Use of Natural Antimicrobials for Control of *Escherichia coli* 0157:H7 In Ground Beef and Dry Fermented Sausages

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

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Department of Food Science

University of Manitoba

Winnipeg, Manitoba, Canada

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To my father

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Organization of the thesis

The thesis is organized to include four manuscripts that are submitted for journal publication and another manuscript was submitted as a congress proceeding (chapters 3-7). The manuscripts are in various stages of publication which are indicated here. These Chapters were standardized for presentation in the thesis format with addition of more graphs and information. Chapter 1 gives an overall introduction to the thesis, Chapter 2 is a comprehensive review of related work and a conclusion is presented as Chapter 8 followed by bibliographic references.

Chapter 3 was published with authorship by P. Muthukumarasamy, Han, J. H, and Holley, R. A. in 2003 as "Bactericidal effects of *Lactobacillus reuteri* and allyl isothiocyanate on *E. coli* O157:H7 in refrigerated ground beef" in the Journal of Food Protection 66(11): 2038-2044.

Chapter 4 was originally presented at the 50th International Congress of Meat Science and Technology, Helsinki, Finland. August 12, 2004. (Abstr.3.30, p.155). and published as a proceeding with authorship by P. Muthukumarasamy, Han, J. H. and Holley, R. A. in 2004 as "Lethal effects of non-deheated (hot) mustard flour on *E. coli* O157:H7 in refrigerated nitrogen packed ground beef".

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2005 as JFS-2005-0345. The original manuscript was expanded with inclusion of more figures to better explain the results of the experiment.

Chapter 6 was submitted for publication in Sept 16, 2005 to the International Journal of Food Microbiology with authorship by P. Muthukumarasamy, and Holley, R. A as "Microbiological and sensory quality of dry fermented sausages containing alginate-microencapsulated *Lactobacillus reuteri*".

Chapter 7 was submitted for publication Nov 24, 2005 to the journal Food Microbiology with authorship by P. Muthukumarasamy and Holley, R. A. as "Survival of *Escherichia coli* O157:H7 in dry fermented sausages containing microencapsulated probiotic lactic acid bacteria".

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Abstract

Lactobacillus (*Lb.*) *reuteri* (which produces an antimicrobial reuterin by anaerobic fermentation of glycerol) and allyl isothiocyanate, AIT (extracted from horse radish) are two potent antimicrobials with activity against Gram positive and Gram negative bacteria. These two antimicrobials were studied for their direct inhibitory effects against *Escherichia* (*E.*) *coli* O157:H7 in refrigerated ground beef. In addition non-deheated mustard flour as a natural source of allyl isothiocyanate was used as an ingredient and studied for its action against *E. coli* O157: H7 in ground beef. Different microencapsulation methods and encapsulating material for protecting *Lb. reuteri* against simulated gastric juice were evaluated and an optimum method and material were determined. The suitability of *Lb. reuteri* and *Bifidobacteria* (*B.*) *longum* cultures as probiotic cultures in dry fermented sausages was studied with or without protecting the cells by microencapsulation. The effects of these probiotic organisms against *E. coli* O157:H7 in dry fermented sausages were also investigated.

Allyl isothiocyanate and *Lb. reuteri* were added separately and in combination and examined for their action against a cocktail of 5 strains of *E. coli* O157:H7 in ground beef stored at 4° C over a period of 25 d. Raw inside round beef roasts were ground using a mechanical grinder and inoculated with two levels of *E. coli* O157:H7 (3 log cfu/g or 6 log cfu/g). To this ground beef AIT at a concentration of 1300 ppm and/or *Lb. reuteri* (plus 250 mmol glycerol/kg meat) at two levels (3 log cfu/g or 6 log cfu/g) were added according to the experimental design and stored at 4° C after flushing the bags with nitrogen. Sampling was done every 5d for *E. coli* O157:H7, total bacteria and lactic acid bacteria.

Lb. reuteri at both levels in the presence of glycerol killed *E. coli* O157:H7 at both

inoculated levels before day 20. AIT eliminated *E. coli* O157:H7 when initially present at 3 log cfu/g and reduced viability > 4.5 log cfu/g when initially present at 6 log cfu/g by the end of the storage period. The combination of *Lb. reuteri* with AIT did not yield synergistic or additive effects against *E. coli* O157:H7. *Lb. reuteri* in the presence of glycerol was highly effective against *E. coli* O157:H7 in ground beef during refrigerated storage (4° C) in modified atmosphere packages.

Deheated (deodorized) mustard flour is incapable of AIT release due to the thermal inactivation of the constitutive enzyme myrosinase that is required for isothiocyanate production from its precursor glucosinolate. Non-deheated mustard flour has active myrosinase and yields AIT following its action on glucosinolates in the presence of moisture. The antimicrobial effect of deheated and non-deheated mustard flour at 10 and 20% in refrigerated ground beef inoculated with a five strain cocktail of *E. coli* O157:H7 was studied and compared with the effects of pure AIT at 1300 ppm.

Non-deheated mustard flour at 20% eliminated *E. coli* O157:H7 after day 3 whereas AIT and 10 % non-deheated mustard flour required 15 and 18d, respectively, to eliminate inoculated *E. coli* O157:H7 from ground beef. Deheated mustard flour at both 10 and 20% failed to eliminate *E. coli* O157:H7 by the end of storage. Total bacterial numbers were significantly reduced ($P< 0.05$) when 20% non-deheated mustard flour or AIT were used, indicating potential extension of ground beef shelf life. Isothiocyanates and probably AIT present in the mustard flour are the agents responsible for the antimicrobial effects of mustard flour.

Dry fermented sausages present a challenging environment for the survival of probiotic bacteria thus making it difficult to supplement these products with these organisms.

Microencapsulation using various polymers has been shown to protect bacterial cells against harsh conditions but a comparative study involving various encapsulating materials and methods had not been published, making it difficult to choose an optimum wall material and method for the microencapsulation of *Lb. reuteri* for addition to dry fermented sausages. A study was undertaken to determine the most suitable method and wall material for microencapsulation of *Lb. reuteri* and resulting cell viability were studied during challenge with simulated gastric juice. Four wall materials, namely alginate, alginate plus starch, κ-carrageenan with locust bean gum, or xanthan with gellan and two methods of microencapsulation (extrusion or phase separation by emulsion formation) were studied for their ability to protect cell viability during gastric acid challenge.

Lb. reuteri when microencapsulated survived better than planktonic cells and survival varied with the type of strain, method of microencapsulation and wall material used. Microencapsulation using alginate and alginate with starch by both extrusion and phase separation were found to offer greater protection ($p< 0.05$) against simulated gastric juice.

