DEVELOPMENT OF A COMPETITIVE EXCLUSION PRODUCT TO REDUCE THE CARRIAGE OF *ESCHERICHIA COLI* O157:H7 IN CATTLE

by

Divya Jaroni

A DISSERTATION

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DEVELOPMENT OF A COMPETITIVE EXCLUSION PRODUCT TO REDUCE THE CARRIAGE OF *ESCHERICHIA COLI* O157:H7 IN CATTLE

Divya Jaroni, Ph. D.

University of Nebraska, 2001

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Several experiments were conducted to select lactic acid bacteria (LAB) for a competitive exclusion product (CEP) that would potentially inhibit *E. coli* O157:H7 in the intestinal tract of live cattle. Fecal samples from cattle that were culture negative for *E. coli* O157:H7 were collected every three weeks over a period of 12 weeks. Lactic acid bacteria were isolated from cattle feces by repeated plating over de Man-Rogosa-Sharpe (MRS)/Lactobacillus Selection (LBS) agar. Six hundred eighty-six pure colonies were isolated and each isolate was tested for inhibition of a four-strain mixture of *E. coli* O157:H7 using agar spot test. Three hundred fifty-five isolates (52%) showed significant inhibition. Seventy-five isolates showing maximum inhibition were screened for bile tolerance by monitoring growth in MRS broth with 0, 0.05, 0.15 and 0.3% oxgall over a 24 h period. Most isolates were tolerant to bile, and were subsequently identified using the Analytical Profile Index (API) system. The following strains of LAB were most commonly identified, *Lactobacillus acidophilus, L. fermentum, L. delbreukii, L. salivarius, L. brevis, L. cellobiosus, Leuconostoc* spp., *and Pediococcus acidilactici.* Well-identified strains were further tested for antibiotic resistance and inhibition towards *E. coli* O157:H7 in manure and rumen fluid. Four of the 19 strains showed susceptibility to all the antibiotics. Seven of the 19 strains (37%) significantly reduced *E. coli* counts in manure and 13 of the 19 strains (68%) significantly reduced *E. coli* counts in rumen fluid

($P<0.05$). Two of the isolates, M35 and L411, that were found closely related to each other by ribotyping analysis, were finally selected for CEP and further tested for acid tolerance and resistance to vancomycin, cephalosporin, and polymixin B. For acid tolerance, growth was monitored at pH 2, 4, 5, and 7 in MRS broth. Both isolates showed acid tolerance and were susceptible to the three antibiotics tested for. The 16S rRNA sequence analysis of M35 revealed its close homology to *L. crispatus*. The developed CEP will be further used in cattle feeding trials.

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CHAPTER I

Literature Review

A. *ESCHERICHIA COLI*

1. *Introduction*

Escherichia coli, which is considered to be part of the normal flora of the intestinal tract of humans and other warm-blooded animals (67), was first described by Dr. Theodor Escherich in 1885 (75). The infant gastrointestinal tract is colonized by beneficial strains of *E. coli* within a few hours after birth (67). This bacterial population in the intestine is known to have a protective effect against pathogenic bacteria and is also necessary for synthesizing appreciable amounts of B-vitamins (230). Generally, *E. coli* strains that colonize the human intestine are harmless when confined to the intestinal lumen (168). However, they may become pathogenic and have the potential to cause infection among immuno-compromised individuals, or when the integrity of the defense barrier system of the intestinal mucosa is compromised (174).

Over the years some *E. coli* strains have developed the ability to cause disease of the gastrointestinal, urinary, or central nervous system even in healthy individuals (174). The diarrheagenic *E. coli* that have been associated with foodborne illnesses are grouped into six categories based on their pathogenic characteristics, clinical features, differences in epidemiology and distinct O:H serogroups (150). The six main categories include, enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EaggEC), enterohemorrhagic *E. coli* (EHEC), and diffusely adherent *E. coli* (DAEC) (171).

