCIG) at a level of 0.1 g/L to MSA (MSA-BCIG) in order to detect the production of β-glucuronidase in bacterial colonies. The use of MUG as a substrate was rejected due to diffusion of the fluorescent product out of the colony, obscuring the actual colonial source of the enzyme. On MSA-BCIG, *E. coli* O157:H7 colonies being sorbitol-negative and β-glucuronidase negative remained white, while sorbitol negative, β-glucuronidase positive colonies turned from green to blue. However, MSA-BCIG medium does not differentiate between β-glucuronidase negative, sorbitol negative colonies which are not *E. coli* , from those that are *E. coli* (Okrend et al., 1990c).

Some *E. coli* other than serotype O157:H7 are NSF as are *Proteus* spp. and *Aeromonas* spp. The use of CR-SMAC reduced the number of NSF colonies that need to be screened through the use of cefixime and rhamnose (Chapman et al., 1991). On MSA-BCIG and MSA-MUG *E. coli* O157:H7 is differentiated from other *E. coli* through their lack of fluorescence (Okrend et al., 1990a; Padhye and Doyle, 1992).

Zadik et al. (1993) incorporated tellurite (2.5 mg/L) and cefixime (0.05 mg/L) into SMAC (CT-SMAC). The minimum inhibitory concentrations (MIC) for cefixime and tellurite were reported to be higher for *E. coli* O157:H7 than for other *E. coli* and for other non-sorbitol fermenting enterics including *Aeromonas* spp., *Plesiomonas* spp., *Morganella*

morganii, *Providencia spp.* and *Hafnia alvei*. As a result CT-SMAC permitted the growth of *E. coli* O157:H7 but inhibited 67% of other strains of *E. coli*. The incorporation of tellurite into SMAC has been reported to have dramatically increased the rate of isolation of *E. coli* O157:H7 from cattle rectal swabs (Zadik et al.,1993).

A recent study investigated the use of selective and differential agars for the isolation of *E. coli* O157:H7 from bovine feces (Wallace and Jones, 1996). Four types of selective agars were used: SD-39 (QA Life Sciences, USA);CT-SMAC; CR-SMAC; CHROMagar (CHROMagar, France). IMS using Dynabeads anti- *E. coli* O157 was also performed according to manufacturer's instructions with the beads being plated onto CT-SMAC.

Both SD-39 and CHROMagar allow the differentiation of *E. coli* O157 from other *E. coli* by colour and involve filtering the sample through an HGMF. On CHROMagar and SD-39 agar, *E. coli* O157:H7 forms characteristic pink colonies while other *E. coli* form blue or colourless colonies (Wallace and Jones, 1996; Bettelheim, 1998b). SD-39 agar contains monensin (0.038 g/L) and novobiocin (0.0075 g/L) as the selective agents and lysine (10 g/L), MUG (0.01 g/L) and sorbitol as the differential agents (Sage and Ingham, 1998).

For the evaluation of CT-SMAC and CR-SMAC, the methods of direct plating and direct plating following enrichment were performed. For SD-39 and CHROMagar, for direct plating and direct plating following enrichment, a one ml aliquot of the appropriate dilution was filtered through an HGMF prior to incubation on the surface of the agar. CHROMagar was incubated at 37° C while SD-39 was incubated at the manufacturer's

recommended temperature of 44.5 ° C. Poor growth of *E. coli* O157:H7 strains, however, has been observed above temperatures of 42 ° C (Raghubeer and Matches, 1990). The enrichment broth used for the evaluation of all of the agars was mTSB supplemented with 20 mg/L novobiocin and incubated for 18 h at 37° C without agitation.

The four isolates which were confirmed as *E. coli* O157:H7, out of the 614 presumptive *E. coli* O157 isolates, were recovered using enrichment in mTSB for 18 h at 37° C followed by filtration using the HGMF procedure and subsequent plating on CHROMagar O157 (Wallace and Jones, 1996). This method was also reported to be slightly more sensitive than IMS in the isolation of *E. coli* O157:H7 from spiked fecal samples. It has been suggested that the plating of pure cultures of *E. coli* O157:H7 isolated from CHROMagar O157 following enrichment onto CT-SMAC or CR-SMAC would be a discriminatory test for the identification of *E. coli* O157:H7 (Wallace and Jones, 1996). Testing for β-glucuronidase production is a useful test for *E. coli* O157:H7 which is commonly used (Besser et al., 1997; Faith et al., 1996; Hancock et al., 1994; Hancock et al., 1997c; Scotland et al., 1991; Shere et al., 1998).

Studies have concluded that CT-SMAC agar is the most effective isolation medium for the detection of typical sorbitol-negative *E. coli* O157:H7 from bovine feces and cattle rectal swabs (Chapman et al., 1994; DeBoer and Heuvenlink, 2000; Sanderson et al., 1995; Zadik et al., 1993). It has been recommended that standardized methods for the isolation of *E. coli* O157:H7 should include at least CT-SMAC in addition to a second isolation medium which is not based on sorbitol fermentation, but is based on β-glucuronidase activity, for example (DeBoer and Heuvelink, 2000).

Isolation Methods

Hydrophobic Grid Membrane Filter (HGMF)- Immunoblot. An assay to isolate *E. coli* O157:H7 from enrichment cultures of foods which was based on hydrophobic grid membrane filtration in combination with an immunoblot procedure utilizing antiserum to verocytotoxin (VT1 and VT2) was developed by Doyle and Schoeni (1987). Samples were selectively enriched in mTSB supplemented with 20 mg/L novobiocin for 18-24 h at 37° C and filtered through a hydrophobic grid membrane. The membranes were incubated on nitrocellulose paper placed on modified trypticase soy agar plates (mTSA) (mTSB + 1.5% agar) for 18-24 h at 37° C. Following incubation, the nitrocellulose paper was treated using an immunoblot procedure to detect Vero cell cytotoxin(s) produced by the colonies on the HGMF. Positive colonies were confirmed using Vero cell cytotoxicity tests, O157 and H7 antisera, and biochemical tests. The procedure effectively isolated as few as 1.5 *E. coli* O157:H7 per g of food. However, it is not routinely used due to its complexity and demand for time (DeBoer and Heuvelink, 2000; Padhye and Doyle, 1992; Vernozy-Rozand, 1997).

Hydrophobic Grid Membrane Filter Enzyme Labeled-antibody Procedure (HGMF-ELA). Todd et al. (1988) developed a method for the detection of *E. coli* O157:H7 in foods and bovine feces based on HGMF and a monoclonal antibody that was specific for *E. coli* O157. In the procedure, the sample is mixed with peptone water, coarse filtered and subsequently filtered through an HGMF. After incubation, the HGMF is immunostained with horseradish peroxidase-labeled monoclonal antibody to *E. coli* O157:H7. The antibody attached to *E. coli* O157 and the addition of substrate and colour

development solution resulted in a visible product (Todd et al., 1988). Since the limit of detection of the method was 10 *E. coli* O157:H7 per g of sample, enrichment was investigated as a means of increasing the sensitivity. It was reported that enrichment in mTSB using the same conditions provided optimum recovery of *E. coli* O157:H7 from meat while enrichment in TSB at 43 °C and agitation (100 rpm) provided optimum recovery of *E. coli* O157:H7 from fecal samples. An increased concentration of bile salts from the mTSB and the feces may have suppressed the growth of *E. coli* O157:H7 (Szabo et al.,1986). It has also been reported that *E. coli* O157:H7 grows poorly at temperatures above 42 ° C (Raghubeer and Matches, 1990). The sensitivity of the assay was less than 1 CFU of *E. coli* O157:H7 per g, a ten-fold increase in the sensitivity as compared to direct plating (Szabo et al., 1990; Todd et al., 1988).

Enzyme Linked Immunosorbent Assays (ELISA). The sandwich - ELISA described by Padhye and Doyle (1991a) has been used for detecting *E. coli* O157:H7 in bovine feces (Garber et al., 1995; Zhao et al., 1995). The ELISA is based on a double antibody sandwich ELISA in which a polyclonal antibody is used as the capture antibody and a highly specific monoclonal antibody (MAb) 4E8C12 for *E. coli* of serotypes O157:H7 and 026:H11 is used as the detection antibody (Padhye and Doyle, 1991b). The procedure involves enrichment of the sample for 16-18 h at 37° C with agitation followed by application of the enrichment culture to the sandwich-ELISA procedure. Isolates are confirmed by plating onto MacConkey-sorbitol agar (MSA) supplemented with 4-methylumbelliferone-β-D-glucuronide (0.1g/L)(MSA-MUG), by serology with O157 and H7 antisera (Difco Laboratories, Detroit, Mich.), and by the Vero cell cytotoxicity assay

(Doyle and Schoeni, 1987).

According to the author, the ELISA can be completed in 3 h and the entire procedure can be conducted in approximately 20 h. The sensitivity of the procedure, determined by using ground beef and dairy products inoculated with *E. coli* O157:H7, is reported to be 0.2 to 0.9 cell per g of food. This procedure is reported to be not only rapid, specific and sensitive but was also easy to perform, making it amenable for use in labs performing routine microbiological testing (Padhye and Doyle, 1991a).

A screening method for detecting and isolating *E. coli* from ground beef using a commercially available reactive disc blot ELISA (Petrifilm™* Test Kit - HEC) was described by Okrend et al. (1990b). The procedure involved enrichment of the sample in modified EC broth (with 20 mg/L novobiocin) with agitation for 6 h at 37 °C. Dilutions of the enrichment culture are subsequently inoculated onto 3M Petrifilm *E. coli* count plates. The Petrifilm plates are incubated at 42 ° C for 18 h, replica plated onto reactive discs and tested for the presence of O157 antigen by an immunoblot assay using antiserum raised against *E. coli* O157:H7 (Okrend et al., 1990b). Isolates were confirmed by plating onto MSA or MSA supplemented with 5-bromo-4-chlor-3-indoxyl-B-D-glucuronide (BCIG). The procedure can be completed in 26-28 h and is reported to have a minimum level of sensitivity of 0.6 *E. coli* O157:H7 per g of food, with 0% false negatives and 2% false positives. All positive samples must be confirmed as *E. coli* O157:H7 since the method uses O157 polyclonal antisera (Okrend et al., 1990b).

Immunomagnetic Separation (IMS). The immunomagnetic separation procedure involves the use of superparamagnetic polystyrene beads, coated with

antibodies specific for *E. coli* O157, in order to separate and concentrate the organism from a mixed enrichment culture (Dynabead anti-*E.coli* O157; Dynal, Oslo, Norway). In the procedure, samples are mixed with the antibody-coated beads, the target organism is captured onto the magnetic particles and the whole complex is removed using a magnetic particle separator. Consequently, *E. coli* O157:H7 is removed from the fecal debris and background microorganisms and the bead-bacteria complex is plated onto selective agar plates (Bennet et al., 1996; Chapman et al., 1994; Okrend et al., 1992;Vernozy-Rozand, 1997).

Comparisons of immunomagnetic separation and direct culture for the detection of *E. coli* O157:H7 in bovine feces have determined that IMS is a sensitive, simple and rapid technique for the isolation of *E. coli* O157:H7 from bovine fecal samples (Chapman et al., 1994; Chapman et al., 1997c; Cubbon et al., 1996). Enrichment culture in BPW-VCC for 6 h at 37 ° C followed by IMS was found to be 100 times more sensitive than direct culture onto cefixime rhamnose sorbitol MacConkey agar (CR-SMAC) and CT-SMAC, and similar in sensitivity to the use of PCR for verotoxin gene carriage (Chapman et al., 1994; Cubbon et al., 1996; Wright et al., 1994).

Bennet et al. (1995,1996) have demonstrated the ability of Dynabeads anti- *E. coli* O157 to detect < 10 cells of *E. coli* in 25 g of minced beef as compared to 10 cells per 25 g using the direct plating method and 24 h enrichment in modified EC medium with novobiocin (Bennett et al., 1995, 1996). In minced beef inoculated with *E. coli* O157:H7, Wright et al., (1994) recovered 2 CFU/g using enrichment culture in BPW-VCC, IMS and subsequent subculturing onto CT-SMAC (Wright et al.,1994). IMS resulted in an

approximate 100-fold increase in the sensitivity of detection of *E. coli* O157:H7 over subculture following enrichment. The use of IMS has allowed for the detection of *E. coli* O157:H7 at a level of 10^2 CFU/g of stool in the presence of a background microflora of 10^7 CFU/g coliforms (Karch et al., 1996).

In contrast, Sanderson et al. (1995) reported that immunomagnetic separation was only slightly more sensitive than overnight enrichment at 37° C in TSBcv followed by plating of 10^{-3} and 10^{-4} dilutions onto CT-SMAC, and they found that this difference was not significant. A sample size of 10g of feces yielded slightly more sensitivity, but not significantly greater than cotton-tipped swab fecal samples of 0.1 g (Sanderson et al., 1995). Larger fecal sample sizes and IMS did not provide considerable increases in the sensitivity of recovery of *E. coli* O157:H7 from bovine feces (Sanderson et al., 1995).

The majority of surveys of feedlots and dairy herds in the United states which have determined the prevalence of *E. coli* O157:H7 in cattle did not utilize IMS. While IMS is commonly used to detect *E. coli* O157:H7 from bovine fecal samples in studies conducted in the United Kingdom (Chapman et al., 1997a; Mechie et al., 1997), one recent study in the United States used IMS to detect *E. coli* O157:H7 in bovine fecal samples in parallel with broth enrichment followed by direct plating. Additional *E. coli* O157:H7 isolates were obtained when IMS was applied to the enrichment broths (Besser et al., 1997).

Studies have reported that non-specific binding of organisms other than *E. coli* O157 to the polystyrene beads and tubes may occur. This non-specific binding was reduced by incorporating detergents such as Tween- 20 into the phosphate buffered saline (PBS) wash solution at a level of 0.002 - 0.05% (Fratamico et al., 1992; Wright et al., 1994).

A recent study has indicated that the nonspecific adsorption often encountered with the immunomagnetic separation method can be reduced using a low ionic-strength solution for IMS and for the washing solution (Tomoyasu, 1998). Milli-Q (MQ) water was treated with cation-exchange resin to make low ionic-strength water. Five grams of analytical grade Chelex 100 chelating ion-exchange resin was mixed with 100 ml of MQ water and allowed to stand at room temperature overnight in order to chelate ions and settle the resin. The resulting low ionic-strength supernatant was evaluated and it was found that the proportion of *E. coli* O157 cells to non-O157 *E. coli* cells increased from 9.6 to 31.4 times compared to results obtained directly with brain heart infusion broth (BHI), MQ water or PBS-Tween 20 (Tomoyasu, 1998).

Ogden et al. (2000) reported that the recovery of *E. coli* O157:H7 was improved ninefold by increasing the enrichment volume tested from 1 ml to 10 ml while maintaining the volume of immunomagnetic beads at 20µl. While the use of 50 ml volumes yielded a threefold increase in recovery an appropriate magnetic particle separator which may be used with this sample volume is yet unavailbale (Ogden et al, 2000).

The use of IMS has been found to be 100 times more sensitive than direct culture in the isolation of *E. coli* O157:H7 , with an increase in sensitivity being achieved through the concentration of *E. coli* O157:H7 relative to the background microflora (Chapman et al., 1994; DeBoer and Heuvelink, 2000). In addition to being rapid and simple to perform, IMS appears to be the most sensitive and cost-effective method for the isolation of *E. coli* O157:H7 from bovine feces (Chapman et al., 1994; Chapman et al., 1997c; Cubbon et al., 1996).

Confirmation of Isolates

O157. Serological identification of *E. coli* O157 can be achieved through the use of Bacto *E. coli* O Antiserum O157 (Difco Laboratories, Detroit, Mich.). The serological technique is based on the reaction of specific antiserum with its homologous O antigen lipopolysaccharide of *E. coli* O157:H7. A portion of an isolated colony grown overnight on blood agar plates is emulsified in a drop of Bacto *E. coli* O Antiserum O157 on a glass slide. The slide is rocked and observed for visible agglutination within 1 minute. Agglutination should be rapid and complete (Difco Laboratories, Detroit, Mich.).

A latex agglutination kit (*Escherichia coli* O157 latex test; Oxoid) consisting of latex particles which have been sensitized with specific rabbit antibody reactive with the *E. coli* O157 antigen is commercially available for the rapid presumptive detection of *E. coli* O157 (March and Ratnam, 1989). The latex test was found to be 100% specific as it correctly identified all of the 230 isolates of *E. coli* O157:H7 (March and Ratnam, 1989). *Escherichia hermannii, Brucella abortus, Brucella melitensis, Yersinia enterocolitica* serogroup 0 : 9, *Salmonella* group N, *Pseudomonas maltophilia* and some other enteric bacteria have been reported to cross react with the O157 antiserum and could therefore be mistaken for *E. coli* O157:H7 (Chart et al., 1993; Doyle, 1991; Perry and Bundle, 1990; Perry et al., 1986; Todd et al., 1988). False-positive identification of *E. coli* O157 can be avoided if the latex controls are routinely used (Borczyk et al., 1990). Since the latex test provides presumptive identification of *E. coli* O157:H7, the complete identification of *E. coli* O157:H7 would require testing for both the O157 and H7 antigens (Sowers et al., 1996).

H7. Numerous studies have used Bacto *E. coli* antiserum H7 (Difco Laboratories, Detroit, Mich.) to complete the serological identification of *E. coli* O157:H7 following presumptive identification with *E. coli* O157 antiserum (Faith et al., 1996; Wells et al., 1991; Zhao et al., 1995). Adequate expression of the H7 antigen is first achieved by several successive transfers of the suspect *E. coli* isolate in motility medium (0.4% agar in nutrient broth). A loopful of the motility medium culture is inoculated into Bacto Veal Infusion Broth (Difco Laboratories, Detroit, Mich.) and incubated at 35° C for 6 -8 h. The culture is then inactivated using formalin to a final concentration of 0.3%. A 0.5 ml aliquot of a 1:500 dilution of the H7 antiserum is then added to 0.5 ml of the test antigen and the tube is incubated in a 50 ° C water bath for 1 h and examined for agglutination. Tubes exhibiting 50% or more of the cells agglutinating are considered to be positive for the H7 antigen (Difco Laboratories, Detroit, Mich.).

Farmer and Davis (1985) have described a simple procedure for the screening of *E. coli* O157:H7 which takes advantage of the properties of its flagellar antigen (H7), and its inability to ferment sorbitol within 24 h (Farmer and Davis, 1985). The H7 antiserum-sorbitol fermentation medium (H7-sorbitol medium) contained D-sorbitol and *E. coli* H7 antiserum as the identifying agents. A colony to be screened is stabbed approximately 5 mm into the center of the dry H7-sorbitol medium. The medium is incubated at 37 ° C for 18-24 h and observed for sorbitol fermentation and immobilization by the H7 antiserum. A titre of 1:4,096 is defined as almost complete immobilization: thus, the dilution of H7 antiserum to be used is set at 1:2,000 (Farmer and Davis, 1985). Since sorbitol fermentation and the presence of the H7 antigen can be determined in a single tube, the

screening method is reported to be very specific. False positives could be easily detected using Bacto *E. coli* O Antiserum O157 (Difco Laboratories, Detroit, Mich.) or *Escherichia coli* O157 latex test (Oxoid Diagnostic Reagents, Hampshire, England).

A commercial latex reagent for the detection of the H7 antigen is available from Remel Microbiology Products. It is reported that the Remel H7 latex reagent has a sensitivity of 96% and a specificity of 100% compared to the standard tube agglutination method of the CDC with H7 antiserum (Sowers et al., 1996). Since the amount of antigen contained in a single colony is often insufficient to produce adequate agglutination of the H7 latex reagent for some strains of *E. coli* O157:H7, additional passages through motility medium are often required to increase the expression of the H7 antigen. This enhancement of antigen expression increased the sensitivity of the Remel H7 latex reagent to 100%. (Sowers et al., 1996).

The presence of the $fliC_{H7}$ gene encoding the H7 flagellar antigen can also be identified using a multiplex PCR assay as described by Gannon et al.(1997). PCR primers which were synthesized based on the $fliC_{H7}$ sequence amplify DNA fragments from all *E. coli* strains in which the H7 antigen is present. In addition to identifying the $fliC_{H7}$ gene, this multiplex PCR identifies vt_{1+2} genes and the $eaeA_{O157}$ gene, thus being specific for the identification of *E. coli* O157:H7 or NM and other EHEC strains (Gannon et al., 1997). The presumptive identification of *E. coli* O157:H7 can be achieved through serological identification, with isolates being confirmed as *E. coli* O157:H7 through the use of PCR.

Verotoxin Production (VT). *E. coli* O157:H7 elaborates toxins that are cytotoxic to Vero cells, a cell line of African Green Monkey kidney cells. The Vero cell assay (VCA)

is a test that detects *E. coli* O157:H7 verotoxins in monolayers of Vero cells. Killing of the Vero cells is considered evidence of the presence of verocytotoxins if the toxin can be neutralized by antisera to toxins (Konowalchuk et al., 1977). Although the VCA is a highly sensitive means of detecting verocytotoxins, the constraints inherent in tissue culture assays in addition to the lack of specificity in the absence of neutralization of VTs have limited its use as a routine microbiological procedure in the detection of *E. coli* O157:H7 (Rahn et al., 1996).

The PCR is an alternative test to the VCA which has recently gained prominence. In comparison to the VCA, PCR is a more rapid and specific procedure with results normally being generated in one day. While the VCA detects toxin production, PCR can detect the toxin gene sequences (Rahn et al., 1996).

A rapid and sensitive method for detecting VT1 and VT2 genes of verocytotoxin-producing *E. coli* (VTEC) using the polymerase chain reaction (PCR) was described by Gannon et al. (1992). The use of one set of oligonucleotide primers to allow amplification of all VT genes can result in several base degeneracies, and such primers have been used unsuccessfully in the past (Karch and Meyer, 1989). Therefore, two sets of oligonucleotide primers were used to amplify the VT genes. The first set of oligonucleotide primers is homologous for *vt1* while the second set was homologous for *vt2* and *vt2* variants (Gannon et al.,1992). Sufficient differences between the two sets of primers allow for the differentiation of *vt1* and *vt2*. Using inoculated ground beef samples, as few as 1 CFU VTEC strain per g was detected using the PCR assay following selective culture in mTSB for 6 h at 42 °C and subsequent DNA extraction. The method is sensitive and rapid, with

the total time required for detection being approximately 9 h. A disadvantage of the technique is that one or more VTEC strains may be present in the broth culture and the serotype information cannot be obtained until the strain is isolated from the broth culture (Gannon et al.,1992).

A PCR procedure for the specific detection of verotoxin genes in *E. coli* was also developed by Pollard et al. (1990). A set of four oligonucleotide probes was derived from sequences of the VT1 and VT2 genes. The probes clearly identified VT1 and VT2 strains of *E. coli* but did not identify VT2 variant producing *E. coli*. The sensitivity of the PCR procedure for the detection of both VT1 and VT2 genes is 1 ng of total nucleic acid (Pollard et al., 1990).

Karch and Meyer, (1989) constructed four synthetic oligonucleotide probes representing different regions of the VT1 gene and one oligonucleotide probe derived from the VT2 gene of *E. coli* O157:H7 strain 933. They were examined for the detection and characterization of VT1 and VT2-producing strains of *E. coli* strains. The probes provided an alternative approach to immunological assays for VT detection and can serve as a useful tool in defining the epidemiology of infection of EHEC (Karch and Meyer, 1989).

Samadpour et al. (1990) evaluated the use of DNA probes for VT1 and VT2 detection of *E. coli* O157:H7 from food and calf fecal samples. Colonies formed following enrichment of the samples overnight in mTSB (TSB + 20 μg/ml vancomycin) at 37 ° C were probed for VTEC by colony hybridization and by dot blot. In the dot blot assay, enrichment cultures spotted on Nytran filters were lysed and then hybridized with radiolabeled probes. An autoradiogram of each filter was then prepared in order to visualize the positive

colonies. The dot blot technique yielded results in 48 h (Samadpour et al., 1990). The colony blot hybridization method involved plating serial dilutions of the enrichment culture on mTSA (TSA + 20 µg/ml vancomycin), which following overnight incubation at 37 °C, were transferred to Whatman filter paper, hybridized and detected by an autoradiogram as in the dot blot assay. VTEC can be detected in 3-4 days using the colony hybridization procedure. Inoculation recovery studies from ground beef and oyster samples found that the procedure could detect 1.3 *E. coli* O157:H7 per g (Samadpour et al., 1990).

Digoxigenin-labeled polynucleotide and oligonucleotide DNA probes were found to be as sensitive and specific for the detection of VTEC as radiolabeled probes (Padhye and Doyle, 1992). Bacterial isolates were spotted onto nylon membranes following overnight growth in nutrient broth. The nylon membranes were subsequently placed on MSA and incubated for 6 h at 37 °C. The cells were then lysed and the released DNA was hybridized with VT probes, thus detecting the VT gene. The digoxigenin-labeled polynucleotide and oligonucleotide probes detected the VT gene in all of 100 VTEC tested (Padhye and Doyle, 1992).

***eae* Gene.** The polymerase chain reaction (PCR) can be used to detect the attaching and effacing (*eae*) gene of *E. coli* O157:H7 (Gannon et al., 1993). A primer pair with homology to the 3' nucleotide sequence of *eae* from *E. coli* O157:H7 appears to be specific for *E. coli* O157:H7 and H- strains. PCR provides a rapid and sensitive means of detecting the *eae* gene in human and bovine isolates of *E. coli* O157:H7 (Gannon et al., 1993).

Characterization of Isolates

The ability to differentiate between bacterial isolates of the same serotype is essential in successfully tracing the source and route of the infection. The combined use of different methods may further assist efforts in determining the relatedness of strains and assess epidemiologic associations. Typing of bacterial isolates is important not only in halting a current outbreak of infection, but in contributing to the knowledge of the spread of infection and in the design of preventive programs (Pitt, 1994).

Ribotyping. Ribotyping is a technique in which labeled probes are used to detect rRNA genes in restriction fragments on a Southern blot. The method requires DNA extraction, digestion and separation of resulting fragments by gel electrophoresis and after Southern blotting, hybridization with a probe to detect probe-target hybrids. The resultant pattern of bands is referred to as a ribopattern or riboprint. rRNA genes are highly conserved and are present in multiple copies in the genome of all bacteria. This allows the use of universal primers and probes such as *E. coli* 16S and 23S rRNA.

Ribotyping provides results which are reproducible and relatively simple to interpret, however, the method is a complex, costly, labor-intensive and time-consuming method (Tarkka et al., 1994). Ribotyping has been shown to be an acceptable typing method for many bacterial pathogens, but has not been found to be useful for discriminating *E. coli* O157:H7 isolates (Martin et al., 1996).

λ-Restriction Fragment Length Polymorphism (RFLP) Analysis. An alternative approach to molecular characterization exploits the polymorphism of λ-bacteriophage sequences surrounding the VT genes in *E. coli* O157:H7 in order to differentiate unrelated

isolates and connect epidemiologically linked isolates of *E. coli* O157:H7. This technique uses λ-bacteriophage to probe DNA from *E. coli* O157:H7 digested with a suitable endonuclease (*PvuII*, *EcoRI*) (Paros et al., 1993; Samadpour et al., 1993).

λ- RFLP analysis provides a very discriminatory, reproducible and stable method for the epidemiologic analysis of strains of *E. coli* O157:H7 (Samadpour et al., 1993). This procedure has linked apparently unrelated cases of human infection through the analysis of random bovine and human isolates in a defined geographic area (Grimm et al., 1995; Paros et al., 1993; Samadpour et al., 1993).

The procedure requires electrophoresis and Southern hybridization making λ-RFLP analysis a time consuming technique. Although λ- RFLP analysis has been reported to be comparable to phage typing in its discriminatory power in characterizing *E. coli* O157:H7 isolates, it should be used in conjunction with an additional typing method (Paros et al., 1993). λ- RFLP requires the analysis of of multiple resulting bands which often makes comparisons difficult (Samadpour, 1995).

Phage Typing. The method of phage typing classifies strains of *E. coli* O157:H7 based on their lytic reactions on agar plates when incubated for 18 h at 37 °C with typing phages (Ahmed et al., 1987; Khakhria et al., 1990). Phage typing is more rapid and less labor intensive than methods such as PFGE. It is a relatively simple and inexpensive technique which is helpful in interpreting data in conjunction with other typing methods such as PFGE. The method can be used as a rapid screen to eliminate the need for performing another procedure (such as PFGE) on unrelated isolates. Phage typing is reproducible and provides an excellent first level of strain discrimination (Ahmed et al.,

1987; Barrett et al., 1994; Khakhria et al., 1990). A change in phage type, however, is also possible during an outbreak and therefore phage typing is best used in conjunction with another typing method. Phage typing is not as sensitive as PFGE for distinguishing outbreak and non-outbreak-related strains (Barrett et al., 1994).

Plasmid Analysis. Plasmid analysis can be useful in identifying strains associated with outbreaks of infection since nearly all *E. coli* O157:H7 carry a 60 MDa plasmid and certain strains carry additional plasmids (Vernozy-Rozand, 1997). Plasmid DNA can be prepared by the alkaline lysis mini-preparation procedure of Heilig et al. (1995) and electrophoresed in 40 mM Tris-acetate, 2 mM EDTA with 1% agarose stained with ethidium bromide. The bands in the gels can then be visualized on an ultraviolet transilluminator (Meng et al., 1995).

In conjunction with other methods such as PFGE and phage typing, plasmid analysis can provide useful information for the characterization of *E. coli* O157:H7 isolates associated with human infection (Ostroff et al., 1989b). Plasmid profiles are useful when plasmids, preferably two or more, are present in most of the isolates examined (Dorn and Angrick, 1991; Meng et al. 1995).

Plasmid profile determinations have some utility in epidemiologic investigations, but the range of different profiles is limited. Plasmid analysis of 100 *E. coli* O157:H7 strains revealed only three basic plasmid profiles (Ratnam et al., 1988). The loss, acquisition or transfer of plasmids as well as conformational changes in plasmids can also lead to difficulties in interpretation. In comparison to pulsed field gel electrophoresis (PFGE), this technique is less discriminatory since plasmids of the same molecular weight

but different DNA sequences are indistinguishable (Meng et al., 1995).

Pulsed Field Gel Electrophoresis (PFGE). Olive and Bean (1999) have referred to PFGE as the "gold standard" of molecular typing methods. The bacterial isolates are grown in broth, combined with molten agarose and poured into small molds, resulting in agarose plugs which contain the whole bacteria. The bacteria in the plug are lysed by enzyme treatment and then the DNA is digested with an infrequently cutting restriction enzyme. The digested bacterial plugs are inserted into an agarose gel and subjected to electrophoresis in which the orientation of the electric field is repeatedly changed and pulsed (Meng et al., 1995). As a result, this method allows for the resolution of large DNA fragments ranging from 10 to 800 kb.

PFGE is a time consuming process with tedious procedures for the purification of intact genomic DNA trapped in agarose, lengthy restriction enzyme digests and extended electrophoresis times (Barrett et al., 1994; Meng et al., 1995). Recently, Gautom (1997) has described a rapid PFGE protocol for the typing *E. coli* O157:H7 which can be carried out in 1 day .

During an outbreak of disease, random genetic events, including point mutations and insertions and deletions of DNA can alter the PFGE pattern making interpretation difficult. Each genetic event typically results in a 2-3 band difference in the PFGE pattern of the isolate as compared to the outbreak strain. Tenover et al. (1995) have presented categories of genetic and epidemiologic relatedness based on small sets of isolates (≤ 30) from outbreaks spanning 1 to 3 months. Isolates can be characterized as closely related or possibly related, when 2-3 or 4-6 fragment differences are observed, respectively. A

difference of 7 or more bands (3 or more genetic events) indicates that the isolate is unrelated to the outbreak strain.

Genomic DNA fingerprinting by PFGE has been shown to be highly discriminatory for the intraspecies characterization of a wide variety of organisms including *E. coli* O157:H7 (Meng et al., 1995). Genomic DNA patterns generated by PFGE are highly specific for different strains of an organism and have significant value in epidemiologic investigations of disease outbreaks (Barrett et al., 1994). PFGE represents the method of choice for the characterization of *E. coli* O157:H7 isolates.

CHAPTER 1

The Effect of Different Grain Diets on Fecal Shedding of *Escherichia coli* O157:H7 by Steers

Abstract

Three groups of six yearling steers (three rumen fistulated plus three non-fistulated) fed one of three different grain diets (85% cracked corn, 15% whole cottonseed/70% barley or 85% barley) were inoculated with 10^{10} CFU *E. coli* O157:H7 strain 3081 and the presence of the inoculated strain was followed in the rumen fluid and feces over a 10 week period. *E. coli* O157:H7 was rapidly eliminated from the rumen of the animals on all three diets but persisted in the feces of some animals up to 67 days post-inoculation, suggesting that the bovine hind-gut is the site of *E. coli* O157:H7 persistence. A significant difference existed in the levels of *E. coli* O157:H7 shed by the animals among diets on days 5, 7, 49 and 63 post-inoculation ($P<0.05$). No significant difference was found between the levels shed among diets on days 9 through 42 inclusive and on day 67 ($P>0.05$). The number of animals which were culture positive for *E. coli* O157:H7 strain 3081 over the 10 week period was significantly higher for the barley fed group (72 of 114 samplings) as opposed to the corn fed group (44 of 114 samplings)($P<0.005$) and the cottonseed/barley fed group (57 of 114 samplings)($P<0.05$). The fecal pH of the animals fed the corn diet was significantly lower ($P<0.05$) than the fecal pH of the animals fed the cottonseed/barley and barley diets likely resulting in a less suitable environment for *E. coli* O157:H7 in the hind-gut of the corn fed animals. *E. coli* O157:H7 strain 3081 was present in 3 of 30 (corn 1/10;

cottonseed 1/10; barley 1/10) animal drinking water samples, 3 of 30 (corn 1/10; cottonseed 0/10; barley 2/10) water trough bio-film swabs, 5 of 30 (corn 0/10; cottonseed 2/10; barley 3/10) feed samples and 30 of 30 manure samples taken from the pens over the entire experimental period. Mouth swabs of the steers were also culture positive for *E. coli* O157:H7 strain 3081 in 30 of 180 samples (corn 7/60; cottonseed 4/60; barley 19/60) taken over the 10 week period. Minimizing environmental dissemination of *E. coli* O157:H7 in conjunction with diet modification may reduce numbers of *E. coli* O157:H7-positive cattle. This chapter was published in the Journal of Food Protection , Vol. 63, No. 11, 2000, Pages 1467-1474 (Buchko et al., 2000a).

Introduction

Since 1982, when enterohemorrhagic *Escherichia coli* O157:H7 was first identified as a human pathogen, it has been implicated in numerous outbreaks of hemorrhagic colitis (HC) and life-threatening hemolytic uremic syndrome (HUS) (Ratnam et al. 1988; Riley et al. 1983). Epidemiological investigations demonstrate that cattle, both beef and dairy, are a principal reservoir of *E. coli* O157:H7 (Hancock et al. 1994; Wells et al., 1991). This association is further supported by numerous field surveys and trace-back studies which link *E. coli* O157:H7 directly and indirectly with bovine sources (Faith et al., 1996; Hancock et al., 1994; Wells et al., 1991). Although contaminated and improperly cooked ground beef has been implicated as the primary vehicle of transmission (Griffin and Tauxe, 1991; Hancock et al., 1994) foods such as radish sprouts (Itoh, 1998), apple cider (Besser et al., 1998), unpasteurized milk (Chapman et al.,1993; Mechie et al.,1997), mayonnaise (Raghubeer et al.,1997), yoghurt (Morgan et al.,1993), venison jerky (Keene et al.,1997) and water (Ackman et al.,1997) have also been linked to *E. coli* O157:H7 outbreaks.

Fecal shedding of *E. coli* O157:H7 in cattle herds is widespread and intermittent in nature (Hancock et al., 1994; Wells et al., 1991). It is well known that the season and the age of the animal have a significant effect on the level and duration of fecal shedding of *E. coli* O157:H7 by cattle and other ruminants. Several studies have reported peak fecal shedding of *E. coli* O157:H7 during the spring and summer months (Hancock et al.,1997; Mechie et al.,1997; Wells et al., 1991). It has also been reported that weaned dairy calves and yearling beef cattle at slaughter are more likely to shed the organism in their feces than adult cattle (Garber et al.,1995; Harmon et al.,1999; Van Donkersgoed et al.,1999). In

addition to these factors, a number of recent studies suggest that diet also influences the fecal shedding of *E. coli* O157:H7 by cattle (Dargatz et al.,1997; Garber et al.,1995; Hancock et al., 1994; Herriot et al.,1998).

Grain feeding is among the dietary factors which are considered to be important in respect to *E. coli* O157:H7 fecal shedding. Beef cattle are commonly fed energy-rich grain diets during the finishing periods of beef production prior to slaughter and fecal shedding of *E. coli* O157:H7 at slaughter is considered to be a significant source of contamination for beef (Van Donkersgoed et al.,1999). Rapid fermentation of grains lowers the ruminal and intestinal pH of cattle, favoring acid-resistant *E. coli* such as *E. coli* O157:H7. While it has been reported that grain- feeding as opposed to hay-feeding favors acid resistant *E. coli*, a more recent study has indicated that the acid sensitivity of *E. coli* O157:H7 is not affected by the diet of cattle (Diez-Gonzalez et al.,1998; Hovde et al.,1999). Much debate still exists concerning these findings and it is not clear if the cattle feeding industry will advocate management changes until the issue resolves.

Information provided by numerous farm surveys suggest that high energy grain diets are associated with herd *E. coli* O157:H7 infection status. Presently, conflicting reports based on survey data exist in regards to the association of whole cottonseed and the fecal shedding of *E. coli* O157:H7 (Dargatz et al.,1997; Garber et al.,1995; Hancock et al., 1994; Herriot et al.,1998). It was noted in one study that no association existed between the feeding of corn and the fecal shedding of *E. coli* O157:H7 while barley feeding was associated with an increase in *E. coli* O157:H7 isolated from fecal pats of feedlot cattle (Dargatz et al.,1997). While these dietary components have been identified as having an

effect on *E. coli* O157:H7 fecal shedding, the exact mechanism by which an association has resulted has not been experimentally verified. It is possible that not only the energy content of the diet is important but also the manner in which the feed is fermented. The high starch content of grains such as corn and high oil content of cottonseed may shift the site of fermentation from the rumen to the hind-gut (Doreau and Ferlay, 1994). This could alter conditions in the hind-gut which may affect the survival of gram negative organisms such as *E. coli* O157:H7.

The objective of the present study was to determine if the type of grain fed had an effect on the fecal shedding of *E. coli* O157:H7 in steers. A secondary objective was to assess the possible role of the environment of the cattle in the maintenance and dissemination of *E. coli* O157:H7.

Materials And Methods

Experimental Animals. Eighteen Hereford-Angus cross yearling steers (nine rumen-fistulated and nine non-fistulated) were used in the experiment. Rumen cannulas were inserted 4 weeks prior to beginning the experimental work. The animals were divided into three treatment groups of six animals (three rumen fistulated plus three non- fistulated) per group. One treatment group was used for each of the three diets. Each group of six animals was housed in a separate, sheltered outdoor pen, an arrangement which prevented nose-to-nose contact between animals in different pens. A separate water trough, feed manger and mineral box were present in each pen. Housing, care and procedures for all steers were approved by the Animal Care Committee (A.D.R.I., Lethbridge, Alberta).