Experiments were also conducted to find whether *Lb. reuteri* viability was better protected in dry fermented sausages by direct addition or after microencapsulation in alginate wall material with microcapsules prepared by either extrusion or emulsion. *Pediococcus* (*P.*) *pentosaceus* and *Staphylococcus* (*S.*) *carnosus* were used as starter cultures for fermentation. The sausage batter was stuffed in fibrous casings and fermented with smoking at ≤ 26° C and 88% relative humidity (RH) for 72 h according to the degree.hour guidelines of Agriculture and Agri-Food Canada and then dried at 75 % RH and 13° C for 25 d. The pH, water activity (a_w) and levels of *Lb. reuteri*, *P. pentosaceus* and *S. carnosus* were monitored during fermentation and drying. The sensory qualities of sausages that contained

free or microencapsulated *Lb. reuteri* as a co-culture were also studied.

The pH of sausages dropped from 5.8 to 4.8 by the end of fermentation and the a_w dropped from 0.97 to 0.89 at the end of drying in all treatments. There were no significant differences among treatments in terms of pH, a_w or in *P. pentosaceus* and *S. carnosus* numbers. Microencapsulation was found to maintain *Lb. reuteri* viability during processing of dry fermented sausages. Planktonic *Lb. reuteri* cells were reduced by > 2.5 log cfu/g, while microencapsulated *Lb. reuteri* cells were reduced only by $\leq$ 0.5 log at the end of 25 d drying. The sensory quality of the sausages across sausage treatments did not differ significantly and varied between 7.1 to 7.3 (like moderately) on a 9 point Hedonic scale for overall acceptability, appearance, flavor and texture.

The effect of probiotic *B. longum* and *Lb. reuteri* both as planktonic and microencapsulated cells either alone or in combination were studied as natural antimicrobials against a 5 strain cocktail of *E. coli* O157:H7 in dry fermented sausages. Sausages were manufactured as in previous experiments observing the degree.hour guidelines of Agriculture and Agri-Food Canada. The survival of *E. coli* O157:H7 and the probiotics was monitored during dry sausage manufacture. The pH and water activity decreased from 5.7 and 0.98 to 4.9 and 0.88 at the end of fermentation and drying, respectively. In the control, *E. coli* O157:H7 decreased by 1.7 log cfu/g at the end of the 27d drying period. In sausages treated with planktonic *Lb. reuteri* or *Lb. reuteri* with *B. longum* a 2.8 to 3 log cfu/g reduction in *E. coli* O157:H7 numbers was found at the end of drying. Addition of planktonic *B. longum* resulted in a 1.9 log reduction in *E. coli* O157:H7 whereas microencapsulated *Lb. reuteri* or *B. longum* alone or in combination did not yield significant reductions in the *E. coli* O157:H7 population. Numbers of planktonic *Lb. reuteri* and *B.*

longum decreased during sausage processing, but microencapsulation was shown to protect them significantly during the manufacture of dry fermented sausages. Microencapsulation of probiotics like *Lb. reuteri* and *B. longum* may be employed for supplementing dry fermented sausages with probiotics but their value as antimicrobials against *E. coli* O157:H7 is limited.

Chapter 1

Introduction

The incidence of *E. coli* O157:H7 has increased significantly in recent years and the presence of *E. coli* O157:H7 in finished product is considered a Health 1 concern by Health Canada. Thus, the organism is considered an adulterant in food that warrants recall and immediate condemnation of the finished products. *E. coli* O157:H7 causes life threatening complications such as hemorrhagic colitis in children, the elderly and the immuno-compromised, and only few cells are required to cause infection (Buchanan and Doyle 1997). *E. coli* O157:H7 which is frequently present in the cattle gastrointestinal (GI) tract often enters human food chain via contaminated meat, milk and manure (Hussein and Sakuma, 2005). Foodborne infections account for > 50% of the total *E. coli* O157:H7 cases in North America (Rangel et al. 2005). Since the herd prevalence of *E. coli* O157:H7 is invariably high (Zhao et al. 1995), it makes prevention of meat contamination during slaughter almost impossible. One of the options for preventing these pathogens from persisting in the food chain is through proper processing of meat and meat products.

Ground beef is often responsible for transmission of *E. coli* O157:H7 in North America (Health Canada, 2000a), and numerous outbreaks traced to contamination of improperly cooked ground beef have been reported (Getty et al. 2000). Dry fermented sausages, which had previously (13 years ago) been considered shelf stable and safe due to their high acid content (pH $\leq$ 4.6), presence of salt, nitrite and low water activity ($\leq$ 0.88), have more recently been shown (Glass et al. 1992) to be vulnerable to *E. coli* O157:H7 contamination. Only after the outbreak in 1994 in Washington and California,

was *E. coli* O157:H7 considered a pathogen of concern in these types of products. The United States Department of Agriculture (USDA) Food Safety and Inspection Service developed guidelines that required the processors of dry and semi-dry sausage to validate at least a 5 log-unit reduction in the number of *E. coli* O157:H7 cells in sausages (Reed, 1995). The potential for more outbreaks warrants rethinking on how to satisfy current *E. coli* O157:H7 reduction requirements, especially for products that do not receive heat treatments such as dry fermented sausages. A variety of methods involving altered processing conditions (fermentation and drying), changes in concentrations of curing agents, variations in storage conditions, thermal inactivation, freezing, irradiation, high pressure processing, use of protective cultures, and the use of chemical and natural antimicrobials have been evaluated for their ability to achieve the 5 log reduction in *E. coli* O157:H7 numbers. The effectiveness of some of these treatments is outlined in the literature review. Post fermentation heating sufficient to validate a 5 log-unit reduction in *E. coli* O157:H7 (Hinkens et al. 1996) may not yield organoleptically satisfactory products. Heating, apart from causing undesirable changes in texture and taste, also increases the cost of production and therefore is not considered suitable for pathogen control (Lahti et al. 2001, Johnson et al. 2000). Thus, there is a need to explore alternative methods to control *E. coli* O157:H7 in these products.

In recent years, there has been an increase in the number of studies on the role of lactic acid (produced by lactic acid bacteria, LAB), in inhibiting pathogenic microorganisms in food, and this approach is consistent with consumer preference for use of natural and health-promoting supplements rather than chemical preservatives.

