NOTE TO USERS

EVALUATION OF THE USE OF LACTIC ACID BACTERIA TO CONTROL PATHOGENS ON ALFALFA SPROUTS

by

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The Use of Lactic Acid Bacteria to Reduce Foodborne Pathogens on Alfalfa Sprouts

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EVALUATION OF THE USE OF LACTIC ACID BACTERIA TO CONTROL PATHOGENS ON ALFALFA SPROUTS

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Several studies have investigated the control of pathogens on alfalfa sprouts and some treatments have been shown to be effective in reducing pathogen populations. However, control methods investigated thus far only provide pathogen control at a given point in the sprouting process leaving the sprouts vulnerable at other times during sprouting and storage. Competitive inhibition of pathogens with LAB (lactic acid bacteria) may provide pathogen control throughout the sprouting process and up to consumption. The purpose of this study was to isolate and identify LAB with the intention of adding selected isolates to alfalfa sprouts to inhibit the growth of pathogens during sprouting and storage. Fifty-eight LAB isolates were obtained from alfalfa seeds and sprouts. Based on the agar spot test, all pathogens were inhibited by 32 (55%) of the isolates, *Salmonella enterica* by 56, *Escherichia coli* O157:H7 by 49, and *Listeria monocytogenes* by 41. The isolates were identified using the Analytical Profile Index for carbohydrate utilization, and the isolate L7 was identified using 16s rRNA sequence analysis. Isolate L7 and an isolate previously obtained, D3, were evaluated for competitive inhibition of pathogens in laboratory media. Pathogen populations were significantly reduced by day 5. The isolates were added to seed soak water with pathogen inoculated seeds. During sprouting and storage, LM showed a

significant reduction compared to the Control while EC and SE were consistently higher than the Control, though significance was borderline. For this reason, the interactions of these LAB cultures and EC and SE need to be elucidated by further study. The effects of LAB on Gram negative bacteria have been largely overlooked in the literature. It is suggested that in competitive inhibition studies, the effects of LAB on Gram negative pathogens be addressed concurrently with Gram positive pathogens.

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I. LITERATURE REVIEW

A. Problem Statement

Lactic Acid Bacteria (LAB) and their metabolites have been used for many years in the control of pathogens in fermented meat, vegetable, and dairy products. Recently, LAB have been shown to be effective in controlling some pathogens in nonfermented meat and vegetable products. In addition to competition between the LAB and other microorganisms for carbohydrates and other nutrients present, the LAB may produce several products that may inhibit pathogens, including: organic acids, hydrogen peroxide, carbon dioxide, and bacteriocins.

Sprouts have a diverse flora which, at times, has included some pathogens. Sprouts are of high nutritional value but in light of recent outbreaks, consumers maybe reluctant to maintain or incorporate sprouts into their diets. Studies have investigated treatments to control pathogens on sprouts and sprout seeds. These treatments include: competitive inhibition, seed washes, sprout washes, gaseous treatment, ozonation, and irradiation.

The goal of this work is to isolate and identify LAB from alfalfa sprouts and partially characterize substances produced that are inhibitory to *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* subsp. *enterica* in order to use these isolates to control the pathogens on alfalfa sprouts.

B. Natural Sprout Flora

Splittstoesser et al. (1983) evaluated microbial populations on alfalfa sprouts after 3 days of germination. Average aerobic plate counts were 5.2 x 10^8 CFU/g of sprouts. Average coliform counts were 9.7 x 10^7 CFU/g of sprouts and average micrococci/staphylococci counts were 8.3 x 10^4 CFU/g of sprouts. They also found that of the 62 isolates examined, 22% were gram positive catalase positive rods, 74% were gram negative catalase positive rods, and 4% were of micrococci morphology.