Diet and Feeding. All diet formulations were on a dry matter basis and were fed

at a level of 2.5 - 3.0% of body weight. The three experimental diets were cracked corn, whole cottonseed/barley and barley. The corn diet consisted of 85% cracked corn and 15% alfalfa silage. The cottonseed/barley diet consisted of 15% whole cottonseed, 70% barley and 15% alfalfa silage. Current recommendations for the feeding of whole cottonseed to cattle suggest up to 150 g/kg of diet in order to avoid gossypol toxicity and any deleterious effects on rumen microorganisms (Arieli, 1998). The barley diet, which represented a standard finishing ration for feedlot animals in Alberta, Canada, consisted of 85% barley and 15% alfalfa silage. A 10% Beef Finisher Supplement (Unifeed, Okotoks, Canada) which contained minerals, Vitamins A, D, E plus monensin sodium at a level of 528.8 mg/kg, was incorporated into each of the diets at a level of 4% of the total ration. All diets were mixed on site and sufficient feed was prepared for a 10 day period and placed in an enclosed shed. The rations were fed *ad libitum* once per day with free access to water and a 2:1 (calcium:phosphorus) cattle mineral mixture (New Life Feeds, Calgary, Canada). Animals were conditioned to the control and experimental diets for a period of three weeks prior to inoculation with *E. coli* O157:H7.

Animal Inoculation. *E. coli* O157:H7 strain 3081 (kindly made available by W.C. Cray, National Animal Disease Center, Ames, Iowa) was used in order to facilitate the recovery of the organism from the animals following inoculation on selective media. The strain is resistant to 100 μg/ml ampicillin and 100 μg/ml kanamycin and produces verotoxin 1(VT1) and VT2. Strain 3081 was grown in 100 ml of tryptic soy broth (TSB) (BDH, Toronto, Canada) for 16 h at 37°C. Enumeration of *E. coli* O157:H7 strain 3081 from the TSB was performed using spectrophotometric readings at 600 nm (UltraSpec Plus

4054, Pharmacia, Baie d'Urfe', Canada) which were confirmed by standard dilution plating in triplicate onto sorbitol MacConkey agar (SMAC)(Oxoid, Nepean, Canada) supplemented with 2.5 mg/L potassium tellurite (Dynal, Lake Success, U.S.A.), 0.05 mg/L cefixime (Dynal), 100 µg/ml ampicillin (Sigma, Oakville, Canada) and 100 µg/ml kanamycin (Sigma) to yield CT-KASMAC. Cefixime has been reported to inhibit the growth of non-sorbitol-fermenting *Proteus* spp., while tellurite inhibits non-O157 *E. coli* and other non-sorbitol -fermenting enterics, minimizing the incidence of false positives (Bennett et al.,1995; Zadik et al., 1993). Each steer was inoculated with 50 ml of a culture containing 10^9 CFU/ml of *E. coli* O157:H7 strain 3081 using a sterile 60 cc. syringe (Fisher, Nepean, Canada) and gavage (using a stomach tube). Oral inoculation was followed by delivery of three 100 ml aliquots of phosphate-buffered saline, pH 7.4 (PBS; 15 mM KH_2PO_4, 8 mM Na_2HPO_4, 137 mM NaCl, 2.6 mM KCl).

Rumen Fluid and Fecal Sampling. Rumen fluid (50 - 100 ml) and fecal samples (5 - 10 g) were collected on the day of inoculation, three times a week for 4 weeks and then once a week for 6 weeks, for a total of 10 weeks. Rumen fluid samples from the nine fistulated steers were obtained by accessing the rumen directly through the cannula. Fecal samples were collected by digital rectal retrieval. All samples were collected using sterile latex gloves, with a new glove being used for each sample. Samples were placed in 118 ml sterile polypropylene specimen containers (Fisher) and transported to the laboratory where microbiological analysis was started within 1 h.

Volatile Fatty acid (VFA) Analysis and pH Determinations. A 2.5 ml sample of rumen fluid was acidified with 0.5 ml of 25% metaphosphoric acid (Sigma) and frozen

at -80 ° C for later VFA analysis. Thawed rumen fluid samples were clarified by centrifugation at 1320 g and 21 ° C for 30 min prior to VFA analysis by gas chromatography. Concentrations of acetic, butyric, propionic, iso-butyric , iso-valeric and valeric acids were determined by gas chromatography using a Varian Star 3400, 8100 Autosampler gas chromatograph (Varian Canada, Mississauga, Canada) equipped with a flame ionization detector and a Supelco column (SP-1200 packing; 183 cm x 0.2 cm column, inner diameter). The carrier gas was helium flowing at a rate of 25 ml/min. The analysis was isothermal for 4 min at 125 ° C with an injection temperature of 175 ° C and detector temperature of 195 ° C. VFAs were identified and automatically quantified from chromatograph peak areas as a result of previous calibration with external standards.

Rumen fluid pH determinations were made from each fistulated animal immediately following removal of the sample from the rumen. Fecal pH was recorded for an approximately 10 g sample following addition and mixing with 10 ml de-ionized water. The pH of the fecal samples obtained from each animal was taken once a week within 1 h of collection.

Mouth Swabs. Sterile swabs were used to obtain saliva samples from the inner cheek of each animal once a week. Swabs were immediately placed into 10 ml of modified tryptic soy broth (mTSB) containing 20.0 mg/L novobiocin (Sigma),1.5 g/L bile salts 3 (Difco, Ottawa, Canada), 1.5 g/L dipotassium phosphate (Sigma) and 30 g/L TSB (BDH), and transported to the laboratory for analysis within 1 h. Novobiocin has been reported to reduce the growth of Gram-positive organisms while having no adverse effect on Gram-negative organisms such as *E. coli* O157:H7 (Doyle and Schoeni, 1987; Sage and Ingham,

1998).

Environmental Sampling. Feed, water and manure samples were taken from each pen once a week. Swabs of the water trough were taken at the water-trough interface (bio-film). Water samples (approximately 100 ml) were obtained from each water trough following thorough mixing of the water in the trough. A 10 g sub-sample of feed was obtained from 5 pooled samples of approximately 20 g each. Feed piles which were mixed weekly were sampled following mixing but prior to feeding. The feed in the mangers was sampled approximately 4 h after animal access. Manure samples of approximately 20 g were obtained from three areas of each pen and pooled. All samples were placed in 118 ml sterile polypropylene specimen containers and transported to the laboratory for analysis within 1 h.

Isolation and Enumeration of *E. coli* O157:H7. The methods used for detection and quantification of *E. coli* O157:H7 were modeled after those used by Cray and Moon (1995) . To test for the presence of *E. coli* O157:H7 prior to inoculation, rumen fluid and fecal samples were cultured using direct and dynabead isolation and plated onto SMAC supplemented with 2.5 mg/L potassium tellurite and 0.05 mg/L cefixime (CT-SMAC) and CT-KASMAC.

For direct isolation 1.0 g of feces or 1.0 ml of rumen fluid was placed into 9 ml PBS. The sample was vortex-mixed and 100 µl was plated onto CT-SMAC and CT-KASMAC. Plates were incubated for 18-24 h at 37°C . Three sorbitol negative colonies from each plate were tested for the O157 antigen by agglutination with O157 antiserum (Difco). Multiplex PCR assays as described by Gannon et al. (1997) were used in order to

positively confirm the presence of *E. coli* O157:H7. The H7-specific primers used in combination with primers which target the VT1 and VT2 genes of *E. coli* O157:H7 and the *E. coli* O157:H7 *eaeA* gene, allow for the specific identification of *E. coli* O157:H7 in the O157 specific multiplex PCR assay (Gannon et al., 1997).

For dynabead isolation approximately 1.0 ml of the fecal or rumen fluid suspension previously added to 9 ml PBS was placed into 9 ml of mTSB and incubated for 6 h at 37°C. Following this enrichment step, immunomagnetic separation (IMS) of *E. coli* O157 was performed using Dynabeads anti-*E. coli* O157 (Dynal) according to the manufacturer's instructions. A 50 ul aliquot of the sample-exposed antibody-coated bead suspension was plated onto single plates of CT-KASMAC and CT-SMAC and the plates were incubated for 18-24 h at 37 ° C. Three sorbitol negative colonies from each plate were tested for the O157 antigen by agglutination with O157 antiserum. The presence of VT, $eaeA_{spec}$ and *fliC* genes was confirmed using multiplex PCR as in direct isolation.

For quantification and detection of *E. coli* O157:H7 *strain* 3081 following inoculation, a 1.0 g sample of feces or a 1.0 ml aliquot of rumen fluid was placed in 9 ml PBS. Samples were then vortex-mixed and serially diluted in PBS. A 100 µl aliquot from each dilution was then plated evenly onto CT-KASMAC in triplicate using a glass spreader. Once a week samples were plated onto CT-SMAC and CT-KASMAC in order to detect non-3081 *E. coli* O157:H7 strains. Plates were incubated for 18-24 h at 37°C. Three sorbitol negative colonies from each plate were tested for the O157 antigen by agglutination with O157 antiserum. Multiplex PCR (O157 specific) assays were used as a verification tool on one rumen fluid and one fecal sample a week in which the experimental strain was

recovered (*E. coli* O157:H7 *strain* 3081). Enrichment and dynabead isolation was performed on fecal and rumen fluid samples if *E. coli* O157:H7 strain3081 was not detected by direct plating.

Isolation of *E. coli* O157:H7 from Environmental Samples and Mouth Swabs. Water samples (100 ml) were filtered through a Corning 150 ml disposable bottle top filter (0.2 μm) (Fisher). The filter was then removed with a sterile scalpel and placed into 90 ml of mTSB. For feed and manure samples, 10 g was placed into 90 ml mTSB. Mouth swabs and water- trough bio-film swabs were placed into 10 ml mTSB immediately following sampling. All samples were mixed well and incubated for 18-24 h at 37° C. Dynabead isolation was performed and a 50 ul aliquot of the antibody-coated bead suspension was plated onto CT-KASMAC and CT-SMAC and incubated for 18-24 h at 37° C. Suspect colonies were confirmed using O157 antiserum and O157 specific multiplex PCR as described previously.

Statistical Analysis. For enumeration of *E. coli* O157:H7 *strain* 3081, fecal and rumen fluid samples were plated in triplicate onto selective media. Analysis of variance was performed using the SAS General Linear Models procedure (SAS Institute Inc., Cary, N.C.). The data was analyzed as a split-plot in time with diet as the main plot and day as the subplot. Animals nested within diet were used as the error term to evaluate diet. The least significant difference (LSD) test was used to determine the differences among means where significant effects were observed ($P<0.05$). Chi-square analysis was used to determine if differences in numbers of animals culture positive for *E. coli* O157:H7 among treatments were significant ($P<0.05$).

Results

Fecal Shedding of *E. coli* O157:H7 *strain* 3081. Pre-inoculation fecal samples from animals on each of the three diets were negative for *E. coli* O157:H7 using direct and dynabead isolation. The numbers of *E. coli* O157:H7 strain 3081 shed in the feces of the animals decreased over the first 14 days post-inoculation for steers on all three diets. Thereafter, variation existed in the magnitude of shedding among animals with numbers of *E. coli* O157:H7 recovered ranging from 7.8×10^4 CFU/g to undetectable (Figure 1.1).

On days 1 and 2 following inoculation with *E. coli* O157:H7 strain 3081, no significant difference existed in the levels of *E. coli* O157:H7 shed in the feces of the animals on the three diets ($P>0.05$). Five days post-inoculation the levels of *E. coli* O157:H7 strain 3081 shed by the animals on the corn diet were significantly lower than the levels shed by the animals on the cottonseed/barley and barley diets ($P<0.01$). On day 7, levels shed by animals on the corn diet were significantly lower than from animals on the cottonseed/barley diet ($P<0.05$). No significant difference was found between the levels shed among diets on days 9 through 42 inclusive and on day 67. On day 49, levels shed by the animals on the corn and cottonseed/barley diets were found to be significantly lower than the levels shed by the animals on the barley diet ($P<0.05$). A difference in levels also existed between the animals on the cottonseed/barley and barley diets on day 63, with levels of *E. coli* O157:H7 shed by the animals fed cottonseed/barley being significantly lower than levels shed by animals fed barley ($P<0.05$).

The frequency with which animals were culture positive for *E. coli* O157:H7 *strain* 3081 over the entire experimental period was significantly higher for the barley fed animals

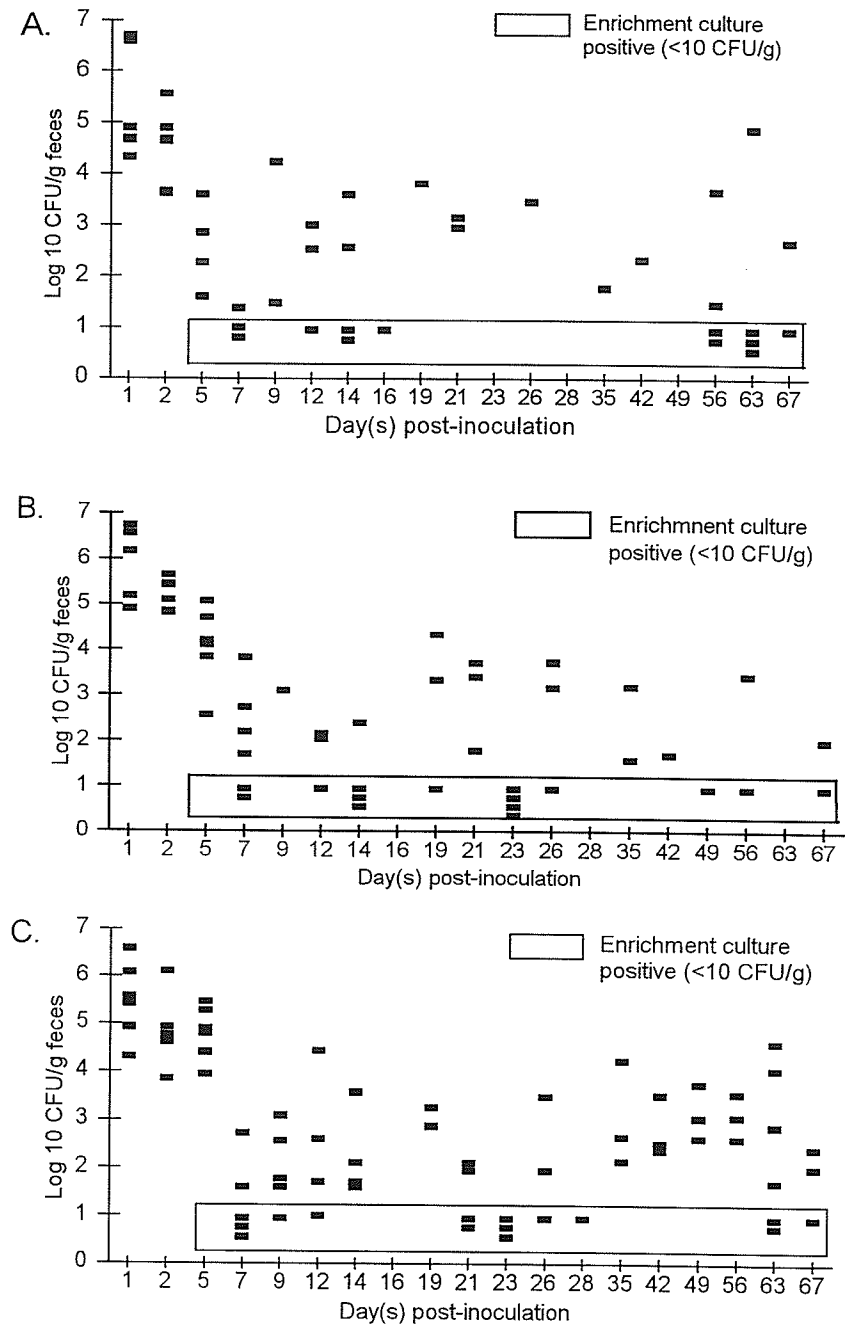

Figure 1.1. Levels of *E. coli* O157:H7 strain 3081 shed in the feces of steers fed three different grain diets. Animals were inoculated with 10^{10} CFU of *E. coli* O157:H7 strain 3081 on day 0 (n = 6 for each of the three diets). A, corn (85% cracked corn, 15% alfalfa silage); B, cottonseed/barley (15% whole cottonseed, 70% barley, 15% alfalfa silage); C, barley (85% barley, 15% alfalfa silage). Each symbol represents an individual animal. Symbols in the enrichment culture positive box represent animals which were culture negative by direct plating but were culture positive using enrichment followed by dynabead isolation.

(72 of 114 samplings) as compared to the corn fed animals (44 of 114 samplings)($P<0.005$) and the cottonseed/barley fed animals (57 of 114 samplings)($P<0.05$) (data not shown). No significant difference existed between the number of animals shedding *E. coli* O157:H7 in the corn fed group as compared to the cottonseed/barley fed group ($P>0.05$). All animals on the corn diet were culture negative on days 23, 28 and 49. All animals on the cottonseed diet were culture negative on days 16, 28 and 63. All animals on the barley diet were culture negative for *E. coli* O157:H7 *strain* 3081 on day 16 but at least one animal in the barley fed group remained positive for *E. coli* O157:H7 for the duration of the experimental period (Figure 1.2).

The presence of non-3081 strains of *E. coli* O157:H7 was detected on one sampling occasion 19 days post-inoculation. The feces of two animals in the cottonseed fed group and one animal in the corn fed group were found to be culture positive for *E. coli* O157:H7 but not the inoculated strain. The isolates, which were recovered on CT-SMAC and failed to grow on CT-KASMAC, were confirmed as *E. coli* O157:H7 using latex agglutination and multiplex PCR.

Isolation of *E. coli* O157:H7 *strain* 3081 from Rumen Fluid. The inoculated strain disappeared rapidly from the rumen fluid with the animals on all three diets culture negative for *E. coli* O157:H7 *strain* 3081 on day 16 post-inoculation (data not shown). The inoculated strain was not subsequently recovered from rumen fluid for the duration of the experimental period with the exception of rumen fluid from one animal in the cottonseed group which was positive for *E. coli* O157:H7 on day 26. Non-3081 strains of *E. coli* O157:H7 were not recovered from the rumen fluid of any of the animals on the three diets

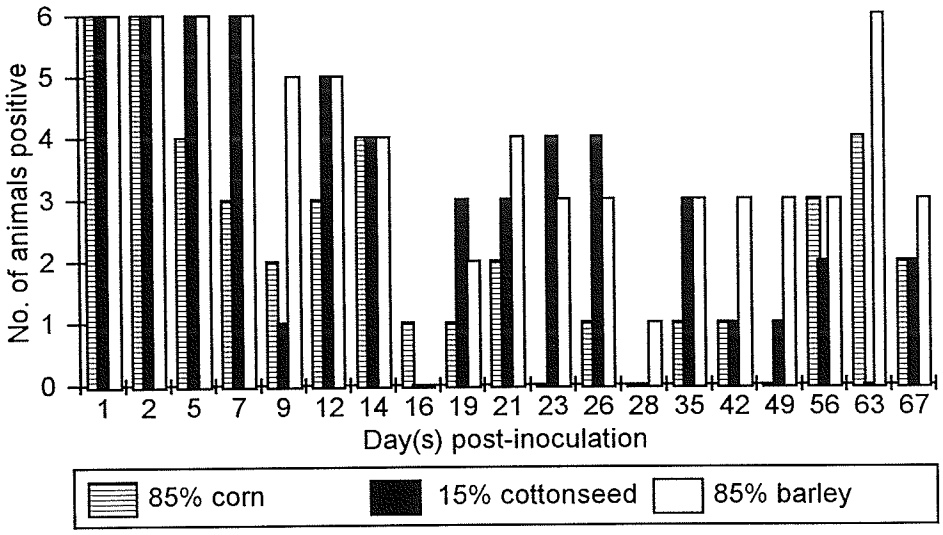

Figure 1.2. The number of animals on three different grain diets in which *E. coli* O157:H7 strain 3081 was isolated from fecal samples. Animals were inoculated with 10^{10} CFU of *E. coli* O157:H7 strain 3081 on day 0 (n=6 for each of the three diets). A, corn (85% cracked corn, 15% alfalfa silage); B, cottonseed/barley (15% whole cottonseed, 70% barley, 15% alfalfa silage); C, barley (85% barley, 15% alfalfa silage).

during the entire experimental period. There was no significant difference in the mean levels of *E. coli* O157:H7 *strain* 3081 recovered from the rumen fluid of the animals on the barley, cracked corn or cottonseed diets ($P>0.05$).

Rumen Fluid pH and Volatile Fatty Acid (VFA) Analysis. The rumen fluid pH for animals on the corn diet ranged between 5.6 and 6.6; for the cottonseed/barley diet between 5.9 and 7.0, and for the barley diet between 5.9 and 6.8 (Figure 1.3A). The mean VFA concentrations in the rumen fluid of the animals over the entire experimental period were 107.4 ±3, 107.9 ±3 and 104.81 ±2 mmol/L for the corn, cottonseed/barley and barley diets respectively. Acetate and propionate were the predominant VFAs detected in the rumen fluid of the fistulated steers on all three diets, accounting for 50 and 35% of the total VFAs respectively. The remaining 15% consisted of butyrate, valerate, iso-valerate and iso-butyrate (data not shown).

Fecal pH. The animals on the corn diet had significantly lower fecal pH values than the animals on the barley and cottonseed/barley diets ($P<0.05$) (Figure 1.3B). The mean fecal pH values of the animals on the corn diet were between 5.4 and 6.3, while the mean fecal pH values of the animals on the cottonseed/barley and barley diets were between 6.6 and 7.5, over the entire experimental period (Figure 1.3B).

Prevalence of *E. coli* O157:H7 *strain* 3081 in Mouth Swabs and Environmental Samples. The inoculated strain was recovered from 30 of 180 (16.7%) mouth swab samples. *E. coli* O157:H7 was recovered most frequently from the mouth swabs of the animals fed the barley diet with 19 of 60 (31.7%) culture positive samples. In comparison, 7 of 60 (11.7%) and 4 of 60 (6.7%) culture positive samples were obtained

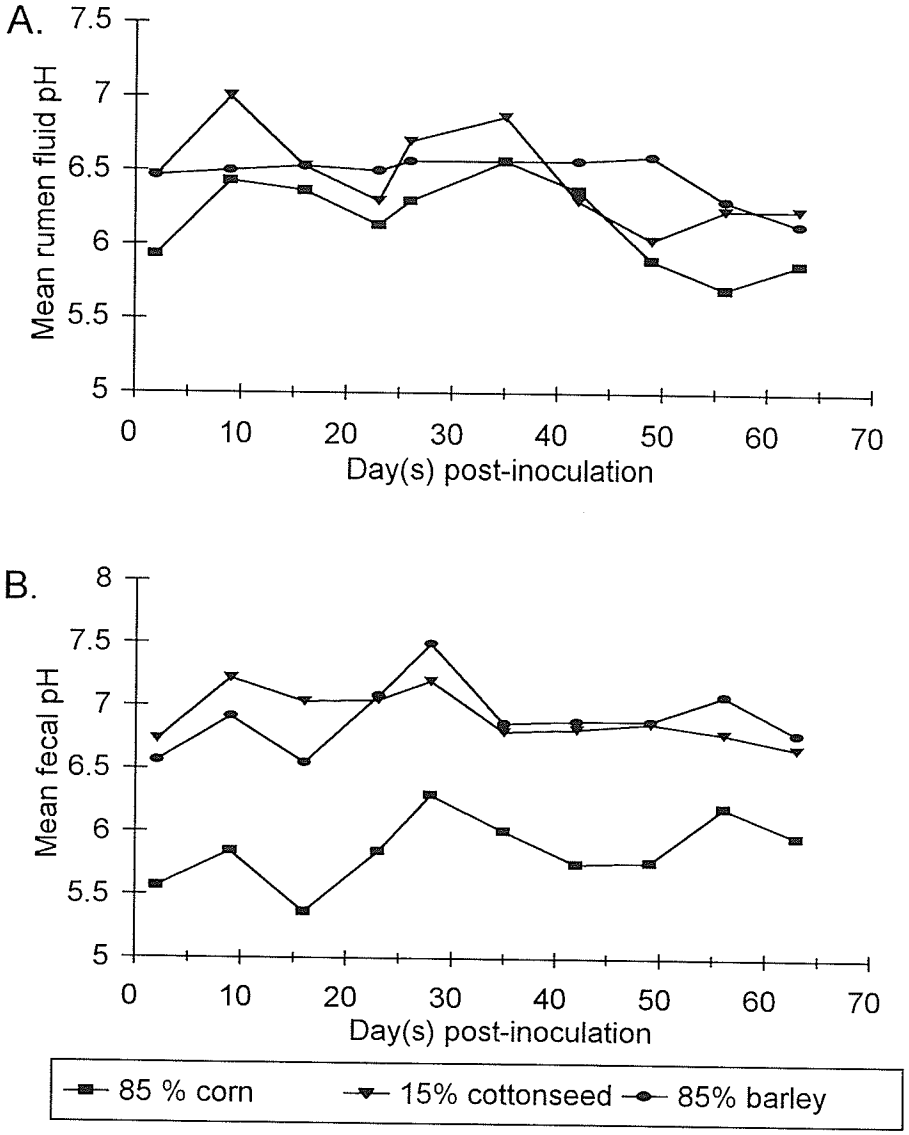

Figure 1.3. The mean pH of rumen fluid and fecal samples from steers fed diets of corn (85% cracked corn, 15% alfalfa silage), cottonseed and barley (15% whole cottonseed, 70% barley, 15% alfalfa silage), and barley (85% barley, 15% alfalfa silage). (A) Rumen fluid (n = 3); (B) fecal samples (n = 6). Steers were inoculated with 10^{10} CFU *E. coli* O157:H7 strain 3081 on day 0.

from the animals fed the corn and cottonseed/barley diets, respectively (Table 1.1). Mouth swabs from each of the six animals in the barley fed group were culture positive for *E. coli* O157:H7 at least once over the entire experimental period. The inoculated strain was not recovered from the mouth swabs of two animals in the corn fed group and three animals in the cottonseed fed group. Culture positive mouth swab samples were found throughout the experimental period.

The animal drinking water was culture positive for *E. coli* O157:H7 a total of three times during the experimental period, all of these occurring within 14 days post-inoculation. The water in the barley fed pen was culture positive for a non-3081 strain of *E. coli* O157:H7 while the water in the corn and cottonseed/barley fed pens was culture positive only for the inoculated strain. *E. coli* O157:H7 strain 3081 was recovered from 3 of 30 (10%) water-trough bio-film swab samples. The organism was isolated from the water-trough bio-film swabs on day 12 from the corn fed pen, and on days 26 and 61 from the barley fed pen. The inoculated strain was recovered from five of a total of 30 (16.7%) feed samples following animal access; three (10%) of these positive samples were recovered from the barley fed pen and two (6.7%) from the cottonseed/barley fed pen. The culture positive samples were recovered on days 6, 54 and 61 post-inoculation from the barley fed pen. The feed from the cottonseed/barley fed pen was culture positive on days 12 and 54 post-inoculation. Manure samples taken from the pen floors of each of the diet groups over the entire experimental period were 100% positive for *E. coli* O157:H7 *strain* 3081 (Table 1.1).

Table 1.1. Recovery[a] of *E. coli* O157:H7 strain 3081 from mouth swabs of experimentally inoculated steers and their environment using Dynabead isolation following overnight enrichment in mTSB at 37 °C.

Diet	Mouth swabs	Water	Water-trough Biofilm	Feed[b]	Manure
Corn	7/60	1/10	1/10	0/10	10/10
Cottonseed	4/60	1/10	0/10	2/10	10/10
Barley	19/60	1/10[c]	2/10	3/10	10/10
Total positive	30/180	3/30	3/30	5/30	30/30

[a] number culture positive for *E. coli* O157:H7/number of samples

[b] samples were obtained from feed mangers 4 h after animal access

[c] non-3081 strain of *E. coli* O157:H7.

Discussion

The pattern of fecal shedding observed in the steers following oral inoculation with *E. coli* O157:H7 was similar to that found in previous studies. There was a steady decline in number of *E. coli* O157:H7 in the feces for the first 14 days post-inoculation with numbers being highly variable thereafter (Brown et al., 1997; Cray and Moon, 1995; Harmon et al.,1999). The animals remained healthy throughout the entire experimental period in agreement with observations by others that cattle are asymptomatic carriers of *E. coli* O157:H7 (Hancock et al., 1994; Wells et al., 1991).

Two studies have found a negative association with the feeding of whole cottonseed and the incidence of *E. coli* O157:H7 (Garber et al.,1995; Hancock et al., 1994), while two similar studies have failed to find an association (Dargatz et al.,1997; Herriot et al.,1998). The findings of the present study indicate that no significant difference in the levels of *E. coli* O157:H7 fecal shedding occurred on most sampling days in steers fed 15% whole cottonseed/70% barley as compared to 85% corn or 85% barley.

It has been reported that the use of barley in the diets of cattle is associated with an increased likelihood in the isolation of *E. coli* O157:H7 from the fecal pats of feedlot cattle (Dargatz et al.,1997). The significantly greater number of animals in the barley fed group shedding *E. coli* O157:H7 as opposed to the corn and cottonseed/barley fed groups in the present study would support such findings ($P<0.05$). However, further investigation is warranted in order to define the dynamics of the association between barley feeding and *E. coli* O157:H7 fecal shedding. The feeding of barley and corn are strongly regional practices. There appears to be a geographic distribution of *E. coli* O157:H7 infection, with

outbreaks and sporadic cases being more common in the northern than southern states of the United States and more common in western as opposed to eastern Canada (Griffin and Tauxe, 1991; Nataro and Kaper, 1998; Slutsker et al., 1997; Waters et al., 1994). It is possible that geographical differences in rates of human infection with *E. coli* O157:H7 are related to different regional cattle feeding practices and resultant levels of animal carriage of *E. coli* O157:H7.

Although no significant difference existed in the levels of *E. coli* O157:H7 shed by the animals on each of the three diets on most days, a highly significant difference existed in the number of animals culture positive for *E. coli* O157:H7 fed the corn diet as opposed to the number of animals culture positive for *E. coli* O157:H7 fed the barley diet ($P<0.005$). Animals in the corn fed group also had significantly lower fecal pH values than animals in the cottonseed/barley or barley fed groups ($P < 0.05$) (Figure 1.3B). It has been shown that barley has a very rapid rate of digestion in the rumen, while much of the starch in corn escapes rumen degradation and is digested in the small intestine or fermented in the large intestine (Orskov, 1986). It is possible that the lower fecal pH in combination with inhibitory fecal VFAs of the animals fed the corn diet make the large intestine a less suitable site for the proliferation of *E. coli* O157:H7 as opposed to conditions in the large intestine of the cottonseed/barley or barley fed animals.

In the present study, *E. coli* O157:H7 did not persist in the rumen, or at least not within the detection limits of the sampling and culture method used. It is possible that pH in conjunction with VFA concentrations aided in the rapid elimination of *E. coli* O157:H7 from the rumen of the animals on all three diets. While *E. coli* O157:H7 strain 3081 was

detected in the rumen fluid of one animal after 16 days post-inoculation, the organism was recovered from the feces of 0/6 to 6/6 of the animals in each diet group for up to 67 days post-inoculation. This implies that growth and maintenance of the organism is more likely to occur in the hind-gut as opposed to the rumen. Although attachment followed by subsequent release of *E. coli* 157:H7 from the rumen wall is possible, this would seem unlikely since *E. coli* O157:H7 does not appear to colonize the mucosal surface of the rumen (Brown et al., 1997; Cray and Moon, 1995). The isolation of *E. coli* O157:H7 from the rumen fluid of one animal in the cottonseed/barley fed group on day 26 post-inoculation was an exception to this general finding. However, it is not known if the organism was present in the rumen fluid as a result of reactivation or simply resulted from recent ingestion of the organism present in feed or water. It is also interesting to note that fecal samples from this rumen-positive animal were culture negative for *E. coli* O157:H7 on day 16 and remained culture negative for the remainder of the experimental period.

The frequent rise in the prevalence of *E. coli* O157:H7 in cattle and the incidence of *E. coli* O157:H7 associated food-borne illness during the summer months suggests that environmental replication plays a key role in the epidemiology of infections (Hancock, 1997). Feed, water and manure were all potential sources of *E. coli* O157:H7 strain 3081 for animal re-infection. The present study began in February and ended in June and it is possible that warmer temperatures late in the study may have contributed to the growth and spread of *E. coli* O157:H7 in the animal environment. The study also simulated a feedlot environment and it is possible that under such conditions the spread of *E. coli* O157:H7 was promoted, rendering the cattle more susceptible to re-infection (Hovde et al.,1999).

E. coli O157:H7 can persist in water trough sediments for periods of at least 4 months and may even be able to replicate in this environment (Hancock, 1997). The isolation of *E. coli* O157:H7 in the water and water trough bio-film swabs in our study suggests that contaminated water troughs may be a site where *E. coli* O157:H7 is maintained and from which it may be spread among animals (Faith et al., 1996; MacDonald et al., 1996). A recent study has presented evidence which suggests that oral contamination as opposed to fecal contamination resulted in the dissemination of *E. coli* O157:H7 through animal drinking water (Shere et al., 1998). The frequent isolation of *E. coli* O157:H7 strain 3081 from the mouth swabs of the animals suggest that the organism may be able to persist or even proliferate in the alkaline saliva in the mouth of cattle long enough to be spread to feed, water or other cattle. The positive mouth swab samples from animals with negative rumen fluid samples strongly suggests that regurgitation of rumen contents was not the source of *E. coli* O157:H7 in the mouth swabs of the steers. It is interesting to note that *E. coli* O157:H7 strain 3081 was recovered more often from the mouth swabs and environmental samples of the barley fed group, despite the fact that all of the animals were inoculated with the same number of *E. coli* O157:H7. It is possible that the barley fed animals were exposed to higher levels of *E. coli* O157:H7 in their environment due to the greater number of animals shedding the organism compared to the number of animals shedding *E. coli* O157:H7 in the corn and cottonseed/barley fed groups (Figure 1.2). It is also possible that once excreted, the survival of *E. coli* O157:H7 was enhanced in the feces of barley fed animals. The feces of the animals in the corn and cottonseed/barley fed groups may have been more antagonistic to the survival of *E. coli* O157:H7. While selectivity for

E. coli O157:H7 has been increased with the use of CT-KASMAC, it is recognized that injured microorganisms may be present but escape detection because they are unable to form colonies on selective media. As a result, viable counts of *E. coli* O157:H7 may be an underestimation of actual numbers present (Bennett et.,1995; Hurst, 1977; Zadik et al., 1993).

This study demonstrates that the control of fecal shedding of *E. coli* O157:H7 in cattle requires a multi-faceted approach. Although no significant difference existed in the levels of *E. coli* O157:H7 shed by the animals on each of the three different grain diets on most days, it appears that a diet of 85% cracked corn results in significantly fewer animals shedding *E. coli* O157:H7 as compared to an 85% barley diet. Oral survival of *E. coli* O157:H7strain 3081 in steers and its survival in feed, water and manure raise the question of environmental survival of *E. coli* O157:H7 in response to diet (ie. the type of manure). Recurrent exposure to environmental sources of *E. coli* O157:H7 may play an important role in the maintenance and spread of the organism among animals. Targeting environmental sources contributing to the establishment and dissemination of *E. coli* O157:H7 in herds, in addition to modification of diet, may result in the reduction of this deadly pathogen on the farm.

CHAPTER 2

The Effect of Fasting and Diet on Fecal Shedding of *Escherichia coli* O157:H7 by Cattle

Abstract

Cattle naturally infected with *Escherichia coli* O157:H7 were used to assess the effects of diet and feed withdrawal on the fecal shedding of *E. coli* O157:H7. Animals were fed an 80% concentrate diet (80% barley and 20% alfalfa silage), fasted for 48 h, fed a 100% forage diet (alfalfa silage), fasted for 48 h, and subsequently re-fed 100% forage (alfalfa silage). There were no differences in the numbers of animals positive for the shedding of *E. coli* O157:H7 when fed an 80% barley diet or an all-forage diet ($P>0.05$) or during the fasting periods following each diet ($P>0.05$). Upon re-feeding an all-forage diet following a 48 h fast, animals positive for *E. coli* O157:H7 shedding increased ($P<0.05$), with 42.5% of the animals shedding the pathogen after five days. Re-feeding 100% forage following fasting appeared to have increased the number of animals shedding *E. coli* O157:H7 in their feces, which may have been influenced by diet in addition to fasting. This chapter was published in the Canadian Journal of Animal Science, Vol. 80, 2000, Pages 741- 744 (Buchko et al., 2000b).

Introduction

Escherichia coli O157:H7 has evolved as an important food-borne pathogen since its initial description in 1982 and outbreaks associated with *E. coli* O157:H7 have been reported with increased frequency (Riley et al., 1983). Although contaminated ground beef and raw milk have been implicated as the primary vehicles of transmission, foods of non-bovine origin have also been linked to *E. coli* O157:H7 outbreaks (Griffin and Tauxe, 1991).

Epidemiological investigations in addition to numerous surveys have demonstrated that cattle are a principal reservoir of *E. coli* O157:H7 (Griffin and Tauxe, 1991; Hancock et al., 1994; Zhao et al.,1995). Fecal shedding of *E. coli* O157:H7 is intermittent in nature and the organism does not cause disease in cattle (Besser et al., 1997). Fecal contamination of carcasses with *E. coli* O157:H7 during animal production and slaughter is assumed to be the main source of the organism in products such as ground beef (Buchanan and Doyle, 1997; Elder et al., 2000). Transmission of *E. coli* O157:H7 to other food products is thought to be the result of contact with contaminated bovine products (Griffin and Tauxe, 1991).

It has been suggested that diet may play an important role in the fecal shedding of *E. coli* O157:H7 in cattle. In a study conducted using sheep inoculated with *E. coli* O157:H7, a change from a high energy, low fibre diet to a low energy, high fibre diet was found to increase *E. coli* O157:H7 fecal shedding while a change in diet in the opposite direction resulted in a decrease in shedding (Kudva et al., 1997). Similarly, steers experimentally inoculated with *E. coli* O157:H7 and fed hay were found to shed *E. coli*

O157:H7 for an average of 39 to 42 days while grain-fed cattle were culture positive for 4 days (Hovde et al., 1999).

While it has been reported that fasting alone has no significant effect on the shedding of *E. coli* O157:H7, diet and manner of feeding prior to feed withdrawal may affect fecal shedding of the pathogen (Cray et al., 1998; Harmon et al., 1999; Kudva et al., 1997). A study using calves inoculated with *E. coli* O157:H7 found that while fasting reduced rumen volatile fatty acid (VFA) concentrations and increased rumen pH, it had no significant effect on numbers of *E. coli* O157:H7 present in the rumen or shed in the feces as a result of feed withdrawal (Harmon et al., 1999). This suggests that the bovine hind-gut, as opposed to the rumen, is likely the key niche for the proliferation of *E. coli* O157:H7. The objective of the present study was to determine if fasting in conjunction with diet composition subsequent to fasting, had a significant effect on the number of cattle shedding *E. coli* O157:H7 in their feces.

Materials And Methods

Forty Hereford x Angus yearling steers and heifers from a herd naturally infected with *E. coli* O157:H7 were divided into four pens of 10 animals with an equal number of heifers and steers in each pen. Animals in all 4 pens were fed an 80% concentrate diet (80% barley and 20% alfalfa silage), fasted for 48 h, fed a 100% forage diet (alfalfa silage), fasted for 48 h, and subsequently re-fed 100% forage (alfalfa silage). Animals were worked up gradually to the 80% barley diet over a period of 5 weeks. Once adapted, the animals remained on their respective diets for a minimum of two weeks to ensure a stable rumen environment existed prior to being fasted. All diets were formulated on a dry matter basis

and rations were fed *ad libitum* once per day at the same time each day. The animals were housed in an outside feedlot with free access to water and a mineral supplement (Unifeed, Okotoks, AB). The experiment was conducted during the months of September, October and November and no extreme weather conditions were observed. Housing, care and procedures for all animals were in accordance with guidelines of the Canadian Council on Animal Care (1993).

Fecal samples of approximately 10 g were collected from each animal twice a week at the same time each day while they were on either the 80% barley or 100% alfalfa silage diet. During the 48 h fasting period, samples were collected at 12, 24, 36 and 48 h following the last missed feeding time. Fecal samples were collected directly from the rectum of cattle using a new sterile latex glove for each sample, placed into Cary-Blair transport medium (Dalynn, Calgary, AB) and transported to the laboratory for analysis within 1 h.