In this research two naturally occurring antimicrobials, namely reuterin produced by *Lactobacillus reuteri* and allyl isothiocyanate extracted from horse radish, were used as intervention strategies to control *E. coli* O157:H7 during processing and refrigerated storage of ground beef. Another aspect of this study involved the use of probiotic organisms in dry fermented sausages. With the advent of microencapsulation techniques, dry fermented sausages may have potential as a carrier of probiotic organisms. The survival of two probiotic organisms, *Lb. reuteri* and *B. longum*, was studied in dry fermented sausages with or without microencapsulation. The study also focused on the use of *Lb. reuteri* to control *E. coli* O157:H7 during processing of these types of products.

The main objective of this research was to determine whether the above mentioned natural antimicrobials could be successfully used for reduction of *E. coli* O157:H7 in refrigerated ground beef and dry fermented sausages. Apart from yielding safe products, the development of novel probiotic products providing beneficial health effects should have a positive impact in the marketplace.

The long term objective was to develop a validated method for use by the meat industry to achieve the desired reduction of *E. coli* O157:H7 numbers, thereby preventing costly product recalls, satisfying regulatory requirements, and improving confidence in product safety.

Chapter 2

Review of Literature

2.1 *E. coli* O157:H7 morphology and characteristics

E. coli O157:H7 is a Gram negative, rod shaped, facultatively anaerobic organism which is a pathogenic variant of *E. coli*. "O" defines the somatic antigen (157) and "H" the flagellar antigen present. *E. coli* O157:H7 which is classified as an enterohemorrhagic *E. coli* strain (EHEC), unlike generic *E. coli,* cannot ferment sorbitol within 24h and lacks β – glucuronidase enzyme activity (Doyle, 1991). *E. coli* O157:H7 growth temperatures range between 30 to 42° C with optimum growth at 42° C. Thus they are undetectable by enumeration methods involving incubation at 45° C that are traditionally used to detect *E. coli* in foods and water (Doyle, 1991).

E. coli O157:H7 can grow over a pH range of 4.5 to 9.0 with an optimal pH around 7.0 (Glass et al. 1992) but *E. coli* O157:H7 have high acid tolerance, and can even survive 2-7 h exposure to pH 2.5 at 37° C (Benjamin and Dutta 1995, Buchanan and Edelson, 1996). *E. coli* O157:H7 do not grow, but survive a combination of low pH, low water activity (a_w), and high salt and nitrite developed during the fermentation and drying processes used for dry and semi-dry sausage manufacture (Glass et al. 1992, Clavero and Beuchat 1996, Hinkens et al. 1996, Calicioglu et al. 1997).

2.2 Epidemiology of *E. coli* O157:H7

E. coli O157:H7 was not identified as a human pathogen until 1982 (Riley et al. 1983), although symptoms similar to *E. coli* O157:H7 infections such as bloody diarrhea were reported as early as 1975 in California. Still, awareness of the magnitude of *E. coli* O157:H7 illnesses was not recognized until an outbreak linked to undercooked ground

beef involving four states and 732 people in the US was reported in 1993 (Bell et al. 1994). *E. coli* O157:H7 became a nationally notifiable cause of human illness in 1990 in Canada and in 1994 in the US (Todd, 2000, Rangel et al. 2005). Thus reporting infections due to this organism to appropriate federal agencies is mandatory in both countries.

Ruminants and primarily cattle are the principal reservoir of *E. coli* O157:H7, although the organism seldom causes disease in these animals (Caprioli et al. 2005). Transmission is through the fecal-oral route by direct or indirect contamination of food by cattle feces. The organism can survive in the environment for several months and contaminate water or food sources (Getty et al. 2000). *E. coli* O157:H7 infections are common in North America, Japan and Europe, with Canada, the US and the UK reporting most of the cases (Bach et al. 2002). The hemorrhagic uremic syndrome caused by non-O157 strains are common in Australia, Argentina, Chile, Columbia and South Africa (Park et al. 2001). It is estimated that 73,480 *E. coli* O157:H7- related illnesses occur in the US each year leading to an estimated 2,168 hospitalizations and 61 deaths in that country (Mead et al. 1999). In Canada, the incidence was 4.07 to 8.81 per 100,000 Canadians per year for the 10 year period between 1991 and 2000 with 1173 to 2714 cases every year (PHAC, 2003). In the year 1995 alone, the hospitalization rate due to pathogenic *E. coli* in Canada was 365 per 1000 cases, with a mortality rate of 39 per 1000 (Anonymous, 1998).

Foodborne *E. coli* O157:H7 outbreaks account for 52% of the transmission of this pathogen to humans. Other routes of transmission include 14% by direct transmission from individual to individual, 9% by water, 3% by contact with animals on farms and 0.3% by working in a laboratory (Rangel et al. 2005). Twenty one percent of the total

transmission is from unknown sources, indicating the possibility of even higher foodborne transmission. Large community gatherings, picnics, camps, and the food service industry at large are commonly implicated in *E. coli* O157:H7 outbreaks, whereas individual homes are rarely involved.

In the US and Canada, contaminated ground beef is the most common vehicle for transmission of *E. coli* O157:H7 and is responsible for > 40 % of the foodborne outbreaks (Health Canada 2000a, Rangel et al. 2005). Outbreaks of *E. coli* O157:H7 infection through fresh produce and acidic foods such as yogurt, mayonnaise and dry fermented sausages are not uncommon (Table 2.1). *E. coli* O157:H7 outbreaks are more frequent during summer (May to October) in North America probably because of improper cooking during use of the barbeque and the higher seasonal prevalence in cattle (Chinen et al. 2001).

Table 2.1 *E. coli* O157:H7 outbreaks due to various food sources

Foods	**Occurrence**	**Year**	**References**
Ground Beef	Several States, US	2002	CDC (2002)
	Winnipeg	1999	MacDonald et al. (2000)
	Colorado	1997	CDC (1997a)
	California	1993	CDC (1994)
Drinking Water	Walkerton, Ontario	2000	Auld et al. (2004)
	Alpine, Wyoming	1998	Olsen et al. (2002)
Apple cider and apple juice	Southeastern Massachusetts	1991	Besser et al. (1993)
	California, Colorado, and Washington	1996	CDC (1996)
	Connecticut and New York	1996	CDC (1997b)
	Ontario	1998	Tamblyn et al. (1999)
Yoghurt	Northwest England	1991	Morgan et al. (1993)
Dry fermented sausages	Washington and California	1994	CDC (1995a)
	Australia	1995	CDC (1995b)
	British Columbia	1999	MacDonald et al. (2001)
Mayonnaise	Oregon (salad dressings)	1993	Weagant et al. (1994)
Vegetables			
Radish Sprouts	Sakai City, Japan	1996	Michino et al. (1999)
Lettuce	Connecticut and Illinois	1996	Hilborn et al. (1999)
	Montana	1995	Ackers et al. (1998)
Alfalfa sprouts	Michigan and Virginia	1997	Breuer et al. (2001)

2.3 Pathogenesis of *E. coli* O157:H7

E. coli O157:H7 affects young children, the immuno-compromised and the elderly population by causing bloody diarrhea and severe kidney damage leading to hemorrhagic colitis (HC), the hemorrhagic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP) (Buchanan and Doyle, 1997).