Prokopowich and Blank (1991) evaluated the microbial flora of alfalfa seeds and commercially prepared alfalfa sprouts. The seeds and sprouts had aerobic plate count ranges of 3.0 x 10^2 to 4.0 x 10^5 CFU/g of seeds and 2.7 x 10^8 to 2.3 x 10^9 CFU/g of sprouts. The seeds had a coliform count range of zero to 2.3 x 10^1 CFU/g of seeds and the sprouts had a coliform count of >1.1 x 10^4 CFU/g of sprouts. One of 8 seed samples and 4 of 18 sprout samples were found to have coagulase positive *Staphylococcus aureus*.

Thunberg et al. (2002) evaluated retail alfalfa sprouts for *Campylobacter*, *Salmonella*, and *Escherichia coli*. They found that none of the 6 samples tested positive for these pathogens. They also examined 2 samples of sprouts for entero-toxigenic *Bacillus cereus* and *Staphylococcus aureus* and neither pathogen was detected.

Laio and Fett (2001) described natural sprout flora of commercial alfalfa sprouting seeds. Total aerobic plate counts were approximately 4.5 $\log_{10}$ CFU/g and

23% of these microorganisms were fluorescent pseudomonads. Approximately 1 $\log_{10}$ of LAB were also present; however no yeasts were detected.

Moline and Kulik (1997) found that browning of alfalfa sprout roots was caused by *Erwinia herbicola* that survived seed surface sterilization with 1% sodium hypochlorite. Park and Sanders (1990) found that 40% of 20 lots of alfalfa seed tested were positive for *Klebsiella pneumoniae*. This bacterium is usually regarded as a spoilage organism but has been associated with gastroenteritis in humans (Martin et al. 1971). Barak et al. (2002) isolated *Rahnella aquatilis*, *Pseudomonas putida*, and *Pantoea aggiomerans* obtained from commercially produced alfalfa sprouts prior to packaging.

Callister and Agger (1987) showed that *Aeromonas* spp. are almost ubiquitous in grocery store produce. This study included 2 samples of alfalfa sprouts, of which one was *Aeromonas* positive the day of purchase (2.3×10^4 CFU/g) and both samples were positive on days 7 and 14 of storage.

C. THE PATHOGENS

1. *ESCHERICHIA COLI* O157:H7

EHEC (enterohemorrhagic *Escherichia coli*) was first designated a human pathogen in 1982, when *Escherichia coli* O157:H7 was responsible for hemorrhagic colitis in 2 outbreaks associated with hamburgers at a national chain restaurant (CDC 1982). Subsequently, other strains have been associated with EHEC but serotype O157:H7 is the most predominant cause of illness. EHEC produce vero toxins (VT) also termed Shiga-like toxins (SLT) because they are very similar to those produced by *Shigella* (Doyle et al. 1997).

a. Taxonomy and Characteristics

The genus, *Escherichia*, belongs to the family Enterobacteriaceae together with *Enterobacter*, *Citrobacter*, *Klebsiella*, *Salmonella*, *Shigella*, *Serratia*, and *Yersinia*, among others (Orskov 1984). *Escherichia coli* is termed a coliform which is a Gram-negative rod that ferments lactose within 48 h and does not form spores (Batt 2000a). *E. coli*'s molecular percent G + C of DNA is 48-52% (Orskov 1984). They are facultatively anaeorbic, and may be nonmotile or motile with peritrichious flagella. *E. coli* are oxidase-negative chemoorganotrophs with an optimum temperature for growth of 37 °C (Orskov 1984). However, their range for growth is 7-8 °C to 44-46 °C (International Commission on Microbiological Specifications for Foods 5, 1996). Most strains of *E. coli* have pili or fibrillar proteins that extend from their surfaces to the surrounding environment and may have adhesive functions (Orskov 1984).

Benjamin and Datta (1995) tested *E. coli* O157:H7 for its ability to survive at 37 °C in pH levels of 2.5 and 3.0 for 5 h, conditions similar to the human digestive tract. They found that many of the *Escherichia coli* O157:H7 tested were able to survive under these conditions and this may be the reason *Escherichia coli* O157:H7 is able to cause illness with a low infective dose. Waterman and Small (1996) evaluated strains of Shigella-like toxin producing *Escherichia coli* O157:H7 and found that isolates containing the rpoS gene were resistant to pH 2.5 for 2 h. They also suggest this may contribute to the low infective dose necessary to produce illness and may also be associated with higher infection rates by person-to-person contact.