Enteropathogenic *E. coli* are mainly associated with neonatal and infantile diarrhea, although they are also shown to be carried by many adults without exhibiting any symptoms (274). The EPEC strains exhibit their pathogenesis by attaching and

effacing intestinal cells which has been observed in intestinal biopsy specimens from patients and can be reproduced in cell culture (10, 119, 138, 166, 211, 246, 257). The EIEC strains were first shown to be capable of causing diarrhea in volunteer studies conducted by DuPont et al. (70) and, as the name suggests, are highly invasive. They invade the colonic epithelium, multiply within the host cells, and cause necrosis of the epithelial lining resulting in bloody diarrhea (171). They have been usually linked with foodborne and waterborne outbreaks, although person-to-person transmission has also been reported (107). The incidence is thought to be low in developed countries but occasional foodborne outbreaks, such as one restaurant-associated outbreak involving 370 people in Texas, do occur (97). Enterotoxigenic *E. coli* strains are associated with two major clinical syndromes: weanling diarrhea among children in the developing countries (171), and traveler's diarrhea in the United States (65). They colonize the small intestinal mucosa and produce one or more enterotoxins belonging either to heat labile (LT1 or LT2) or heat stable family (STI or STII) (150). Some investigators have reported that ETEC strains may exhibit limited invasiveness in cell cultures, but this has not been demonstrated *in vivo* (73, 74). Enteroaggregative *E. coli* are currently defined as *E. coli* strains that do not produce enterotoxins LT or ST and that adhere to Hep-2 cells in an aggregative pattern (170, 264). They have been associated with diarrhea in developing populations, most prominently in association with persistent diarrhea (≥ 14 days) (61, 109). The fifth group of pathogenic *E. coli*, EHEC, is named after its disease-defining symptom – hemorrhagic colitis (HC) i.e. bloody diarrhea (34). However, not all EHEC infections produce blood in the stools. *E. coli* O157:H7 is one of the most important serotypes belonging to the EHEC group that has been commonly associated with HC

(209) and also hemolytic uremic syndrome (HUS) (129) around the world. Enterohemorrhagic *E. coli* strains exhibit their pathogenesis mainly by the production of Shiga toxin (1 and 2) (175, 220, 237) and adherence to the intestinal cells (2). Little is known about the pathogenesis of DAEC. They adhere to Hep-2 cells by means of a surface fimbria (F1845) which mediates the diffusely adherent (DA) phenotype (25). The limited reports on DAEC suggest that they are commonly associated with watery diarrhea especially in children older than infants (1 year to 4-5 years), and may also be important diarrheal pathogens in the developed world (171).

2. *Escherichia coli O157:H7*

(a) History and Origins:

E. coli O157:H7 was serotyped on the basis of its expression of the "157th" somatic (O) antigen and the "7th" flagellar (H) antigen (162). It was first isolated in 1975 when a woman from California was diagnosed with severe bloody diarrhea (209). The organism was first recognized as an important human pathogen in 1982, when it was implicated in two food-associated outbreaks of HC (209), a distinctive clinical entity characterized by abdominal cramps, bloody stools, and little or no fever (162). The Centers for Disease Control and Prevention (CDC) identified 47 individuals with HC and the illness was epidemiologically linked to the ingestion of hamburgers from the same fast-food restaurant chain. *E. coli* O157:H7 was isolated from the stools of about half the patients, but from none of the healthy controls (209). In the same year an outbreak of bloody diarrhea occurred in a nursing home in Canada, and *E. coli* O157:H7 was isolated from ill patients (236). These outbreak strains produced toxins active on Vero (African

green monkey kidney) and HeLa cells (122, 176). Subsequently, these strains were shown to produce toxins that were similar to Shiga toxin of *Shigella dysenteriae* and distinct from previously described *E. coli* ST and LT toxins (175). In 1983, Karmali and colleagues (129) reported an association between infection with *E. coli* that produce Shiga toxins and post-diarrheal HUS. In recognition of its distinct clinical manifestations, *E. coli* O157:H7 became the first of several serotypes referred to as EHEC, which are now believed to account for more than 90% of all cases of HUS in industrial countries (220).