The infectious dose of *E. coli* O157:H7, although not precisely known, is considered low. Two to 2000 cells may be required to cause illness and the low dose required for causing infections is attributed to the acid tolerance of *E. coli* O157:H7 (Buchanan and Edelson, 1996). Less than 700 cells were reported to be responsible for the large outbreak of *E. coli* O157:H7 in 1993 linked to undercooked hamburger (Tuttle et al. 1999), but Tilden (1996) reported that < 50 cells initiated infections in the outbreak from dry cured salami in 1994.

For pathogenesis to occur, the organism must be ingested either through food, water or personal contact. Next colonization of the organisms in the gut occurs leading to production of one or more phage encoded Shiga-like toxins (Stx1 and Stx 2) and the development of local and systemic disease. Colonization of the mucosal membrane of the intestine is promoted by the attaching and effacing gene which, with other factors such as Stx production and plasmid located pathogenicity islands (PAI) all play roles in virulence of the strain (Caprioli et al. 2005).

E. coli O157:H7 produces Shiga toxins 1 and/or 2 (also called verotoxins 1 and 2) which are proteins of 70, 000 daltons (Dal) molecular weight and are composed of a single A subunit of 32 kDal and five B subunits of 7.7 kDal. The B subunit is responsible for binding to the intestinal receptors and the A subunit blocks protein synthesis by the

intestinal cells (Buchanan and Doyle, 1997). The H7 flagellum may induce production of interleukin 8 and cause infiltration of neutrophils in the mucosal membrane, thus increasing Stx intake across the membrane. Stx and interleukin cause lesions in the blood vessels leading to HC or HUS (Moxley, 2004).

HC occurs 1-2 days after consumption of contaminated foods. Colonization by *E. coli* O157:H7 results in mild fever, abdominal cramps and non-bloody diarrhea due to disruption of intestinal cells. The diarrhea progresses to bloody diarrhea when Shiga toxins damage the blood vessels. Bloody diarrhea with severe abdominal pain and dehydration occurs most commonly in children and the elderly whose immune systems function at lower than normal levels. HC is self limiting and the illness may last for only few days (Tarr et al. 2005).

In some cases HC progresses to HUS which is characterized by destruction of red blood cells leading to anemia, decreased numbers of platelets leading to thrombocytopenia, decreased urine formation, edema and kidney failure. HUS patients often require kidney dialysis and the mortality rate is 3 to 5 %. TTP may also occur mainly in adults, and is characterized by central nervous system damage leading to seizures and strokes in addition to renal damage (Tarr et al. 2005).

2.4 Cattle as a reservoir of *E. coli* O157:H7

As mentioned earlier, ruminants are the main reservoir of *E. coli* O157:H7 (Riley et al. 1983). Cattle were most often reported as a vector but sheep, deer and pigs have occasionally been implicated (Buchko et al. 2000). *E. coli* O157:H7 colonizes the gastrointestinal tract of cattle and are localized in the rumen, reticulum, omasum and colon (Buchanan and Doyle, 1997). Cattle seldom show any symptoms of *E. coli*

O157:H7 presence but can shed up to 10^3 to 10^5 cfu/g intermittently in the feces (Zhao et al. 1995). *E. coli* O157:H7 is shed more frequently during summer compared to winter, thus playing a direct role in increased numbers of outbreaks during summer months (Hancock et al. 1997). The herd prevalence of *E. coli* O157:H7 in dairy cattle reported in various studies in North America was between 0 to 22 % with a medium range of 7 to 8%, although one study reported a 61% herd prevalence (Meyer-Broseta et al. 2001). Individual cattle shedding was reported to depend on the age of the animal with young calves < 8 weeks, calves 2 to 4 months and heifers 4 to 24 months old having a prevalence of 1.5, 1.8 and 2.3 % respectively. Adult dairy and beef cattle shedding of *E. coli* O157:H7 was only 0.7 % of animals whereas feedlot cattle had a shedding prevalence of 1.5 to 3.6 % (Meyer-Broseta et al. 2001).

Products of bovine origin such as ground beef, other beef products, unpasteurized milk and cheese can be contaminated with bovine feces and may be responsible for transmission of *E. coli* O157:H7 to humans (Hussein and Sakuma, 2005). Other modes of transmission include contaminated drinking water, contact with the animal and its environment (feed and manure) and also by person to person contact. Produce or vegetables which had been irrigated or washed with manure-contaminated water or grown in cattle grazing pastures were also reported responsible for transmission of *E. coli* O157:H7 (Hilborn et al. 1999).

2.5 Acid resistance of *E. coli* O157:H7

Foods like dry fermented sausages, mayonnaise, apple cider and yoghurt which were considered safe due to their low pH prior to 1982, were later shown to allow the survival of *E. coli* O157:H7 (Tables 2.1 & 2.2). While *E. coli* O157:H7 cannot grow in

these acidic foods, it can survive for long periods depending on the type of product and the storage temperature (Zhao et al. 1993, Glass et al. 1992). The acid resistance or acid tolerance response of *E. coli* O157:H7 enhances the ability of these organisms to survive in low pH environments which were previously thought lethal to these organisms. The acid resistance property of *E. coli* O157:H7 may contribute to its low infective dose (Gorden and Small, 1993) and the increase in outbreaks and incidence of infection in part may be attributed to occurrence of new strains with enhanced acid resistance (Jordan et al. 1999).

Several mechanisms including expression of acid shock proteins (Heyde and Portalier, 1990), the alternate sigma factor *rpoS* regulated system (Cheville et al. 1996), the acid-induced arginine and other acid-induced oxidative systems, the glutamate dependent system (Lin et al. 1996) or production of the extra-cellular polysaccharide colonic acid (Mao et al. 2001), the involvement of surface polysaccharides (Barua et al. 2002), or activation of the DNA binding protein *dps* (Choi et al. 2000), have been offered as explanations for the acid resistance of *E. coli* O157:H7.