Traditionally, there are four groups of *E. coli* which cause distinct illnesses and possess unique virulence factors: EPEC (enteropathogenic),EHEC (enterohaemorrhagic), EIEC (enteroinvasive), and ETEC (enterotoxigenic) *Escherichia coli.* EHEC is also referred to as STEC for Stx-producing *E. coli* and VTEC for verotoxogenic *E. coli* (Puente and Finlay 2001). Recently, additional groups have been described as EaggEC (enteroaggregative) and DAEC (diffusely adhereant, Batt 2000a).

b. Clinical Features

The infectious dose for EHEC is unknown but it is thought that as few as 10 organisms may cause disease (FDA 2004a). EHEC causes abdominal cramps and watery diarrhea followed by hemhorragic bleeding of the lower gastrointestinal tract (Karmali 1989) and may lead to the sequelae HUS (hemolytic uremic syndrome) or

TTP (thrombotic thrombocytopenic purpura). HUS is a serious sequelae occuring in about 8% of EHEC infections (CDC 2003). HUS is characterized by acute renal failure, low platelet count, and anemia. It affects the mucosal and submucosal layers of the colon causing edema and hemorrhage. Systemic toxemia is thought to mediate HUS with endothelial cells being the target (Richardson et al. 1988). The clinical features of TTP are similar to HUS; however, neurological impairment and fever are more prominent and those in their 30s are most likely to be affected (Karmali 1989).

c. Habitat and Sources

Most *E. coli* strains are not harmful but are useful in the human digestive sytem. They may suppress detrimental bacteria and are also responsible for synthesizing some vitamins (FDA 2004a). *E. coli* occur, most commonly, in the lower intestines of warm blooded animals (Batt 2000a, Orskov 1984, Doyle et al. 1997, Bell and Kyriakides, 1998). Many animals consumed as food may carry serotypes that are pathogenic to humans as normal commensal flora. *Escherichia coli* O157:H7 infection may be spread by direct contact with an infected animal, by person-to-person contact, or through contaminated food. The prevalency of *E. coli* contributes to the inevitablely that it will be found in most raw foods (including meat, milk, and vegetables, Bell and Kyriakides 1998).

d. Virulence

The inability to ferment sorbitol within 24 h and the inability to grow at ≥ 44.5 °C distinguish *Escherichia coli* O157:H7 from most *E. coli* strains (Doyle et al. 1997). In addition, there are three main characteristics associated with EHEC virulence: the ability to form attachment and effacement lesions (DeVinney et al. 1999, Puente and Finlay 2001), the expression of Shiga toxin (Batt 2000a, Puente and Finlay 2001), and the presence of a 60 MD plasmid (Puente and Finlay 2001).

Once inside the gut, EHEC adheres to the intestinal mucosa. A/E (attachment and effacement) lesions are produced in the brush border microvillous membrane (Knutton et al. 1989). The A/E lesions are initialized when EHEC injects effector proteins into host cells which in turn modify the cells function. The effector proteins bind cytoskeletal proteins and cause them to accumulate at the attachment site, inducing the attachment and effacement lesions and creating the formation of a pedestal-like stucture (Kodama 2002). The genes necessary for A/E are encoded on the EHEC chromosome (Jerse et al. 1991, Sperandio et al. 1998) and in the LEE (locus of enterocyte effacement, Sperandio et al. 1998) region which is highly conserved amoung intestinal pathogens that produce A/E lesions (McDaniel et al. 1995).