In order to maximize the detection of *E. coli* O157:H7 from fecal samples, the methods of direct and dynabead isolation were used in parallel on each sample. For direct isolation, samples were plated directly from the Cary-Blair transport medium onto sorbitol MacConkey agar (SMAC) (Oxoid, Nepean, ON) supplemented with 2.5 mg/L potassium tellurite (Dynal, Lake Success, NY) and 0.05 mg/L cefixime (Dynal) (CT-SMAC) using sterile cotton tipped swabs. Plates were incubated at 37° C for 18-24 h. For dynabead isolation, approximately 1.0 ml of the fecal suspension from the Cary-Blair transport medium was placed into 9 ml of modified tryptic soy broth (mTSB) containing 20.0 mg L^{-1} novobiocin (Sigma, Oakville, ON), 1.5 g L^{-1} bile salts 3 (Difco, Ottawa, ON) 1.5 g L^{-1}

dipotassium phosphate (Sigma), and 30 g L^{-1} tryptic soy broth (TSB)(BDH, Toronto, ON) and incubated at 42 ° C for 6h. Immunomagnetic separation (IMS) of *E. coli* O157 was performed using Dynabeads anti-*E. coli* O157 (Dynal) according to the manufacturer's instructions. Following IMS, 50 ul of the bead suspension was plated onto CT-SMAC. Plates were incubated at 37 °C for 18-24 h. Five sorbitol negative colonies from each plate were tested for the O157 antigen by slide agglutination with O157 antiserum (Difco). A fecal sample was considered positive if *E. coli* O157:H7 was isolated by either direct or dynabead isolation, or both. A multiplex PCR assay described by Gannon et al. (1997) was used in the detection of *vt*, *eaeA* and *flic* (H7) genes and in the specific identification of *E. coli* O157:H7.

The experiment was conducted as a repeated measures design where the pens were blocks and the treatment was time. The incidence of *E. coli* O157:H7 fecal shedding in the animals was compared during each diet period and during each 48 h fast. Analysis of variance was calculated using the SAS General Linear Models procedure (SAS Institute, Inc.,1989). Duncan's multiple range test was used to determine differences between treatment means with significance at the 5% level of probability.

Results And Discussion

The number of animals with feces culture positive for *E. coli* O157:H7 while on an 80% barley diet or a 100% alfalfa silage diet (after the first fast) was not different and ranged from 5 (2/40) to 10% (4/40) ($P>0.05$) (Figure 2.1). A 48 h fast following the feeding of an 80% barley diet for two weeks had no significant effect on the numbers of animals which shed *E. coli* O157:H7, with between 0 and 10% of fecal samples being

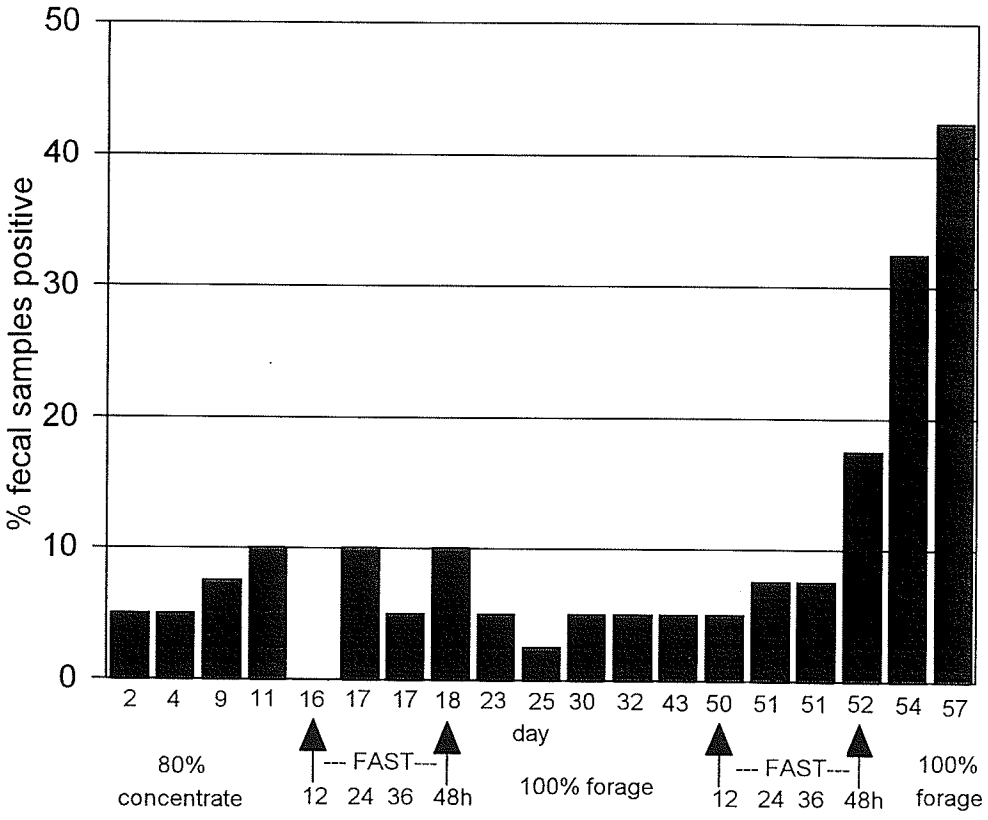

Figure 2.1. A comparison of the prevalence of *E. coli* O157:H7 in the fecal samples of 40 yearling heifers and steers on 80% concentrate (barley) and 100% forage (alfalfa silage) diets and 48 h fasting periods following each diet. Arrows represent the beginning and end of each 48 h fasting period.

culture positive (P>0.05). The organism however, was not recovered from the feces of any of the animals after 12 h of feed withdrawal following the feeding of the 80% barley diet. There was no difference between the numbers of positive fecal samples during the two fasting periods (P>0.05). An increase in *E. coli* O157:H7 positive fecal samples occurred at 48 h after the last missed feeding (22.5%) following the 100% alfalfa silage diet, however, this increase was not significant (P>0.05). Upon re-feeding 100% alfalfa silage following the second 48 h fast, the number of animals positive for *E. coli* O157:H7 increased (P< 0.05), with 32.5% and 42.5% of the fecal samples positive at 3 and 5 days after completion of the fast, respectively (Figure 2.1).

Two animals shed *E. coli* O157:H7 in their feces 14/19 and 10/19 sampling times while in each of four pens there were some animals which were culture negative for *E. coli* O157:H7 during the entire experimental period (data not shown). This is consistent with the pattern of *E. coli* O157:H7 fecal shedding which has been observed in cattle. The bacterium is not always shed at detectable levels and methods used may not consistently detect the organism (Zhao et al., 1995). The prevalence of *E. coli* O157:H7 in herds may be low for extended periods of time with the status changing suddenly (Besser at al., 1997).

Dynabead isolation (enrichment and IMS of *E. coli* O157) proved to be a more sensitive method than direct isolation for the detection of *E. coli* O157:H7 from fecal samples. Samples were culture positive for *E. coli* O157:H7 in 74 of 75 positive samples by dynabead isolation while 22 of 75 positive samples were culture positive by direct isolation (data not shown).

The findings of the present study indicate that fasting does not result in a

significant increase in the fecal shedding of *E. coli* O157:H7 during the fasting period but may influence fecal shedding upon re-feeding. The absence of an increase in the number of fasted animals shedding *E. coli* O157:H7 may be attributed to inhibitory conditions in the rumen, competition by other microorganisms, the presence of bacteriocins, lysis of the bacteria by phage and predation by rumen protozoa (Cray et al.,1998).

The absence of culture positive animals after 12 h of feed withdrawal following the 80% concentrate diet (Figure 2.1) may be influenced by the time of day samples were taken, however, culture positive animals were found at the same time of day following 36 h of feed withdrawal. The more frequent fecal sampling during the fasting period likely demonstrates the intermittent pattern of *E. coli* O157:H7 fecal shedding observed in cattle (Zhao et al.,1995).

The increase in the number of animals shedding *E. coli* O157:H7 after 48 h of feed withdrawal following the 100% alfalfa silage diet and the further increase upon re-feeding alfalfa silage, may be influenced by dietary regime prior to the fast in addition to fasting. Low VFA concentrations, high pH and changes in microflora may have produced conditions in the hind-gut which were conducive to the growth and subsequent fecal shedding of *E. coli* O157:H7, resulting in an increase in the number of culture positive fecal samples. Such conditions may not have occurred during the first 48 h fast following the 80% barley diet or upon feeding 100% alfalfa silage following the first fast. The practice of forage feeding following a fast is not the practice for feedlot cattle destined for slaughter, but can occur with feeder cattle that pass through a sales barn.

The increase in *E. coli* O157:H7 positive animals may also be due to the increased

susceptibility of some animals to colonization by *E. coli* O157:H7 (Cray et al., 1998). Calves fasted prior to inoculation with *E. coli* O157:H7 were shown to be more susceptible to colonization with *E. coli* O157:H7 than animals fasted following inoculation (Cray et al., 1998), suggesting that the acid conditions in the rumen may prevent initial colonization by *E. coli* O157:H7 but have little effect on fecal shedding once the large intestine is colonized (Harmon et al., 1999).

The possibility of environmental contamination and horizontal transmission of *E. coli* O157:H7 in the farm environment can not be overlooked. Numerous studies have isolated the organism in the farm environment and imply that the environment may serve as a reservoir for the maintenance and further dissemination of the organism (Shere et al., 1998). Although the number of animals positive for *E. coli* O157:H7 in each of the four pens was similar, dissemination of the organism throughout each pen may have played a role in increasing the numbers of animals becoming infected. A build up of environmental contamination in the pens may also have contributed to an increase in the number of animals culture positive for *E. coli* O157:H7 late in the study.

In summary, fasting for 48 h and type of diet prior to the fast had no significant effect on fecal shedding of *E. coli* O157:H7 in cattle during the period of feed withdrawal. This suggests that feed withdrawal prior to slaughter should not increase the risk of *E. coli* O157:H7 entering the food chain. However, re-feeding 100% forage following a 48 h fast resulted in a significant increase in the number of animals shedding *E. coli* 0157:H7 which may have been influenced by both fasting and diet. This situation may occur when feeder cattle are moved from one farm to another via a sales barn and may be one of the reasons

for the higher incidence of *E. coli* 0157:H7 shedding by cattle when they first enter the feedlot (Dargatz et al.,1997). More controlled studies are required to assess the effects of diet in conjunction with feed withdrawal on the fecal shedding of *E. coli* O157:H7 in cattle.

CHAPTER 3

The Effect of Monensin Sodium on the Growth and/or Survival of *Escherichia coli* O157:H7 and *E. coli* ATCC 25922

Abstract

Monensin sodium (monensin) is a feed additive commonly used in ruminant diets to improve feed efficiency. The industry wide introduction of ionophore feeding in cattle occurred just prior to the first human cases of *Escherichia coli* O157:H7 foodborne illness. Pure culture studies were conducted to investigate whether monensin might be enhancing the growth and/or survival of the pathogen *E. coli* O157:H7. *E. coli* O157:H7 strain 3081 and *E. coli* ATCC 25922 (non-O157:H7) were cultured in brain heart infusion (BHI) broth containing monensin at 0, 5, 15, 25 and 50 μg/ml for 48 h at 37° C. In triplicate studies, each tube was inoculated with 10^5 CFU/ml of *E. coli* O157:H7 or *E. coli* ATCC 25922 and viable numbers were determined after 0, 6, 12, 24 and 48 h incubation. Monensin significantly ($P < 0.05$) reduced growth of *E. coli* O157:H7 strain 3081 at 50 μg/ml after 24 and 48 h. Monensin did not affect ($P > 0.05$) growth of *E. coli* ATCC 25922 at any of the levels tested. For improving feed efficiency of cattle, monensin is recommended for inclusion at a level of up to 33 ppm of diet dry matter (DM), which equates to 5 to 10 mg/L of ruminal fluid. At these levels, monensin did not affect the growth of *E. coli* O157:H7 strain 3081 or *E. coli* ATCC 25922.

Introduction

The human pathogen *Escherichia coli* O157:H7 is a global public health concern (Griffin and Tauxe, 1991). Although contaminated ground beef has been implicated as the primary vehicle of transmission, foods of non-bovine origin have also been linked to *E. coli* O157:H7 outbreaks (Griffin and Tauxe, 1991). It has been suggested that feed management practices such as the use of ionophores may be associated with fecal shedding of *E. coli* O157:H7 by cattle (Herriott et al., 1998).

Rumensin (monensin sodium) is one of the two most common ionophores used as a feed additive in cattle diets to improve feed efficiency, at a level of up to 33 ppm dry matter (DM) (USDA:APHIS:VS, 1994). It was approved for use in 1975 in feedlot diets and in 1978 for use in cattle on pasture, being used in almost all large feedlot operations shortly thereafter (USDA:APHIS:VS, 1994). Since the implementation of ionophore feeding occurred just prior to the first human cases of *E. coli* O157:H7 foodborne illness in 1982, there has been some concern that ionophores may select for *E. coli* O157:H7 in the bovine gut.

Since ionophores inhibit Gram-positive organisms (Bergen and Bates, 1984). This feature has been exploited in the development of two monensin based media, Lactose Monensin Glucuronate agar (LMG)(Entis and Boleszczuk, 1990) and Plate Count-Monensin-KCl agar (PMK) (Petzel and Hartman,1985), which are selective for Gram-negative bacteria and coliforms (including *E. coli*). While monensin has been reported to have little or no effect against Gram-negative bacteria, the effect of monensin on the growth and/or survival of the pathogen *E. coli* O157:H7 has not been reported.

The objective of the present study was to investigate whether monensin sodium had a stimulatory or inhibitory effect on the growth and/or survival of the human pathogen *E. coli* O157:H7 and a non-pathogenic strain of *E. coli*, ATCC 25922.

Materials and Methods

Bacterial Inoculants. *E. coli* O157:H7 strain 3081 (kindly made available by W.C. Cray, National Animal Disease Centre, Ames, Iowa) and *E. coli* K-12 ATCC 25922 were used in this study. Each strain was grown separately in 100 ml brain heart infusion (BHI) broth (Difco) for 18 h at 37° C. The bacteria were sedimented by centrifugation (4000 x *g*, 12 min), washed three times with 0.1M phosphate-buffered saline, pH 7.2 (PBS), and resuspended in PBS. Cells were adjusted with PBS to an optical density (OD) of 0.5 at 640 nm (approximately 10^8 CFU/ml). The populations of each strain were verified by enumeration on BHI agar (Difco) held at 37 °C for 24 h. An inoculum containing *E. coli* O157:H7 strain 3081 or *E. coli* ATCC 25922 at 10^6 CFU was used.

Monensin Solutions. A monensin stock solution of 1000 µg/ml was prepared by dissolving sodium monensin (Sigma) in 95% ethanol. Monensin was added to tubes of BHI broth to obtain concentrations of 5, 15, 25 and 50 µg/ml monensin in a final volume of 10 ml. Control tubes at each time period contained appropriate amounts of only ethanol to reflect those used in preparation of monensin test concentrations. Tubes of monensin at each concentration and control tubes were inoculated with *E. coli* O157:H7 strain 3081 or *E. coli* ATCC 25922 to obtain a final concentration of 10^5 CFU/ml.

Incubation and Sampling. Monensin tubes inoculated with *E. coli* O157:H7 strain 3081 or *E. coli* ATCC 25922 were mixed thoroughly and incubated at 37° C for 48 h. At

0, 6, 12, 24 and 48 h intervals, tubes and their respective controls were removed and enumerated for *E. coli* O157:H7 strain 3081 or *E. coli* ATCC 25922. Samples (1ml) were serially diluted in 9ml PBS and 100 ul was plated onto BHI agar in duplicate. Plates were incubated for 18 to 24 h at 37 °C prior to the determination of viable numbers. Three independent trials were performed.

Statistical Analysis. All microbiological enumerations were performed in duplicate. Survival was expressed as a percentage of survival in the respective controls. Analysis of variance was performed using the SAS General Linear Models procedure (SAS Institute, Inc., Cary, N.C.) after converting the data to $\log_{10}$ values. The least significant difference test was used to determine the differences among means where significant effects were observed ($P< 0.05$).

Results and Discussion

The growth and/or survival of *E. coli* ATCC 25922 was not affected by monensin at concentrations of 5, 15, 25 and 50 μg/ml (P>0.05)(Figure 3.1A). Percent survival of *E. coli* ATCC 25922 ranged from 95.4 to 100% over the 48 h experimental period. Monensin at concentrations of 5, 15 or 25 μg/ml had no effect on the growth and/or survival of *E. coli* O157:H7 strain 3081 and between 98.2 and 100% survival was exhibited over 48 h (P>0.05). At 50 μg/ml, monensin significantly reduced the growth of *E. coli* O157:H7 strain 3081, with 77.8 and 58.0% survival being exhibited after 24 and 48 h, respectively(P< 0.05) (Figure 3.1B).

Monensin is the ionophore which is most extensively used to improve the efficiency of feed utilization in cattle without producing significant alterations in carcass composition

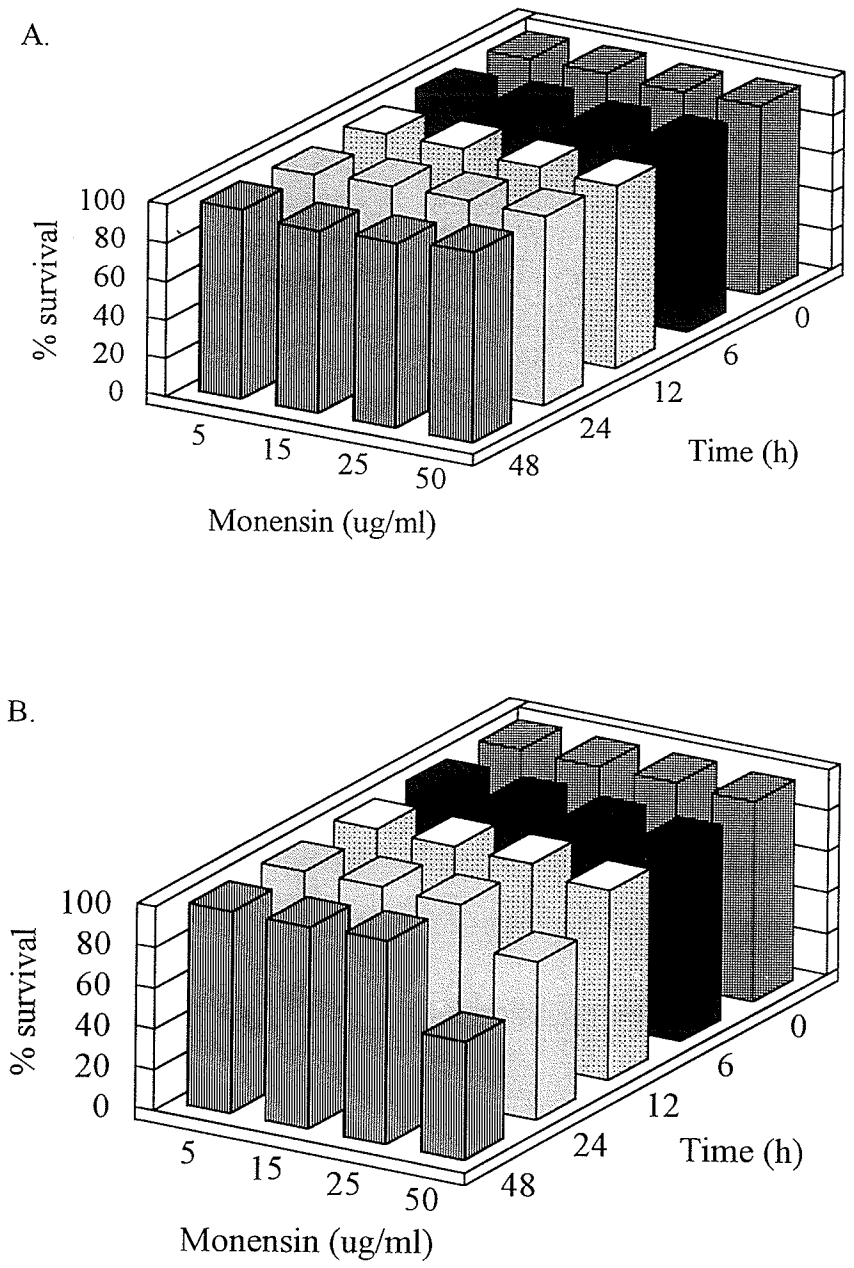

Figure 3.1. The effect of monensin sodium on the growth and/or survival of (A) *E. coli* ATCC 25922; (B) *E. coli* O157:H7 strain 3081. Values represent means of three independent trials.

and dressing percentage. In cattle monensin usually decreases food intake while the rate of body weight gain either is unaffected or improves slightly (Russell and Strobel, 1989; Schelling, 1984; Wallace, 1992). In cattle fed high grain finishing rations, ruminal pH can decrease rapidly due to an increase in lactate-producing bacteria (*Streptococcus* and *Lactobacillus*), often leading to rumen acidosis. Monensin has been shown to be inhibitory to numerous strains of lactic acid-producing rumen bacteria thereby preventing lactic acid accumulation and exerting a modifying effect on the ruminal pH of grain fed animals (Schelling, 1984).

Ionophores or "ion bearers" achieve their effects by modifying the movement of ions across the membranes of rumen microbes (Bergen and Bates, 1984; Schelling, 1984). Monensin has a high selectivity for Na^+ (the predominant extracellular cation in the rumen) but also has the ability to translocate K^+. The proton gradient across the cell membrane of Gram-positive bacteria is dissipated resulting in total depletion of intracellular ATP resulting in cell death (Russell and Strobel, 1989). The end result is the inhibition of certain rumen microbial species and a shift in rumen fermentation patterns leading to a decrease in methane production, an increase in the ratio of propionate to acetate production and a decrease in the degradation of protein to ammonia (Duncan et al., 2000; Richardson et al., 1976; Russell and Strobel, 1989; Wallace, 1992).

Gram-negative bacteria possess an outer membrane which is impermeable to many macromolecules, with porins mediating solute movement. The porins, which form hydrophilic channels through the hydrophobic membrane, have an exclusion limit of approximately 600 daltons. Since ionophores are extremely hydrophobic and have

molecular sizes greater than 500 daltons, the outer membrane serves as a barrier to their entry. As a result, Gram-negative bacteria are generally resistant to ionophores. Gram-positive bacteria, which lack a protective outer membrane, are generally sensitive to ionophores, (Bergen and Bates, 1984; Russell and Strobel, 1989). Some common rumen bacteria such as *Butyrivibrio fibrisolvens* and ruminococci, which stain Gram-negative, have a Gram-positive cell wall structure and are sensitive to ionophores (Russell and Strobel, 1989). Their cell walls contain derivatives of techoic acid, characteristic of Gram-positive cells, and are very thin (12 to 18 nm), which may account for their tendency to stain Gram-negative (Cheng and Costerton, 1977). It has been suggested that because of the inhibitory effect ionophores exert on the Gram-positive populations in the rumen, Gram-negative populations are able to flourish (Bergen and Bates, 1984; Duncan et al., 2000).

While the hypothesis is that the bacterial outer membrane provides the pattern for ionophore resistance, some conflicting observations have been reported (Russell and Strobel, 1989). Studies have indicated that some Gram-negative species are not resistant to high levels of ionophores (Russell and Strobel, 1989; Wallace, 1992). This was evident in the present study, where a significant reduction in the growth of *E. coli* O157:H7 occurred at 50 μg/ml monensin. Ionophores have been shown to increase ion flux in some Gram-negative bacteria, and some Gram-negative bacteria which have been reported to be initially sensitive to ionophores have been able to adapt. There have also been reports to suggest that Gram-positive bacteria can develop resistance to ionophores (Dawson and Boling, 1983; Russell and Strobel, 1989). Such studies suggest that the presence of an outer membrane, as in Gram-negative bacteria, is not the only criteria for resistance among

bacteria to ionophores.

Ionophores were approved by the Food and Drug Administration for use in feedlot diets in 1975 (Russel and Strobel, 1989; USDA:APHIS:VS, 1994). Shortly thereafter, in 1982, *E. coli* O157:H7 was first recognized as a human pathogen (Riley et al., 1983). It was hypothesized that the introduction of ionophores may have selected for *E. coli* O157:H7 in the rumen by inhibiting Gram-positive populations (USDA:APHIS:VS, 1994). It is unknown whether ionophores allow *E. coli* O157:H7 to proliferate in the rumen. It has been reported that the use of ionophores in cattle feed were not associated with fecal shedding of *E. coli* O157:H7 (Dargatz et al., 1997; Garber et al., 1995). Herriot et al. (1998) reported a tentative association between the fecal shedding of *E. coli* O157:H7 by cattle and the use of ionophores in cattle rations. There have been no experimental studies in cattle which have established a direct cause-and-effect relationship between ionophore use and the fecal shedding of *E. coli* O157:H7 (Rasmussen et al., 1999).

The results of the present study indicate that the ionphore monensin exerts an inhibitory effect on *E. coli* O157:H7 strain 3081 at high levels. Results indicate that different serotypes and strains of *E. coli* may be affected by monensin differently. While monensin may inhibit Gram-positive organisms in the rumen and provide a selective advantage for Gram-negative organisms, this may or may not include *E. coli* O157:H7. Other gram-negative organisms such as *Fibrobacter succinogenes* have been reported to grow well in the presence of monensin sodium (Slyter, 1979). This may allow these microorganisms to outcompete organisms such as *E. coli* O157:H7. Rasmussen et al.(1999) have implied that while it may be possible that ionophores alter the microflora of

the rumen in ways that may give *E. coli* a selective advantage, these effects may be subtle and indirect, and to date have been untested.

Monensin fed to cattle at recommended levels of 5.5 to 33 mg/kg of dietary DM would reach a concentration of 5 to 10 ug/ml in the rumen, assuming 70 kg rumen contents and 12 kg feed consumed (Chalupa, 1977; Dennis et al., 1981). The concentration of monensin in the rumen would not approach 25 µg/ml. Monensin affected survival of *E. coli* O157:H7 only at concentrations of 50 µg/ml, and the effect was negative. While monensin sodium does not exert a direct effect on *E. coli* O157:H7 at concentrations typically achieved in the rumen, the indirect effects (positive or negative) of inhibiting the Gram-positive rumen bacteria cannot be overlooked. Further studies are necessary to evaluate the impact of monensin on the competitive success of *E. coli* O157:H7 in the rumen environment and the hindgut.

CHAPTER 4

The Effect of a Lactic Acid Producing Inoculant on the Survival of *Escherichia coli* O157:H7 in Laboratory Ensiled Barley

Abstract

The effect of a lactic acid producing bacterial (LAB) inoculant on the elimination of *E. coli* O157:H7 from barley forage was assessed. Triplicate mini-silos were prepared for 4 treatments and 6 sampling times (1, 3, 7, 15, 30 and 42 days post-ensiling). The treatments were (1) 10^5 CFU/g *Pediococcus pentosaceus* and *Propionibacterium jensenii* (P2), (2)10^5 CFU/g *E. coli* O157:H7 strain 3081 and 10^5 CFU/g *E. coli* Biotype 1 strains 719IE10, 719IE14 and 614ME49 (EC), (3) P2+EC, and (4) the control (inoculated with sterile distilled water). Triplicate mini-silos were opened at each sampling time and samples were taken for pH, volatile fatty acid (VFA) and lactate determinations. Samples were also enumerated for LAB, yeasts and molds, *E. coli* and *E. coli* O157:H7. On days 3 and 7, numbers of *E. coli* O157:H7 in P2+EC were significantly lower than in EC ($P<0.05$). *E. coli* O157:H7 was not detected in P2+EC and EC 7 and 15 days post-ensiling, respectively. Bacteriocins of P2 were not found to be inhibitory to *E. coli* O157:H7 using the agar-spot procedure. Inhibition of *E. coli* O157:H7 was seen at pH 3.55 following the destruction of the bacteriocins from the P2 supernatant using 1 mg/ml proteinase-K or 1 µg/ml chymotrypsin. *E. coli* O157:H7 inoculated into the control silage at a level of 10^3 CFU/g and exposed to aerobic conditions at 22 °C was undetectable after 1 day and remained undetectable for the remainder of the 28 day exposure period. The use of P2

hastened the elimination of *E. coli* O157:H7 from silage likely due to low pH and increased lactate production. Results emphasize the importance of adequate ensiling since *E. coli* O157:H7 may be maintained and spread among cattle through feed.

Introduction

Escherichia coli O157:H7 has been recognized as a important cause of hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura, since its initial description as a human pathogen in 1982 (Riley et al., 1983). Cattle have been implicated as the primary reservoir of the organism and beef carcass contamination has been shown to be correlated to the prevalence of the organism in feces (Elder et al., 2000; Hancock et al., 1994; Wells et al., 1991).

Cattle shed *E. coli* O157:H7 in their feces at levels ranging from 10^2 to 10^5 CFU/g and considerable potential exists for the contamination of cattle feeds on the farm with *E. coli* O157:H7 (Gansheroff and O'Brien, 2000; Lynn et al., 1998; Zhao et al., 1995). It has been demonstrated that *E. coli* O157:H7 can replicate in a variety of cattle feeds *in vitro,* however, studies have not shown whether *E. coli* O157:H7 can survive the ensiling process or in silage (Fenlon and Wilson, 2000; Lynn et al. 1998). While the presence of fermentation acids in silage may be inhibitory to the growth of *E. coli* O157:H7, the characteristic acid resistance of the organism may allow it to survive in silage (Benjamin and Datta, 1995).

Lactic acid bacteria (LAB) are commonly used as bacterial inoculants in the production of silage (Inglis et al, 1999). The LAB become dominant in the silage, increasing lactic acid concentrations and decreasing pH values (McAllister and Hristov, 2000). Antimicrobial compounds such as bacteriocins and hydrogen peroxide are produced by *Lactobacillus* spp. and they may play a role in the preservation of silage. The use of LAB as inoculants may also aid in the elimination and/or persistence of pathogens such as

E. coli O157:H7 from silage.

Increased shedding of *E. coli* O157:H7 by cattle during the summer months is coincident with the increased food borne disease associated with the organism. This seasonal feature suggests that environmental growth may play an important role in the ecology of *E. coli* O157:H7 (Hancock, 1997; Lynn et al., 1998). Contaminated silage may be a means by which *E. coli* O157:H7 is maintained and spread among cattle. As a result, efforts to control *E. coli* O157:H7 in silage may have an impact on the occurrence of diseases associated with this human pathogen. The present experiment was designed to assess the effect of a LAB inoculant on the survival of *E. coli* O157:H7 and *E. coli* Biotype 1 in experimentally inoculated barley forage and to assess the survival of *E. coli* O157:H7 in the subsequently ensiled barley exposed to aerobic conditions.

Materials and Methods

Harvesting, Inoculation, and Ensiling. Whole barley at the soft dough stage was harvested at 35.3% DM in late August, 2000. Barley was swathed, wilted for 2-4 h and chopped (10 mm theoretical length of cut) with a John Deere 6810 forage harvester (John Deere Co., Moline, Ill.). Three random samples of fresh barley forage (~ 1 kg) were collected and stored at - 40 ° C for subsequent chemical analysis.

The experimental inoculants were P2 (which contained *Pediococcus pentosaceus* and *Propionibacterium jensenii*), EC (which contained *E. coli* O157:H7 strain 3081 and a mixture of *E. coli* Biotype 1 strains 719IE10, 719IE14 and 614ME49) and P2 + EC (which contained *P. pentosaceus*, *P. jensenii*, *E. coli* O157:H7 and a mixture of the three strains of *E. coli* Biotype 1). The P2 inoculant, was a commercially available inoculant (Biotal

Canada Ltd.,Calgary, AB) consisting of a minimum bacterial count of 4×10^{10} CFU/g from *P. pentosaceus* and *P. jensenii*. The P2 inoculant was suspended in sterile distilled water (3.0 g/L) and applied to the fresh forage to give an application rate of 1×10^{5} CFU/g of forage.

The *E. coli* inoculant consisted of *E. coli* O157:H7 strain 3081 (kindly made available by W.C, Cray, National Animal Disease Center, Ames, IA) and a mixture of three *E. coli* Biotype 1 strains 719IE10, 710IE14 and 614ME49, which were isolated from cattle manure at the feedlot of the Research Centre in Lethbridge, Alberta. *E. coli* O157:H7 strain 3081 is resistant to 100 µg/ml ampicillin and 100 µg/ml kanamycin and was used to facilitate the recovery of the organism from the silage. The *E. coli* strains were grown separately in trypticase soy broth (TSB) (BDH, Toronto, ON) for 18 h at 37 °C. The bacteria were sedimented by centrifugation (4,000 x g, 12 min), washed three times in phosphate-buffered saline, pH 7.4 (PBS; 15 mM KH_2PO_4, 8 mM Na_2HPO_4, 137 mM NaCl, 2.6 mM KCl) and re-suspended in sterile distilled water. Cells were adjusted with sterile distilled water to an optical density of 0.5 at 640 nm (~ 10^{8} CFU/ml)(UltraSpec Plus 4054, Pharmacia, Baie d'Urfe', QC). The population of *E. coli* O157:H7 cells was verified by enumeration on sorbitol MacConkey agar (SMAC)(Oxoid, Nepean, ON) supplemented with 2.5 mg/L potassium tellurite (Dynal, Lake Success, NY.), 0.05 mg/L cefixime (Dynal), 100 µg/ml ampicillin (Sigma, Oakville, ON) and 100 µg/ml kanamycin (Sigma) to yield CT-KASMAC. The population of *E. coli* cells was further verified by enumeration on MacConkey (MAC)(BDH) agar. The *E. coli* was applied to the chopped barley to achieve a level of 10^{5} CFU/g of *E. coli* O157:H7 strain 3081 and 10^{5} CFU/g of each of the three

strains of *E. coli* Biotype 1.

The chopped barley was weighed and spread onto a plastic sheet in a confined facility. The inoculants were evenly sprinkled onto the barley and mixed thoroughly. The four ends of the plastic sheet were then drawn together and the barley was tumbled inside the sheet for a period of 5 min in order to ensure even distribution of the inoculum. Forage inoculated with an equivalent amount of sterile distilled water served as the control. The forage was ensiled in 72 upright cylindrical mini-silos (h = 37.5 cm, r = 5.75 cm, 2.5 kg capacity) with the forage being compressed into the mini-silos using a hydraulic press. Triplicate mini-silos were prepared for each treatment and the control at each sampling time. The mini-silos were then sealed and stored at 22 °C for a period of 42 d.

Sampling and Analysis. Three arbitrarily selected samples (~25 g) of chopped barley from each of the four treatments (following inoculation but prior to compression in the mini-silos) were collected and enumerated for *E. coli*, *E. coli* O157:H7, lactic acid bacteria (LAB) , and yeasts and molds. Triplicate silos were opened at 1, 3, 7, 15, 30 and 42 days post-ensiling to perform microbial enumerations and chemical analyses.

Microbial Enumerations. Silage (10 g) was added to 90 ml of sterile 70 mM potassium phosphate buffer (pH 7.0) and stomached for 60 s on high in a Stomacher 400 laboratory blender (Seward Medical, London, UK). Serial ten-fold dilutions were prepared in sterile 70 mM potassium phosphate buffer (pH 7.0) for the enumeration of *E. coli*, *E. coli* O157:H7, LAB and yeasts and molds. *E. coli* O157:H7 and *E. coli* were enumerated on CT-KASMAC and MAC respectively. Plates were incubated for 18 h at 37° C. When *E. coli* O157:H7 was no longer detected by spread-plating, enrichment and immunomagnetic

separation (IMS) using Dynabeads anti-*E. coli* O157 (Dynal, Lake Success, NY) were used to detect the organism. Colonies on CT-KASMAC were confirmed as *E. coli* O157 by slide agglutination with O157 antiserum (Difco, Ottawa, ON). Selected colonies on MacConkey were confirmed as *E. coli* by growth and gas production in brilliant green bile broth at 44 °C in addition to using the API20E System (BioMerieux, St. Laurent, QC) to determine indole, methyl red, Voges-Proskauer and citrate reactions, typical of *E. coli* strains, i.e., indole positive, methyl red positive, Voges-Proskauer negative and citrate negative. LAB were enumerated on MRS containing 200 µg/ml cycloheximide with plates being incubated aerobically at 37 °C for 48 h. Yeasts and molds were enumerated on Sabouraud dextrose agar containing 100 µg/ml each of tetracycline and chloramphenicol, following incubation at 25° C for 72 h. For all microbial counts triplicate plates of a range of dilutions were prepared and where possible those dilutions yielding 30 - 300 colonies per plate were counted.

The direct supernatant spot assay (Spelhaug and Harlander, 1989) was used to screen the bacteriocins of the LAB in the P2 inoculant for inhibitory action against *E. coli* O157:H7 strains 3081, E318N, H4420, E32511 and *E. coli* ATCC 25922. The P2 inoculant was grown in MRS broth (BDH) at 37° C for 72 h. Cell-free supernatants were prepared by centrifuging the cultures at 8,000 x *g* for 10 min at 4 °C. The cell free supernatant was divided into two aliquots. In one aliquot, the pH of the cell free supernatant was determined (un-neutralized). In the other aliquot, the pH was adjusted to 6.5 with 3N NaOH (neutralized). Both un-neutralized and neutralized aliquots were then filtered through a 0.20 µm millipore syringe filter (Fisher, Nepean, ON). Un-neutralized and neutralized aliquots

of the supernatant were incubated for 6 h at 37 °C with 1 mg/ml proteinase-K (Sigma), 1 µg/ml chymotrypsin (Sigma), or untreated. Assay agar plates were prepared by adding 1 ml of an overnight culture of *E. coli* O157:H7 strain 3081, E318, H4420, E32511 or *E. coli* ATCC 25922 to 100 ml of molten (45 °C) trypticase soy agar (TSA)(BDH, Toronto, ON). Approximate 10 ml portions of the TSA containing each of the *E. coli* strains were poured into petri-dishes. Plates were then spot inoculated with 20 µl of the neutralized and un-neutralized filtered supernatant, treated and untreated with 1 mg/ml proteinase-K or 1 µg/ml chymotrypsin. Spots of the enzyme solutions only and sterile distilled water served as negative controls. Plates were observed for zones of inhibition following 24 h incubation at 37° C. All assays were performed in duplicate.

Chemical Analysis. Dry matter content of the initial forage samples was determined by drying for 24 h at 105 °C. For pH determinations, 15 g of fresh forage or silage was pre-weighed into a 250 ml beaker and distilled water was added to a final weight of 150 g in order to obtain a 1:10 dilution. Contents of the beaker were transferred into a blender and mixed for 30 s. The suspension was filtered through two layers of cheesecloth and the pH of a 10 - 15 ml sub-sample was immediately measured using an Orion PerpHect Meter, Model 310 (Orion Research Inc., Beverly, MA).

Volatile fatty acids (VFAs) and lactic acid were determined by homogenizing 3 g of fresh forage or silage in 15 ml of distilled water for 30 s in a Polytron homogenizer (Brinkman Instruments, Rexdale, ON). The mixture was centrifuged (10,000 x *g* , 15 min at 4 ° C) and aliquots (5 ml) of the supernatant were deproteinized using 24% (w/v) *m*-phosphoric acid in 2.5 M H_2SO_4 (1ml) and centrifuged at 10,0000 x *g* for 15 min. The

supernatant was mixed with 30 mM crotonic acid (1.0 ml) as an internal standard. Lactic acid was methylated and determined using malonic acid as an internal standard as described by Kudo et al. (1987). Samples were analyzed for volatile fatty acids and lactic acid (column:15 m Nukol fused silica capillary, 0.53 mm I.D., Supelco Canada, Mississauga, ON) on a Hewlett Packard model 5890 Series Plus II gas-liquid chromatograph.