Various other factors affect the potential acid resistance of *E. coli* O157:H7. These include prior adaptation to acid pH (Leyer et al. 1995, Tsai and Ingham, 1997, Hsin-Yi and Chou, 2001), composition of growth media (Casey and Condon, 2002, Diez-Gonzalez and Russell, 1999), prior growth conditions (Buchanan and Edelson, 1996, Jarvis and Russell, 2001, Arnold and Kasper, 1995), growth phase of the cells (Benjamin and Datta, 1995), type of acidulant (Diez-Gonzalez and Russell, 1999), strain to strain variation (Buchanan and Edelson, 1999), temperature of growth (Miller and Kasper, 1994), cell density (Cui et al. 2001, Datta and Benjamin, 1999), aerobic vs. anaerobic

environment (Diez-Gonzalez and Russell, 1999), composition of foods (Yokoigawa et al. 2003), and even differences in cattle diet (Diez-Gonzalez et al. 1998, Hovde et al. 1999). The persistence of an acid resistant state for extended periods and induction of cross protection in acid resistant strains to other environmental stresses such as heat, salt or irradiation is a concern for the food industry which depends on these hurdles to assure safety of foods (Cheville et al. 1996, Lin et al. 1996, Buchanan et al. 1999).

Table 2.2 Survival of *E. coli* O157:H7 in acidic foods

Acidic Foods	pH	Conditions and length of survival	Reference
Apple cider	3.6-4.0	10-31 d at 8° C	Zhao et al. (1993)
	3.7-4.1	14-21 d at 4° C	Miller and Kasper (1994)
	3.56-3.98	> 56 d at 25° C	Ryu and Beuchat (1998)
	3.42-3.75	> 10d at 20° C	Semanchek and Golden (1996)
Yoghurt	4.4-4.6	> 7d after fermentation at 42° C and storage at 4° C.	Massa et al. (1997)
	3.9	> 6d at 7° C	Hsin-Yi and Chou (2001)
	3.92-4.14	≤ 312, 168, 28 and 16 h at 4, 8, 17 and 22° C, respectively	Bachrouri et al. (2002)
	4.0	> 12d	Dineen et al. (1998)
Mayonnaise	3.6-3.9	5° C for 34-55 d and 20° C for 8- 21d.	Zhoa and Doyle (1994)
	3.76-4.44	> 35d at 5° C	Weagant et al. (1994)
	3.86-3.97	≤ 93 d at 5° C	Hathcox et al. (1995)
Fermented sausages	4.8	Fermentation, drying and storage at 4° C for > 2 mos.	Glass et al. (1992)
Dry salami	5.0	> 4d at 5° C	Leyer et al. (1995)
Pepperoni	≤ 4.8	> 28d after fermentation and storage for 28d at 21° C	Faith et al. (1997)
Fruit pulps (Acerola Cajá, Pitanga,Grape, Passion fruit)	2.51-3.26	> 4d at 4° C.	Marques et al. (2001)
Ketchup	3.6	1-7 d at 5° C	Tsai and Ingham (1997)
Orange juice	3.82-3.86	> 56d at 25° C	Ryu and Beuchat (1998)

Table 2.2 Survival of *E. coli* O157:H7 in acidic foods (continued..)

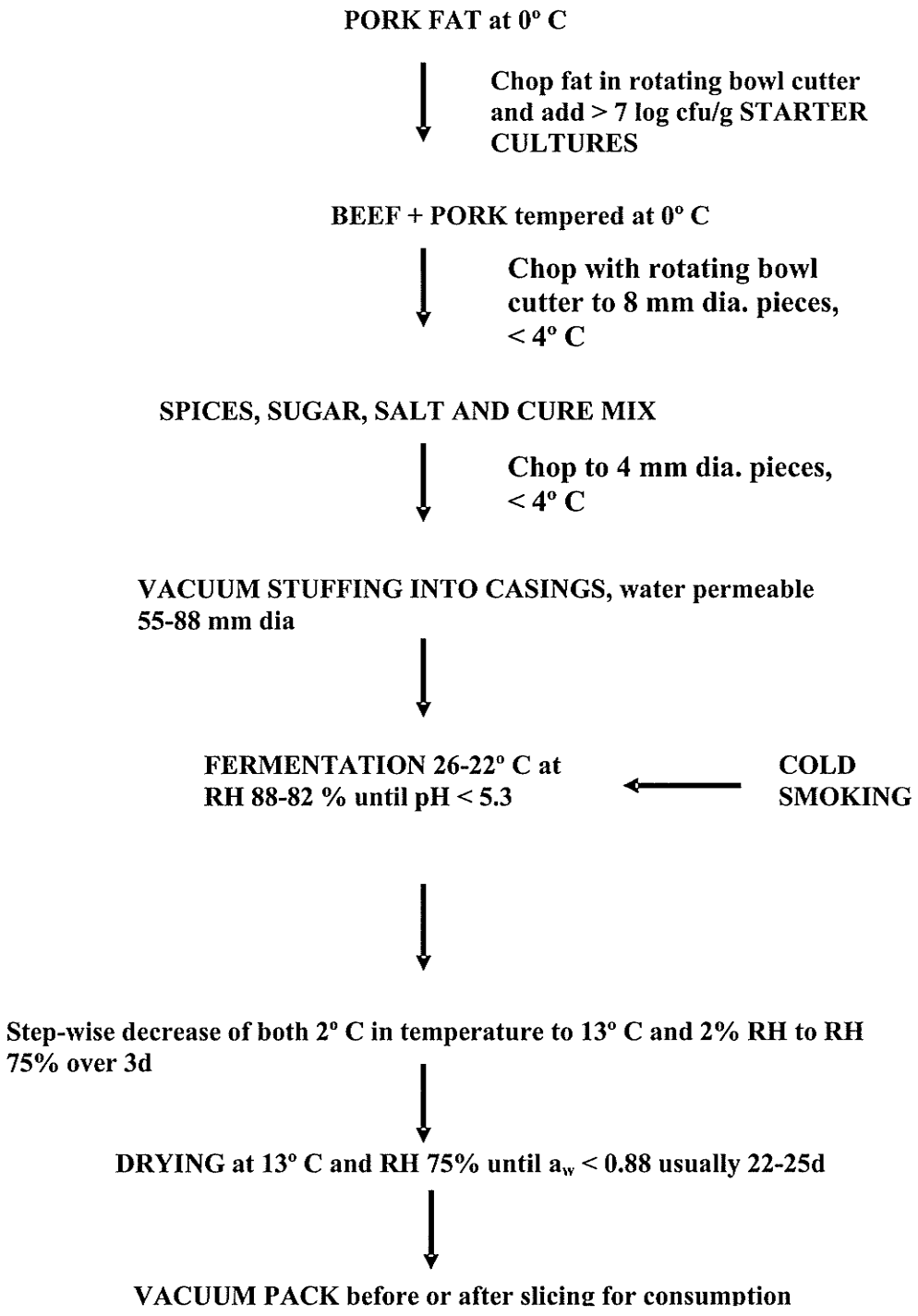
Acidic Foods	pH	Conditions and length of survival	References
Mango Juice Asparagus juice Yakult	3.2 3.6 3.6	> 8d at 7° C > 20d at 7° C > 6d at 7° C	Hsin-Yi and Chou (2001)
Salad dressing	4.51	> 17d at 4° C	Raghubeer et al. (1995)
Egg plant salad	4.0	> 13 d at 10° C	Skandamis and Nychas (2000)
Fermented milk	4.0-4.4	24h fermentation at 37-43° C	Ogwaro et al. (2002)
Cheddar Cheese	4.95-5.2	processing and ripening for > 158d	Reitsma and Henning (1996)
Feta cheese and Telemes Cheese	4.81-5.10	36-44d in feta cheese and 30-40d in Telemes cheese after 24h fermentation, ripening at 16° C (pH 4.6) and storage at 4° C	Govaris et al. (2002)