The Shiga toxins produced by *Shigella dysenteriae* type 1 show close homology with the toxins produced by cytotoxic *E. coli* and are the most likely progenitor for *E. coli* Shiga toxins (Batt 2000a). Whittam et al. (1993) suggest that an *E. coli* O55:H7-like progenator cell gave rise to EHEC. *E. coli* O55: H7, which causes

infantile diarrhea, already posesed the factors necessary for attachment and effacement. Evolution of EHEC may have occurred when the progenator cell aquired the genes necessary to produce Shiga-like toxins by horizontal transfer, possibly by a prophage.

Two Shiga-like toxins have been found in EHEC: Stx1 and Stx2 (Puente and Finlay 2001). Stx are AB_5 toxins with 1 enzymatic, A, subunit of 32 kDa and 5 binding, B, subunits of 7.7 kDa. The toxin's B subunits bind to the cell surface at a specific glycolipid receptor (Ling et al. 1998). The toxins enter the cell by endocytosis and are trasported to the Golgi apparatus, the entoplasmatic reticulum, and the nuclear envelope (Sandvig et al. 1992). The A subunit inhibits the binding of tRNA to the rRNA in the cytoplasm thus protein synthesis is blocked and causes death of the host cell (Saxena et al. 1989).

Escherichia coli O157:H7 carries a 60 MDa plasmid, termed "pO157" or "the large plasmid". The functions of this plasmid have not been completely elucidated but it does play a role in virulence. Its DNA has been completely sequenced and found to contain 100 open reading frames, of which 42 gene products were similar to known proteins, 19 gene products were possible virulence factors, and 22 had no similarity to known proteins (Burland et al. 1998).

Several of the genes that may encode virulence factors have been described. Schmidt et al. (1995) discovered a hemolysin that was responsible for the enterohemolytic phenotype and which was found to be reactive with sera of patients with HUS. Brunder et al. (1996) described a gene coding for a catalase peroxidase

and designated it KatP. This enzyme was shown to be is closely associated with the occurance of the EHEC hemolysin in Shiga-like toxin producing strians of *Escherichia coli* O157:H7. Brunder et al. (1997) identified an autotransporter serine protease desinated EspP. It was capable of degredation of human coagulation factor, V, which contributes to hemorrhage and was detected in sera of those diagnosed with EHEC infection. Burland et al. (1998) identified a putative cytotoxin active site shared with the large clostridial toxins family and toxic proteins associated with *Clostridium difficile*. It encodes an enterohemolysin and a fimbrial antigen that may assist with colonization of the gut (Nataro and Kaper 1998). Tatsuno et al. (2001) found that full adhereace of EHEC to intestinal epithelial cells was dependent on the protein products of the toxB gene of p0157. Lathem et al. (2002) found that StcE, which is a metalloprotease, is secreted by a type II sectetion system. It may be capable of causing inflammatory and coagulation responses which can lead to tissue damage, intestinal edema, and vascular problems. In addition to virulence genes, pO157 encodes many other genes. These genes include: type II secreation pathway genes (Schmidt et al.1997), regulation and expression genes, replication and maintenane genes, insertion sequences, and some antibiotic resistance genes (Burland et al., 1998).

2. *LISTERIA MONOCYTOGENES*

Listeria monocytogenes was discovered almost 100 years ago and is the agent that causes listeriosis. It was observed to cause a mononucleosis-like infection in

rabbits and guinea pigs and was not isolated in humans until 1929. Until 1981 *L. monocytogenes* was not recognized as an important foodborne pathogen (Martin and Fisher 2000).

a. Taxonomy and Characteristics

Listeria belongs to the *Clostridium* subbranch of the Gram positive bacteria along with *Staphylococcus*, *Streptococcus*, *Lactobacillus*, and *Brochothrix* (Rocourt and Cossart 1997). *Listeria* has a low G + C content of 36-38% (Seeliger and Jones 1986). The genus *Listeria* are short rods that may appear coccoid and occur singly or in short chains. They are facultative anaerobes and their metabolism on glucose yields mainly lactate. *Listeria* are catalase positive and oxidase negative. Their optimum growth temperature is 30 –37° C but when grown at 20-25° C they produce peritrichous flagella and are motile (Bergey 1994).