E. coli O157:H7 is closely related, genetically, to *E. coli* O55:H7, an EPEC strain that is associated with infantile diarrhea (273). Similar to the EPEC strain, *E. coli* O157:H7 can adhere to epithelial cells and produce characteristic 'attaching and effacing' lesions (171). However, acquisition of additional virulence traits and the transition from O55 to O157 antigen are believed to have occurred as a result of lateral transfer and recombination (77). According to LeClerc et al. (144) *E. coli* O157:H7 may be particularly adept at incorporating foreign DNA as a result of intrinsically high rates of defects in DNA repair mechanisms

(b) Microbiology of *E. coli* O157:H7:

Most biochemical characteristics of *E. coli* O157:H7 isolates are typically the ones exhibited by other *E. coli* strains, for example, fermentation of lactose (101). However, there are some differences that set *E. coli* O157:H7 apart. These include, sorbitol fermentation (159, 271), β-glucuronidase activity (63, 250), and production of a hemolysin belonging to the α-hemolysin family (23). Unlike 80-95% of *E. coli* of human

origin, *E. coli* O157:H7 does not ferment sorbitol within 24 hours (159) and, unlike 92-96% of *E. coli*, it does not produce β-glucuronidase which is the basis for a rapid fluorogenic assay for *E. coli* (250). It also produces a hemolysin similar to the *E. coli* α-hemolysin which is encoded by the large virulence plasmid (pO157) of *E. coli* O157 strains (219). In 1988, Beutin et al. (23) had described a new type of hemolysin called enterohemolysin, and it was shown to be closely associated with Shiga-toxin-producing *E. coli* including *E. coli* O157:H7. However, further studies by Schmidt et al. (219), led to the discovery of a new EHEC hemolysin, encoded on the virulence plasmid, that was shown to be responsible for the enterohemolytic phenotype and was related but not identical to α-hemolysin.

Another important characteristic of *E. coli* O157:H7 is its inability to grow well at 44-45.5°C, the usual temperature for detecting *E. coli* in food and water (66, 200). The organism does not exhibit any unusual resistance to heat (63). The D-values for *E. coli* O157:H7 have been estimated to be 270, 45, 24, and 9.6 seconds at 57.2, 60, 62.8, and 64.3°C respectively (63). D'Aoust and co-workers (54) determined that pasteurization of milk (72°C, 16.2 s) can kill more than 10^4 CFU/ml of *E. coli* O157:H7. As demonstrated by Raghubeer and Matches (200), the temperature range for *E. coli* O157:H7 growth and gas production in EC (*E. coli*) medium within 48 hours is 19.3 to 41°C. However, Palumbo et al. (187) found that the upper temperature for *E. coli* O157:H7 growth was culture medium-dependent; all strains grew in BHI broth at 45°C, but six of sixteen strains did not grow in EC broth. The minimum growth temperature for *E. coli* O157:H7 under otherwise optimal conditions is approximately 8-10°C (201). Doyle and Schoeni (63) demonstrated that it can also survive well in ground beef during frozen storage with

no significant changes in numbers at -80°C and -20°C for up to nine months. The number of outbreaks associated with eating ground beef, combined with the fact that the organism does not grow well at high temperatures but survives freezing, suggests that undercooked meat is the likely reason for *E. coli* O157:H7 infections.

The pathogen's survival in acidic foods is also of particular importance because of the fact that several outbreaks have been associated with low levels of *E. coli* O157:H7 surviving in acidic foods such as apple cider, fermented sausages, and apple juice. The organism grows equally well at pH 5.5 and 7.5 but growth rates decline at lower pH values, the minimum pH for growth being 4.0-4.5 (33). The organism has been experimentally shown to survive for several weeks to months in a variety of acidic foods, including mayonnaise (280), sausages (44), apple cider (279), and cheddar cheese (207) with increased rate at refrigerated temperatures.