2.6 Ground beef, hamburger and dry fermented sausages

Ground beef is generally made from trimmings of less popular cuts of beef and occasionally from tender cuts. The trim is ground in a mechanical grinder so that the grinding action tenderizes the beef. The fat content in ground beef is regulated and for regular ground beef should not be > 30 % (FSIS, 2002). Fat present in ground beef improves the overall flavor and reduces its dryness. Spices and seasonings may be added to ground beef and hamburger but phosphates, water and binders are seldom allowed by law (FSIS, 2002). The only difference between a hamburger and ground beef is that the former can have added fat but fat should not be added to ground beef. Ground beef alone accounts for > 40 % of the total meat consumption in the US (Kennedy et al. 2000).

Dry fermented sausages originated in the Mediterranean region, thousands of years ago due to warmer dry climates there that favor drying of meat (Lücke, 1998). Dry fermented sausages are traditionally prepared by addition of spices, cure mix containing salt and nitrate/nitrite and sugars to chopped meat which are fermented with LAB cultures after stuffing into natural or artificial casings and followed by a long air drying process. Dry fermented sausages are most often smoked in Northern areas of Europe and the combination of low pH due to production of lactic acid, the presence of salt and nitrite, low water activity and smoke contribute to protect safety and shelf life stability even at room temperature. In the United States dry and semi-dry fermented sausages should have a pH of < 5.0 and a moisture/protein ratio of less than 1.9:1 and 3.1:1, respectively (FSIS, 2005). A flow chart for preparation of dry sausages is presented in Fig 2.1. There are over 350 varieties of dry sausages in Germany alone and the yearly per capita consumption there is > 5kg (Varnam and Sutherland, 1995).

Fig 2.1 Flowchart for the manufacture of dry fermented sausages



2.7 Regulations for processors of ground beef

In the United States, *E. coli* O157:H7 is not tolerated in ground beef and if contaminated with the organism, the beef is considered to be adulterated (FSIS, 1999) and must be recalled and condemned. In 2002 all processors of raw beef products were required to reassess their Hazard Analysis and Critical Control Point (HACCP) plan taking in to account the *E. coli* O157:H7 occurrence data and to estimate the likelihood of *E. coli* O157:H7 presence during processing. Processors involved in grinding of beef were asked to validate Critical Control Points (CCPs) and include *E. coli* O157:H7 or were required to have a prerequisite program in place to prevent entry of contaminated product into the plant.

The Food Safety and Inspection Service (FSIS) encourages raw beef testing for *E. coli* O157:H7 and when a sample is presumptive positive (awaiting final results) or confirmed positive, the processor can move the product out of the establishment only under appropriate control for disposition to a landfill or for rendering. If the processor wishes to further process the positive lot to eliminate the pathogen, the contaminated material can only be moved under company or USDA seal. The Office of Public Health and Safety (OPHS) samples ground beef from processors routinely for FSIS verification testing as well as for follow-up or trace-back. Establishments are required to hold the lot that was sampled until the FSIS results are reported negative. Beef that is imported is also subject to FSIS verification testing. If a sample tested by FSIS is positive, a decision is taken regarding retention, detention or recall of the product, and is followed by a reassessment of the HACCP plan. FSIS will ensure the establishment takes corrective

measures and will conduct follow up sampling. If retail ground beef tests positive during FSIS verification testing, the sampled lot must be recalled from the retail market.

In Canada raw ground beef processors test samples for *E. coli* O157:H7 although it is voluntary and not required by the Meat Inspection Act (Health Canada 1999). Health Canada considers that routine testing for *E. coli* 0157:H7 is not required as its prevalence in ground beef is too low (0.3 % to 1.5 %) for testing to be informative or accurate. Health Canada encourages enumeration of generic *E. coli* levels for possible fecal contamination in ground beef and samples should not have > 100 cfu/g from 5 samples from a lot or sub-lot representing different stages of manufacturing. When ground beef samples are found positive for *E. coli* O157:H7 and if the generic *E. coli* levels is > 100 cfu/g or unknown, a product recall to the consumer level is recommended. However, if a product is positive for *E. coli* O157:H7 and the generic *E. coli* levels are < 100 cfu/g (indicating proper handling) only a retail level recall is recommended. Raw beef that is recalled at the consumer level should be destroyed without contaminating the environment, but products that are recalled at the retail level may be reprocessed under Canadian Food Inspection Agency (CFIA) supervision by heat treatment > 71° C to eliminate *E. coli* O157:H7. Health Canada also recommends review of Good Manufacturing Practices and the plant HACCP plan as a follow-up to positive *E. coli* O157:H7 results in fresh beef products.

With the prevalence of *E. coli* O157:H7 being high in cattle, contamination of carcasses is more likely. Therefore, intervention strategies are essential at that level in order to control these pathogens in beef in order to prevent outbreaks, costly recalls and foodborne illness.

2.8 Guidelines and regulations governing dry fermented sausage manufacture

Prior to 1992 when guidelines for the safe manufacture of fermented dry and semi-dry (uncooked) sausages were published by the American Meat Science Association and adopted by Agriculture Canada (AAFC, 1992, CFIA, 1999), periodic contamination of these products by *Salmonella* and *Staphylococcus* occurred. By specifying the maximum time sausage meat mixes could be held > 15.6° C before the required pH of 5.3 was reached during fermentation, threats from these two groups of organisms were largely eliminated. The "degree·hour" requirements in the guidelines specified a limit of combined temperature and time, where, if temperature above 15.6° C were increased, the allowable time at that temperature was decreased. For example, the established degree·hour factor for fermentation at < 33° C is 665 degree·hours and this decreases to 555 if fermentation takes place at 33° C to 37° C. Following the guideline then at 26° C, 64h are allowed to reach pH 5.3 (26-15.6=10.4; 665÷10.4 = 64), but this drops to 32h for fermentations done at 33° C (33-15.6 = 17.4; 555÷17.4 = 32). To achieve dependable compliance with the guidelines LAB starter cultures or the acidulant glucono-delta-lactone were almost exclusively adopted for raw meat sausage fermentations in North America.