Survival of *E. coli* O157:H7 in Barley Silage Exposed to Aerobic Conditions. Fifty four 2.5 L styrofoam containers (3 per mini-silo x 3 treatments x 6 sampling times) were filled with 400 g of the 42 d silage from the EC , P2+EC and control mini-silos and exposed to air at 22° C. Silage from one of the control mini-silos was inoculated with 10^3 CFU/g of *E. coli* O157:H7 strain 3081 in order to determine the effect of exposure to air on the survival of low levels of *E. coli* O157:H7 in silage. Eighteen 2.5 L styrofoam containers were filled with the inoculated silage and exposed to air at 22 °C. Triplicate containers for each treatment were emptied after 1, 3, 7, 14, 21 and 28 d exposure for enumeration of *E. coli* O157:H7.

Statistical Analysis. Data were analyzed using the general linear model (GLM) procedure of the SAS Institute Inc. (1988). Data were analyzed using the following statistical model:

$$Y_{ijk} = M + D_i + T_j + DT_{ij} + e_{ijk}$$

where Y_{ijk} is the observation; M is the mean; D_i is the day (1, 3, 7, 15, 30 and 42); T_j is the effect of treatment (P2, EC, P2 + EC, Control); DT_{ij} is the interaction between day and treatment and e_{ijk} is the residual error. Differences between reported least square means were determined using the least square linear hypothesis test (SAS Institute, 1988).

Results

Microbial Enumerations During Ensiling. Populations of LAB were similar among treatments prior to ensiling (P>.05) (Table 4.1). LAB populations during the ensiling period are illustrated in Figure 4.1A. Populations of LAB in P2 and P2+EC peaked after 3 days of ensiling, while populations of LAB in the control and EC silage peaked on day 7. On days 3 and 7 populations in P2 and P2+EC were higher than populations in the control and EC silage (P<0.05). On day 42, LAB populations in all four treatments were significantly different (P< 0.05).

Yeast populations were similar among the four treatments prior to ensiling (P>0.05)(Table 4.1). Molds were detected at levels $\leq$100 CFU /g throughout the ensiling period. In the P2 and P2+EC silage, yeasts were undetectable on day 3 but increased steadily to 9.8×10^{3} and 6.7×10^{4} CFU/g respectively on day 42. Numbers of yeasts in the control silage were undetectable following 15 days of ensiling. Numbers of yeasts in EC, P2 and P2+EC were similar on day 42 (P>0.05)(Figure 4.1B).

No *E. coli* was detected in the P2 and control silage prior to ensiling (Table 4.1). Numbers of *E. coli* recovered from P2+EC and EC were similar prior to ensiling indicating consistent inoculant application and distribution (P>0.05). Numbers of *E. coli* subsequently decreased in P2+EC and EC to 4.46×10^{2} and 1.7×10^{6} CFU/g, respectively, on day 7. The numbers of *E. coli* detected in P2+EC were significantly lower than numbers detected in the control and EC silage. On day 15 through 42, *E. coli* was not detected in P2+EC and EC (Figure 4.2A).

Prior to ensiling, *E. coli* O157:H7 was not detected in the barley forage, P2 or

Table 4.1. Microbial numbers from barley forage after inoculation, but prior to ensiling[1].

	Inoculants			
	P2	P2 + EC	EC	Control[2]
	log 10 CFU/g			
LAB[3]	6.99[a]	7.02[a]	7.00[a]	7.01[a]
Yeasts and molds[4, 5]	6.41[a]	6.36[a]	6.36[a]	6.43[a]
E. coli[6]	0[a]	5.88[b]	5.85[b]	0[a]
E. coli O157:H7[7]	0[a]	5.10[b]	5.31[b]	0[a]

[1] Symbols: P2: 10^5 CFU/g *Pediococcus pentosaceus* and *Propionibacterium jensenii*; P2 + EC: 10^5 CFU/g *E. coli* O157:H7 strain 3081, 10^5 CFU/g *E. coli* Biotype 1 strains 719IE10, 719IE14 and 614ME49; EC: 10^5 CFU/g *E. coli* O157:H7 strain 3081 and 10^5 CFU/g *E. coli* Biotype 1 strains 719IE10, 719IE14 and 614ME49.

[2] Control (inoculated with sterile distilled water)

[3] Lactic acid bacteria grown on MRS agar with cycloheximide

[4] Yeasts and molds grown on Sabouraud dextrose agar containing tetracycline and chloramphenicol

[5] Molds were not detected or colony forming units were too few on the 10^0 dilution for valid enumeration

[6] *E. coli* grown on MacConkey agar

[7] *E. coli* O157:H7 grown on sorbitol MacConkey agar containing cefixime, tellurite, ampicillin and kanamycin

[ab] Means within a row followed by different letters differ ($P<0.05$)

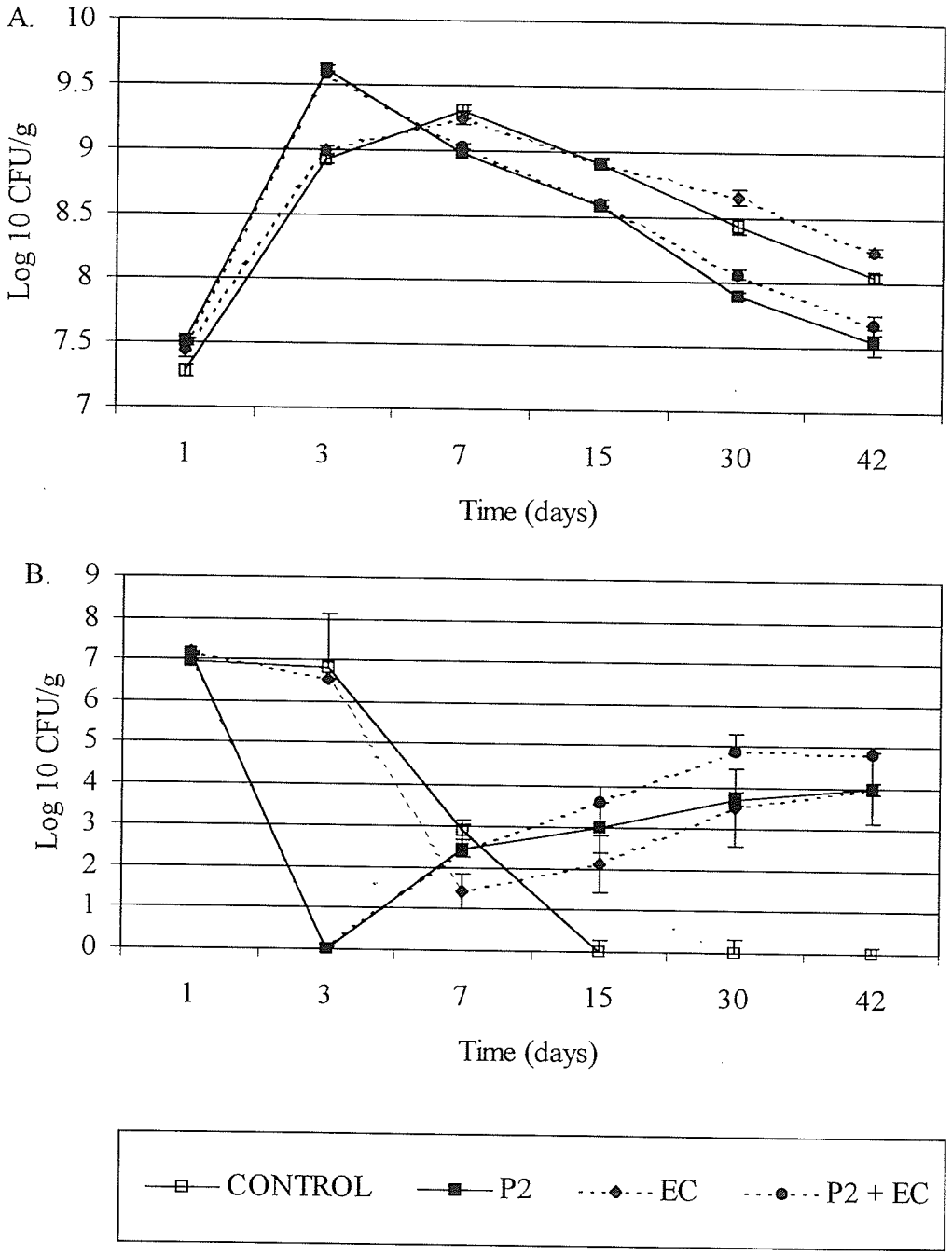

Figure 4.1. Changes in the populations of (A) LAB (B) and, yeasts and molds in control and inoculated barley during the 42 day ensiling period. Bars represent the standard error of the mean. Where not shown, error bars are within symbols.

control silage (Table 4.1). Numbers of *E. coli* O157:H7 strain 3081 declined rapidly in both the EC and P2+EC silage. On days 3 and 7 numbers of *E. coli* O157:H7 detected in P2+EC were lower than EC ($P<0.05$). Following day 3 and day 7, *E. coli* O157:H7 was not detected from P2+EC and EC, respectively (Figure 4.2B).

No zones of inhibition were produced by the neutralized cell free supernatant (pH 6.5) of the P2 inoculant when treated with proteinase-K (1 mg/ml)(pH 6.5+P-K) or chymotrypsin (1 μg/ml)(pH 6.5+C) for 6 h at 37° C or untreated (pH 6.5), and screened for inhibitory action against *E. coli* O157:H7 strains 3081, E318N, H4420, E32511 and *E. coli* ATCC 25922. When the un-neutralized supernatant (pH 3.55) was treated with proteinase-K (1 mg/ml)(pH 3.55+P-K) or chymotrypsin (1 μg/ml)(pH 3.55+C), zones of inhibition ranging from 7-9 mm were observed for each of the *E. coli* strains assayed. Zones of inhibition were also produced by the untreated supernatant, pH 3.55 (Table 4.2). No zones of inhibition were produced by the enzyme solutions.

Chemical Analysis During Ensiling. The DM content of the initial barley forage was determined to be 35.3%. The pH of the four treatments during the 42 d ensiling period are shown in Figure 4.3. The pH of P2 and P2+EC decreased more rapidly than the pH in EC and the control. No difference in pH among the four treatments was seen until day 3, at which time the pH of P2 and P2+EC was lower than that of EC and the control ($P<0.05$). No difference was observed in pH among the four treatments on sampling days 14, 30 and 42 ($P>0.05$). The pH of the silage after 42 days of ensiling ranged from 3.74 to 3.9 among the treatments (Figure 4.3).

Acetate was the predominant VFA produced during the ensiling period in all four

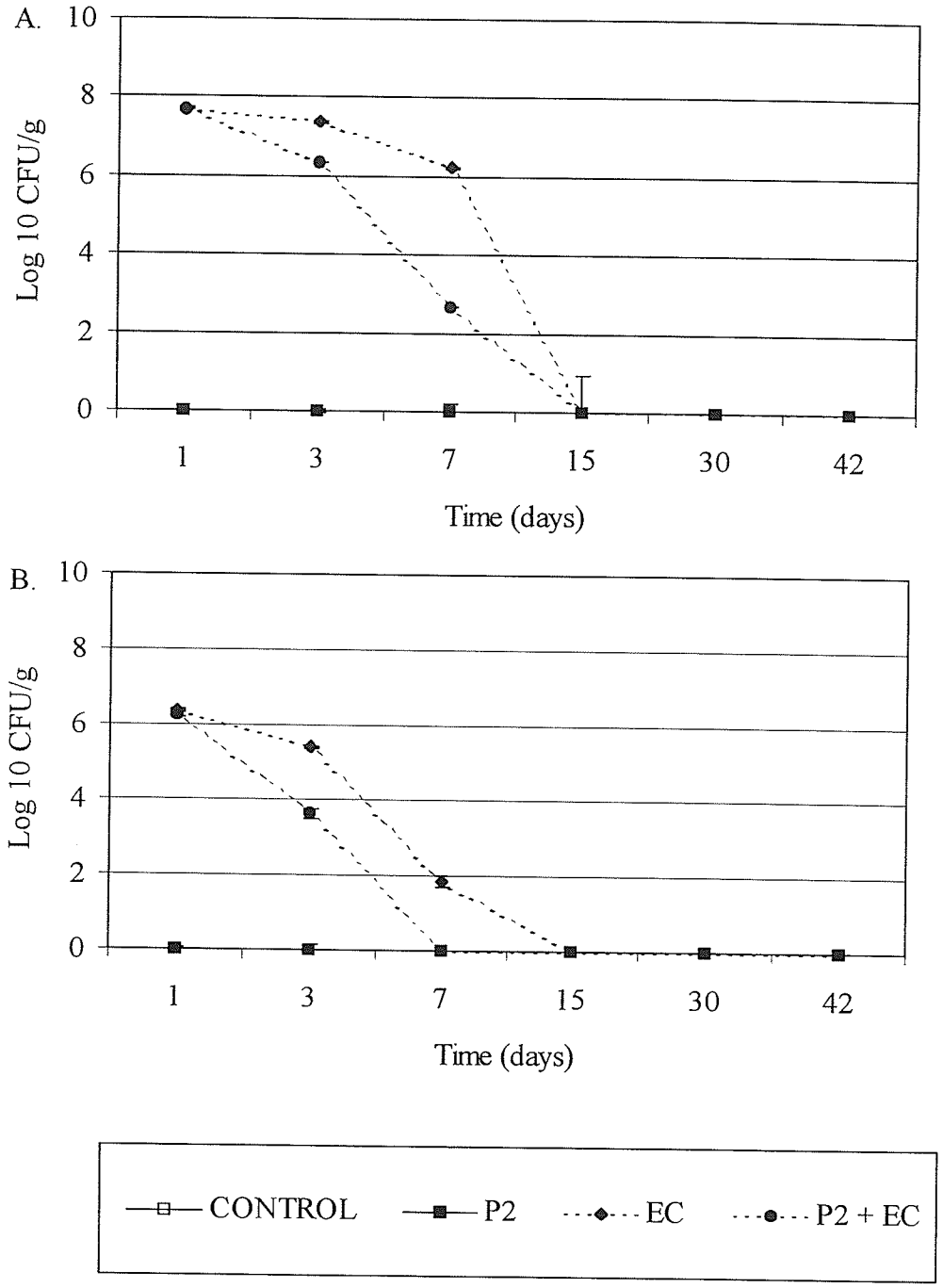

Figure 4.2. Changes in the populations of (A) *E. coli* (B) and, *E. coli* O157:H7 strain 3081 in control and inoculated barley forage during the 42 day ensiling period. The control and P2 yielded identical results at 0 $\log_{10}$ CFU/g in this figure, both A and B. Bars represent the standard error of the mean. Where not shown, error bars are within symbols.

Table 4.2. Detection of bacteriocin activity of the P2 inoculant against *E. coli* O157:H7 strains 3081, E318N, H4420, E32511 and *E. coli* ATCC 25922 [1].

	Treatment of cell-free supernatant of P2 inoculant					
E. coli strain	pH 6.5	pH 6.5 + P-K	pH 6.5 + C	pH 3.55	pH 3.55 + P-K	pH 3.55
E. coli O157:H7 strains:	Zones of inhibition (mm)					
3081	N[2]	N	N	7[3]	7	8
E318N	N	N	N	7	7	8
H4420	N	N	N	8	8	7
E32511	N	N	N	6	7	8
E. coli ATCC 25922	N	N	N	9	9	8

[1] Symbols: P2: 10^5 CFU/g *Pediococcus pentosaceus* and *Propionibacterium jensenii*; P-K: 1 mg/ml proteinase-K; C: 1 µg/ml chymotrypsin.

[2] No zone of inhibition present

[3] Values represent the mean of duplicate determinations

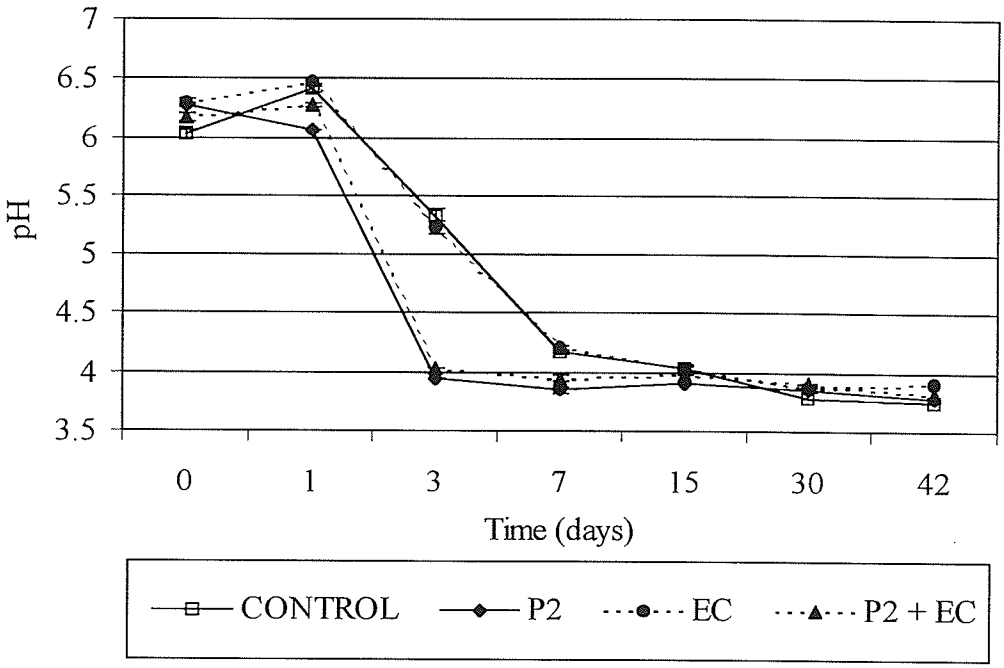

Figure 4.3. Changes in pH of control and inoculated barley forage during the 42 day ensiling period. Bars represent the standard error of the mean. Where not shown, error bars are within symbols.

treatments (Figure 4.4). Concentrations of VFA and acetate were significantly higher in EC and the control as compared to P2 and P2+EC on days 3, 7, 15, 30 and 42 ($P< 0.05$). On days 15 and 30, VFA and acetate concentrations were significantly higher in EC as compared to P2, P2+EC and the control.

Lactate levels increased significantly in P2 and P2+EC 3 days post-ensiling as compared to EC and the control($P<0.05$)(Figure 4.5). Lactate concentrations peaked in all four treatments on day 7 post-ensiling with 9.27, 10.94, 9.64 and 9.53 mM being recovered from the control, P2, P2+EC and EC, respectively. Lactate concentrations were higher in P2 and P2+EC on all sampling days with the exception of day 42 ($P<0.05$), at which time the lactate concentration in EC was significantly higher than all the other treatments (Figure 4.5).

Survival of *E. coli* O157:H7 in Barley Silage Exposed to Aerobic Conditions. *E. coli* O157:H7 was not detected from EC, P2+EC or the control over the entire 28 d period of aerobic exposure. In the barley silage inoculated with 10^3 CFU/g of *E. coli* O157:H7 strain 3081 and exposed to air at 22 ° C, the organism was detected on days 0 and 1. After 1 day of exposure to air at 22 °C, *E. coli* O157:H7 was undetectable and remained undetectable for the remainder of the 28 day exposure period (data not shown).

Discussion

Results of the present study suggest that 10^5 CFU/g *E. coli* O157:H7 will be eliminated from barley forage 15 days post-ensiling (Figure 4.2B). The use of the bacterial inoculant, P2 , eliminated *E. coli* O157:H7 from the silage following 7 days of ensiling. The mixture of three strains of *E. coli* Biotype 1, was eliminated from the silage

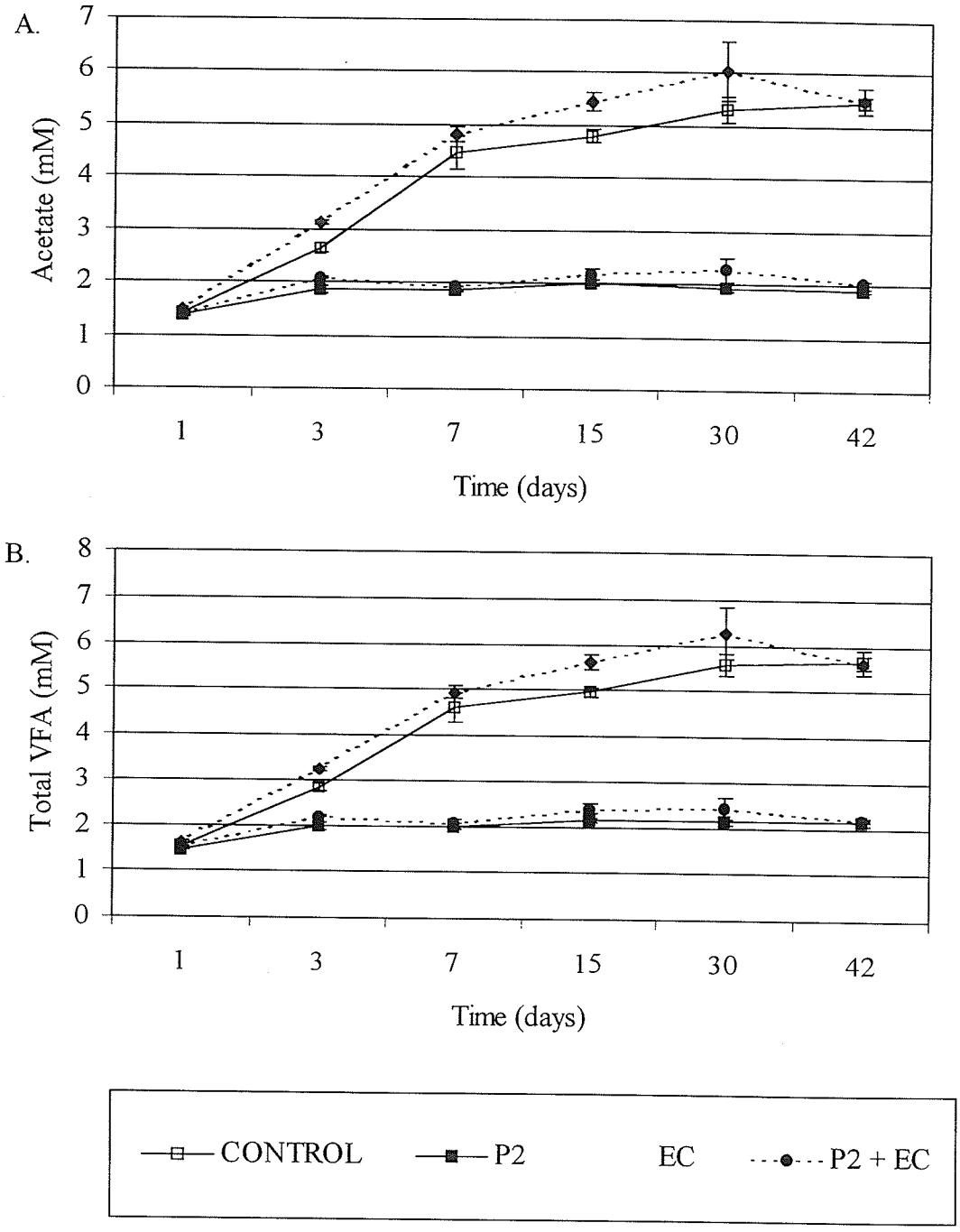

Figure 4.4. Changes in concentrations of (A) acetate (B) and, total VFAs in control and inoculated barley forage during the 42 day ensiling period. Bars represent the standard error of the mean. Where not shown, error bars are within symbols.

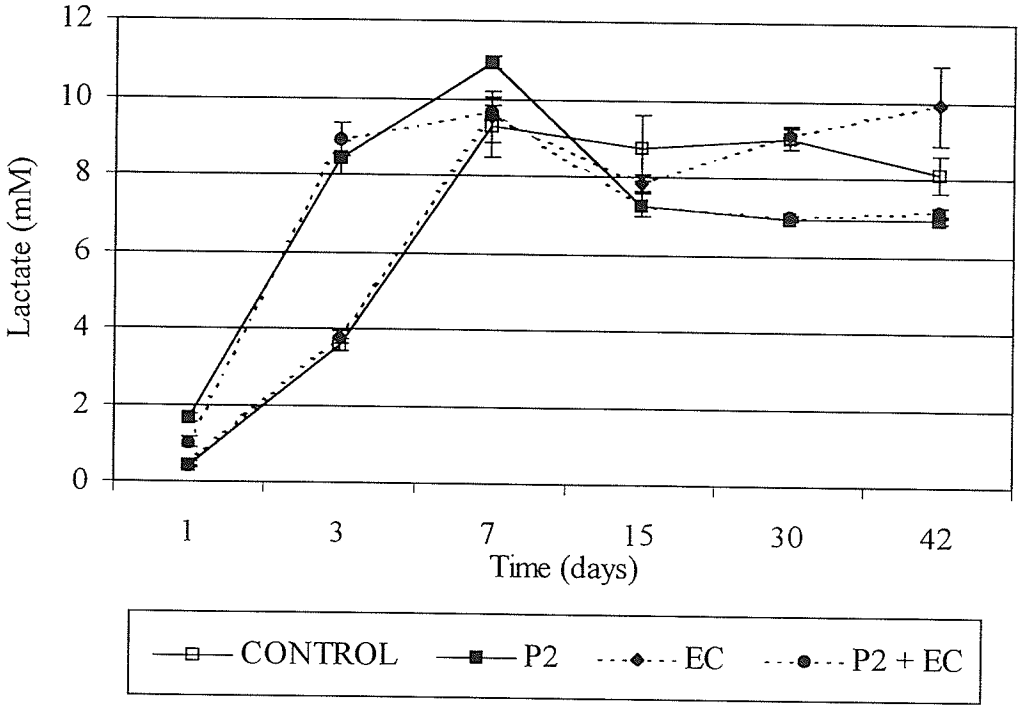

Figure 4.5. Changes in concentrations of lactate in control and inoculated barley forage during the 42 day ensiling period. Bars represent the standard error of the mean. Where not shown, error bars are within symbols.

in P2 + EC and EC after 15 days of ensiling (Figure 4.2A).

The addition of LAB inoculants at ensiling are intended to ensure rapid and vigorous fermentation that results in faster accumulation of lactic acid and lower pH values at earlier stages of ensiling (Cai et al., 1999; McAllister and Hristov, 2000). This was evident in the present study as levels of lactate were significantly higher in P2 and P2+EC as compared to EC and the control 3 days post-ensiling ($P<0.05$)(Figure 4.5). Increased concentrations of lactate may have resulted in the earlier elimination of *E. coli* O157:H7 from P2+EC as compared to EC.

Ensiling is an uncontrolled fermentation process, the success of which depends upon the creation of anaerobic conditions in addition to the presence of sufficient LAB and adequate fermentable carbohydrate in the forage prior to ensiling (McAllister and Hristov, 2000; Rooke, 1990). Once anaerobic conditions are established, the homofermentative LAB become the predominant bacteria with sufficient lactic acid produced to lower pH and inhibit the activity of undesirable organisms through the toxicity of the undissociated acids (Cai et al., 1999; Fitzsimons et al., 1992; McAllister and Hristov, 2000; Rooke, 1990).

The ability of LAB to inhibit various Gram-negative and Gram-positive bacteria may also be due to the production of hydrogen peroxide and bacteriocins (Chateau et al., 1993). However, bacteriocins of *P. pentosaceus* and *P. jensenii* in P2 may not have inhibitory effects on *E. coli* O157:H7 as observed by lack of zones of inhibition when the neutralized cell free supernatant of P2 was spot inoculated on agar plates containing *E. coli* O157:H7 and *E. coli* ATCC 25922. The presence of zones of inhibition when the bacteriocin in the cell free supernatant of P2 was destroyed using the enzymes proteinase-K

and chymotrypsin, suggests that the inhibition of *E. coli* O157:H7 and *E. coli* was possibly due to a low pH (3.55) as a result of lactic acid production (Table 4.2).

The inhibitory effect of weak organic acids, such as lactic acid, is based on the pH, the pK_a of the acid, the antimicrobial activity of the undissociated form of the acid, and specific effects of particular acids. The cell membranes of bacteria are reported to be relatively impermeable to the anions formed by weak-acid dissociation, but are readily permeated by the undissociated acid. It is thought that the undissociated molecules pass through the cell membrane and release protons, lowering internal pH in the process (Bearson et al., 1997). The influx of protons is thought to uncouple growth and drain cellular energy resources (Ujas and Ingham, 1998). Salmond et al. (1984) have reported that inhibition is two-fold, consisting of specific inhibition of an unidentified metabolic function by the undissociated acid, and a generalized inhibition as a result of acidification of the cytoplasm.

Cattle are a primary reservoir of *E. coli* O157:H7, with the organism being shed in the feces at a level of 10^2 to 10^5 CFU/g (Chapman et al., 1997; Hancock et al., 1994; Wells et al., 1991; Zhao et al., 1995). When uncomposted manure from cattle is used as fertilizer for forage crops there is a risk of the spread of pathogens to feeds (Ostling and Lindgren, 1991). *E. coli* O157:H7 may survive on manured crops and subsequently be present on the forage which is used for ensiling. It has been reported that *E. coli* O157:H7 can survive in soil which contains rooted grass for periods in excess of 130 days (Maule, 2000). When anaerobic conditions are not strictly maintained during the ensiling period *E. coli* O157:H7 may be present in silage. Fenlon and Wilson (2000) have reported that *E.*

coli O157:H7 can grow in poorly ensiled forage from an initial inoculum of 10^3 CFU/g to $> 10^6$ CFU/g within 13 d. As a result, even a small inoculum of *E. coli* O157:H7 surviving on the forage prior to ensiling could result in substantial numbers of *E. coli* O157:H7 being ingested by the animals.

In the present study, *E. coli* O157:H7 inoculated into barley silage at a level of 10^3 CFU/g was undetectable after 1 day of aerobic exposure and remained undetectable for 28 days. This suggests that conditions present in adequately ensiled barley are not conducive to the growth and/or survival of the organism. However, incorporation of silage into a total mixed ration (TMR) and subsequent fecal contamination of the feed by cattle may allow survival and growth of *E. coli* O157:H7. Lynn et al. (1998) reported that *E. coli* O157:H7 was able to grow in a variety of TMRs *in vitro*. The increased isolation rate of *E. coli* O157:H7 from the feces of cattle during the summer months suggests that environmental growth of the organism may play a role in the increased carriage rate of *E. coli* O157:H7 by cattle (Hancock, 1997; Lynn et al., 1998). Fecal contamination of forage in addition to poor silage management may therefore be a factor in the persistence of *E. coli* O157:H7 in ruminants (Fenlon et al., 2000).

Barley silage is the principal forage which is fed to feedlot cattle in Western Canada (Inglis et al., 1999; McAllister et al., 1995). Adequate ensiling is of utmost importance in eliminating *E. coli* O157:H7 and other pathogens and ensuring good quality silage. Ensiling for a minimum of 15 days eliminated 10^5 CFU/g of *E. coli* O157:H7 from barley silage, while the addition of the LAB inoculant P2 resulted in the elimination of *E. coli* O157:H7 after 7 days. Producers often start to feed cattle from their silage pits before the ensiling

process is finalized. Thus, there may be a risk of this early silage acting as an inoculant of *E. coli* O157:H7 for feedlot cattle. The use of P2 may shorten the time of *E. coli* O157:H7 survival on barley forage and thus allow silage to be fed to cattle earlier in the ensiling process without the risk of spreading *E. coli* O157:H7 to cattle.

CHAPTER 5

The Effect of Acetic and Propionic Acid on the Survival of *Escherichia coli* O157:H7 in Water

Abstract

Drinking water may be a means by which *E. coli* O157:H7 is spread and maintained in cattle herds. The effect of 0.25 and 0.50% acetic or propionic acid on the growth and/or survival of *E. coli* O157:H7 in water was assessed. The treatments were 0.25% acetic acid (A25), 0.50% acetic acid (A50), 0.25% propionic acid (P25), 0.50% propionic acid (P50), and distilled water (control). In three independent trials, triplicate bottles of A25, A50, P25, P50 and the control were inoculated with 10^7 CFU *E. coli* O157:H7 and incubated at 4, 22 and 30° C for a 14 day period. At 4 °C, a significant reduction in numbers of *E. coli* O157:H7 was observed in all four treatments over the 14 day period ($P<0.05$). Numbers recovered from A50 were lower than P25 and P50, but not significantly so ($P>0.05$). The organism was eliminated from all four treatments following 1 and 3 days of incubation at 30 and 22 ° C, respectively. Both A50 and P50 were more effective than A25 and P25 in reducing the levels of *E. coli* O157:H7 in water, with inhibition being more effective at higher temperatures. The potential exists for the use of weak acids in water troughs as a means of controlling *E. coli* O157:H7 in animal drinking water and thus reducing the incidence of this deadly pathogen in cattle.

Introduction

Escherichia coli O157:H7 first came to prominence as a foodborne pathogen in 1982, following two outbreaks of hemorrhagic colitis in the United States associated with undercooked hamburger patties (Ratnam et al., 1988; Riley et al., 1983). Since then, human illness associated with *E. coli* O157:H7 has been reported with increasing frequency, worldwide (Griffin and Tauxe, 1991; Parry and Palmer, 2000). While *E. coli* O157:H7 infections have been primarily associated with contaminated bovine products such as ground beef and unpasteurized milk, an increasing number of outbreaks have been associated with other food products and water (Ackman et al., 1997; Bell et al., 1994; Besser et al., 1993; Chapman et al., 1993a; Michino et al., 1999; Morgan et al., 1993).

Epidemiological investigations have revealed that cattle, both dairy and beef, are the primary reservoirs of *E. coli* O157:H7 (Hancock et al., 1994; Wells et al., 1991; Zhao et al., 1995). The organism is shed in the feces of healthy cattle and contamination of carcasses during slaughter and processing is likely the manner in which beef becomes contaminated (Besser et al., 1997; Buchanan and Doyle, 1997; Chapman et al., 1993b; Elder et al., 2000).

E. coli O157:H7 has been isolated from the farm environment. The seasonal pattern of *E. coli* O157:H7 shedding by cattle, with increased prevalence during the summer months, suggests that environmental replication plays a key role in the ecology of the organism (Hancock, 1997; MacDonald et al., 1996; Shere et al., 1998).

The isolation of *E. coli* O157:H7 from animal drinking water suggests that this may be the means by which the organism is maintained and spread among cattle (Faith et al., 1996; Hancock et al., 1998; Rice and Johnson, 1999; Shere et al., 1998). Numerous studies

have reported that *E. coli* O157:H7 can survive in water for extended periods of time (Maule, 2000; Kerr et al., 1999; Rice et al., 1992; Wang and Doyle, 1998;Warburton et al., 1998). The detection of *E. coli* O157:H7 in the mouth swabs of steers implies that the organism may be able to survive in the alkaline saliva of cattle long enough to be spread to feed, water and other cattle (Buchko et al., 2000a). Control of *E. coli* O157:H7 in drinking water may be a means of reducing the spread of the organism among cattle.

The objective of the present study was to assess the effects of 0.25% and 0.50% acetic and propionic acid on the survival of *E. coli* O157:H7 in water. If cattle find these concentrations of acids palatable, their incorporation into water troughs may be a means to control *E. coli* O157:H7 in drinking water.

Materials and Methods

Bacteria. *E. coli* O157:H7 strain 3081 (kindly made available by W.C. Cray, National Animal Disease Center, Ames, Iowa) was used in this study. The strain is resistant to 100 µg/ml ampicillin and 100 µg/ml kanamycin. Strain 3081 was grown in 100 ml of tryptic soy broth (TSB) (BDH, Toronto, ON) for 18 h at 37 °C. The bacteria were sedimented by centrifugation (4000 x *g*, 12 min), washed three times with 0.1M phosphate-buffered saline, pH 7.2 (PBS), and resuspended in PBS. Cells were adjusted with PBS to an optical density (OD) of 0.5 at 640 nm (approximately 10^8 CFU/ml)(UV-1201 Spectrophotometer, Mandel Scientific Co. Ltd., Guelph, ON). The populations of *E. coli* O157:H7 were confirmed by standard dilution plating in duplicate onto tryptic soy agar (TSA) (BDH) containing 100 µg/ml ampicillin (Sigma, Oakville, ON) and 100 µg/ml kanamycin (Sigma) to give TSA-AK. Plates were incubated at 37° C for 24 h prior to the

determination of viable numbers. An inoculum containing 10^7 CFU *E. coli* O157:H7 strain 3081 was used.

Acetic and Propionic Acid Solutions. Stock solutions of 5% acetic acid (v/v)(Sigma) and 5% propionic acid (Sigma) were prepared using distilled water and filtered through a 0.45 um bottle top filter (Fisher, Nepean, ON.). Stock solutions of each of the acids were added to distilled water in sterile bottles to obtain a concentration of 0.25% acetic acid (A25), 0.50% acetic acid (A50), 0.25% propionic acid (P25) and 0.50% propionic (P50), in a total volume of 100 ml. Triplicate bottles were prepared for each concentration of acid, one for each of the three incubation temperatures of 4, 22 and 30 °C. A control (100 ml distilled water) was included for each temperature of incubation.

Inoculation and Sampling. All treatments (A25, A50, P25, P50) and the control were inoculated with 10^7 CFU/ml of *E. coli* O157:H7 strain 3081 and incubated at 4, 22 or 30 °C. A 1 ml aliquot was withdrawn on days 0, 1, 3, 7 and 14 for the enumeration of *E. coli* O157:H7. Three replicates of the experiment were performed.

Bacterial Counts. Samples were serially diluted in PBS and 100 ul aliquots were spread plated onto TSA-AK, in duplicate. Plates were incubated at 37 °C for 18-24 h prior to the determination of viable numbers. When the organism was no longer detected by spread plating, enrichment and immunomagnetic separation using Dynabeads-anti *E. coli* O157 (Dynal, Lake Success, NY) were used. A 1 ml aliquot of a 1:10 dilution of the sample was added to 9 ml of modified TSB (mTSB) containing 20 mg/L of novobiocin (Sigma), 1.5 g/L of bile salts 3 (Difco, Ottawa, ON), and 30 g/L TSB (BDH) and incubated for 6 h at 37 °C. Following this enrichment step, immunomagnetic separation (IMS) of *E. coli*

O157 was performed using Dynabeads anti-*E. coli* O157 (Dynal) according to the manufacturer's instructions. A 50 µl aliquot of the suspension was plated onto TSA-AK and the plates were incubated for 18-24 h at 37 ° C. Three sorbitol- negative colonies from each plate were tested for agglutination with O157 antiserum (Difco).

Statistical Analysis. Three independent replicates were performed and averages of data are reported. Analysis of variance was performed using the SAS General Linear Models procedure (SAS Institute, Inc., Cary, NC) after converting the data to $\log_{10}$ values. The least significant difference test was used to determine the differences among means where significant effects were observed ($P< 0.05$).

Results

Numbers of *E. coli* O157:H7 steadily declined in A25, P25, A50 and P50 over the 14 day incubation period at 4 °C (Figure 5.1A). On day 14, numbers of *E. coli* O157:H7 recovered from A25 and P25 were higher than numbers recovered from A50 and P50 ($P<0.05$), with numbers recovered from all four treatments being lower than those in the control ($P<0.05$). In the control, a 0.5 $\log_{10}$ reduction in initial numbers was seen over the 14 day incubation period. The greatest reduction in numbers of *E. coli* O157:H7 over the 14 day incubation period at 4 ° C was observed in A50 and P50 (Figure 5.1A).

Following 1 day of incubation at 22 °C, levels of *E. coli* O157:H7 isolated from A25, P25, A50 and P50 were lower than the control ($P<0.05$). At this time, levels of *E. coli* O157:H7 recovered from P50 and A50 were similar ($P>0.05$), but levels of the organism recovered from P50 were lower than A25 and P25 ($P<0.05$). *E. coli* O157:H7 was not recovered from any of the four treatments on day 3 and for the remainder of the 14 day

incubation period. A 0.75 $\log_{10}$ reduction was observed in the control over the 14 day period (Figure 5.1B).