(c) Virulence Factors and Pathogenesis:

Several factors have been associated with the virulence of *E. coli* O157:H7. One of the most important virulence characteristics is its ability to produce one or more Shiga toxins. The first of these, Shiga toxin 1, first reported as Verotoxin by Konowalchuck et al. (137) in 1977, is immunologically indistinguishable from Shiga toxin produced by *Shigella dysenteriae* type 1 (171, 175) and is also identical in biological activities i.e. cytotoxicity to HeLa cells, mouse lethality and enterotoxicity (175). The second, Shiga toxin 2, is a more divergent molecule, with only 56% amino acid homology with Shiga toxin 1 (220, 237). Both toxins consist of a single A subunit and five B subunits (177, 277). The B subunit provides tissue specificity by binding to globotriaosylceramide

(Gb3), a glycolipid of unknown function that is abundant in the cortex of the human kidney and is present in primary human endothelial cell cultures (153, 266). After endocytosis, the A subunit enzymatically inactivates the 60S ribosomal subunit, thus blocking protein synthesis (162, 180). The identification of Gb3 as the functional receptor of B subunit explains the etiologic role of *E. coli* O157:H7 in HUS, in which endothelial cells of the renal vasculature are the principal sites of damage (185). It was demonstrated in 1983 that the genes controlling the production of the two toxins are bacteriophage encoded (178, 221, 227). It is believed that the bacteriophage was acquired by the *E. coli* O157:H7 strains directly or indirectly from *Shigella* (34).

Production of Shiga toxins in itself is not sufficient to cause disease. *E. coli* O157:H7 has other characteristics that help make it virulent and deadly. Adhesion to the epithelial cells lining the intestinal tract may be one of the important aspects of the organism's pathogenic potential (101). The type of adherence demonstrated by *E. coli* O157:H7 to animal and tissue culture cells is referred to as 'attaching and effacing' (166). This is characterized by intimate adherence to the enterocyte and dissolution of the brush border at the site of attachment, with the formation of an "attaching and effacing lesion" (2, 166). During this process the bacteria make and secrete a number of proteins that affect the underlying human gut cells (2). One of these proteins that is of particular interest is intimin that acts as bacterial anchor by attaching itself to specific receptors on the human cells (2). Within the enterocyte, filamentous actin accumulates at the site of attachment and the enterocyte membrane may cup the bacteria, forming a pedestal-like structure (139). Attachment to mucosal surfaces prevents loss of bacteria into the environment (19) and promotes the delivery of toxins to euakryotic cell surfaces in a

concentrated manner (247, 278). Other factors thought to contribute to the virulence of *E. coli* O157:H7 include a 60MDa virulence plasmid (pO157) and the locus of enterocyte effacement (LEE) (162). The involvement of the 60MDa plasmid in adherence has been suggested but the reports on its exact role are conflicting (128, 253, 256, 267). It has also been suggested that the pO157 encodes a hemolysin that, in concert with specialized transport systems, may allow *E. coli* O157:H7 to use blood released into the intestine as a source of iron (142). The LEE contains genes for adhesion molecule intimin and for other factors important to the production of attaching-effacing lesions (168).

(d) Epidemiology:

In 1993-1994, CDC estimated *E. coli* O157:H7 to cause about 10,000-20,000 illnesses and close to 250 deaths per year (29, 78). However, according to a recent estimate by CDC, *E. coli* O157:H7 causes more than 73,000 human infections annually in the United States (163), a number much higher than that estimated in the past. Human infections with *E. coli* O157:H7 have also been reported in about 30 other countries including Canada (269), United Kingdom (168), Mexico (51), China (276), Argentina (156), Belgium (195) and Scotland (211, 269). These incidents have occurred in homes, day care centers, swimming pools, schools, nursing homes, and in fast-food and other restaurant operations. Although large outbreaks involving hundreds of individuals have attracted the most attention, sporadic *E. coli* O157:H7 infections comprise the major disease burden of this pathogen and appear to be more common in Canada than in the United States (101).