When controlled fermentations to pH 5.3 were followed, problems from the major known pathogens were eliminated. Subsequent drying (usually a month at 12-16° C) and the accompanying additional slight pH reduction determine the temperature stability of the final products. Sausages are considered shelf-stable at room temperature after drying if they have a pH $\leq$ 4.6 or water activity (a_w) $\leq$ 0.85. If the combined pH and a_w are $\leq$ 5.3 and 0.90, respectively, they are also shelf-stable at room temperature. Usually after

fermentation (green room treatment) sausages are compliant with the combined pH and a_w requirements and the degree·hour limits do not apply during the drying phase. Drying end points for commercial products divide them into two different types of sausage. Dry (uncooked) fermented sausages have about 35% moisture and a moisture/protein ratio of 1.9. They qualify as shelf-stable at room temperature (eg. Hungarian dry or Genoa salami). Semi-dry sausages have about 50% moisture and a moisture/protein ratio of 2.3. These latter require refrigeration and consist of a heterogeneous group (eg. pepperoni and various other salami types) that may have undergone only brief fermentation, and depending on pH may be cooked before drying at <15° C to achieve shelf-life and safety goals.

While the above guidelines essentially eliminated uncooked dry and semi-dry sausages as vehicles for *Staphylococcus*, *Salmonella* and *Listeria* transfer to humans (Farber et al. 1993), however, *E. coli* O157:H7 was shown to survive the manufacturing process (Glass et al. 1992). Two years later it was proven beyond doubt that *E. coli* O157:H7 could survive in uncooked dry and semi-dry sausages and cause foodborne illness when an outbreak of *E. coli* O157:H7 was linked to commercially produced dry cured salami in Washington and California in December 1994 (CDC, 1995a).

The outbreak prompted the United Sates Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) to develop new regulations governing manufacture of these products that required the processors of dry and semi-dry sausage to adopt a processing step that was sufficient to kill at least 5 log cfu/g of *E. coli* O157:H7 in sausages (Reed, 1995). A US Blue Ribbon task force on fermented sausages conducted extensive research on different manufacturing processes to control this pathogen in

fermented sausages. After the outbreaks due to contaminated raw fermented sausages in Australia (1995) and Canada (1998, 1999) the Canadian Food Inspection Agency's (CFIA) Meat Hygiene Directive (1999) adopted the FSIS rule on fermented sausage manufacture to control *E. coli* O157:H7. One of the following five options was required by both these regulatory agencies to achieve the intended 5 log reduction of *E. coli* O157:H7 numbers: (1) employ a heat processing step at 145° F (62.8° C) for 4 min; (2) use combined methods that are sufficient to validate a 5 log reduction in *E. coli* O157:H7; (3) conduct a "hold and test" of finished product; (4) adopt a HACCP program to include raw batter testing plus use of a process yielding a 2 log reduction in *E. coli* O157:H7; (5) adopt a manufacturing process that is known to kill 5 log viable *E. coli* O157:H7.

The option to develop alternate manufacturing processes that can reduce *E. coli* O157:H7 numbers by 5 log is one area that should be explored as the other options are not satisfactory from the processors point of view. Heat processing at 62.8 ° C may result in loss of identity of these products which are traditionally consumed uncooked. Adopting a "hold and test" or HACCP approach requires qualified professionals and is not always economically feasible due to the high sampling intensity required for raw batter testing.

2.9 Control of *E. coli* O157:H7

Contamination of beef with animal feces containing *E. coli* O157:H7 occurs during slaughtering operations particularly during evisceration and hide removal. *E. coli* O157:H7, if present before grinding, can be distributed throughout the meat matrix and thus adequate heat treatment of every particle of beef is required for elimination of the pathogen. Inadequate cooking of ground beef hamburger patties, particularly during

barbeques often leads to outbreaks of *E. coli* O157:H7. Many intervention strategies have been used for reducing the incidence of *E. coli* O157:H7 in ground beef and dry fermented sausages and this has resulted in an overall reduction in *E. coli* O157:H7 infection in the US (CDC, 2004). Strategies to control *E. coli* O157:H7 in ground beef and beef products should include a farm to fork approach in order to successfully reduce the incidence of *E. coli* O157:H7 in ground beef.

2.9.1 Control of *E. coli* O157:H7 on the farm

Proper hygiene in the farm environment is a key to reduce the levels of *E. coli* O157:H7 at the farm and adopting HACCP in animal production systems may result in reducing the shedding of *E. coli* O157:H7 by cattle (Buchanan and Doyle, 1997). Cattle diet has been associated with fecal shedding of *E. coli* O157:H7 (Buchko et al. 2000, Diez-Gonzalez et al. 1998, Hovde et al. 1999, Kudva et al. 1995) although it is still a subject of controversy (Magnuson et al. 2000). Diez-Gonzalez et al. (1998) reported that grain feeding increases the colonic content of volatile acids thereby providing an opportunity for *E. coli* to adapt to low pH before being shed in feces. They found that cattle fed with a high grain diet shed a higher percentage of *E. coli* which were 100,000 fold more acid resistant those from cattle fed with hay. They concluded that hay feeding before slaughter may help in reduce the acid resistance of *E. coli* and by extrapolation, *E. coli* O157:H7. A year later, Hovde et al. (1999) showed that no significant variation in acid resistance properties of *E. coli* O157:H7 could be detected between steers fed with grain or hay. Diet change, fasting and stress, all influence the shedding of *E. coli* O157:H7 from cattle (Bach et al. 2002). Use of probiotics to competitively exclude pathogenic *E. coli* O157:H7 and vaccination are two approaches attempted for reducing

the carriage of *E. coli* O157:H7 (Zhao et al. 1998, Potter et al. 2004, Younts-Dahl et al. 2004,) although bacteriophages (Kudva et al. 1999) and colicins (Murinda et al. 1996, Schamberger et al. 2004) have also been used. Zhao et al. (1998) studied 18 isolates from cattle feces that inhibited *E. coli* O157:H7 *in vitro.* A mixture of the 18 isolates was administered orally to calves and *E. coli* O157:H7 was not recovered from the rumen of animals treated with the probiotic isolates. The results indicated that administration of selected probiotic bacteria to cattle prior to exposure with *E. coli* O157:H7 reduced the frequency of *E. coli* O157:H7 carriage in animals.