After 1 day of incubation at 30° C, *E. coli* O157:H7 was not recovered from A25, P25, A50 or P50. A 5.5 $\log_{10}$ reduction in the numbers of *E. coli* O157:H7 was seen in all four treatments after 1 day and the organism was not recovered from any of the four treatments for the remainder of the 14 day period. A 2.75 $\log_{10}$ reduction in *E. coli* O157:H7 was observed in the control after 14 days at 30 °C (Figure 5.1C).

The pH values of A25, P25, A50, P50 and the control prior to inoculation with *E. coli* O157:H7 strain 3081 are presented in Table 5.1 The pH determinations for A25, A50 and P25, P50 were below the respective pKa values for acetic and propionic acid of 4.75 and 4.90.

Discussion

The effectiveness of weak acids as inhibitors of bacterial growth has been reported to be enhanced as pH is decreased and/or incubation temperature is increased (Abdul-Raouf et al., 1993; Brudzinski and Harrison, 1998; Conner and Kotrola, 1995; Entani et al., 1998; Ujas and Ingham, 1998). This was evident in the present study. At 4 °C the organism was recovered from all four treatments after 14 days while at 22 and 30 °C, 10^5 CFU/ml of *E. coli* O157:H7 was eliminated from all four treatments after 3 and 1 day, respectively (Figure 5.1). Possible explanations for the increased survival of *E. coli* O157:H7 in the presence of weak acids at low temperatures include transient induction of protective proteins, decreased rates of enzymatic reactions and proton permeability, and decreased fluidity of membrane lipids. Influx of undissociated organic acids may also be more rapid

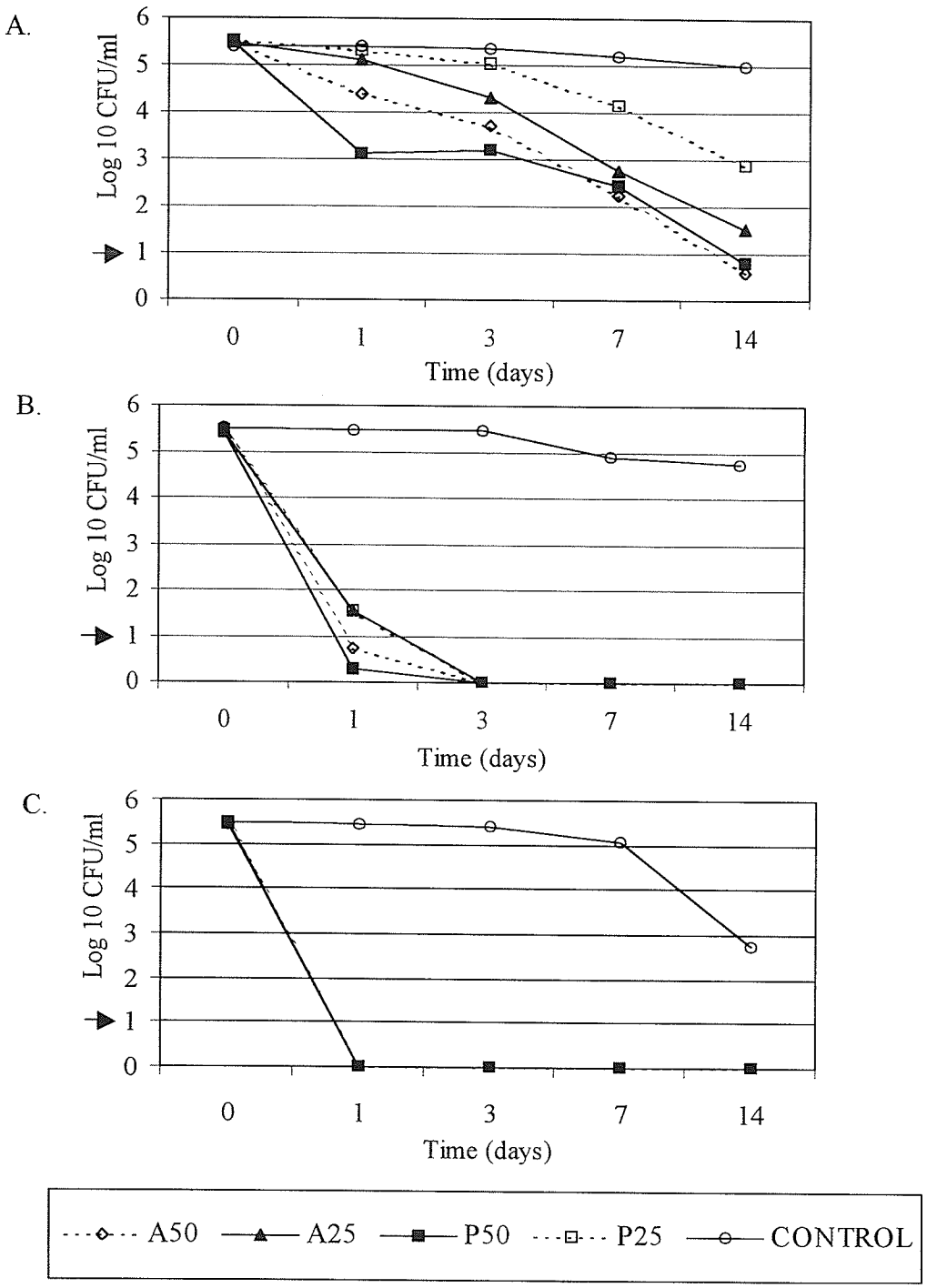

Figure 5.1. Survival of *E. coli* O157:H7 strain 3081 in 0.50% acetic acid (A50), 0.25% acetic acid (A25), 0.50% propionic acid (P50), 0.25% propionic acid (P25), and distilled water (Control) at (A) 4 °C; (B) 22 °C; (C) 30 °C. Arrows indicate detection by enrichment (<10 CFU/ml).

Table 5.1. Determinations of pH made on solutions of 0.25 and 0.50% acetic acid, 0.25 and 0.50% propionic acid, and distilled water prior to inoculation with *E. coli* O157:H7 strain 3081.[1]

	Treatment			
Control [2]	A25	A50	P25	P50
6.47 [3] (0.00)[4]	2.74 (0.02)	2.67 (0.00)	2.79 (0.01)	2.73 (0.00)

[1] Symbols: A25: 0.25% acetic acid; A50, 0.50% acetic acid; P25, 0.25% propionic acid; P50, 0.50% propionic acid.

[2] Control (100 ml of distilled water)

[3] Values represent the mean of 3 determinations

[4] Values in parenthesis are standard errors of the mean (SEM)

at high temperatures when none of the protective effects associated with cold temperatures are present (Uljas and Ingham,1998).

The antimicrobial effect of weak acids has been explained by the ability of undissociated acid molecules to pass through the cell membrane and release protons (Conner and Kotrola, 1995; Salmond et al., 1984). The cell membrane is relatively impermeable to the anions formed by weak-acid dissociation but is readily permeated by the undissociated acid. At levels of pH below their pKa weak acids remain mainly undissociated allowing them to penetrate the cell membrane. The pH of the 0.25 and 0.50% acetic and propionic acid solutions were all well below their respective pKa values of 4.75 and 4.90, suggesting that they remained mainly in the undissociated form (Table 5.1). Once inside the cell, dissociation likely occurred resulting in the lowering of the internal pH of the cell below the growth range (Brudzinski and Harrison, 1998; Ujas and Ingham, 1998). This influx of protons is also thought to drain the energy resources of the cell by dissipating the proton motive force (McKellar and Knight, 1999; Salmond et al., 1984). It has been suggested that the weak acid effect on intracellular pH is greater than the inhibition of transport by reducing the ΔpH component of the proton motive force. Overall, growth inhibition due to weak acids has been proposed to be the result of two components; specific inhibition of an unidentified metabolic function caused by the undissociated acid, and a generalized inhibition caused by acidification of the cytoplasm (Salmond et al., 1984).

At 4 °C, enhanced survival was observed in A25 and P25, which had slightly higher pH values than A50 and P50 (Figure 5.1A, Table 5.1). Bacteria generally maintain their cytoplasmic pH (pHi) constant despite variations in the pH of their environment (pHo).

This results in a large transmembrane pH gradient (ΔpH) in growing cells as pHo is lowered leading to greater inhibition by weak acids, since the amount of acid dissociating in the cytoplasm is larger. As a result, the potential effect of the weak acid accumulation on pHi increases as pHo is lowered, with inhibition being due to the hydrogen ion concentration (Salmond et al., 1984).

A significant correlation between pHi and growth inhibition has been reported, with growth inhibition being strongly correlated with the concentration of the undissociated acid (Salmond et al., 1984). Acids of a low pKa have been reported to be more effective at collapsing the pH gradient than those of higher pKa. The pKa of acetic acid (4.75) is slightly lower than that of propionic acid (4.90). While numbers of *E. coli* O157:H7 declined more rapidly in P50 as compared to A50 at 4 °C, the greatest reduction over the 14 day period was exhibited in A50, but this difference was not significant ($P>0.05$) (Figure 5.1A). At 22 ° C following 1 day of incubation, numbers of *E. coli* O157:H7 isolated from both A50 and P50 were significantly lower than the control ($P<0.05$)(Figure 5.1B). This suggests that A50 and P50 may be effective in eliminating *E. coli* O157:H7 from water troughs on the farm at temperatures typically experienced during the summer months.

In addition to having a slightly lower pKa than propionic acid, acetic acid is thought to be more inhibitory due to its lower molecular weight as compared to propionic acid, which facilitates its entry into the cells (Abdul-Raouf et al., 1993). In the present study, P50 appeared to be slightly more effective than A50 in reducing numbers of *E. coli* O157:H7 in water, but not significantly so ($P>0.05$). Duncan et al. (1999) have reported that a concentration of 100mM propionate is the most effective of the volatile fatty acids

(VFAs) in inhibiting *E. coli* O157:H7 under anaerobic conditions.

In *in vitro* studies, *E. coli* O157:H7 has been found to survive in water for long periods of time (Rice et al., 1992; Wang and Doyle, 1998). Rice and Johnson (1999) found that in cattle drinking water inoculated with 10^3 CFU/ml of *E. coli* O157:H7, the organism survived at 5 and 15 °C for 6 days, with higher numbers of organism being consistently recovered at the lower temperature. In cattle drinking water inoculated with 10^2 CFU/ml of *E. coli* O157:H7, the pathogenic organism survived for 4 and 8 days at 15 and 5 °C, respectively (Rice and Johnson, 1999). Similar observations were made in the present study, with enhanced survival of *E. coli* O157:H7 being exhibited at lower temperatures.

Water intake for cattle ranges from 3.6 - 4.1 L/kg DM per day (Squires, 1988). For an animal consuming 15 kg DM this equals 54 - 61.5 L of water per day. Salivary secretions (120 L/day) buffer the rumen fermentations of cattle to a pH of between 5.5 and 7.5 (Owens and Goetsch, 1988). Despite variations in the microbial populations and differences in feed intake, ruminal VFA proportions are remarkably stable with molar ratios (moles of acetic acid: propionic acid: butyric acid) usually being 65:25:10 with roughage diets and 50:40:10 with concentrate diets. VFA concentrations vary between 70 and 150 mM with lower concentrations in animals fed roughage diets (50-100 mM) as opposed to concentrate diets (80 - 150 mM). As a percentage of ruminal fluid the VFA total is between 0.5 and 1.5% and VFAs are continually being absorbed from the rumen (Owens and Goetsch, 1988; Russell and Diez-Gonzalez, 1998). Considering the outflow of VFAs and the buffering capacity of the rumen, an intake of 54 - 61.5 L of 87 mM (0.50%) acetic acid or (67.5 mM) (0.5%) propionic acid in drinking water may have little effect on rumen

fermentation.

Results of the present study indicate that the incorporation of 0.50% acetic or 0.50% propionic acid into the drinking water of cattle, especially during the summer months, may be a means to control *E. coli* O157:H7 in drinking water. Further studies, however, are required to determine the palatability of these weak acid solutions to livestock, their effects on rumen VFA concentrations, the subsequent acid tolerance response of the organism, their effects on the shedding of *E. coli* O157:H7 in the feces, and the carriage of the organism in the mouths of cattle.

CHAPTER 6

The Survivability of *Escherichia coli* O157:H7 in an Artificial Rumen System (Rusitec)

Abstract

An artificial rumen system (Rusitec) was used to assess the effects of barley silage, barley, clover and timothy hay diets on the growth and/or survival of 10^7 CFU/ml *E. coli* O157:H7. Two Rusitec units were used, each equipped with eight fermenters, with two fermenters in each unit assigned to a different diet (n=4). *E. coli* O157:H7 was eliminated from the barley silage, barley, clover and timothy diets at 60, 72, 120 and 144 h post-inoculation, respectively. At 36, 48 and 60 h numbers of *E. coli* O157:H7 recovered from the barley silage and barley diets were significantly lower than numbers recovered from the timothy and clover diets ($P<0.05$). Following 72 and 84 h, levels of *E. coli* O157:H7 recovered from the timothy diet were significantly higher than numbers recovered from the barley and clover diets ($P<0.05$). The organism was recovered from the feed residue in the fermenters fed all four diets but persisted in the feed of the barley silage fed fermenters 60 h after it was undetectable in the rumen fluid. The type of forage in addition to ability to associate with feed particles may affect the survival of *E. coli* O157:H7 in the rumen and numbers passing into the colon and subsequently resulting in fecal shedding.

Introduction

Escherichia coli O157:H7 has evolved as a public health concern worldwide, since its identification as a human pathogen in 1982 (Riley et al., 1983). The organism has been reported to be highly virulent, causing diarrhea, HC and HUS. Effects of these illnesses are severe particularly in children under the age of 5 and the elderly (Buchanan and Doyle, 1997; Riley et al., 1983). The majority of cases of *E. coli* O157:H7 infection have been traced back to undercooked ground beef and raw milk. Between the years of 1982 and 1997, 100 outbreaks of *E. coli* O157:H7 infection have occurred in the United States, with 52% being attributed to bovine food products (WHO, 1997). However, a number of outbreaks have been associated with other food products and water (Chapman et al., 1993b; Griffin and Tauxe, 1991; Hancock et al., 1994).

E. coli O157:H7 may be shed in the feces of healthy cattle at levels ranging from 10^2 to 10^5 CFU/g (Boyce et al., 1995; Zhao et al., 1995). Fecal shedding is intermittent in nature with peak levels of the organism shed during the summer months (Besser et al., 1997; Chapman et al., 1997b; Hancock et al., 1997a; Van Donkersgoed et al., 1999). While initial studies isolated *E. coli* O157:H7 from cattle at a rate of $\leq 3\%$, more recent studies using immunomagnetic separation have revealed that the prevalence of the organism in cattle feces may be much higher than initially reported (Dargatz et al., 1997; Hancock et al., 1994;Wells, 1991). Chapman et al. (1997b) isolated *E. coli* O157:H7 from 13.4% of beef cattle and 16.1 % of dairy cattle at slaughter in a study in England, with the monthly prevalence in cattle being as high as 36.8% during the summer months. *E. coli* O157:H7 has also been isolated from 7.5 to 28% of fecal samples from beef cattle at slaughter in

North America (Elder et al., 2000; Van Donkersgoed et al., 1999).

It has been suggested that the hindgut of cattle is the site of *E. coli* O157:H7 colonization and the main source for fecal shedding of the organism (Brown et al., 1997; Rasmussen et al., 1999; Whipp et al., 1994). However, to establish itself in the hindgut the organism must first survive passage through the rumen, where it may persist at low levels and be subsequently passed into the colon, where further growth may occur (Harmon et al., 1999). It has been reported that populations of *E. coli* O157:H7 in the rumen are not significantly different in *E. coli* O157:H7 inoculated calves fed a high concentrate than those fed a high roughage diet (Tkalcic et al., 2000).

The objectives of the present study were to: (i) assess the effects of three types of high forage diets and a high grain diet on the survival of *E. coli* O157:H7 in rumen fluid in an artificial rumen fermentation system (Rusitec), (ii) to characterize the disappearance of *E. coli* O157:H7 in rumen fluid using the Rusitec, (iii) and, to develop a model for use in subsequent studies in assessing the effect of various antimicrobial substances on *E. coli* O157:H7 in the rumen.

Materials and Methods

Bacterial Inoculant. *E. coli* O157:H7 strain 3081 (kindly made available by W.C. Cray, National Animal Disease Center, Ames, Iowa) was used in order to facilitate the recovery of the organism from the rumen fluid. *E. coli* O157:H7 strain 3081 is resistant to 100 µg/ml ampicillin and 100 µg/ml kanamycin and produces verotoxin 1 (VT1) and verotoxin 2 (VT2). *E. coli* O157:H7 strain 3081 was grown in 100 ml of tryptic soy broth (TSB) (BDH, Toronto, ON) for 18 h at 37 °C. Enumeration of *E. coli* O157:H7 strain

3081 from the TSB was performed using spectrophotometric readings at 640 nm (UltraSpec Plus 4054, Pharmacia, Baie d'Urfe', QC), which were confirmed by standard dilution plating in triplicate onto sorbitol MacConkey agar (SMAC)(Oxoid, Nepean, ON) supplemented with 2.5 mg/L potassium tellurite (Dynal, Lake Success, NY.), 0.05 mg/L cefixime (Dynal), 100 μg/ml ampicillin (Sigma, Oakville, ON) and 100 μg/ml kanamycin (Sigma), to yield CT-KASMAC. Each fermenter was inoculated with a 10 ml culture containing 10^9 CFU/ml of *E. coli* O157:H7 strain 3081.

Diet Preparation. Four experimental diets were used: (i) rolled barley grain; (ii) barley silage; (iii) timothy hay; (iv) sweet clover hay. Dry matter content of the feed was determined by drying for 24 h at 105° C. All diets were ground through a 5- mm screen and 10 g (DM basis) of each feed was placed into nylon bags (80 x 160 mm; 51 μm pore size).

Apparatus and Culture Conditions. The rumen simulation technique (Rusitec) developed by Czerkawski and Breckenridge (1977) was used in this study. Two artificial rumen systems, each equipped with eight fermenters was used in order to achieve four replications of each treatment diet (Figures 6.1, 6.2). The nominal volume in each vessel was 820 ml. Rumen fluid and solid digesta was obtained from four rumen fistulated Hereford heifers maintained on an 80% barley grain and 20% barley silage diet, and pooled. The heifers from which the inoculum for the Rusitec were obtained were cared for in accordance with the guidelines set down by the Canadian Council on Animal Care (1993). Rumen fluid was filtered through four layers of cheesecloth to partition it into liquid and solid fractions. To begin the experiment, each fermenter was filled with 820 ml of filtered rumen fluid determined to be pH 6.6. One nylon bag containing 20 g of solid digesta and

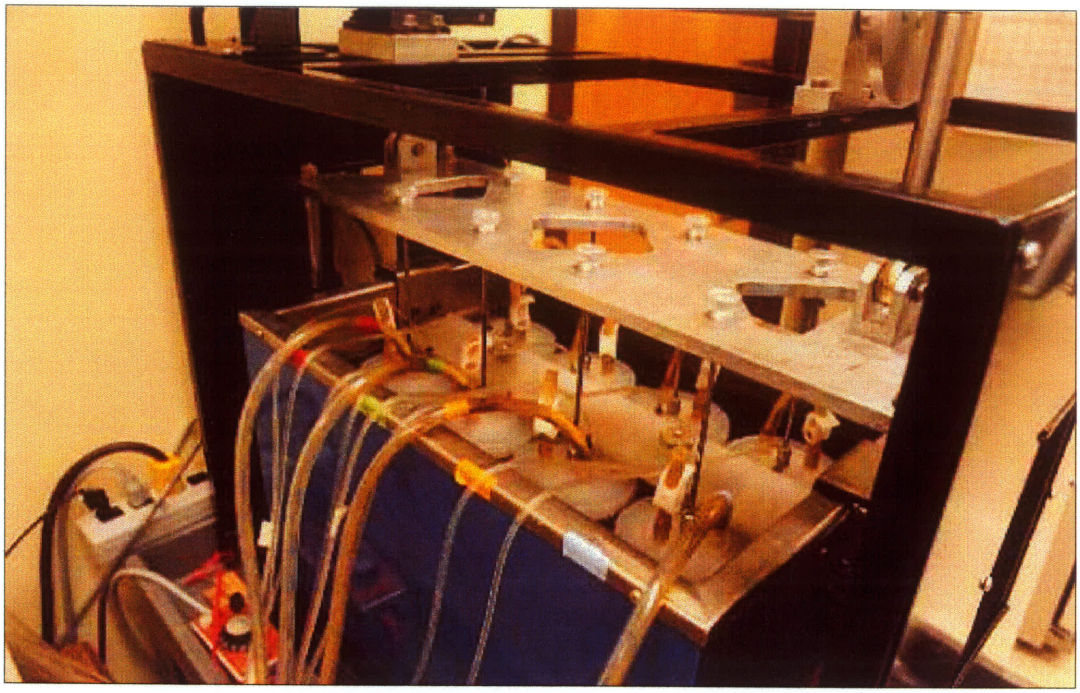

Figure 6.1. An artificial rumen system (Rusitec).

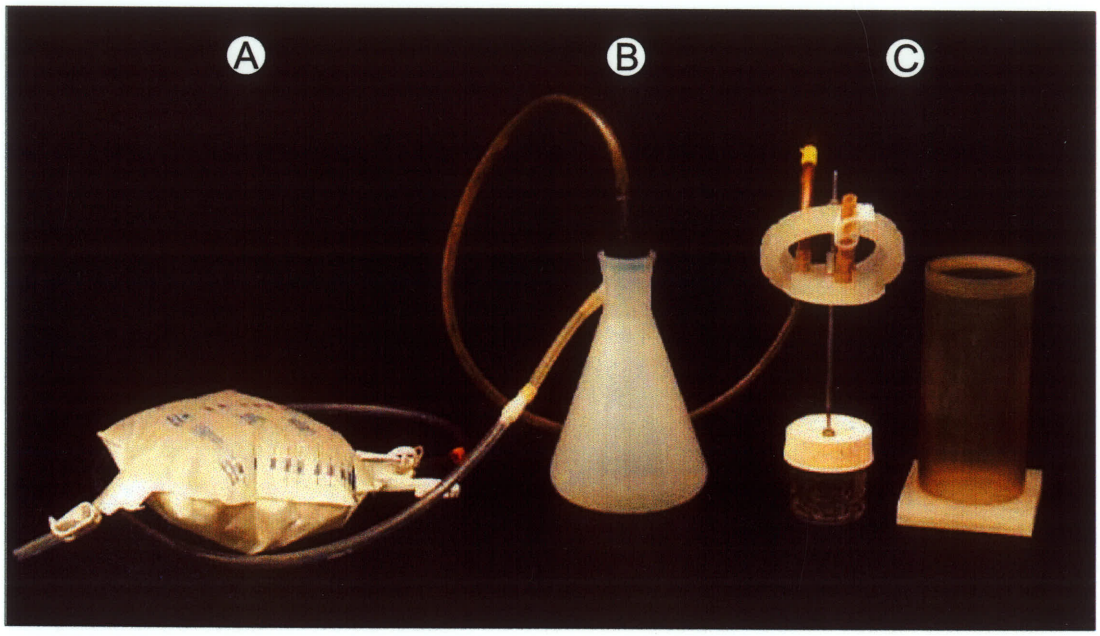

Figure 6.2. Components of an artificial rumen system (Rusitec); (A) gas bag for collection of gases resulting from bacterial fermentation; (B) flask for collection of effluent from fermentation vessels; (C) fermentation vessel (820 ml capacity) containing rumen fluid and feed bags.

one nylon bag containing the experimental diets (feed bags) were also placed in each fermenter. After 24 h, the nylon bag containing the solid digesta was replaced by a fresh feed bag. Thereafter, one feed bag was replaced daily, so that each feed bag remained in the fermenter for 48 h. Fermenters received a continuous infusion of artificial saliva (pH 8.1; McDougall, 1948) at a rate of 0.32 ml/min. Throughout the setup of the fermenter units and during daily replacement of feed bags, the fermenters were flushed with oxygen-free CO_2 to foster anaerobiosis (Hungate, 1950). Fermenter liquid pH, vessel volume and production of gas and effluent were monitored daily prior to feed bag replacement, in order to monitor fermentation.

Inoculation and Sampling. Following 9 days of adaptation, an inoculum containing 10^{10} CFU of *E. coli* O157:H7 strain 3081 was added to each fermenter in order to obtain a final concentration of 10^{7} CFU/ml of *E. coli* O157:H7. Fermenter rumen fluid samples were collected 30 min post-inoculation and then, at 12 h intervals for 120 h, followed by 24 h for a subsequent 120 h. Used feed bags were individually bagged in Whirl Pak bags (Fisher, Nepean, ON) for the subsequent isolation of *E. coli* O157:H7 strain 3081.

Enumeration of *E. coli* O157:H7 strain 3081. To test for the presence of *E. coli* O157:H7 in the rumen fluid prior to inoculation, enrichment culture followed by immunomagnetic separation (IMS) was perfomed using Dynabeads anti-*E.coli* O157 (Dynal). Briefly, a 1 ml sample of rumen fluid was added to 9 ml of phosphate buffered saline (PBS), pH 7.4, and vortexed. A 1 ml aliquot was then added to 9 ml of modified TSB (mTSB) containing 20 mg/ L of novobiocin (Sigma), 1.5 g/L of bile salts 3 (Difco,

Ottawa, ON), 1.5 g/L of dipotassium phosphate (Sigma) and 30 g/L of TSB (BDH), and incubated for 6 h at 37° C. Following this enrichment step, IMS of *E. coli* O157 was performed using Dynabeads anti-*E. coli* O157 according to the manufacturer's instructions. A 50 µl aliquot of the sample-exposed, antibody-coated bead suspension was plated onto single plates of SMAC supplemented with 2.5 mg/L potassium tellurite (Dynal) and 0.05 mg/L cefixime (Dynal), to give CT-SMAC. Media were incubated for 18 to 24 h at 37 °C.

For enumeration of *E. coli* O157:H7 strain 3081 following inoculation, a 1 ml sample of rumen fluid was placed in 9 ml of PBS. Samples were vortexed and serially diluted in PBS. A 100 ul aliquot from a series of dilutions was then plated onto CT-KASMAC in duplicate using a glass spreader. Media were incubated for 18 to 24 h at 37 ° C. Three sorbitol- negative colonies from each plate were tested for the O157 antigen by agglutination with O157 antiserum (Difco). Enrichment and IMS using Dynabeads *anti-E. coli* O157 was performed when *E. coli* O157:H7 strain 3081 was no longer detected by plating on CT-KASMAC.

For the detection of *E. coli* O157:H7 from the feed residue, a 1 g sample of the residue was homogenized using a Polytron Homogenizer PT10/35 (Brinkman, Switzerland) in 9 ml PBS and cultured for *E. coli* O157:H7 strain 3081 using overnight enrichment in mTSB at 37° C and IMS (Dynabeads anti-*E. coli* O157), as previously described. Feed residues were analyzed daily for *E. coli* O157:H7 following the inoculation of the organism into the fermenters.

Statistical Analysis. For enumeration of *E. coli* O157:H7 from rumen fluid, samples were plated in duplicate onto CT-KASMAC. Analysis of variance was performed

using the SAS Mixed Model procedure using the compound symmetry covariance structure (SAS Institute, Inc., Cary, NC). The repeated measures data were analyzed as a split-plot in time with diet as the main plot and time as the subplot. Fermenters nested within diet were used as the error term to evaluate diet. The least significant difference (LSD) test was used to determine the differences among means where significant effects were observed ($P<0.05$).

Results

Isolation of *E. coli* O157:H7 from Rumen Fluid. The rumen fluid used as the inoculum for the fermenters was negative for the presence of *E. coli* O157:H7 using enrichment and IMS. There was no difference in the numbers of *E. coli* O157:H7 recovered from the rumen fluid of the fermenters fed all four diets at 0 and 12 h post-inoculation ($P>0.05$). At 24 h post-inoculation, levels of *E. coli* O157:H7 recovered from the barley fed fermenters were lower than levels recovered from the barley silage, clover or timothy diets ($P<0.05$)(Figure 6.3). At this time, numbers of *E. coli* O157:H7 recovered from the barley, barley silage, clover and timothy hay fed fermenters were 3.35, 4.77, 5.36 and 6.07 $\log_{10}$ CFU/ml, respectively .

At 36, 48 and 60 h numbers of the inoculated strain recovered from the barley silage and barley diets were significantly lower than numbers recovered from the timothy hay and clover diets ($P<0.05$). The highest numbers of the organisms were isolated from the rumen fluid of the timothy hay diet, with 6.23, 5.58 and 5.05 $\log_{10}$ CFU/ml being recovered 36, 48 and 60 h post-inoculation, respectively. Numbers isolated from the clover diet during this time period were higher than those isolated from the barley and barley silage diet

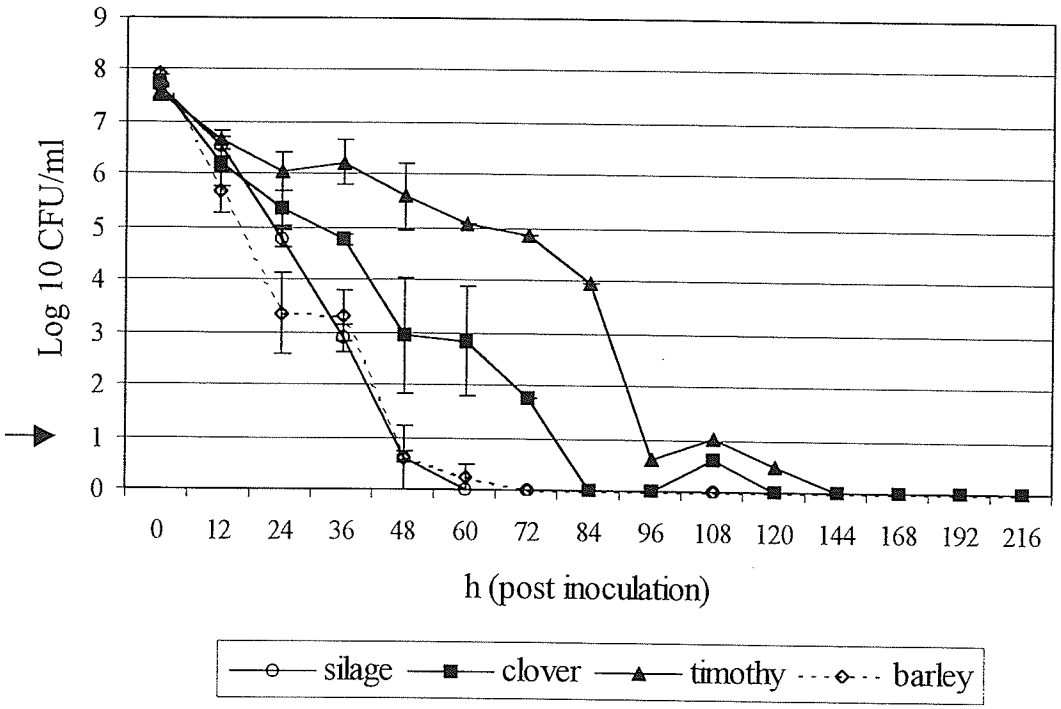

Figure 6.3. The survival of *E. coli* O157:H7 in rumen fluid in an artificial rumen system (Rusitec) fed four different diets, following an initial inoculum of 10^7 CFU/ml of *E. coli* O157:H7 strain 3081 at 0 h. Bars represent the standard error of the mean. Where not shown, error bars are within symbols. The arrow indicates detection by enrichment (<10 CFU/ml).

($P<0.05$), but lower than those isolated from the timothy hay diet ($P<0.05$). At 60 h, *E. coli* O157:H7 was not recovered from the fermentation vessels fed the barley silage diet and enrichment was required to detect *E. coli* O157:H7 from the barley fed fermenters. However, at 60 h post-inoculation, 2.8 and 5.05 $\log_{10}$ CFU/ml of *E. coli* O157:H7 were recovered from the clover and timothy fed fermenters, respectively (Figure 6.3).

Following 72 and 84 h, levels of *E. coli* O157:H7 recovered from the timothy hay diet were higher than numbers recovered from the barley or clover diets ($P< 0.05$). *E. coli* O157:H7 was not recovered from the barley diet 72 h post-inoculation. Following 84 h, no significant difference was observed in the levels of *E. coli* O157:H7 recovered from the timothy hay or clover diet ($P>0.05$). *E. coli* O157:H7 was not recovered from the clover diet 120 h post-inoculation, but persisted in the fermentation vessels fed the timothy diet until 144 h (Figure 6.3).

Isolation of *E. coli* O157:H7 from Feed Residues. *E. coli* O157:H7 strain 3081 was recovered from the feed residues in the fermentation vessels fed all four experimental diets. The organism was recovered from the feed residues of the barley and silage fed fermentation vessels up to and including 60 and 108 h post-inoculation, respectively. *E. coli* O157:H7 was isolated from the feed of the timothy hay and clover fed fermenters 120 h post-inoculation (Table 6.1). In the silage fed fermenter *E. coli* O157:H7 was detected in the feed residue for 60 h after it was undetectable in the rumen fluid. In the barley grain and timothy hay fed fermenters, *E. coli* O157:H7 was recovered from the feed residue 12 and 24 h, respectively, after the organism was no longer detected in the fermenter. Elimination of *E. coli* O157:H7 from the feed residue in the clover fed fermenters

Table 6.1. Recovery of *E. coli* O157:H7 strain 3081 from feed residues in an artificial rumen system (Rusitec) using Dynabeads anti-*E. coli* O157 following overnight enrichment in mTSB at 37 °C[1].

Time (h)[2]	Diet			
	Silage	Clover	Timothy	Barley
12	4	4	4	4
36	4	4	4	4
60	4	4	4	4
84	3	4	4	0
108	2	1	1	0
120	0	1	3	0
144	0	0	0	0
168	0	0	0	0
192	0	0	0	0
216	0	0	0	0
240	0	0	0	0

[1] Data are presented as number of feed residues culture positive for *E. coli* O157:H7 strain 3081/four fermenters sampled

[2] Time represents h post-inoculation of fermenters with 10^7 CFU/ml *E. coli* O157:H7 strain 3081

coincided with its elimination from the rumen fluid at 120 h (Table 6.1, Figure 6.3).

The pH values of the rumen fluid in the fermenters over the 20 day experimental period were 6.66 $\pm$ 0.05, 6.75 $\pm$ 0.05, 6.61 $\pm$ 0.07 and 6.41 $\pm$ 0.12 for the barley silage, clover, timothy and barley diets, respectively (data not shown).

Discussion

In the present study, there was a rapid decline in the numbers of *E. coli* O157:H7 isolated from the rumen fluid of the fermentation vessels fed all four diets (Figure 6.3). This steady decrease in the numbers of *E. coli* O157:H7 isolated from the rumen following inoculation with *E. coli* O157:H7 has been observed in numerous *in vivo* studies (Brown et al., 1997; Harmon et al., 1999; Tkalcic et al., 2000).

Tkalcic et al. (2000) observed no differences in rumen populations of *E. coli* O157:H7 in calves fed high concentrate (concentrate mix) or high roughage (Bermuda grass hay) diets, even though the rumen fluid from the animals fed the high roughage diet had lower concentrations of VFAs and a higher pH than the rumen fluid from the animals fed the high concentrate diet. In the present study, significantly higher numbers of *E. coli* O157:H7 were recovered from the clover and timothy diets 36, 48 and 60 h post-inoculation and from the timothy diet 72 and 84 h post-inoculation, as compared to the barley and barley silage diets (Figure 6.3). Although the VFAs of the rumen fluid were not measured in the present study, the pH of the rumen fluid fed the clover (pH 6.75), timothy (pH 6.61) and barley silage (pH 6.66) diets were higher than that of the barley grain diet (pH 6.41), over the entire experimental period. However, the infusion of artificial saliva into the fermentation vessels at a rate of 0.32 ml/min buffered bacterial fermentations to a pH of

between 6 and 6.5, similar to conditions *in vivo* (Czerkawski and Breckenridge, 1977; McDougall, 1948). The artificial saliva also represented an approximate 50-55% volume replacement in the fermentation vessels in a 24 h period resulting in the reduction of *E. coli* O157:H7 from the rumen fluid over time.

In the present study, *E. coli* O157:H7 was eliminated from the fermentation vessels fed the barley silage and barley grain diets 60 and 72 h post-inoculation, respectively, while the organism persisted in the clover and timothy hay fed fermenters 120 and 144 h post-inoculation, respectively (Figure 6.3). This suggests that forage type affects the survival of *E. coli* O157:H7 in the rumen and that barley silage created conditions in the fermenters which were more antagonistic to the survival of *E. coli* O157:H7 than the clover or timothy diets.

The recovery of *E. coli* O157:H7 from the feed residue in the feed bags indicates that the organism may associate with particulate matter in the rumen. This may allow the organism to persist for longer periods of time in the rumen and serve as a reservoir for re-introduction in the colon and subsequent fecal shedding. The type of feed may also influence the degree of association. Results of the present study indicate that *E. coli* O157:H7 association with barley silage may represent a means by which the organism may be maintained at low levels in the rumen. *E. coli* O157:H7 was detected in the feed of the barley silage fed fermenters 60 h after it was no longer detected in the rumen fluid (Table 6.1).

Diet may result in varying inhibitory conditions in the rumen which limit the growth of *E. coli* O157:H7 and its subsequent passage through the abomasum and into the colon

and cecum. It has been reported that conditions in the rumen of animals fed a high concentrate diet may favor the development of acid resistance, allowing greater numbers of *E. coli* O157:H7 to survive passage through the acidic abomasum and proliferate in the colon (Tkalcic et al. 2000). This was supported by *in vitro* studies in which a higher percentage of acid tolerant *E. coli* O157:H7 cells were found in rumen fluid from steers fed a high concentrate diet as compared to a high roughage diet. However, proliferation of *E. coli* O157:H7 was favored in the rumen fluid from animals fed a high roughage diet as compared to a high concentrate diet *in vitro*, but this same effect was not demonstrated *in vivo* (Tkalcic et al., 2000).

Conflicting results have been reported by Hovde et al. (1999) who found that hay (100% timothy) fed steers shed *E. coli* O157:H7 for longer periods of time than grain (90% corn and 10% alfalfa) fed steers and irrespective of their diet, the bacteria were equally as acid resistant. However, numbers of *E. coli* O157:H7 in the rumen of the animals was not determined in the study by Hovde et al. (1999).

Results of the present study suggest that barley silage and barley provide conditions in the rumen which are more antagonistic to the survival of *E. coli* O157:H7 than clover and timothy. It was also shown that grain, in addition to forage type, may have an effect on the survival of *E. coli* O157:H7 in the rumen and numbers subsequently introduced into the colon. The association of *E. coli* O157:H7 with feed particles in the rumen may also allow the organism to persist at low or undetectable levels in the rumen. Conflicting reports on the survival and growth of *E. coli* O157:H7 in the rumen, and its development of acid tolerance warrant further investigations on the varying inhibitory conditions in the

rumen which limit the growth of *E. coli* O157:H7 and limit or influence its subsequent passage into the colon and cecum.