The genus has been recoginzed to have 6 species and 2 lines of descent. *Listeria monocytogenes*, *L. ivanovii*, *L. innocua* are closely related, as are, *L. grayi* (which includes the former species *L. murrayi*), *L. seeligeri*, and *L. welshimeri* (Rocourt and Cossart 1997). Of these, *L. ivanovii* is pathogenic to animals but *L. moncytogenes* is pathogenic to both humans and animals (Martin and Fisher 2000). There are thirteen serovars, of which 1/2a, 1/2b, and 4b are resposible for the majority of human cases (Rocourt and Cossart 1997). The serotype, 4b, is predominant in the United States, Canada, and Europe (Martin and Fisher 2000).

Growth and survival of *L. monocytogenes* is influenced by the parameters of temperature, pH, and acid type. *L. monocytogenes* has a broad temperature range for growth (<1 °C to <50 °C) and can grow at pH ranges of 4.0-9.5 (Martin and Fisher 2000). *L. monocytogenes* is very hardy for a pathogen that does not form spores, and is able to resist the effects of freezing, drying, and heat quite well (FDA 2004b). The undissociation of the hydrogen ion is related to the degree of detrimental effect that the acid has on *L. monocytogenes.* Acetic acid has the most deliterious effect followed by lactic acid, and then citric acid (Martin and Fisher 2000). *L. monocytogenes* is capable of growth in 10% NaCl and is able to survive in higher concentrations (Martin and Fisher 2000).

Schaack and Marth (1988a) inoculated *Streptococcus cremoris* or *Streptococcus lactis* into skim milk and allowed to it ferment for 15 h at 21 or 30 °C. *S. lactis* lowered the pH more that did *S. cremoris* and *L. monocytogenes* did not grow when the pH dropped below 4.75. *L. monocytogenes* did not grow at all with 5% *S. lactis* at 30 °C but declined in numbers with the combination of 5% *S. cremoris* at 30 °C.

In another study by Schaack and Marth (1988b), *L. monocytogenes* in skim milk cultures and yogurt cultures was evaluated. *L. monocytogenes* was able to survive with *Streptococcus thermophilus* starter culture incubation at 37 and 42 °C for 15 h in skim milk, but was unable to grow. When incubated with *Lactobacillus bulgaricus*, *L. monocytogenes* was only able to survive between 9 and 15 h of incubation and a decrease in pH below 4.0 resulted in rapid death of *L.*

monocytogenes. In the yogurt mix, *L. monocytogenes* was able to increase by 1 order of magnitude.

L. monocytogenes is a psychrotroph whose growth is only slowed by lower tempertures. Membre et al. (1999) determined that *L. monocytogenes* held in optimal and suboptimal media at temperatures of 4 and 7 °C for 3 and 5 days was capable of growth at 7 °C without a lag phase. This suggests that refrigeration is not an effective control intervention for inhibiting *L. monocytogenes* growth under actual processing and industry storage conditions.

Dykes and Withers (1999) found that *L. monocytogenes* subjected to starvation and stored at 4 °C for 4 weeks was able to grow on selective agar only when undiluted. However, when nonselective media was used, *L. monocytogenes* grew in the lowest dilutions performed (10^1 CFU/mL). They suggest, that in order to detect all *L. monocytogenes* capable of growth in food, a non-selective enrichment step should be included when attempting to detect *L. monocytogenes* that my have undergone sublethal injury.

b. Clinical Features

The infectious dose of *L. monocytogenes* is unknown but is thought to vary with an individual's suseptibility and the strain (FDA 2004b). Those most at risk for listeriosis are pregnant women, neonates, and those with compromised immune systems (Slutsker and Schuchat 1999). Doses as low as 1000 organisms are believed to be capable of causing disease (FDA 2004b). *L. monocytogenes* has been reported to