CHAPTER 7

The Effect of DC22 on *Escherichia coli* O157:H7 in an Artificial Rumen System (Rusitec) and in Experimentally Inoculated Sheep

Abstract

The effect of a bacteriophage, DC22, on the survival of *E. coli* O157:H7 in an artificial rumen system (Rusitec) and in experimentally inoculated sheep was assessed. DC22 was found to be specific for *E. coli* O157:H7, with 24 of 24 *E. coli* O157:H7 strains assayed being sensitive to DC22, while 15 non-O157 strains of enterohemorrhagic *E. coli* were not sensitive to the bacteriophage. In triplicate studies using clarified rumen fluid, the multiplicity of infection (MOI) of DC22 on *E. coli* O157:H7 was determined to be $> 10^4$ plaque forming units (PFU)/CFU. In the artificial rumen system (Rusitec), 10^4 CFU/ml of *E. coli* O157:H7 strain 3081 were eliminated from the fermenters (n=4) 4 h following the administration of 10^5 PFU of *E. coli* O157:H7/CFU of DC22 ($P<0.05$). *E. coli* O157:H7 persisted in the control fermenters (n=4) for up to 168 h post-inoculation. Two groups of six wethers were fasted for 48 h and experimentally inoculated orally with 10^8 CFU of *E. coli* O157:H7 strain E318N. On day 2 post-inoculation, one group was inoculated with 10^5 PFU/CFU of DC22 (DC22-treated group) and the other group was inoculated with an equivalent amount of SM buffer (control group). There was no significant difference in the levels of *E. coli* O157:H7 shed by wethers in the DC22- treated group or control group over a 30 day period ($P>0.05$). The large number of viable particles required for infection of *E. coli* O157:H7 by DC22 suggests that the bacteriophage possessed low antibacterial activity

against *E. coli* O157:H7. Non-specific adsorption of DC22 may also have reduced numbers available for *E. coli* O157:H7 infection. A minimum concentration of DC22 sufficient to result in the elimination of *E. coli* O157:H7 in the gastrointestinal tract of the wethers did not appear to be maintained.

Introduction

The human pathogen *Escherichia coli* O157:H7 has become a global public health concern since its recognition in 1982, and outbreaks associated with *E. coli* O157:H7 have been reported with increased frequency (Riley et al., 1983). Although undercooked ground beef and unpasteurized milk have been implicated as the primary vehicles of transmission, numerous other foods and water have been linked to *E. coli* O157:H7 outbreaks (Bell et al., 1994; Borczyk et al., 1987; Ostroff et al., 1990; Padhye and Doyle, 1992; Swerdlow et al., 1992; Wells et al., 1982). Among these are fresh-pressed apple cider (Besser et al., 1993), yoghurt (Morgan et al., 1993), mayonnaise (Raghubeer et al., 1995; Weagent et al., 1994), alfalfa sprouts (CDC, 1997), radish sprouts (Hara-Kudo et al., 1997; Itoh et al., 1998), lettuce (Ackers et al., 1998), cantaloupe (Beucat, 1996) and dry-cured salami (CDC, 1995).

Cattle, both dairy and beef, are considered the primary reservoir of *E. coli* O157:H7 (Griffin and Tauxe, 1991; Hancock et al., 1994; Wells et al., 1991; Zhao et al., 1995). Fecal shedding of *E. coli* O157:H7 by cattle is intermittent and seasonal in nature, with peak levels shed during the summer months (Besser et al., 1997; Hancock et al., 1997; Mechie et al., 1997; Van Donkersgoed et al, 1999; Wells et al., 1991). Numbers of *E. coli* O157:H7 shed in the feces of cattle have been reported to range from 10^2 to 10^5 CFU/g of feces (Zhao et al., 1995). Contamination of carcasses with feces during slaughter and processing is likely the manner in which beef becomes contaminated. Following this, the organism is subsequently consumed in undercooked beef (Buchanan and Doyle, 1997; Chapman et al., 1993b; Elder et al., 2000). A simulation study conducted by Jordan et al.(1999) found that pre-slaughter intervention strategies, such as the use of agents which would reduce the

numbers of *E. coli* O157:H7 shed in the feces of cattle, would have the greatest impact on reducing the contamination of carcasses with *E. coli* O157:H7.

Since the discovery of bacteriophages in the early 1900's by Twort and d'Herelle, they have been used successfully to control bacterial pathogens such as *Salmonella*, *Shigella* and *Staphylococcus* in humans (Alisky et al., 1998; Barrow and Soothill, 1997; Sulakvelidze et al., 2001). Bacteriophages have also been shown to control enteropathogenic *E. coli* infection in mice (Smith and Huggins, 1982), calves, piglets and lambs (Smith and Huggins, 1983; Smith et al.,1987). Bacteriophages specific for *E. coli* O157:H7, isolated from the feces of cattle and sheep, were found to eliminate *E. coli* O157:H7 in laboratory studies (Kudva et al., 1999). Recently, Waddell et al., (2000) have successfully used O157-specific bacteriophages as a means of reducing the period of *E. coli* O157:H7 fecal shedding in calves. Bacteriophage therapy may be an effective means of controlling *E. coli* O157:H7 in ruminants.

The objectives of the present study were (i) to assess the effectiveness of a particular bacteriophage (DC22) for its specificity and sensitivity for *E. coli* O157:H7, (ii) to assess the effectiveness of DC22 in reducing *E. coli* O157:H7 in an artificial rumen fermentation (Rusitec) system, (iii) and, to assess the ability of DC22 to reduce the fecal shedding of *E. coli* O157:H7 by sheep.

Materials and Methods

The Specificity and Sensitivity of *E. coli* O157:H7 to DC22. Forty bacterial strains (see Table 7.1) were tested for susceptibility to DC22. These strains included a variety of enterohemorrhagic *E. coli* (EHEC), including *E. coli* O157:H7 strains of different

Table 7.1. The sensitivity of Enterohemorrhagic *E. coli*, including *E. coli* O157:H7 and Enteropathogenic *E. coli* to DC22.

Bacterial isolate	Serotype	Origin	Titer[1]
Enterohemorrhagic *E. coli* isolates			
LRH-69	O157:H7 PT[2]14	Human	10.67 ± 0.06^{abc}
LRH-70	O157:H7 PT 14	Human	10.73 ± 0.15^{ab}
E32511	O157:NM PT 31	Human	10.90 ± 0.00^{ab}
E319	O157:H7 PT 1	Human	10.77 ± 0.12^{ab}
E321	O157:H7 PT 4	Human	10.83 ± 0.06^{ab}
E318N	O157:H7	Human	10.82 ± 0.50^{ab}
HS99-1	O157:H7 PT 14	Bovine	10.70 ± 0.16^{ab}
HS99-2	O157:H7 PT 14	Bovine	10.70 ± 0.10^{abc}
HS99-3	O157:H7 PT 14	Bovine	10.70 ± 0.00^{abc}
HS99-4	O157:H7 PT 14	Bovine	10.60 ± 0.20^{bcd}
H4420	O157:H7 PT 87	Bovine	10.83 ± 0.56^{ab}
3081	O157:H7 PT 43	Bovine	10.93 ± 0.06^{a}
ECI-565	O157:H7 PT 23	Bovine	10.33 ± 0.20^{def}
ECI-590	O157:H7 PT 49	Bovine	10.37 ± 0.16^{def}
ECI-596	O157:H7 PT 31	Bovine	10.10 ± 0.26^{fg}
ECI-600	O157:H7 PT 27	Bovine	10.00 ± 0.10^{g}
ECI-603	O157:H7 PT 49/Aty[3]	Bovine	10.37 ± 0.06^{def}

Table 7.1. *(Continued)*

E. coli strain	Serotype	Origin	Titer[1]
ECI-605	O157:H7 PT 1	Bovine	10.37 ± 0.12^{def}
ECI-607	O157:H7 PT 1	Bovine	10.13 ± 0.15^{efg}
ECI-651	O157:H7 PT 32	Bovine	10.17 ± 0.60^{efg}
ECI-652	O157:H7 PT 32	Bovine	10.27 ± 0.12^{ef}
ECI-654	O157:H7 PT Aty[3]	Bovine	10.20 ± 0.00^{efg}
ECI-660	O157:H7 PT 8	Bovine	10.17 ± 0.06^{efg}
43426	O103:H25	Human	-
9291	O103:H2	Human	-
44717	O111:H12	Human	-
5529	O103:H4	Human	-
52133	O111:K58	Human	-
55184	O2:NM	Human	-
44131	O26:H11	Human	-
33264	O145:H-	Human	-
35280	O103:H2	Human	-
52050	O111:NM	Human	-
5520	O111:K58	Human	-
5432	O103:H2	Human	-

Table 7.1. *(Continued)*

E. coli strain	Serotype	Origin	Titer[1]
Enteropathogenic *E. coli* isolates			-
EC990984	O55:H7	Human (EPEC)	$10.10 + 0.10^{fg}$
4582	O26:H11	Human (EPEC)	-
Other *E. coli* isolates			
1879S1	O157:H7	Porcine (VTEC)	$10.43 + 0.06^{cde}$
PVT91	O157:KV17:F4	Porcine	-
25922	Not available	Difco reference strain	-

[1] Mean $\log_{10}$ pfu/ml of DC22 $\pm$ standard deviation (n=3)

[2] Phage type

[3] Atypical phage type

[abcdefg] Means within a column followed by different letters differ ($P<0.05$)

phage types, and enteropathogenic *E. coli* (EPEC) strains. Each of the EHEC and EPEC strains were grown separately in trypticase soy broth (TSB) (BDH, Toronto, ON) for 18 h at 37 °C. The sensitivity and specificity of each strain of EHEC and EPEC to DC22 was determined using the plaque titration assay as described by Sambrook et al.(1989). Briefly, a range of DC22 dilutions, prepared in sterile salt-magnesium (SM) buffer (5.8 g/L NaCl, Sigma; 2 g/L $MgSO_4 \cdot H_20$, Sigma; 50 ml/L 1 M Tris·Cl (pH 7.5), Sigma; 5 ml/L 2% gelatin, Difco), were plated onto Luria- Bertani (LB) agar (Difco, Ottawa, ON) along with 10^7 CFU of each of the forty bacterial strains suspended in 0.75 % LB agar, in duplicate. The plates were allowed to harden at room temperature for 15 min prior to being inverted and incubated at 37 °C for at least 12 h. Only dilutions with plaque counts between 5 and 300 were used for the titer calculation.

Preparation of plate lysate stocks was performed using the soft agar overlay technique (Sambrook et al., 1989). *E. coli* O157:H7 strain H4420 PT 87 was found to give the highest titer when used to propagate DC22. The bacteriophage DC22 was diluted in SM buffer and 10^5 plaque forming units (PFU) were combined with 10^7 CFU of *E. coli* O157:H7, incubated for 20 min at 37 °C, added to molten (45 °C) 0.75% LB agar and poured onto prewarmed (37 °C) LB agar plates. After 6-8 h of incubation, following confluent lysis, the soft top agar was harvested and the plate was rinsed with SM buffer. A 0.01 volume of chloroform was added to the soft agar slurry which was incubated at 37 °C with shaking for 15 min prior to centrifugation at 4000 × *g* for 10 min to recover the supernatant containing the bacteriophage (lysate). Plate lysate stocks were also prepared without the addition of chloroform in order to determine the sensitivity of DC22 to

inactivation by chloroform.

Large-scale lysates of DC22 were prepared by liquid infection as described by Sambrook et al., 1989. Briefly, a 500 ml volume of NZCYM broth, (per liter: 10 g NZ amine, Sigma; 5 g NaCl, Sigma; 5 g yeast extract, Difco; 1 g casamino acids, Sigma; 2 g $MgSO_4$·7H20, Sigma) was inoculated with 1 ml of an overnight culture of *E. coli* O157:H7 strain H4420 and incubated at 37 °C with good aeration until the OD_{640} of the culture reached 0.5. The culture was then inoculated with 10^{10} PFU of DC22 and incubated at 37 °C with aeration until lysis was observed. A 0.01 volume of chloroform was added to the lysed culture which was incubated at 37 °C with aeration for a further 15 min prior to concentration.

The method for the concentration of DC22 which yielded the highest titre was the protocol described by Blattner et al., 1977. Briefly, the lysed culture was centrifuged at 17,700 × g for 10 min and a polyethylene glycol 8000 (PEG 8000)(Sigma) solution was added to the supernatant and incubated overnight at 4 °C. The supernatant was centrifuged at 3000 × g for 10 min and the PEG 8000 was removed from the thick white precipitate using 1 M potassium chloride (KCl) (Sigma). The KCl was removed by centrifugation at 12,100 × g for 10 min at 4 °C and the supernatant containing the phage was filtered through a 0.45 μm filter followed by one of 0.20 μm pore size.

DC22 stocks were treated with chloroform as before, stored at 4 °C and titered immediately prior to their use. LB plates prepared to determine whether any bacteria were present in the DC22 stocks were consistently negative. Strains of *E. coli* O157:H7 used for the propagation of DC22 were tested for development of resistance to DC22. *E. coli*

O157:H7 was separated from 24 h cultures of *E. coli* O157:H7 and DC22 and titered using the plaque titration assay as described previously. Susceptibility and resistance to DC22 was indicated by plaque formation and the absence of plaque formation, respectively.

Three independent replicates were performed in the determination of the sensitivity and selectivity of *E. coli* O157:H7 to DC22 and averages of the titers were reported. Analysis of variance was performed using the SAS General Linear Models procedure (SAS Institute, Inc., Cary, NC) after converting the data to $\log_{10}$ values. The least significant difference test was used to determine the differences among means where significant effects were observed ($P < 0.05$).

The Effect of DC22 on the Survival of *E. coli* O157:H7 in an Artificial Rumen System (Rusitec). *E. coli* O157:H7 strain 3081 (kindly made available by W. C. Cray, National Animal Diseases Center, Ames, Iowa) was used as the bacterial inoculant in an artificial rumen system (Rusitec). *E. coli* O157:H7 strain 3081 is resistant to 100 μg/ml ampicillin and 100 μg/ml kanamycin which facilitated the recovery of the organism from the rumen fluid following inoculation. Prior to inoculation, the strain was grown in TSB for 18 h at 37 °C. The bacteria were sedimented by centrifugation (4,000 x g, 12 min), washed three times in phosphate-buffered saline, pH 7.4 (PBS; 15 mM KH_2PO_4, 8 mM Na_2HPO_4, 137 mM NaCl, 2.6 mM KCl) and re-suspended in PBS. Cells were adjusted with PBS to an optical density of 0.5 at 640 nm (~ 10^8 CFU/ml)(UltraSpec Plus 4054, Pharmacia, Baie d'Urfe', QC). The population of *E. coli* O157:H7 strain 3081 cells was verified by enumeration on sorbitol MacConkey agar (SMAC)(Oxoid, Nepean, ON) supplemented with 2.5 mg/L potassium tellurite (Dynal, Lake Success, NY.), 0.05 mg/L

cefixime (Dynal), 100 µg/ml ampicillin (Sigma, Oakville, ON) and 100 µg/ml kanamycin (Sigma) to yield CT-KASMAC.

To determine the optimal multiplicity of infection (MOI) for DC22, *E. coli* O157:H7 strain 3081 was incubated with DC22 in rumen fluid. Clarified rumen fluid (5000 x g, 5 min followed by 12,000 x *g* for 30 min) with supplementary glucose (0.2% w/v) (Sigma), tryptone (0.1% w/v)(Difco) and cysteine-HCl (20 ml/L of a 2.5% w/v solution) (Sigma) was dispensed to serum vials and equilibrated with CO_2, using oxygen-free CO_2. *E. coli* O157:H7 strain 3081 was added to the serum vials to a final concentration of 10^4 CFU/ml. DC22 was added to the serum vials to achieve desired levels of 10^2, 10^3 and 10^4 PFU/CFU. Phage-free (containing only *E. coli* O157:H7) and cell-free (containing only DC22) serum vials of rumen fluid were used as controls. Serum vials were incubated at 39 °C and cultured for *E. coli* O157:H7 on CT-KASMAC at 0, 8, 12, 24 and 48 h post-inoculation. An MOI of 10^4 PFU/CFU resulted in a 94% reduction in *E. coli* O157:H7 numbers after 24 h of incubation at 39 °C which decreased to 89% after 48 h. Since 10^4 PFU/CFU did not result in complete inhibition of *E. coli* O157:H7 in rumen fluid at 39 °C and a 1 log drop in the titer of DC22 was observed over 24 h, an MOI of 10^5 PFU/CFU was used in subsequent tests in the artificial rumen system.

The rumen simulation technique (Rusitec) developed by Czerkawski and Breckenridge (1977) was used in this study. A Rusitec equipped with eight fermenters (four per treatment, two treatments), each of 820 ml nominal capacity, was used in this study. Inoculum was obtained from two rumen fistulated Hereford heifers maintained on an 80% barley grain and 20% barley silage diet. Rumen fluid was filtered through four

layers of cheesecloth to partition it into liquid and solid fractions. To begin the experiment, each fermenter was filled with 820 ml of filtered rumen fluid, pH 6.6. One nylon bag (80 x 160 mm; 51 um pore size) containing 20 g of solid digesta and one nylon bag containing 10 g of feed (80% barley and 20% silage on a DM basis, ground < 6mm) were also placed in each fermenter. After 24 h, the nylon bag containing the solid digesta was replaced by a fresh feed bag. Thereafter, one feed bag was replaced daily, so that each feed bag remained in the fermenter for 48 h. Fermenters received a continuous infusion of artificial saliva (pH 8.1) at a rate of 0.32 ml/min (McDougall, 1948). Throughout the setup of the fermenter units and during daily replacement of feed bags, the fermenters were flushed with oxygen-free CO_2 (Hungate, 1950) to foster anaerobiosis. Fermenter liquid pH, vessel volume and production of gas and effluent were monitored daily prior to feed bag replacement, in order to monitor fermentation. Fermenter units were fed for a period of 8 days prior to inoculation with 10^7 CFU *E. coli* O157:H7 strain 3081. The heifers from which the inoculum for the Rusitec was obtained were cared for in accordance with the guidelines set down by the Canadian Council on Animal Care (1993).

Following 8 days of adaptation, an inoculum containing 10^7 CFU of *E. coli* O157:H7 strain 3081 was added to each fermenter in order to obtain a final concentration of 10^4 CFU/ml of *E. coli* O157:H7. The two treatments were *E. coli* O157:H7 strain 3081 and *E. coli* O157:H7 strain 3081 + DC22 at a MOI of 10^5 PFU/CFU. The DC22, suspended in SM buffer, was inoculated into the fermenter 8 h following the inoculation of *E. coli* O157:H7 strain 3081. An equivalent amount of SM buffer containing no DC22 was inoculated into the control fermenters. Fermenter liquid samples were collected 30 min

post-inoculation, at 4, 8, 12 and 24 h and daily thereafter for enumeration of *E. coli* O157:H7 and DC22.

For the enumeration of *E. coli* O157:H7 strain 3081 from rumen fluid samples following inoculation, a 1.0 ml sample of rumen fluid was placed in 9 ml PBS, vortex-mixed and serially diluted in PBS. A 100 µl aliquot from the appropriate dilutions was plated onto CT-KASMAC in duplicate using a sterile glass spreader. Plates were incubated for 18-24 h at 37°C prior to the determination of viable numbers. Three sorbitol negative colonies from each plate were tested for the O157 antigen by agglutination with O157 antiserum. Enumeration of DC22 from the rumen fluid was performed using the plaque titration assay as described previously (Sambrook et al., 1989).

For enumeration of *E. coli* O157:H7 from feed residues, the contents of the feed bag (~10 g) was weighed, added to 90 ml PBS and homogenized in an Osterizer 10 speed laboratory blender for 60 s to dislodge particle-associated bacteria. For enumeration of *E. coli* O157:H7 from the feed bag, 100 ml of PBS was added to a stomacher bag containing the empty feed bag and stomached for 60 s using a Stomacher 4000 Laboratory Blender (Seward Medical, London, UK). Samples were serially diluted in PBS and spread plated onto CT-KASMAC in duplicate. When the organism was no longer detected in the rumen fluid, feed residues, or feed bags by spread plating, enrichment in mTSB and immunomagnetic separation (IMS) using Dynabeads anti- *E. coli* O157 was performed according to manufacturer's instructions.

Analysis of variance was performed using the SAS Mixed Model procedure using the spatial model for covariance structure (SAS Institute, Inc., Cary, NC). The repeated

measures data were analyzed as a split-plot in time with treatment as the main plot and time as the subplot. Fermenters nested within treatment were used as the error term to evaluate treatment. The least significant difference (LSD) test was used to determine the differences among means where significant effects were observed ($P<0.05$).

The Effect of DC22 on the Fecal Shedding of *E. coli* O157:H7 in Sheep. Twelve Romanov wethers (4 months of age) were used in the 30 day study. The animals were divided into 2 groups of 6 animals. Each group of 6 animals was housed in a separate climate controlled room containing 2 pens, with 3 wethers in each pen. Wethers were fed a barley based diet (Table 7.2)(McAllister et al., 1996) and had free access to feed and water throughout the experimental period. Individual water ports and creep feeders (four per pen) were available in each pen. Animals were adapted to their diets for a 2 week period. Feed was withdrawn for a 48 h period prior to inoculation with *E. coli* O157:H7 strain E318N. Feed was also withdrawn from three animals in each group for 48 h commencing day 20, in order to assess the effects of feed withdrawal on the fecal shedding of *E. coli* O157:H7 and DC22. Housing, care and handling procedures for the wethers was approved by the Animal Care Committee (Lethbridge Research Centre, Lethbridge, AB).

E. coli O157:H7 strain E318 N (kindly made available by A. Borczyk, Enteric Reference Laboratory, Ministry of Health, Toronto, Ontario) was used to facilitate the recovery of the organism from the animals following inoculation. The organism is resistant to 40 μg/ml nalidixic acid, produces verotoxin 1 (VT1) and verotoxin 2 (VT2) and is susceptible to DC22. The bacterium was grown in TSB for 18 h at 37 ° C, sedimented by centrifugation (4,000 x g, 12 min), washed three times in PBS and re-suspended in PBS.

Table 7.2. Composition of diet fed to wethers.

Ingredients (% as fed)	
Whole-grain barley	77.5
Canola meal	6.8
Soybean meal	5.5
Alfalfa	6.5
Beet molasses	1.0
Calcium carbonate	1.2
Sheep mineral[1]	1.0
Maxi-Pel [2]	0.5
Vitamins ADE [3]	0.025

[1] Containing 93.1% NaCl; . 55% Mg; .33% Zn; .27% Mn; .03% Cu; .005% Se.

[2] Feed pellet binder (Mountain Minerals Co. Ltd., Vancouver, BC)

[3] Containing 10,000 IU/g vitamin A; 1,250 IU/g vitamin D; 10 IU/g vitamin E.

Enumerations of *E. coli* O157:H7 strain E318N from the PBS were performed using spectrophotometric readings at 640 nm (UltraSpec Plus 4054, Pharmacia, Baie d'Urfe', QC), and viable numbers were confirmed by standard dilution plating in triplicate onto SMAC supplemented with 2.5 mg/L potassium tellurite (Dynal), 0.05 mg/L cefixime (Dynal), and 40 μg/ml nalidixic acid (Sigma) to give CT-SMACnal. Each wether was orally inoculated with 50 ml of PBS containing 10^8 CFU *E. coli* O157:H7 E318N using a sterile 60-ml syringe (Fisher, Nepean, ON) and stomach tube. Inoculation was followed by delivery of two 50 ml aliquots of PBS. On day 2 post-inoculation, the 6 wethers in the DC22-treated group were inoculated with 10^{13} PFU of DC22 suspended in SM buffer, using a sterile 60-ml syringe and stomach tube. Oral inoculation was followed by the administration of two 50 ml aliquots of SM buffer. The 6 control wethers were orally inoculated with an equivalent amount of SM buffer.

Fecal samples were collected from the lambs by digital rectal retrieval using sterile latex gloves, with a new glove being used for each sample. Samples were placed in sterile polypropylene specimen containers (Fisher) and transported to the laboratory for analysis within 1 h. Fecal samples were taken at the same time daily for 8 days post-inoculation, followed twice weekly thereafter for a total time period of 30 days. To test for the presence of *E. coli* O157:H7 prior to inoculation, fecal samples were cultured using enrichment in mTSB followed by immunomagnetic separation (IMS) using Dynabeads anti-*E. coli* O157, and plating onto SMAC supplemented with 2.5 mg/L potassium tellurite and 0.05 mg/L cefixime (CT-SMAC) and CT-SMACnal. Plates were incubated for 18-24 h at 37 °C.

For the enumeration of *E. coli* O157:H7 E318N in the fecal samples following

inoculation, a 1.0 g sample of feces was placed in 9 ml PBS, vortex-mixed and serially diluted in PBS. A 100 µl aliquot from a range of dilutions was plated onto CT-SMACnal in duplicate using a sterile glass spreader. Plates were incubated for 18-24 h at 37 °C prior to the determination of viable numbers. Three sorbitol negative colonies from each plate were tested for the O157 antigen by agglutination with O157 antiserum. Multiplex PCR (O157 specific) assays were used as a verification tool on one fecal sample a week in which the experimental strain was recovered (*E. coli* O157:H7 strain E318N) (Gannon et al., 1997). Enrichment and IMS using Dynabeads anti- *E. coli* O157 was performed on fecal samples when *E. coli* O157:H7 E318N was no longer detected by direct plating.

Feed samples and water port swabs were taken once a week and cultured for the inoculated strain. Swabs of the water ports were placed into 10 ml of mTSB. About 25 g of sample from each of the four feeders per pen was pooled and a sub-sample of 10 g of feed was placed into 90 ml of mTSB. Samples were mixed and incubated for 18-24 h at 37 °C. IMS of *E. coli* O157 using Dynabeads anti- *E. coli* O157 was performed and a 50 µl aliquot of the antibody-coated bead suspension was plated onto CT-SMACnal and incubated for 18-24 h at 37 °C. Three sorbitol-negative colonies from each plate were tested for the O157 antigen by agglutination with O157 antiserum (Difco). Multiplex PCR (O157-specific) assays were used as a verification tool on one fecal sample a week in which the experimental strain (*E. coli* O157:H7 strain E318N) was recovered (Gannon et al., 1997).

The titre of DC22 in the fecal samples of the wethers in the treatment and control groups was determined using the plaque titration assay commencing on Day 3 and on each sampling day thereafter (Sambrook et al., 1989). A 1 ml aliquot of the 1:10 fecal dilution

in PBS was centrifuged at 10,000 × *g* for 10 min in order to sediment the fecal material. The supernatant was then filtered using a 0.20 μm syringe filter (Fisher) and titered for DC22 using *E. coli* O157:H7 E318N. Fecal samples were tested for the presence of *E. coli* O157-specific bacteriophages prior to inoculation of the wethers with DC22.

Analysis of variance was performed using the SAS Mixed Model procedure using the spatial model for the covariance structure (SAS Institute, Inc., Cary, NC). The repeated measures data were analyzed as a split-plot in time with diet as the main plot and time as the subplot. Animals nested within treatments were used as the error term to evaluate treatment. The least significant difference (LSD) test was used to determine the differences among means where significant effects were observed ($P<0.05$).

Results and Discussion

Phage Specificity and Sensitivity of *E. coli* O157:H7 to DC22. The morphology of the phage DC22 is presented in Figure 7.1. The phage possesses a polyhedral head which is 0.12 μm in diameter and a 0.15 μm tail. The presence of a contractile tail sheath indicates that DC22 may be designated as a group A bacteriophage, according to Bradley (1967). The insensitivity of DC22 to inactivation by chloroform, as observed in the preparation of plate lysate stocks of the phage in the presence and absence of chloroform, indicates that DC22 is not composed of a significant amount of lipid (Bradley and Rutherford, 1975).

The titers of DC22 on the forty EHEC and EPEC strains are presented in Table 7.1. DC22 lysed all of the twenty four *E. coli* O157:H7 strains which were assayed, with the titers ranging from 10.00 to10.90 $\log_{10}$ PFU/ml. An example of a scanning electron micrograph of *E. coli* O157:H7, strain E318N, is presented in Figure 7.2.

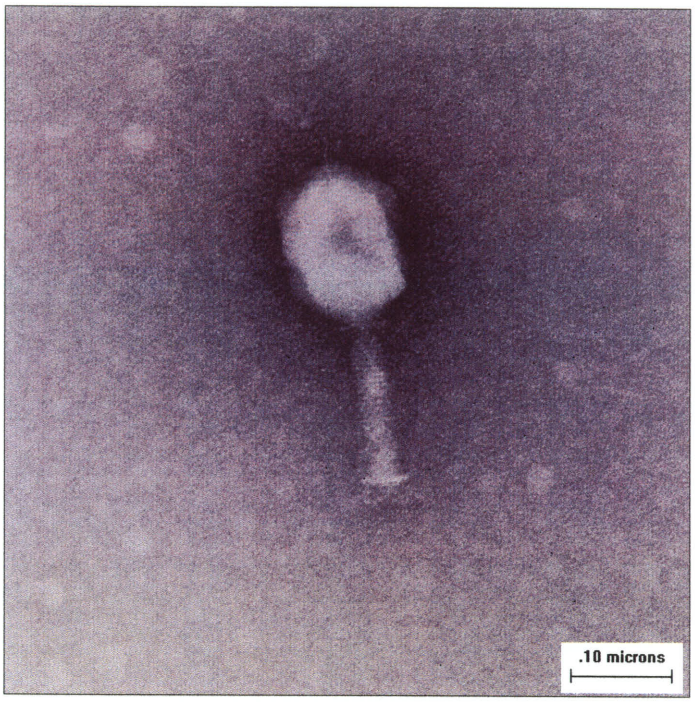

Figure 7.1. Transmission electron micrograph of the bacteriophage DC22 negatively stained with uranyl acetate.

The adsorption of phages to bacterial cells is the first step of infection and is dependent on the presence of highly specific receptors on the bacterial cell wall to which the phage tail fibers bind (Adams, 1959; Hadas et al., 1997). Results indicate that while DC22 appears to be specific for *E. coli* O157:H7, the phage receptor may not be the O157-antigen since *E. coli* strain PVT91 O157:KV17:F4 was not lysed by DC22. In contrast, the EPEC strain O55:H7, a putative progenitor of *E. coli* O157:H7, was lysed by DC22. This suggests that the receptor for DC22 is a cell wall constituent which is common to *E. coli* O157:H7 and *E. coli* O55:H7. The flagellar antigen likely does not play a role in phage adsorption of DC22 to *E. coli* O157:H7, since *E. coli* O157:NM strain E32511 also was susceptible to DC22 (Table 7.1). Further studies are required in order to establish the receptor for DC22.

Kudva et al. (1999) reported that phage infection was influenced by the nature of the host cell O157 lipopolysaccharide (LPS) in three O157-specific phages isolated from ovine and bovine fecal samples. It has been reported that phage attachment sites on bacterial cells do not necessarily correspond to the antigenic sites on the cell (Ronner and Cliver, 1990). Surface exposed LPS and the porin protein OmpF, have been shown to serve as phage receptors in *E. coli* K-12 (Traurig and Misra, 1999). A cooperative interaction between OmpC and LPS was required for the efficient binding of phage AR1, which specifically infects *E. coli* O157:H7 (Ronner and Cliver, 1990; Yu et al., 2000).

The Effect of DC22 on the Survival of *E. coli* O157:H7 in the Rusitec. The MOI of DC22 necessary for lysis of *E. coli* O157:H7 was higher than expected. Approximately 10^4 PFU/CFU did not eliminate *E. coli* O157:H7 from clarified rumen fluid. However, *E.*

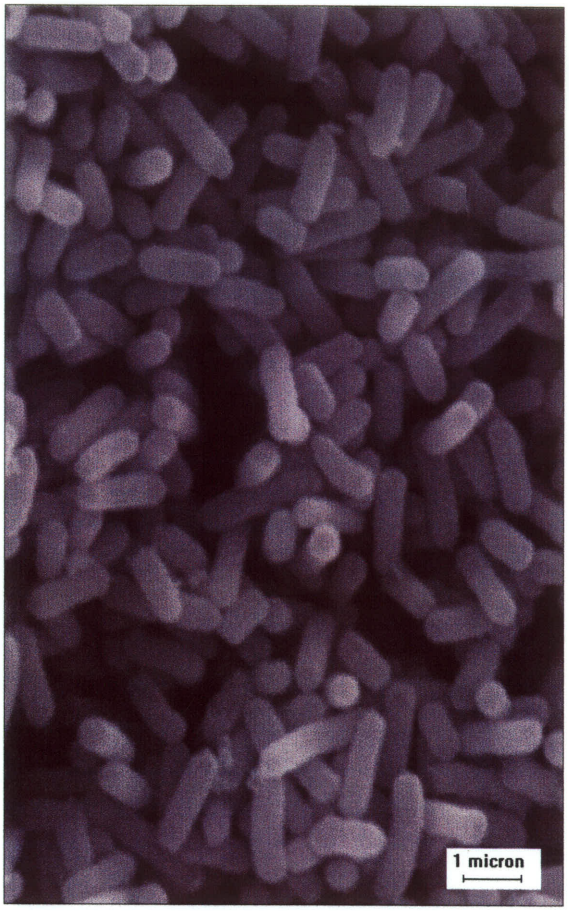

Figure 7.2. Scanning electron micrograph of *Escherichia coli* O157:H7 strain E318N.

coli O157:H7 was eliminated from the rumen fluid in the fermenters 4 h after the addition of DC22 at a MOI of 100,000:1. In the control fermenters, numbers of *E. coli* O157:H7 declined steadily until 120 h post-inoculation, at which time enrichment and IMS was required in order to detect the organism. The inoculated strain was detected in the control fermenters at 168 h post-inoculation (Figure 7.3).

The phage DC22 at a MOI of 10^5 PFU/CFU was successful in eliminating *E. coli* O157:H7 strain 3081 from rumen fluid within 4 h of its inoculation into the fermenters. The temperature of the fermenters (39 °C) in addition to agitation under anaerobic conditions were conducive to phage attachment and lysis of *E. coli* O157:H7 within the fermenters. The lack of replication of DC22 indicates (Figure 7.4) that the phage did not replicate and form infective particles in *E. coli* O157:H7 in the rumen fluid. It is possible that the high MOI resulted in premature lysis of *E. coli* O157:H7 without phage progeny being liberated. This phenomenon has been reported to occur when bacteria are attacked by a very large number of phage particles. Lysis of the bacteria is due to damage to the host cell membrane by the phage particles rather than to infection in the usual manner (Adams, 1959).

Preliminary *in vitro* experiments, however, indicated that while a 10^4 PFU/CFU MOI resulted in a reduction in *E. coli* O157:H7, re-growth of the uninhibited organisms may have resulted in a subsequent increase in the levels of *E. coli* O157:H7. This is in agreement with a study conducted by Kudva et al.(1999) who found that no single phage was able to successfully eliminate *E. coli* O157:H7 from cultures *in vitro*. A mixture of three O157-specific phages was necessary to eliminate *E. coli* O157:H7 from cultures, with aeration, an incubation temperature of 37 °C and a high MOI being factors which were

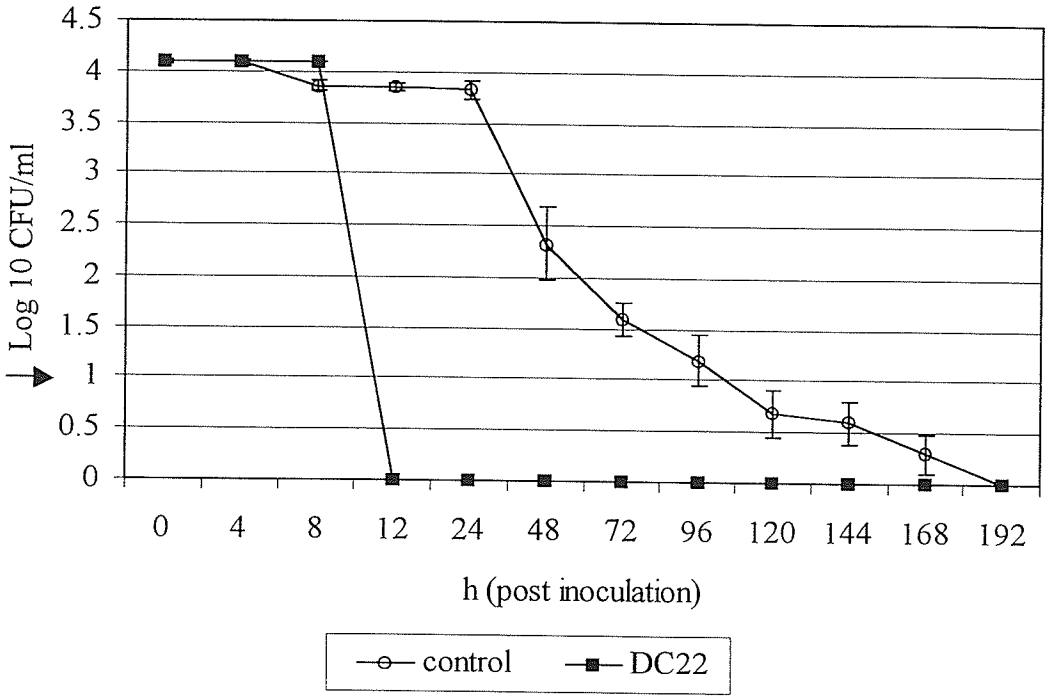

Figure 7.3. The recovery of *E. coli* O157:H7 from rumen fluid in an artificial rumen system (Rusitec) following (1) inoculation with 10^4 CFU/ml of *E. coli* O157:H7 strain 3081 at time 0 (control) (2) and, inoculation with 10^4 CFU/ml of *E. coli* O157:H7 strain 3081 at time 0 and DC22 at a MOI of 100,000:1 at 8 h post-inoculation (DC22). Bars represent the standard error of the mean. Where not shown, error bars are within symbols. The arrow indicates detection by enrichment (<10 CFU/ml).

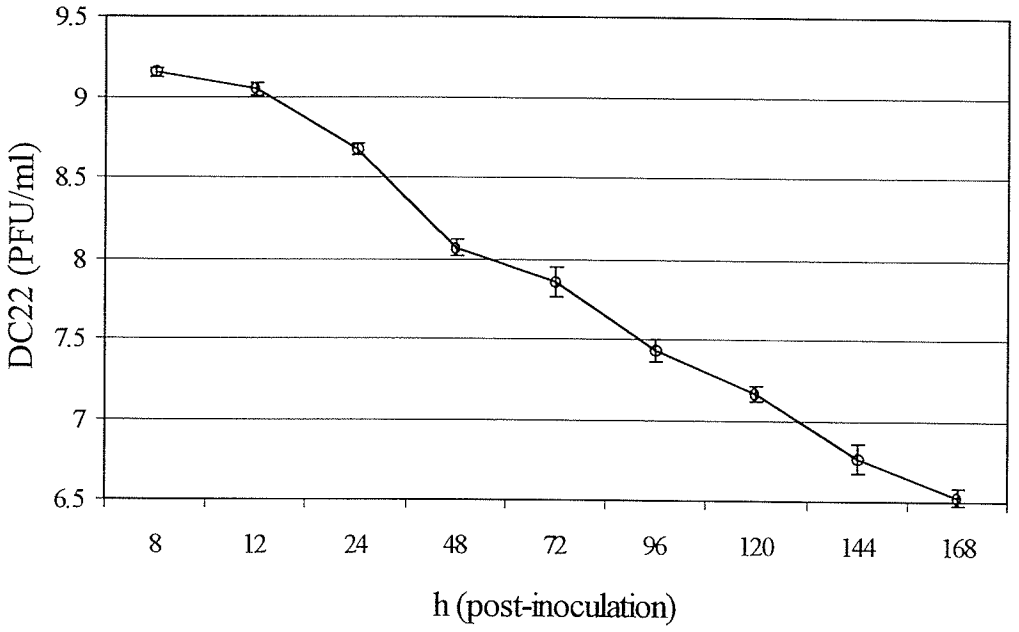

Figure 7.4. The reduction of DC22 from the rumen fluid of fermenters in an artificial rumen system (Rusitec) following inoculation of 10^4 CFU/ml of *E. coli* O157:H7 strain 3081 at 0 h and DC22 at a MOI of 100,000:1 at 8 h post-inoculation. Bars represent the standard error of the mean. Where not shown, error bars are within symbols.

critical for rapid cell lysis (Kudva et al., 1999). While aeration is important in increasing the opportunity for phage-bacterium interaction and cell infection (Kudva et al., 1999), it appears that the mixing which occurred in the fermenters of the Rusitec under anaerobic conditions was sufficient to allow for adsorption of the phage particles to the bacteria.

There was a steady decrease in levels of DC22 recovered from the rumen fluid in the DC22- treated fermenters over the 168 h experimental period. The infusion of artificial saliva (pH 8.1) at a rate of 0.32 ml/min served to buffer the bacterial fermentations within the fermentation vessels to a pH of between 6 and 6.5, simulating rumen fermentations *in vivo*. A one $\log_{10}$ reduction in levels of DC22 in the fermenters was observed 48 h post-inoculation, while an additional one $\log_{10}$ decrease was seen after 120 h. A 2.5 $\log_{10}$ reduction in the levels of DC22 was observed in the rumen fluid over 168 h. This suggests that DC22 is stable in rumen fluid at a pH of 6-6.5 for extended periods of time. The dilution rate of the Rusitec (0.32 ml/min), which represents an approximate 50-55% volume replacement in the fermentation vessels in 24 h, likely resulted in the steady decline in numbers of DC22 (Figure 7.4) and *E. coli* O157:H7 (Figure 7.3) recovered from the rumen fluid over time.

E. coli O157:H7 was detected in the feed residues and feed bags in the control fermenters 24 h post-inoculation at levels of 1.83 and 1.73 $\log_{10}$ CFU/g, respectively. Numbers of *E. coli* O157:H7 recovered from the feed and feed bags declined after this time, with similar numbers being recovered from both feed and feed bags. Enrichment and IMS was required to recover *E. coli* O157:H7 from the feed and feed bags 72 h post-inoculation and thereafter. The organism was not recovered from the feed or feed bags after 144 h post-

inoculation. *E. coli* O157:H7 was not detected in the feed or feed bags of the DC22 treated fermenters during the entire experimental period (Figure 7.5).

The Effect of DC22 on the Fecal Shedding of *E. coli* O157:H7 in Sheep. Preinoculation fecal samples taken from both groups of wethers were negative for *E. coli* O157:H7 using enrichment followed by IMS using Dyanbeads anti-*E. coli* O157. The numbers of *E. coli* O157:H7 strain E318N shed in the feces of the wethers in both the control and DC22-treated group decreased during the first 13 days after inoculation. In the control group, *E. coli* O157:H7 was not detected in the feces of five of the six animals on day 13, and thereafter. An increase in the numbers of *E. coli* O157:H7 was observed in one of the animals 13 and 16 days post-inoculation. Numbers of *E. coli* O157:H7 then decreased in the feces of this animal and the organism was undetectable 30 days post-inoculation (Figure 7.6A). In the DC22 treated group, numbers of *E. coli* O157:H7 decreased over the first 13 days post-inoculation and then increased in three of the six animals over the next 14 days. The organism was undetectable in the feces of all six animals 27 days post-inoculation (Figure 7.6B). Animals in the control and the DC22-treated group were all culture negative for *E. coli* O157:H7 strain E318N 30 days post-inoculation. There was no significant difference in the numbers of *E. coli* O157:H7 shed by the wethers in the control or DC22-treated group during the 30 day experimental period ($P>0.05$). Feed withdrawal on days 20 and 21 had no significant effect on the subsequent fecal shedding of *E. coli* O157:H7 strain E318N in the control or the DC22 treated group on days 23, 27 or 30 ($P>0.05$)(Figure 7.6). Results of a multiplex PCR assay used in the detection of *vt*, *eaeA* and $fliC_{H7}$ genes in the specific identification of *E. coli* O157:H7 to

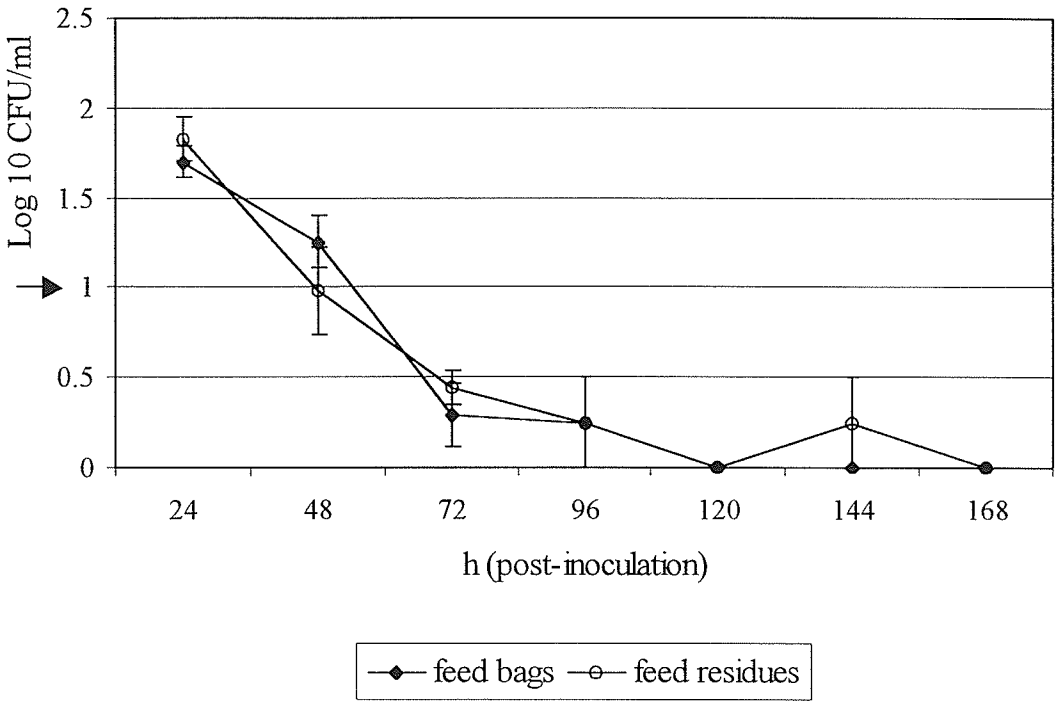

Figure 7.5. Isolation of *E. coli* O157:H7 strain 3081 from the feed residues and feed bags of the control fermenter (only *E. coli* O157:H7) following inoculation of 10^4 CFU/ml of *E. coli* O157:H7 at 0 h. Each symbol represents the mean of four determinations. Bars represent the standard error of the mean. Where not shown, error bars are within symbols. The arrow represents detection by enrichment (< 10 CFU/g).

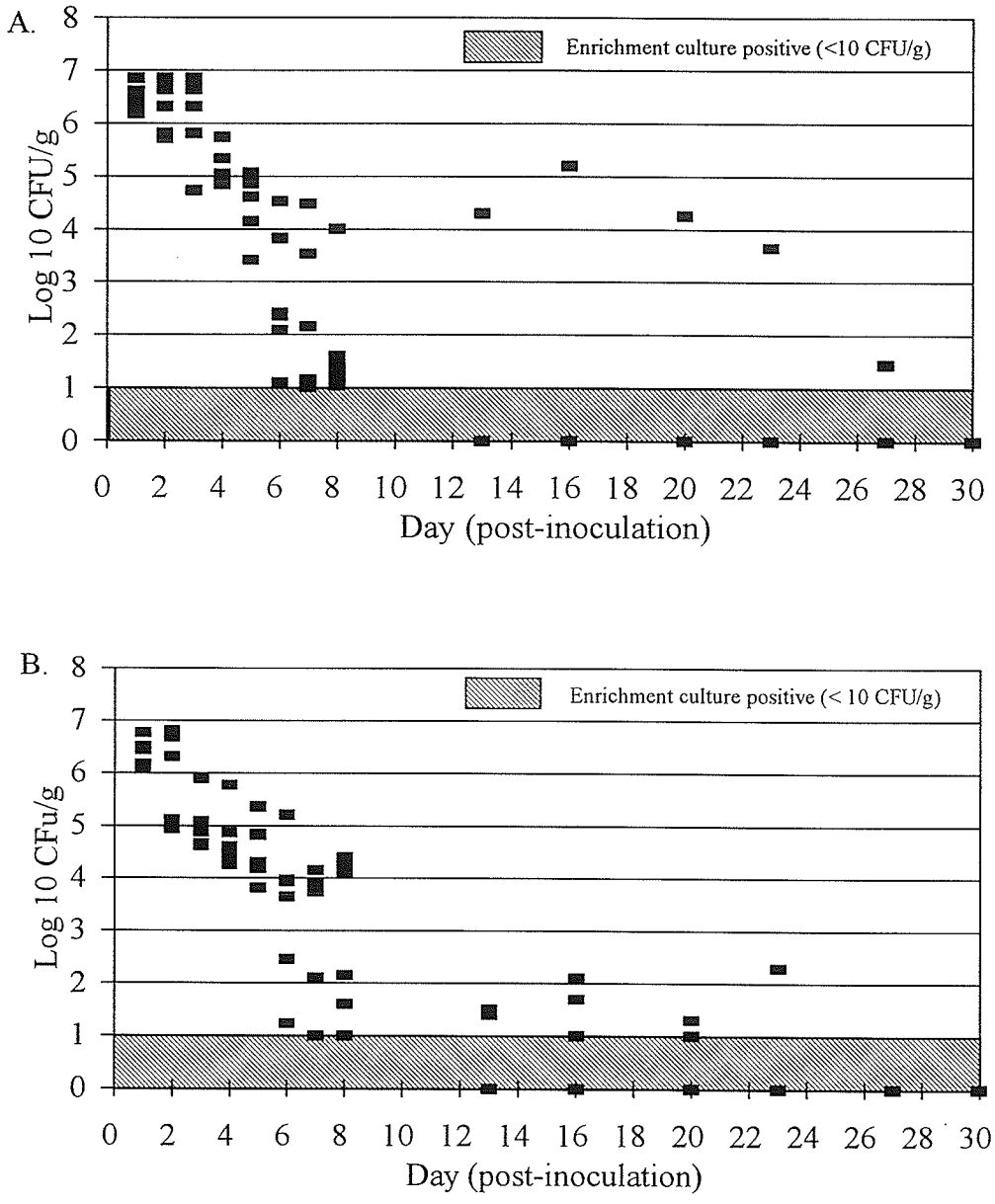

Figure 7.6. Levels of *E. coli* O157:H7 strain E318N shed in the feces of wethers in (A) the control group (B) and, DC22 treated group (n = 6 for each group). Wethers were inoculated with 10^{8} CFU *E. coli* O157:H7 strain E318N on day 0. DC22 (10^{13} PFU) was administered to the wethers in the treatment group on day 2. Each symbol represents an individual animal.

confirm isolates obtained from the wethers as *E. coli* O157:H7 are presented in Figure 7.7.

A rapid decrease in the levels of DC22 recovered from the feces of the wether was observed following administration of DC22 two days post-inoculation. DC22 was not detected in the fecal samples of any of the wethers following 8 days post-inoculation. Bacteriophages were not detected in the feces of the control group over the 30 day experimental period. Feed withdrawal on days 20 and 21 had no effect on the shedding of DC22 in the feces of the treated wethers (Figure 7.8). *E. coli* O157:H7 strain E318N was not detected from the feed or water port samples taken during the experimental period.

While DC22 exerted an inhibitory effect on *E. coli* O157:H7 in rumen fluid in the Rusitec, a high MOI was necessary indicating either a low bactericidal activity of the phage or ineffective attachment of DC22 to receptors on the host cell. DC22 had no effect on the fecal shedding of *E. coli* O157:H7 by wethers. In the Rusitec, an enclosed but continuous fermentation system, DC22 was likely at a sufficient MOI to result in complete lysis within 4 h (Figure 7.3), prior to any reduction in numbers of DC22 or *E. coli* O157:H7 in the fermenters due to volume replacement as a result of the infusion of artificial saliva. The administration of DC22 to wethers 2 days post-inoculation with *E. coli* O157:H7 likely resulted in a concentration of DC22 which was insufficient to sustain lysis of *E. coli* O157:H7 in the gastrointestinal tract of the wethers. This may have been due to a reduction in numbers of DC22 due to non-specific binding in the gastrointestinal tract in addition to the low bactericidal activity of the phage.

Some phages do not always lyse bacteria, but establish a non-lytic presence in the host cell without producing any progeny virions (Adams, 1959). The host cell, which is said

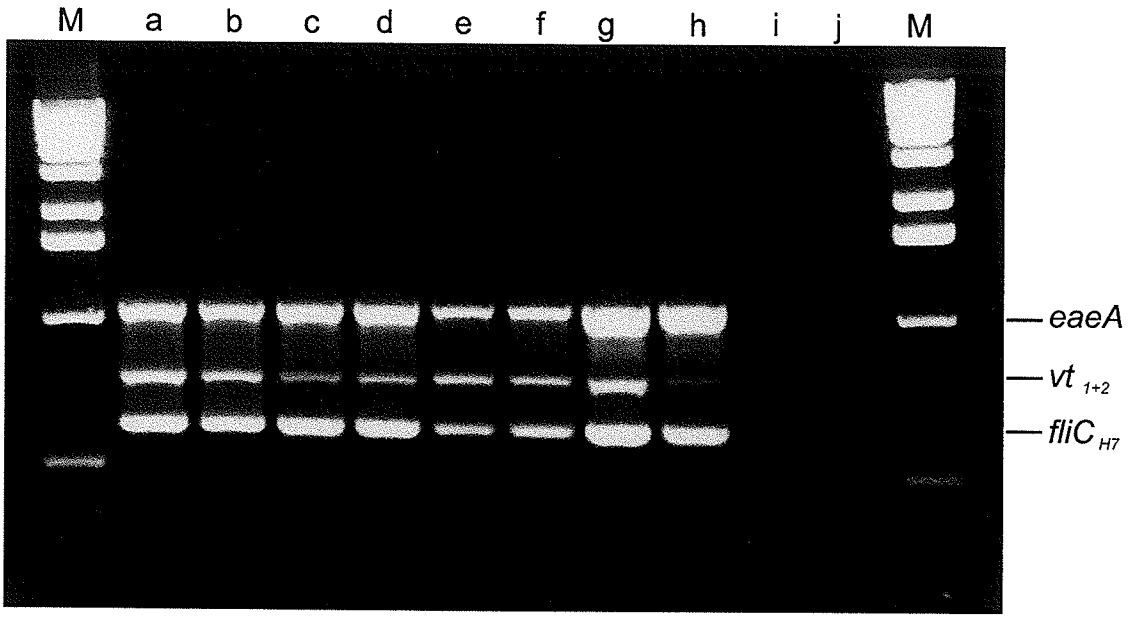

Figure 7.7. Multiplex PCR (O157-specific) assays used in the confirmation of *E. coli* O157:H7 isolates from wethers orally inoculated with *E. coli* O157:H7 strain E318N on d 0. Lanes: M, molecular size markers (1-kb ladder; Gibco-BRL); a, wether #491/d 5; b, wether #504/d 5; c, wether #521/d 5; d, wether #544/d 5; e, wether #572/d 5; f, wether #613/d 5; g, E318N O157:H7; h, 3081 O157:H7; i, DH5α O?R:K12; and j, blank.

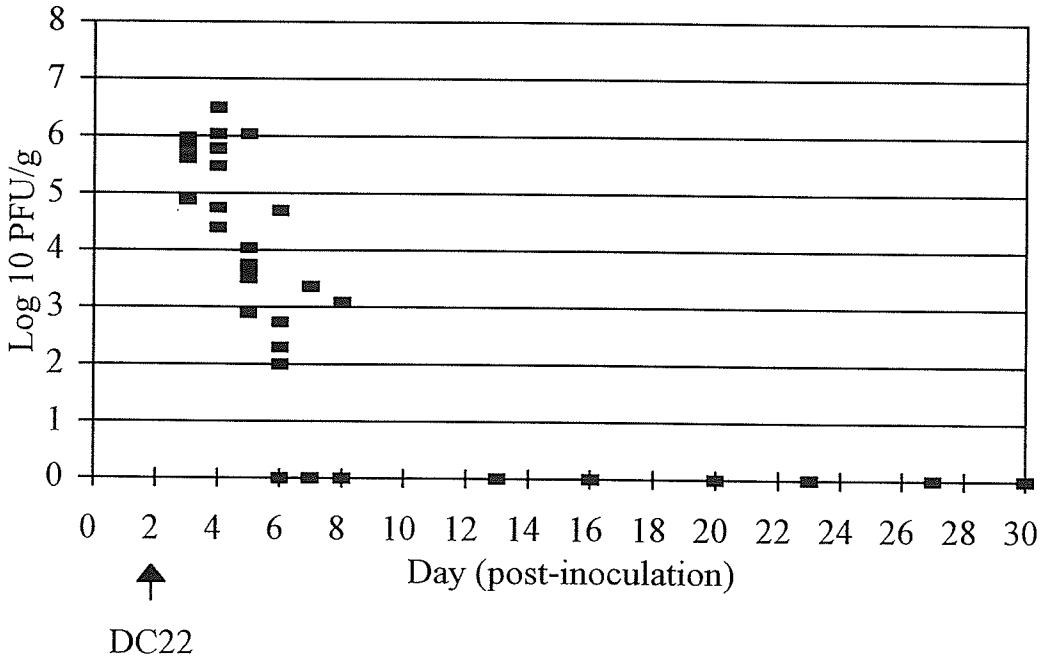

Figure 7.8. The shedding of DC22 in the feces of wethers following the administration of 10^{13} PFU DC22 on day 2 (n = 6). Wethers were inoculated with 10^{8} CFU of *E. coli* O157:H7 strain E318N day 0. Each symbol represents an individual animal.

to be lysogenic, continues to grow and divide, with replication of the phage genome or prophage being coordinated with that of the host chromosome. The prophage is maintained in the host cell by integration into the host chromosome or as an extrachromosomal plasmid (Adams, 1959; Barrow and Soothill, 1997). In the lysogenic state, the host cell resists infection by a second phage of the same or similar type, in a process known as phage immunity. Although the capacity of a phage to lysogenize is dependent on environmental factors, it is genetically controlled. The frequency of the lysogenic response increases as the MOI increases (Adams, 1959). Preliminary experiments suggest that DC22 does not establish a lysogenic state in *E. coli* O157:H7 strain E318N. However, it is possible that DC22 may have established a lysogenic state in a proportion of *E. coli* O157:H7 present in the gastrointestinal tract of the wethers resulting in a decrease in the numbers of phage particles available for infection.

Waddell et al. (2000) were successful in reducing the period of *E. coli* O157:H7 fecal shedding in calves using a mixture of six *E. coli* O157- specific bacteriophages. Smith and Huggins (1983) used a mixture of two phages to protect calves, piglets and lambs from experimental *E. coli* diarrhea. Kudva et al. (1999) found that in *in vitro* experiments a mixture of three O157-specific phages were successful in eliminating *E. coli* O157:H7 from cultures while no single phage could completely kill an *E. coli* O157:H7 culture. While DC22 used alone was unsuccessful in reducing the fecal shedding of *E. coli* O157:H7 in wethers, this phage used in combination with other phages may be effective in reducing *E. coli* O157:H7 fecal shedding.

An important application of bacteriophage therapy would be the reduction of fecal

shedding of *E. coli* O157:H7 by cattle prior to slaughter. However, most studies have shown that phage therapy is most effective when applied before or together with the infective bacteria (Smith and Huggins, 1983). Waddell et al. (2000) successfully reduced the fecal shedding period of *E. coli* O157:H7 in calves when the mixture of 6 phages was administered -7, -6, -1, 0 and 1 day following inoculation with 10^{9} CFU of *E. coli* O157:H7. The application of phages prior to infection may not feasible in regards to *E. coli* O157:H7 in cattle, since the organism is often acquired soon after birth (Gyles, 1986). However, a reduction of *E. coli* O157:H7 fecal shedding by ruminants using bacteriophage therapy involving several phage strains may minimize subsequent carcass contamination with *E. coli* O157:H7 and reduce the incidence of human foodborne illness associated with this pathogen.

CHAPTER 8

The Effects of Propionibacteria and *Saccharomyces cerevisiae* on the Growth and/or Survival of *Escherichia coli* O157:H7

Materials and Methods

The Effect of Bacteriocins of *Propionibacterium freudenreichii* and *P. acidipropionici* on *E. coli* O157:H7. *Propionibacterium freudenreichii* P99 and *P. acidipropionici* P42 (kindly provided by T. Rehberger, Waukesha, WI) used in this study, were found to produce bacteriocins inhibitory to one strain of *E. coli* O157:H7 (Mao, 1994). The indicator strains were *E. coli* O157:H7 strains 3081 (kindly made available by W.C. Cray, National Animal Disease Center, Ames, IO), H4420, E32511, and *E. coli* ATCC 25922. All cultures were stored as frozen stocks at - 80 °C in their appropriate media supplemented with 20% glycerol.

Propionibacteria were routinely propagated in sodium lactate broth (NLB) that consisted of 1.0 % yeast extract (Difco), 1.0 % tryptic soy broth (TSB)(BDH, Toronto, ON), and 1.0 % sodium lactate syrup (Difco)(Hofherr et al., 1983). Cultures were incubated at 32 °C under anaerobic conditions using a BBL anaerobic GasPak plus system (BBL, Oakville, ON).

Nonfat milk medium (NFM), used for bacteriocin production, contained 10% commercial nonfat milk powder and 0.1 % yeast extract. The NFM media was autoclaved at 121 °C for 15 min, cooled to 30 °C, and acidified to pH 5.3 with 10% lactic acid (Sigma, Oakville, ON) prior to use.

The indicator strains of *E. coli* O157:H7 (3081, H4420 and E32511) and *E. coli* ATCC 25922 were grown separately in TSB for 18-24 h at 37 °C. A 1 % inoculum was transferred to TSB and growth was monitored spectrophotometrically until an optical density (OD) of 0.5 at 640 nm was reached (~10^8 CFU/ml). Indicator cultures were used in the preparation of assay agar plates.

The direct supernatant spot assay was used to screen the bacteriocins of *P. freudenreichii* P99 *and P. acidipropionici* P42 for inhibitory action against *E. coli* O157:H7. The two strains of propionibacteria were grown separately in NLB at 32 °C for 18-24 h under anaerobic conditions (GasPak plus). A 1% inoculum of each strain was then transferred to NFM medium and tubes were incubated anaerobically at 32 °C for 4 days. Cell-free supernatants were prepared from cultures by centrifugation at 8000 × g for 10 min. The pH of the supernatant was then adjusted to 6.5 with 3 N NaOH. The supernatant was filtered through a 0.2 µm sterile syringe filter (Fisher, Nepean, ON). Assay agar plates were prepared by adding 1 ml of the indicator culture to 100 ml of molten (45 °C) tryptic soy agar (TSA)(BDH). A 10 ml portion of TSA containing indicator culture was then poured into a 100 x 15 mm petri dish (Fisher). Each propionibacteria strain supernatant was spot inoculated (20 µl) four times in an equidistant manner in the periphery of the plate with 20 µl of sterile distilled water being placed in the center as a negative control. Duplicate plates were prepared for each strain of propionibacteria for each of the four *E. coli* indicator strains. The plates were incubated for 18-24 h at 37 °C prior to examination for zones of inhibition.

The Effect of Levucell SB20 on the Growth and/or Survival of *E. coli* O157:H7.

The yeast, *Saccharomyces cerevisiae* subsp. *boulardii* strain I-1079 was isolated from Levucell SB20 (L'Allemand Biochemicals, Milwaukee, WI) and viable numbers were determined. The product (1g) was re-hydrated in 9 ml of PBS, serially diluted, and a range of dilutions was spread plated onto Sabouraud Dextrose agar (Difco). Plates were incubated for 5 days at 21° C prior to the determination of viable numbers.

E. coli O157:H7 strains 3081 (resistant to 100 µg/ml ampicillin and 100 µg/ml kanamycin), H4420nal (resistant to 200 µg/ml nalidixic acid), H4420 and E32511, and *E. coli* ATCC 25922 were grown separately in TSB for 18-24 h at 37 °C. A 1 % inoculum was transferred to TSB and growth was monitored spectrophotometrically until optical density (OD) reached 0.5 at 640 nm (10^8 CFU/ml).

To test for inhibitory activity of *S. cerevisiae*, 10 µl of a 1:1000 dilution of the re-hydrated culture was spot-inoculated onto Sabouraud Dextrose agar resulting in approximately 10^4 CFU per spot. Each plate contained four inoculations of *S. cerevisiae* spotted in an equidistant manner. A 10 µl spot of sterile distilled water in the center of the plate served as a negative control. Plates were allowed to dry for 2 h at room temperature after which they were inverted and incubated for 5 days at 21 °C. Following incubation, the plates were inverted and a 4.25 cm diameter Whatman No. 1 filter paper (Fisher) was placed in the lid. The filter paper was soaked with 0.3 ml of chloroform (Sigma) and the dish closed for 30 minutes. The surface of the plate was overlaid with 5 ml of Luria-Bertani (LB)(Difco) medium containing 0.75 % agar inoculated with 10^7 CFU of *E. coli* O157:H7 strain 3081. Duplicate plates were prepared for *E. coli* O157:H7 strains 3081, H4420,

E32511, and *E. coli* ATCC 25922. The overlaid plates were incubated for 18-24 h at 37 °C prior to the examination for zones of inhibition.

The effect of Levucell SB20 on the survival of *E. coli* O157:H7 in rumen fluid was determined using *E. coli* O157:H7 strains 3081 and H4420nal. Rumen fluid was obtained from a rumen fistulated Hereford heifer fed an 80% barley and 20% barley silage diet. The rumen fluid was strained through four layers of cheesecloth and the expressed liquid was clarified by centrifugation at 5000 × g for 5 min followed by centrifugation at 20,000 × g for 20 min. The pH of the clarified rumen fluid was determined. The rumen fluid with added glucose (0.2% w/v)(Sigma), tryptone (0.1% w/v) (Difco) and cysteine-HCl (20 ml/L of a 2.5% w/v solution)(Sigma) was dispensed to serum vials. Levucell SB20 was added to duplicate serum vials containing rumen fluid to obtain final concentrations of 0, 0.5, 1.0, 1.5 and 2.0 g/100 ml of rumen fluid. An inoculum of 10^6 CFU *E. coli* O157:H7 strain 3081 or 10^6 CFU *E. coli* O157:H7 strain H4420nal was added to each concentration of Levucell SB20. The populations of *E. coli* O157:H7 in the inoculum were confirmed by standard dilution plating onto TSA supplemented with 100 µg/ml ampicillin and 100 µg/ml kanamycin (TSA-AK) for the enumeration of *E. coli* O157:H7 strain 3081, and TSA supplemented with 200 µg/ml nalidixic acid (TSA-NAL) for the enumeration of *E. coli* O157:H7 strain H4420nal. The final volume of each serum vial was 100 ml. Serum vials were equilibrated with CO_2, sealed and incubated at 39 °C. At 0, 8, 12, 24 and 48 h post-inoculation a 2 ml sample of rumen fluid was removed from each serum vial using a 10cc syringe and 22G1 short bevel needle (Fisher). A 1 ml aliquot was serially diluted in PBS and plated onto TSA-AK for the enumeration of *E. coli* O157:H7 strain 3081, or TSA-NAL

for the enumeration of *E. coli* O157:H7 strain H4420nal. Plates were incubated for 18-24 h at 37 °C prior to the determination of viable numbers. Enrichment in modified TSB (mTSB), which contained 20 mg/L novobiocin (Sigma), 1.5 g/L bile salts 3 (Difco), 1.5 g/L dipotassium phosphate (Sigma), and 30 g/L TSB (BDH), for 6 h at 37 °C, followed by immunomagnetic separation (IMS) using Dynabeads anti- *E. coli* O157 (Dynal, Lake Success, NY) was performed according to the manufacturer's instructions, when the organism was no longer detected by spread plating. Suspect colonies were confirmed as *E. coli* O157 by agglutination with O157 antiserum (Difco). Three independent experiments were performed.

The rumen simulation technique (Rusitec) developed by Czerkawski and Breckenridge (1977) was used to determine the effect of Levucell SB20 on *E. coli* O157:H7 strain 3081 in continuous culture. Twelve fermenters (four per treatment, three treatments) were used in the study, each of 820 ml nominal capacity. Inoculum was obtained from two rumen fistulated Hereford heifers maintained on an 80% barley grain and 20% barley silage. Rumen fluid was pooled and filtered through four layers of cheesecloth to partition it into liquid and solid fractions. The rumen fluid and digesta was confirmed negative for the presence of *E. coli* O157:H7 by enrichment in mTSB followed by IMS using Dynabeads anti-*E. coli* O157. To begin the experiment, each fermenter was filled with 820 ml of filtered rumen fluid, pH 6.6. One nylon bag (80 x 160 mm; 51 um pore size) containing 20 g of solid digesta and one nylon bag containing 10 g of feed (80% barley and 20% silage on a DM basis, ground < 6mm) were also placed in each fermenter. After 24 h, the nylon bag containing the solid digesta was replaced by a fresh feed bag. Thereafter,

one feed bag was replaced daily, so that each feed bag remained in the fermenter for 48 h. Fermenters received a continuous infusion of artificial saliva (pH 8.1)(McDougall, 1948) at a rate of 0.32 ml/min. Throughout the setup of the fermenter units and during daily replacement of feed bags, the fermenters were flushed with oxygen-free CO_2 to foster anaerobiosis (Hungate, 1950). Fermenter liquid pH, vessel volume and production of gas and effluent were monitored daily prior to feed bag replacement, in order to monitor fermentation. Fermenter units were fed for a period of 8 days prior to inoculation with 10^7 CFU *E. coli* O157:H7 strain 3081. The heifers from which the inoculum for the Rusitec were obtained were cared for in accordance with the guidelines set down by the Canadian Council on Animal Care (1993).

Following 8 days of adaptation, an inoculum containing 10^7 CFU of *E. coli* O157:H7 strain 3081 was added to each fermenter in order to obtain a final concentration of 10^4 CFU/ml of *E. coli* O157:H7. The three treatments, added 8 h after inoculation of the fermenters with *E. coli* O157:H7 strain 3081, were (1) 0.5% Levucell SB20 (SB20-0.5), (2) 2% Levucell SB20 (SB20-2), (3) and, the control (no Levucell SB20). Fermenter liquid samples were collected 30 min post-inoculation, at 4, 8, 12 and 24 h and daily thereafter for a total of 192 h, for subsequent enumeration of *E. coli* O157:H7.

For the enumeration of *E. coli* O157:H7 strain 3081 from rumen fluid samples following inoculation, a 1.0 ml sample of rumen fluid was placed in 9 ml PBS, vortex-mixed and serially diluted in PBS. A 100 µl aliquot from the appropriate dilutions was plated onto CT-KASMAC in duplicate using a glass spreader. Plates were incubated for 18-24 h at 37°C prior to the determination of viable numbers. Three sorbitol negative

colonies from each plate were tested for the O157 antigen by agglutination with O157 antiserum.

For enumeration of *E. coli* O157:H7 from the feed residues, the contents of the feed bag (~10 g) were weighed, added to 90 ml PBS and homogenized for 60 s. For enumeration of *E. coli* O157:H7 from the feed bag, 100 ml of PBS were added to a stomacher bag containing the empty feed bag and stomached for 60 s. Samples were serially diluted in PBS and spread plated onto CT-KASMAC in duplicate. Enrichment in mTSB and immunomagnetic separation (IMS) using Dynabeads anti-*E. coli* O157 were performed when the organism was no longer detected in the rumen fluid , feed residue, or feed bags by spread plating.

Analysis of variance was performed using the SAS Mixed Model procedure using the spatial model for covariance structure (SAS Institute, Inc., Cary, NC). The repeated measures data were analyzed as a split-plot in time with treatment as the main plot and time as the subplot. Fermenters nested within treatment were used as the error term to evaluate treatment. The least significant difference (LSD) test was used to determine the differences among means where significant effects were observed ($P<0.05$).

Results and Discussion

The effect of bacteriocins of *P. freudenreichii* and *P. acidipropionici* on *E. coli* O157:H7. No zones of inhibition were observed when cell free supernatants of *P. freudenreichii* P99 and *P. acidipropionici* P42 were tested for inhibitory action against *E. coli* O157:H7 strains 3081, H4420, E32511, and *E. coli* ATCC 25922. Mao (1994) reported *P. freudenreichii* P99 and *P. acidipropionici* P42 produced bacteriocins which were

inhibitory to *E. coli* O157:H7. In the present study, it appeared that the bacteriocins of *P. freudenreichii* and *P. acidipropionici* were not inhibitory to the indicator strains selected or an insufficient amount of bacteriocin was secreted under the conditions tested. Bacteriocin production by the two strains of propionibacteria may also have been lost upon subculture. Bacteriocins have been reported to have a narrow spectrum of activity targeted to species related to the producer strain (Grinstead and Barefoot, 1992). Bacteriocin production can be medium specific (Tagg et al., 1976), and this may place limitations on the use of propionibacteria strains in the control of *E. coli* O157:H7 in the gastrointestinal tract of cattle.

The Effect of Levucell SB20 on the Growth and/or Survival of *E. coli* O157:H7

Levucell SB20 exhibited inhibitory action against *E. coli* O157:H7 strains 3081, H4420, E32511 and *E. coli* ATCC 25922. Zones of inhibition of 15 mm were observed around the spot inoculations of *S. cerevisiae* overlaid with molten agar containing *E. coli* O157:H7 strain 3081. Zones of inhibition observed with *E. coli* O157:H7 strain E32511, H4420 and *E. coli* ATCC 25922 were 18, 19 and 13.5 mm, respectively. Results suggest that metabolites produced by *S. cerevisiae* strain I-1079 were successful in inhibiting *E. coli* O157:H7.

The effect of Levucell SB20 on the growth and/or survival of *E. coli* O157:H7 strain H4420nal is presented in Figure 8.1A. Levucell SB20, at concentrations of 0.5, 1.0, 1.5 or 2.0 g/100ml of rumen fluid, had no effect on the growth of *E. coli* O157:H7 strain H4420nal in rumen fluid until after 24 h of incubation at 39 °C. Inhibition of *E. coli* O157:H7 occurred only after 48 h of incubation at 39 °C with the percent survival of *E.*

coli O157:H7 decreasing with increasing concentrations of Levucell SB20. The survival of the inoculated strain of *E. coli* O157:H7at 0.5 and 1.0 g Levucell SB20/100 ml of rumen fluid was 87.8% and 11.0%, respectively. The organism was not recovered from the serum vials containing 1.5 or 2.0g Levucell SB20 per 100 g of rumen fluid.

Similar results were observed with *E. coli* O157:H7 strain 3081 (Figure 8.1B). There was no effect on numbers of *E. coli* O157:H7 recovered from concentrations of Levucell SB20 of 0.5, 1.0, 1.5 or 2.0 g/100ml of rumen fluid after 24 h of incubation at 39 °C. Inhibition was only observed at 48 h of incubation at 39 °C. Enhanced survival *E. coli* O157:H7, however, was exhibited at concentrations of 0.5 and 1.0 g Levucell SB20/100 ml of rumen fluid with 100 and 64% survival being observed, respectively. At 1.5 and 2.0 g/100 ml, *E. coli* O157:H7 strain 3081 was not recovered from the rumen fluid following 48 h of incubation at 39 °C. Levels of *E. coli* O157:H7 strain 3081 isolated from the controls remained constant throughout the 48 h incubation period.

In the Rusitec, there was a steady decline in numbers of *E. coli* O157:H7 strain 3081 in SB20-0.5, SB20-2 and the control, with no difference in the numbers of *E. coli* O157:H7 recovered among the three treatments over the 192 h experimental period (Figure 8.2)($P>0.05$). There was a slight increase in numbers of *E. coli* O157:H7 in SB20-2 12 h post-inoculation as compared to SB20-0.5 and the control, however, this increase was not significant ($P>0.05$). *E. coli* O157:H7 was not recovered from SB20-0.5 and SB20-2 144 and 168 h post-inoculation, respectively. *E. coli* O157:H7 strain 3081 was recovered from the control fermenters 168 h post-inoculation.

Numbers of *E. coli* O157:H7 recovered from the feed residues 24 h post-inoculation

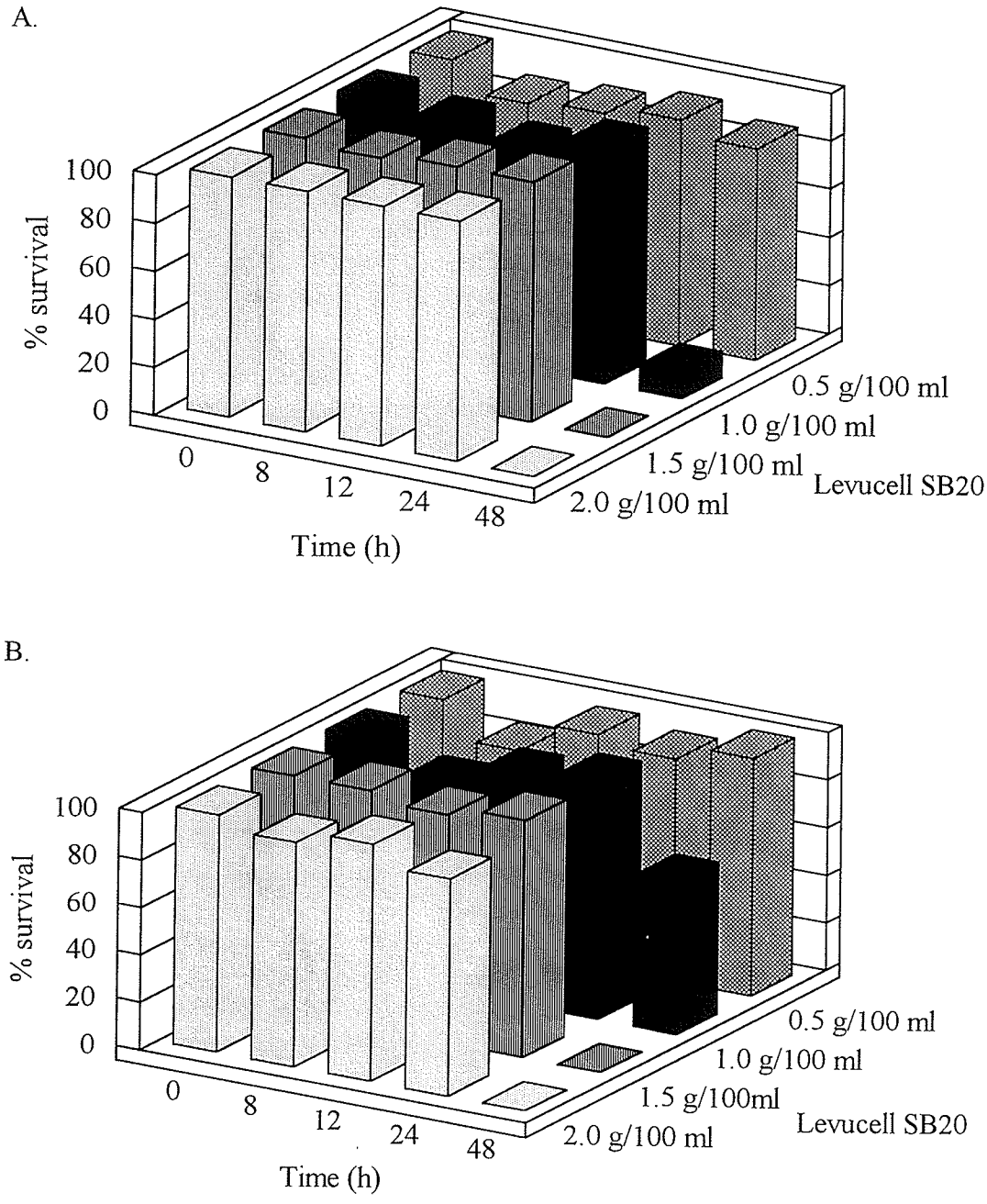

Figure 8.1. The effect of Levucell SB20 on the growth and/or survival of (A) *E. coli* O157:H7 strain H4420nal; (B) *E. coli* O157:H7 strain 3081. Rumen fluid was inoculated with 10^4 CFU/ml of *E. coli* O157:H7 at time 0 and incubated at 39 °C for 48 h.

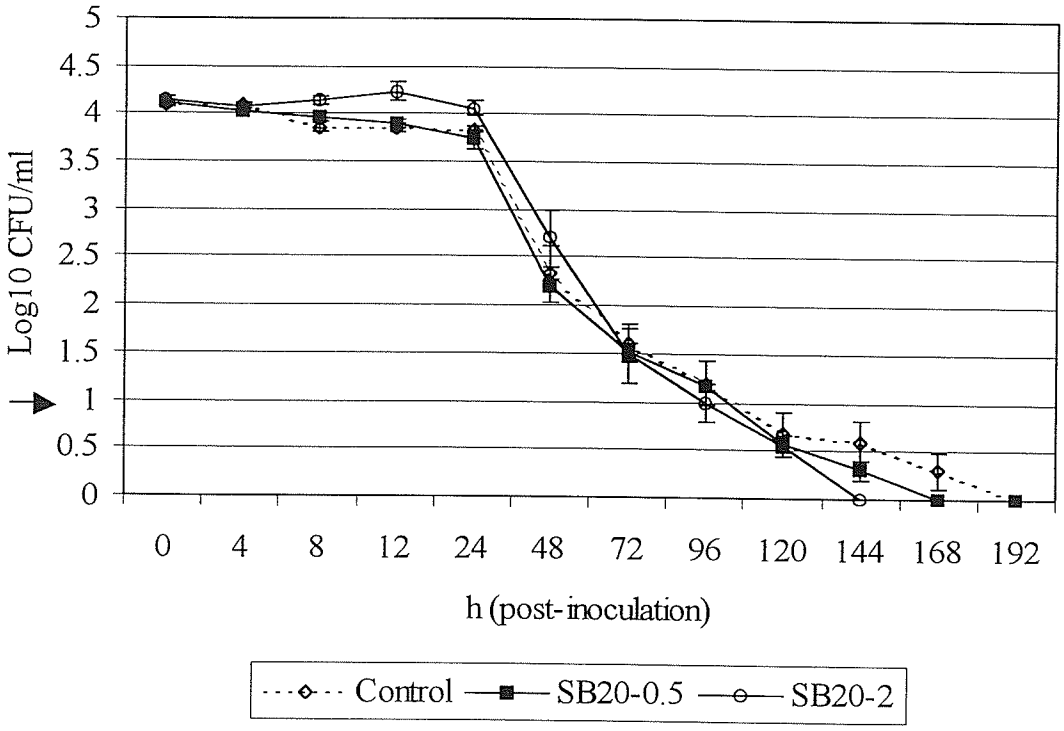

Figure 8.2. The effect of Levucell SB20 on the survival of *E. coli* O157:H7 strain 3081 in an artificial rumen system (Rusitec). Fermenters were inoculated with 10^4 CFU/ml of *E. coli* O157:H7 at time 0. The treatments (1) 0.5% LevucellSB20 (SB20-0.5); (2) 2% Levucell SB20 (SB20-2); (3) control (no Levucell SB20), were administered 8 h post-inoculation. Bars represent the standard error of the mean. Where not shown, error bars are within symbols. The arrow indicates detection by enrichment (<10 CFU/ml).

were similar among treatments (P>0.05). *E. coli* O157:H7 was recovered from the feed in SB20-2, SB20-0.5 and the control until 96, 120, and 168 h post-inoculation, respectively. Numbers of *E. coli* O157:H7 strain 3081 detected in the feed residues declined rapidly over the 192 h experimental period and enrichment and IMS was required to detect *E. coli* O157:H7 from the feed residues 48 h post-inoculation (Figure 8.3).

Numbers of *E. coli* O157:H7 isolated from the feed bags 24 h post-inoculation were similar (P>0.05). Enrichment and IMS was required to detect *E. coli* O157:H7 from SB20-2, SB20-0.5 and the control 72 h post-inoculation. *E. coli* O157:H7 was undetectable from the feed bags in all three treatments 120 h post-inoculation. (Figure 8.4).

Levucell SB20 is a concentrated form of active dry yeast (20×10^9 CFU/g *S. cerevisiae*) which claims to promote the growth of beneficial lactic acid bacteria in the digestive tract while inhibiting the proliferation of undesirable bacteria. The inhibitory effect Levucell SB20 exerted on *E. coli* O157:H7 in the agar spot assays and following 48 h incubation in rumen fluid was not observed in the Rusitec. It is evident that metabolites produced by S. *cerevisiae* strain I-1079 exert an inhibitory effect of *E. coli* O157:H7, however, the production of these metabolites may not occur in the mixed microflora of the rumen or diffusion of these metabolites in the rumen fluid may reduce their effectiveness against *E. coli* O157:H7. The abrupt inhibition of *E. coli* O157:H7 in the clarified rumen fluid in serum vials following 48 h of incubation at 39 °C may have been a result of the production of inhibitory substances by S. *cerevisiae* or it may have been the result of nutrient depletion in the rumen fluid. The lack of an effect of Levucell SB20 on *E. coli* O157:H7 in the Rusitec suggests any inhibitory substances which may have been produced

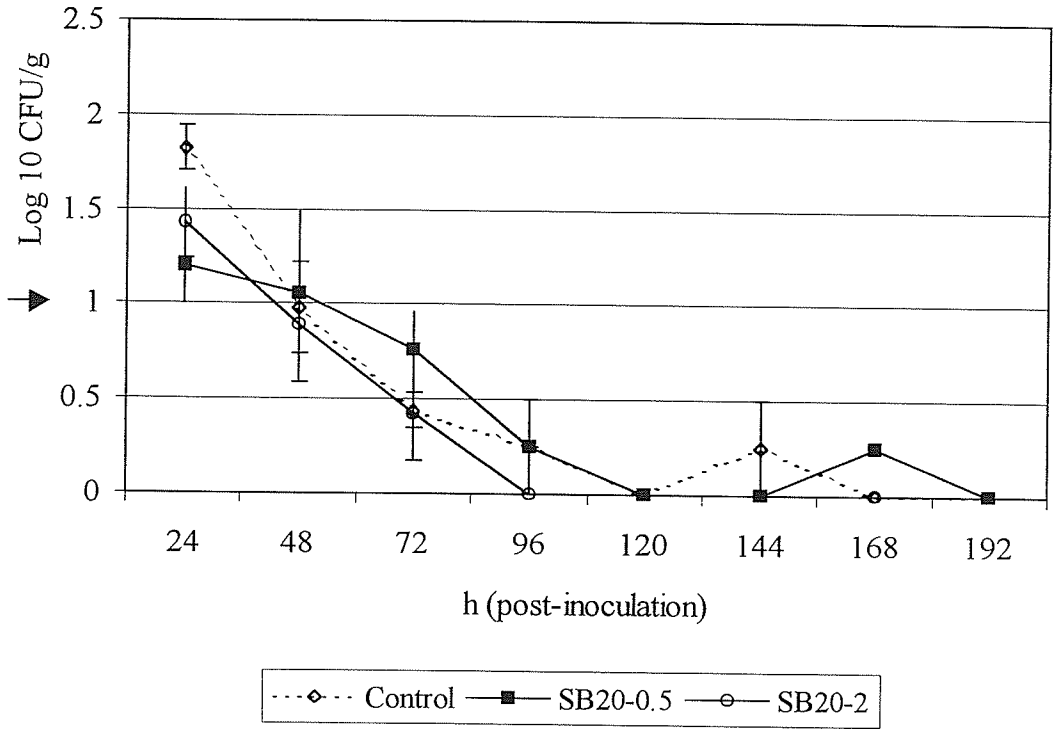

Figure 8.3. Recovery of *E. coli* O157:H7 strain 3081 from feed residues in an artificial rumen system (Rusitec). Fermenters were inoculated with 10^4 CFU/ml of *E. coli* O157:H7 at time 0. The treatments (1) 0.5% Levucell SB20 (SB20-0.5); (2) 2% Levucell SB20 (SB20-2); (3) control (no Levucell SB20), were administered 8 h post-inoculation. Bars represent the standard error of the mean. Where not shown, error bars are within symbols. The arrow indicates detection by enrichment (<10 CFU/g).

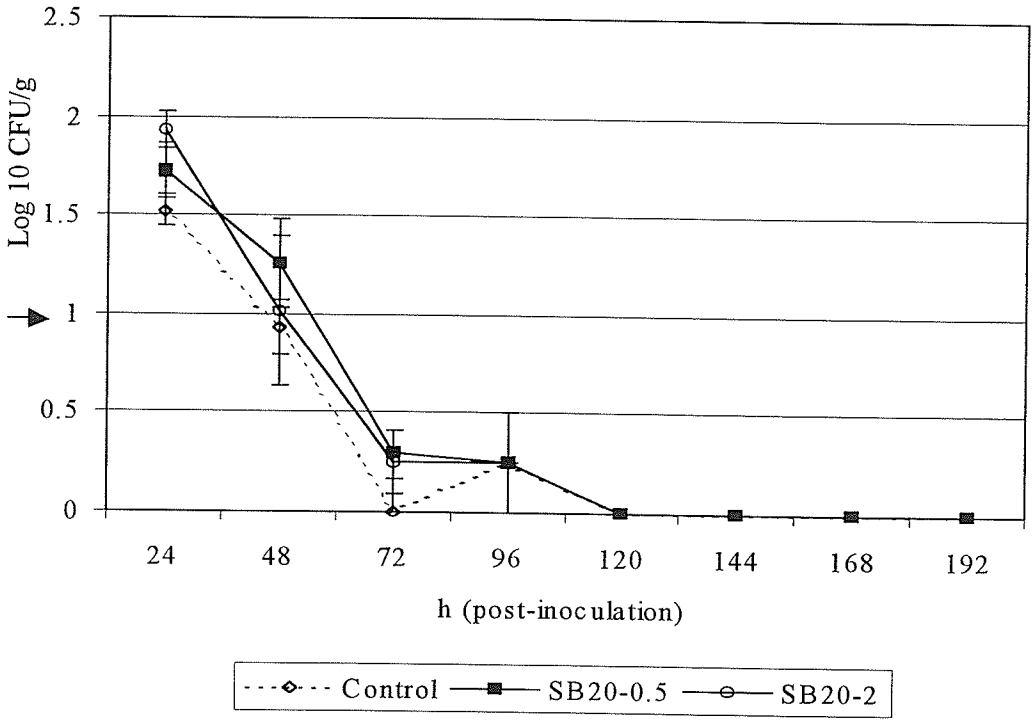

Figure 8.4. Recovery of *E. coli* O157:H7 strain 3081 from feed bags in an artificial rumen system (Rusitec). Fermenters were inoculated with 10^4 CFU/ml of *E. coli* O157:H7 at time 0. The treatments (1) 0.5% LevucellSB20 (SB20-0.5); (2) 2% Levucell SB20 (SB20-2); (3) control (no Levucell SB20), were administered 8 h post-inoculation. Bars represent the standard error of the mean. Where not shown, error bars are within symbols. The arrow indicates detection by enrichment (< 10 CFU/g).

by S. *cerevisiae* in rumen fluid were of insufficient quantity to result in the inhibition of *E. coli* O157:H7.

Probiotic bacteria are those which beneficially affect the host animal by improving its intestinal microbial balance, including the elimination or reduction of undesirable microorganisms (Fuller, 1989; Zhao et al., 1998). The possibility of an association, positive or negative, between yeast supplementation in the diets of cattle and the fecal shedding of *E. coli* O157:H7 has not been considered in the literature to date. However, yeast additives have been widely used as growth promotants in ruminant diets, with strains of *S. cerevisiae* being most commonly used (Wallace, 1992; Yoon and Stern, 1995). Their use, in addition to the use of probiotic bacteria, has been attempted in order to replace the widespread use of antibiotic and synthetic chemical feed supplements (Fuller, 1989; Nisbet and Martin, 1991).

Yeast additives have been shown to improve ruminant production by stimulating the growth and activities of a number of specific groups of microorganisms within the rumen (Wallace, 1992; Williams and Newbold, 1990; Yoon and Stern, 1995). This stimulation has resulted in greater concentrations of cellulolytic and lactic-acid utilizing bacteria and has been reflected by greater concentrations of anaerobic bacteria in the rumen. These activities have also resulted in decreased lactic acid concentrations, lower pH, decreased ammonia concentrations, enhanced digestive processes, and increased microbial protein synthesis in the rumen. Viable counts of bacteria have increased with their addition and the improved stability of the bacterial population has given rise to other benefits such as better fibre breakdown (Nisbet and Martin, 1991; Wallace, 1992).

It has been reported that *S. cerevisiae* does not grow in the rumen (Wallace, 1992). *S. cerevisiae* is known to produce enzymes such as amylases, proteases, lipases and cellulases, however, it is unknown whether these enzymes are secreted and are active in the rumen. It is possible that *S. cerevisiae* strain I-1079 did not secrete metabolites inhibitory to *E. coli* O157:H7 in the rumen. Yeast additives are also a source of B vitamins and may contain several other unidentified growth or stimulatory factors. There is no direct evidence that yeast supplementation affects the digestion or metabolism in the lower gut (Wallace, 1992).

The stimulatory effects which *S. cerevisiae* exerts on rumen microorganisms may not have an effect on the growth and or/survival of *E. coli* O157:H7 in cattle. The lack of a stimulatory effect of *S. cerevisiae* on *E. coli* O157:H7 suggests that yeast supplementation in the diets of cattle likely does not enhance the growth of *E. coli* O157:H7 and contribute to the fecal shedding of the organism.

DISCUSSION AND CONCLUSIONS

Numerous studies have reported conflicting results in terms of the effects of high grain or high forage diets on the fecal shedding of *E. coli* O157:H7 in cattle (Diez-Gonzalez et al., 1998; Hovde et al., 1999; Magnusson et al., 2000; Tkalcic et al., 2000). High grain diets are considered to be important in regards to *E. coli* O157:H7 fecal shedding since beef cattle are commonly fed high grain diets during the finishing prior to slaughter. Fecal shedding of *E. coli* O157:H7 at slaughter is considered to be a significant source of contamination of beef (Van Donkersgoed et al., 1999).

The three grain diets, 85% corn, 15% whole cottonseed and 70% barley, and 85% barley had no effect on populations of *E. coli* O157:H7 in the rumen or those shed in the feces of experimentally inoculated steers ($P < 0.05$). *E. coli* O157:H7 was rapidly eliminated from the rumen of the animals on all three diets 16 days post-inoculation, but persisted in the feces of the animals for up to 67 days post-inoculation. The pH of the rumen fluid and the VFA concentrations were similar among the three diets. This implied not only that the growth and maintenance of *E. coli* O157:H7 was more likely to occur in the hindgut as opposed to the rumen, but also that there was no consistent relationship between rumen and fecal populations of *E. coli* O157:H7.

The concentrations and relative proportions of VFAs in the rumen vary according to diet and pattern of feeding. They typically are a combined total of acetate, propionate and butyrate of between 50 and 150 mM, with an inverse relationship existing between VFA concentrations and pH (Hungate, 1966). Harmon et al. (1999) found that despite fluctuations in rumen VFAs, there was no correlation between daily rumen VFA

concentrations and rumen or fecal numbers of *E. coli* O157:H7. Tkalcic et al. (2000) reported that varying rumen pH and VFA concentrations in animals fed high concentrate and high roughage diets resulted in no effect on rumen proliferation and fecal shedding of *E. coli* O157:H7. Conditions in the colon and cecum have a more direct influence on the shedding of *E. coli* O157:H7 in the feces than conditions in the rumen, implying that the hindgut of cattle is the site of *E. coli* O157:H7 proliferation and a reservoir for fecal shedding of the organism, (Grau et al., 1969; Rasmussen et al., 1993; Tkalcic et al., 2000; Whipp et al., 1994).

Two recent studies have provided evidence that *E. coli* O157:H7 colonizes the colonic mucosa of cattle. Potter and Finlay (2000) suggested that strains of *E. coli* O157:H7 are able to attach to bovine intestinal epithelial cells of cattle using a unique mechanism mediated by the proteins Tir, intimin, EspA and EspB. Dean-Nystrom et al. (1998) demonstrated that intimin was required in order for *E. coli* O157:H7 to produce AE lesions in newborn calves. Baehler and Moxley (2000) found that AE lesions developed on colonic and rectal mucosal explants, from steers inoculated with *E. coli* O157:H7, providing evidence that mucosal epithelium of the large intestine may be the site of infection that contributes to the carriage of *E. coli* O157:H7 in cattle. Phillips et al. (2000) reported that the formation of AE lesions in human and bovine mucosa *in vitro* was restricted to follicle-associated epithelium of Peyer's patches. This suggests that attachment of *E. coli* O157:H7 to bovine epithelial cells may be important in the asymptomatic carriage of the organism in cattle, and may provide a reservoir for the fecal shedding of the organism these animals.

Although no difference existed in the levels of *E. coli* O157:H7 shed by the animals on each of the three diets on most days, significantly fewer animals were culture positive for *E. coli* O157:H7 when fed the corn as opposed to the barley diet ($P<0.005$). Increased hindgut fermentation in the corn fed animals and the resulting decrease in fecal pH, in combination with inhibitory fecal VFAs, may have made the large intestine a less suitable site for the proliferation of *E. coli* O157:H7, than when cottonseed or barley were fed. This suggests that dietary components unaffected by rumen action may result in increased fermentation in the colon, and yield conditions inhibitory to the growth of *E. coli* O157:H7 and reduce fecal shedding of the organism. The development of techniques to simulate and evaluate conditions in the hindgut would allow for a better understanding of the effects of dietary components on the growth and/or survival of *E. coli* O157:H7 in the hindgut. This may subsequently lead to the use of diet as a means to control *E. coli* O157:H7 fecal shedding.

The incorporation of barley into the diets of cattle has been reported to be associated with an increased isolation of *E. coli* O157:H7 from the fecal pats of feedlot cattle (Dargatz et al., 1997). While a greater number of animals in the barley- fed group shed the organism as compared to the cottonseed and corn fed groups, further studies are required in order to define the dynamics of the relationship between barley feeding and the fecal shedding of *E coli* O157:H7. It is possible that geographic differences in rates of human infection with *E. coli* O157:H7 are related to regional cattle feeding practices and resultant levels of animal carriage of *E. coli* O157:H7 (Griffin and Tauxe, 1991; Nataro and Kaper, 1998; Slutsker et al., 1997; Waters et al., 1994).

In the Rusitec experiments, *E. coli* O157:H7 persisted for a longer period of time in fermenters fed timothy and clover as compared to barley and barley silage diets. Different forages may result in varying inhibitory conditions in the rumen which limit the survival of *E. coli* O157:H7 and its subsequent passage through the abomasum and into the colon and cecum. This has important implications in regards to diet and *E. coli* O157:H7 fecal shedding by cattle. While comparisons have been made between high grain and high forage diets, there may be differences in the effects among types of forage diets as well as types of grain, and conditions in the rumen and colon which may subsequently affect the growth and survival of *E. coli* O157:H7.

E. coli O157:H7 may attach to feed particles in the rumen in addition to being freely present in rumen fluid. The extent of the attachment may be dependent on the type of feed. *E. coli* O157:H7 was recovered from the feed residues in the Rusitec fermenters fed barley, barley silage, timothy and clover diets, however, the organism persisted in the feed residue of the barley silage fed fermenter 60 h after it was undetectable in the rumen fluid. Since bacteria rapidly form protective biofilms upon attachment to surfaces (Kumar and Anand, 1998), association with feed particulates may provide an avenue for the organism to remain viable until it reaches the large intestine.

McCowan et al. (1978) have suggested that bacteria present in low numbers, or that are incapable of surviving free in the rumen, may avoid being washed out of the rumen by adhering to the mucosa. By adhering to the epithelial wall, the bacteria have access to a number of potential substrates. Metabolites present in the rumen would be continually made available to the adherent bacteria due to the rhythmic contractions of the reticulo-

rumen, while they would also have access to metabolites transported through the epithelium (McCowan et al., 1978). *E. coli* O157:H7 has been reported to associate with the mucosal surface of rumen tissue samples (Brown et al., 1997). In the sampling of the fistulated steers fed three different grain diets, we sampled rumen fluid only, which was cultured for the presence of *E. coli* O157:H7. As a result, it is likely that any *E. coli* O157:H7 present in the rumen as adherent bacteria were not detected.

Rasmussen et al (1993) observed unrestricted growth of *E. coli* O157:H7 in rumen fluid from fasted cattle, but *E. coli* O157:H7 grew poorly in rumen fluid from well-fed animals. The authors suggested that growth inhibition was correlated with a reduction in pH and an increase in VFA concentrations. Tkalcic et al. (2000) found that the growth of *E. coli* O157:H7 *in vitro* was favored in the rumen fluid of animals fed a high roughage diet as compared to a high concentrate diet, however, similar observations were not made *in vivo*. Harmon et al. (1999) did not observe a correlation between fluctuating rumen VFA concentrations and rumen or fecal numbers of *E. coli* O157:H7. This suggests that the interaction between pH and VFAs may not be the only reason for low numbers of *E. coli* O157:H7 in the rumen.

Very little is known about the nutrition of *E. coli* in the rumen. It has been reported that *E. coli* grows on protein digests or peptides, and is unable to use native proteins (Brecher and Moehlman, 1991). It has been suggested that *E. coli* may have to compete for access to peptides provided by the proteolytic microorganisms in the rumen (Wallace and Brammall, 1985). Since *E. coli* comprises approximately 1% of the culturable microorganisms present in the rumen, their survival depends upon successful competition

for nutrients with the commensal anaerobes in the rumen (Hungate, 1966).

The rumen microenvironment is complex and variable. Many different factors present in the rumen could potentially affect the survival of *E. coli* O157:H7. Competition for nutrients and production of inhibitory metabolites by rumen microflora, as well as different rumen redox potential and changes in microflora composition, appear to be important factors affecting viability of *E. coli* O157:H7 in the rumen (Harmon et al., 1999). It is likely that changes induced by different diets are complex, affecting both the rumen and colon. Dietary changes, changes in competing microorganisms, and parasitism may act together to create conditions favorable for the proliferation and shedding of the organism (Harmon et al., 1999).

It has been suggested that fasting has no effect on the fecal shedding of *E. coli* O157:H7. Cray et al. (1998) found that fasting for 48 h following inoculation with 10^{10} CFU of *E. coli* O157:H7 did not result in an increase in the fecal shedding of *E. coli* O157:H7. Fasting prior to inoculation with *E. coli* O157:H7, however, resulted in an increased susceptibility to infection along with an increase in the numbers of *E. coli* O157:H7 shed. A study using calves inoculated with *E. coli* O157:H7 found that while fasting reduced rumen volatile fatty acid concentrations and increased rumen pH, it had no significant effect on numbers of *E. coli* present in the rumen or shed in the feces as a result of feed withdrawal (Harmon et al., 1999).

In the present study, between 2.5 and 10% of steers and heifers, in a herd naturally infected with *E. coli* O157:H7, were culture positive for *E. coli* O157:H7 when fed 80% concentrate (barley) or 100 % forage (alfalfa silage). Feed withdrawal for 48 h following

an 80% concentrate or 100 % forage diet had no effect on the number of animals which were culture positive for *E. coli* O157:H7 during the 48 h fasting period, confirming that fasting had no significant effect on the shedding of *E. coli* O157:H7 (Cray et al, 1998; Harmon et al., 1999; Kudva et al., 1997). Interestingly, the organism was not recovered from the feces of the cattle 12 h after withholding the 80% concentrate diet, with the number of animals positive for *E. coli* O157:H7 returning to normal levels at 48 h. This suggests that fasting on finishing diets up to 12 h may reduce the number of *E. coli* O157:H7 positive animals going to slaughter.

While fasting alone has no significant effect on the shedding of *E. coli* O157:H7, diet composition prior to feed withdrawal may have an effect on the fecal shedding of *E. coli* O157:H7 (Cray et al, 1998; Harmon et al., 1999; Kudva et al., 1997). We observed an increase in the number of animals shedding *E. coli* O157:H7 at 48 h after the last missed feeding following the 100% alfalfa silage diet, however, this increase was not significant ($P>0.05$). Upon re-feeding 100% alfalfa silage following the second 48 h fast, the number of animals positive for *E. coli* O157:H7 increased ($P<0.05$) to 42.5% at 5 d after completion of the fast. Conditions in the hind-gut were likely conducive to the growth and subsequent fecal shedding of *E. coli* O157:H7, resulting in an increase in the number of culture positive fecal samples. Such conditions may not have occurred during the first 48 h fast (following the 80% barley diet) or upon feeding 100% alfalfa silage following the first fast. The practice of forage feeding following a fast is not normal for feedlot cattle destined for slaughter, but can occur with feeder cattle that pass through a sales barn.

Magnuson et al.(2000) reported that cattle with slower rates of intestinal cell

proliferation in the cecum and distal colon were culture positive for *E. coli* O157:H7 significantly longer than cattle with faster cell proliferation rates. They developed a fasting-re-feeding regime which increased the rate of intestinal cell proliferation in the colon and cecum and induced the clearance of *E. coli* O157:H7 from the bovine gastrointestinal tract. This suggests that the crypts of the lower gastrointestinal tract mucosa may provide a niche for the growth of *E. coli* O157:H7. Increasing the rate of gastrointestinal cell proliferation in the cecum and colon may physically remove this niche as the outer layer is sloughed off (Magnuson et al., 2000). Determination of the association between increased intestinal cell proliferation and the clearance of *E. coli* O157:H7 may allow for dietary manipulation to be used as a means of controlling *E. coli* O157:H7 fecal shedding in animals prior to slaughter.

Supplementation of ruminant diets with growth promotants such as the ionophores, with the goal of improving feed efficiency, has become common practice. Since they became approved for use in the United States the late 1970s, ionophores have been used in essentially all large feedlot operations. The implementation of ionophore feeding occurred just prior to the first cases of *E. coli* O157:H7 infection, and there were concerns that ionophores may alter conditions in the bovine intestinal tract in such a way that selects for *E. coli* O157:H7 (USDA: APHIS:S, 1994). Based on survey data, it has been reported that ionophore use had no effect on the fecal shedding of *E. coli* O157:H7 (Dargatz et al., 1997; Garber et al., 1995). Herriot et al. (1998), however, reported a tentative association between the use of ionophores in cattle rations and *E. coli* O157:H7 fecal shedding.

Conflicting observations in regards to the effects of ionophores on Gram-positive and Gram-negative bacteria have been reported. It has been implied that Gram-positive

organisms can develop resistance to ionophores and that the presence of an outer membrane, as in Gram-negative bacteria, is not the only criterion for resistance among bacteria to ionophores. While ionophores may inhibit Gram-positive organisms in the rumen and provide a selective advantage for Gram-negative organisms, this may not include *E. coli* O157:H7 (Dawson and Boling, 1983; Russell and Strobel, 1989).

In preliminary studies which focused on the screening of specific agents for their effects on the growth of *E. coli* O157:H7, we found that one of the most commonly used ionophores, monensin sodium, did not affect the growth of *E. coli* O157:H7 at levels typically found in the rumen (5 to 10 μg/ml) (Chalupa, 1977; Dennis et al., 1981). Controlled animal studies, however, are required in order to determine if there is a relationship between the feeding of ionophores and the fecal shedding of *E. coli* O157:H7 by cattle.

The supplementation of ruminant diets with probiotic organisms is fast becoming an alternative to the use of antibiotics and synthetic chemical feed supplements (Fuller, 1989; Nisbet and Martin, 1991). The goal in using probiotic bacteria is to beneficially affect the host animal by improving its intestinal microbial balance, including the elimination or reduction of undesirable bacteria. This is commonly achieved through their colonization of sites which are normally colonized by undesirable bacteria, and through the production of inhibitory metabolites (Fuller, 1989; Zhao et al., 1998).

We found that two strains of propionibacteria, *Propionibacterium freudenreichii* P99 and *P. acidipropionici* P42, did not produce metabolites inhibitory to *E. coli* O157:H7 strains in the medium tested. *S. cerevisiae* subsp. *boulardii* produced metabolites which

were inhibitory to *E. coli* O157:H7 in the spot agar assay, however, no inhibition of *E. coli* O157:H7 was observed in the continuous culture conditions of the Rusitec. This suggests that the production of metabolites by *S. cerevisiae* may not occur in the mixed microflora of the rumen or the diffusion of metabolites in the rumen in addition to the dilution of the rumen fluid due to the infusion of saliva (0.3 ml/min) may have reduced their effectiveness against *E. coli* O157:H7.

Probiotic bacteria have been successfully used in reducing the period of fecal shedding in experimentally inoculated calves by Zhao et al. (1998). Seventeen *E. coli* isolates and one *Proteus mirabilis*, isolated from cattle fecal and intestinal tissue samples, produced metabolites inhibitory to *E. coli* O157:H7, *in vitro*. When these 18 isolates were administered to calves orally inoculated with *E. coli* O157:H7, the period of fecal shedding was reduced as compared to calves which did not receive the probiotic bacteria. Although the mechanism of inhibition was not determined, results suggest that many strains of bacteria are likely effective inhibitors of *E. coli* O157:H7 through the production of inhibitory metabolites (Zhao et al., 1998)..

The seasonality of *E. coli* O157:H7 shedding by cattle suggests that environmental replication plays a key role in the persistence of the organism (Faith et al., 1996; Hancock et al., 1998). *E. coli* O157:H7 has been isolated from animal drinking water in several studies (Faith et al., 1996; Shere et al., 1998; Hancock et al., 1998). It has been found to persist in water trough sediments for periods of up to four months and may even replicate in this environment (Hancock, 1997). We isolated *E. coli* O157:H7 from water troughs and the water trough biofilm in the environment of experimentally inoculated steers fed three

different grain diets. *E. coli* O157:H7 was also recovered from the mouth swabs of the steers, suggesting that the organism may be able to survive in the alkaline saliva (pH 8.1) of cattle long enough to be transferred to water, feed and other cattle.

Inhibition of *E. coli* O157:H7 in animal drinking water through the incorporation of 0.5% propionic and 0.5% acetic acid may be a means to control the spread of the organism among cattle. At 22 and 30 °C, both acids eliminated *E. coli* O157:H7 from water samples within 3 and 1 day, respectively, but the organism was still detectable up to 14 days at 4 °C. Due to the buffering effects of ruminant saliva and the relatively small proportion VFAs represent (0.5 - 1.5%) of total rumen fluid, it is unlikely that the intake of 0.5% acetic or propionic acid in drinking water will affect rumen fermentations. However, further studies are required in order to determine the possible effects on rumen fermentations and to determine the palatability of these weak acids to cattle.

The contamination of feed also represents a possible means of transfer of *E. coli* O157:H7 among cattle. Lynn et al., (1998) found that a variety of cattle feeds were able to support the growth of *E. coli* O157:H7 *in vitro*. *E. coli* O157:H7 was isolated from the feed of the experimentally inoculated steers in the cottonseed and barley fed groups, suggesting that the organism can survive in feed long enough to be transferred to other animals.

Fenlon and Wilson (2000) reported that *E. coli* O157:H7 was able to grow in aerobically spoiled silage. We found that adequate ensiling for a minimum of 15 days eliminated 10^5 CFU/g of *E. coli* O157:H7 from barley forage, and that re-growth of the organism subsequently was not observed when exposed to aerobic conditions. Producers often start to feed from their silage pits before the ensiling process is complete. Thus, there

is a risk of the early silage acting as an inoculant of *E. coli* O157:H7 for feedlot cattle. The use of the lactic acid bacteria inoculant, P2, shortened the survival time of *E. coli* O157:H7 in the silage by 8 days, allowing the silage to be fed safely to cattle, earlier.

Although epidemiological investigations in animal populations have concentrated on the bovine reservoir of *E. coli* O157:H7, the organism has been isolated from other animals and even birds. This makes the control of *E. coli* O157:H7 in the environment particularly difficult (Beutin et al., 1996; Chapman et al., 1996a,b,c; Fegan and Desmarchelier, 1999; Hancock et al., 1998; Heuvelink et al., 1998; Keene et al., 1997; Kudva et al., 1996; Rice et al., 1995; Wallace et al., 1997). Hancock et al. (1998) suggested that the presence of *E. coli* O157:H7 in other animal sources in addition to the environment may mean that cattle represent an incidental host for the organisms, being continually re-infected through environmental sources. Feed and water may become contaminated by other animal and bird sources of *E. coli* O157:H7 resulting in the possible transfer of the organism back to cattle. This suggests that a cycle of infection may exist as a result of recurrent exposure of cattle to environmental sources of *E. coli* O157:H7. This may be the basis for the intermittent shedding of *E. coli* O157:H7 among cattle in addition to the establishment and spread of the organism in herds.

While cattle form only one link in the ecological cycle of the organism, few studies have been conducted to identify environmental sources of *E. coli* O157:H7. Once *E. coli* O157:H7 is voided from cattle it must survive in the environment until a new host is found. Studies have shown that *E. coli* O157:H7 can survive in water, manure, and soil for extended periods of time (Kudva et al.,1998; Maule, 2000; Rice and Johnson, 2000; Wang

and Doyle, 1998; Wang et al., 1996). The survival of *E. coli* O157:H7 in ovine manure from experimentally inoculated sheep (Kudva et al.,1998) and the isolation of *E. coli* from animal drinking water and feed (Faith et al., 1996; Shere et al., 1998), emphasize the need for more intensive environmental sampling for *E. coli* O157:H7 to characterize the importance of the organism in the animal environment.

A simulation study conducted by Jordan et al. (1999) found that the intervention strategies which are most likely to have the most significant impact on the contamination of carcasses with *E. coli* O157:H7 are vaccination and the use of agents which may reduce the fecal shedding of *E. coli* O157:H7 by cattle. The bacteriophage, DC22, exhibited specificity for *E. coli* O157:H7 and was successful in eliminating 10^4 CFU/ml of *E. coli* O157:H7 in the Rusitec at a MOI of 100,000:1. The large number of viable particles required for infection of *E. coli* O157:H7 suggested that the bacteriophage possessed low antibacterial activity against *E. coli* O157:H7. DC22 had no effect on the levels of *E. coli* O157:H7 shed by wethers when the bacteriophage was administered at a MOI of 100,000:1 two days following the inoculation of the wethers with *E. coli* O157:H7. Lack of inhibition was likely due to insufficient quantities of the active bacteriophage having access to *E. coli* O157:H7. Non-specific binding of the bacteriophage in the gastrointestinal tract of the wethers may also have resulted in a reduction in its numbers.

Waddell et al. (2000) were successful in reducing the duration of *E. coli* O157:H7 shedding by calves using a mixture of six *E. coli* O157:H7 specific bacteriophages administered -7, -6, -1, 0 and 1 day following inoculation with *E. coli* O157:H7. In *in vitro* studies, Kudva et al. (1999) found that a mixture of three phages was successful in

eliminating *E. coli* O157:H7 from cultures, but no single phage was equally as effective. Smith and Huggins, (1983) reported that phage therapy was most effective when applied before or together with the infective bacteria. While DC22 alone was unsuccessful in reducing the fecal shedding of *E. coli* O157:H7 in experimentally inoculated wethers, in combination with other bacteriophages it may prove effective in controlling the fecal shedding of *E. coli* O157:H7 by ruminants.

The isolation of *E. coli* O157:H7 from the animal environment, in addition to the seasonal and intermittent nature of *E. coli* O157:H7 shedding by cattle, suggests that environmental replication may play a key role in the ecology of the organism (Hancock et al., 1998; MacDonald et al., 1996; Shere et al., 1998; Wells et al., 1991). Farm-management practices resulting from intensification may aid in the maintenance and spread of *E. coli* O157:H7 within the farm environment. The survival of *E. coli* O157:H7 in feed, water, manure and soil implies that the organism survives well in the environment once voided from the host (Hancock et al., 1998; Kudva et al., 1998; Lynn et al., 1998; Maule, 2000; Rice and Johnson, 2000; Wang et al., 1996). The effective control of *E. coli* O157:H7, therefore, requires control of *E. coli* O157:H7 not only at the bovine source, but also control in the animal environment.

Recent studies suggest that the attachment of *E. coli* O157:H7 to bovine epithelial cells in the hindgut likely provides a reservoir for the fecal shedding of the organism and is the site of *E. coli* O157:H7 colonization (Magnuson et al., 2000; Potter and Finlay, 2000). However, once voided into the environment, *E. coli* O157:H7 survives for extended periods of time and this may allow the organism to be transferred back to cattle. This

suggests that a cycle of re-infection of cattle by *E. coli* O157:H7 from the environment must be broken to effectively control the problem. The presence of *E. coli* O157:H7 in other farm animals and wild animals and birds, confounds the problem. Intervention strategies which focus on the reduction of fecal shedding of *E. coli* O157:H7 by cattle in addition to targeting environmental sources of the organism, may yield satisfactory control of *E. coli* O157:H7 on the farm. Novel control measures, with the goal of reducing the fecal shedding of *E. coli* O157:H7 in cattle, in addition to minimizing its presence in the environment, are essential in order to reduce the presence of this deadly pathogen in food.

RECOMMENDATIONS

Recommendations for future research based on this study are:

- ❑ Assess the significance of *E. coli* O157:H7 in the environment in the viable but nonculturable state (VBNC).
- ❑ Evaluate the indirect effects of monensin on *E. coli* O157:H7 in animals experimentally inoculated with *E. coli* O157:H7. Clarification of any relationship between ionophore feeding and the fecal shedding of *E. coli* O157:H7 by cattle is needed.
- ❑ Determine the effects of the incorporation of monensin into a barley diet as compared to a corn diet, and *E. coli* O157:H7 fecal shedding.
- ❑ Assess the use of feed supplements introduced at the same time as ionophores, such as selenium, on the survival of *E. coli* O157:H7 in the bovine gastrointestinal tract and the shedding of *E. coli* O157:H7 by cattle.
- ❑ Assess dietary factors such as the feeding of soybean meal, clover and corn silage on the shedding of *E. coli* O157:H7 by cattle.
- ❑ Evaluate the effects of the incorporation of oils such as canola, sunflower and safflower into the diets of cattle and their effects on *E. coli* O157:H7 fecal shedding.
- ❑ Determine the feasibility of encapsulating dietary components and bacteriophages in order that they may pass unaffected through the rumen and abomasum and be liberated in the hindgut where they may be more effective in reducing numbers of *E. coli* O157:H7.
- ❑ Investigate the effects of a mixture of *S. cerevisiae* strains on the growth and/or

survival of *E. coli* O157:H7 in experimentally inoculated animals.

- ❑ Isolate, characterize and assess the use of a mixture of virulent bacteriophages on the fecal shedding of *E. coli* O157:H7 in experimentally inoculated animals as a means to reduce *E. coli* O157:H7 shedding following colonization.
- ❑ Assess the effectiveness of bacteriophages in the control of *E. coli* O157:H7 in animal drinking water through their use in water troughs.
- ❑ Determine if *E. coli* O157:H7 exists as a biofilm on the epithelial wall of the rumen, cecum and colon through the examination of tissue samples of animals experimentally inoculated with *E. coli* O157:H7. This will also aid in establishing the involvement of the cecum in the shedding of *E. coli* O157:H7 by cattle.
- ❑ Develop a model which will simulate conditions in the cecum and colon (VFA, pH, microorganisms) in order to determine their effect on the growth and/or survival of *E. coli* O157:H7. Cecum and colon fistulated cattle may allow for conditions in the cecum and colon to be monitored prior to the development of an *in vitro* model (artificial cecum and colon).
- ❑ Examine the potential value of synbiotics (prebiotics and probiotics) in reducing the frequency and extent of fecal shedding of *E. coli* O157:H7 and other VTEC.
- ❑ Investigate the effects of the presence of other verocytotoxin-producing *E. coli* (VTEC) in cattle on the shedding of *E. coli* O157:H7 by cattle.
- ❑ Confirm epithelial attachment of *E. coli* O157:H7 in the bovine hindgut through the use of *E. coli* O157:H7 mutants which do not possess the eaeA, Tir , EspA or EspB genes, thought to be necessary for attachment.

- Evaluate dietary modification and components for their effectiveness in stimulating intestinal cell proliferation in the cecum and colon of cattle.
- Determine the mechanism by which increased intestinal cell proliferation in the cecum and colon of cattle is associated with the clearance of *E. coli* O157:H7.

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