Proper feeding practices and use of interventions such as probiotics may reduce the frequency and level of *E. coli* O157:H7 shed by cattle.

2.9.2 Pre-slaughter treatment

The pre-slaughter fecal load of *E. coli* O157:H7 on the hide of cattle plays an important role in the contamination of carcasses (Fegan et al. 2005). Carcasses are most frequently contaminated during hide removal operations, by equipment and manual handling. Pre-slaughter water washes of animals were shown to significantly reduce the levels of *E. coli* O157:H7 on the animal hide (Byrne et al. 2000). A combination of water wash and the antimicrobial cetylpyridinium chloride (CPC) was found to reduce the incidence of *E. coli* O157:H7 on hides thereby minimizing the risk of contaminating carcasses during evisceration (Bosilevac et al. 2004). Chemical dehairing before removal of hides has also been shown to reduce the incidence of *E. coli* O157:H7 on carcasses (Nou et al. 2003). Various studies involving use of hide washing cabinets, ozonated and electrolyzed water washing, steam treatment of hides at 80° C, and use of trisodium

phosphate were shown to be successful in reducing the *E. coli* O157:H7 levels on hides (Dickson et al. 1994, Bosilevac et al. 2005a, 2005b, McEvoy, 2001).

2.9.3 Post-slaughter carcass treatment

Trimming of carcass areas that are contaminated with feces is a common method used in slaughtering plants to reduce *E. coli* O157:H7 levels (Hardin et al. 1995), but trimming results in reduction of final carcass yield. Vacuuming of the contaminated area with steam has been successful and is widely used in the industry (Koohmaraie et al. 2005). Carcass washing with organic acids was proposed as a means to reduce the *E. coli* O157:H7 levels on carcasses (Cutter and Siragusa, 1994, Dorsa et al. 1998, Castillo et al. 2001). Castillo et al. 2001 used a 4 % L-lactic acid spray for 30s in combination with a water wash during pre- and post-chill on outside round roasts (as a model for carcasses) artificially contaminated with *E. coli* O157:H7. They found that *E. coli* O157:H7 was reduced by > 5 log on the beef surface during pre-chill and by an additional 2 log during post-chill treatment. They also reported that treatment of carcasses with lactic acid resulted in reduced *E. coli* O157:H7 numbers in ground beef prepared from the outside round roasts.

Hot water washing of carcasses with or without organic acids such as lactic or acetic acids has been shown to reduce numbers of *E. coli* O157:H7 (Dorsa et al. 1996). Use of steam pasteurization cabinets are now common in the meat industry (Phebus et al. 1997) and have been shown to reduce aerobic plate count numbers, Enterobacteriaceae, total coliforms and *E. coli* numbers when temperatures of 85 to 87.8° C were used (Retzlaff et al. 2005). Arthur et al. (2005) showed that use of low dose /low penetration electron beams at 1 kGy with penetration of 15 mm eliminated 4 log cfu/cm^2 of *E. coli*

O157:H7. Since any single method of intervention has not been sufficient to eliminate *E. coli* O157:H7 from carcasses, combinations of the above methods were proposed (Bacon et al. 2000).

2.9.4 Control of *E. coli* O157:H7 in beef trim

Beef trim serves as the raw material for ground beef and so the quality of ground beef directly depends on the quality of the trim (Murphy and Seward, 2004). Contamination during grinding from equipment or personnel is also common. A combination of water wash, lactic acid treatment at 2%, and hot air treatment of beef trim was shown to reduce numbers of aerobic bacteria, coliforms, psychrotrophic bacteria, and LAB in ground beef prepared and stored for 20d. The use of ozonated water at 1% for 15 min, 200 ppm chlorine dioxide, 5 % acetic acid, or 5% gluconic acid on beef trim have also been shown to reduce the *E. coli* levels in ground beef prepared from treated trim and stored during display at 4° C (Stivarius et al. 2002a, 2002b)

2.9.5 Control of *E. coli* O157:H7 in ground beef

2.9.5.1 Thermal treatment of ground beef

Heat treatment has been shown to be very successful in eliminating *E. coli* O157:H7 from ground beef (Doyle and Schoeni, 1984, Juneja et al. 1997). Heat treatment of ground beef to an internal temperature of 71.1° C (160 F) is recommended both by Health Canada and the FSIS. Clavero et al. (1998) reported that ground beef heated to an internal temperature of 68.3° C for 40 s was sufficient to inactivate 99.99 % *E. coli* O157:H7. Various authors have reported different D and Z values for *E. coli* O157:H7 in ground beef because different procedures for heat treatment and enumeration were used (Juneja et al. 1997, OrtaRamirez, 1997, Line et al. 1991).

Line et al. (1991) studied the D and Z values of *E. coli* O157:H7 in ground beef with different fat content. D values for *E. coli* O157:H7 in regular beef (30% fat) were shown to be significantly higher than in lean ground beef (2%). D Values at 125, 135, 145° F were 78.2, 4.1 and 0.3 min for lean beef and 115.5, 5.3 and 0.5 min for regular beef, respectively, when organisms were enumerated using plate count agar containing 1% sodium pyruvate. Fat tended to protect *E. coli* O157:H7 during cooking. The storage temperature of meat, product composition, pH, heat shock, type of strain used, and prior acid habituation were also factors reported to influence the heat sensitivities of *E. coli* O157:H7 (Juneja and Novak, 2003, Ahmed et al. 1995, Jackson et al. 1996, Williams and Ingham, 1997, Ryu and Beuchat, 1999, Smith et al. 2001, Murano and Pierson, 1992).

2.9.5.2 Irradiation

Use of irradiation of food products for preservation has been approved since 1982 at a maximum dose of 10 kGy by the FAO/IAEA/WHO joint committee on the wholesomeness of irradiated food (Giroux and Lacroix, 1998). Since 1997, the United States has allowed the use of irradiation as a means to inactivate pathogens in fresh and frozen beef at a level of 4.5 kGy and 7.0 kGy, respectively. Strict labeling regulations requiring the use of statements such as "treated with radiation" or "treated by irradiation" along with the international symbol for irradiation (the radura) (FDA, 1997) are in place. Although the Food and Drug Administration (FDA) has approved irradiation after carefully evaluating the safety of the irradiation process over many years (including the resulting chemical and nutrient changes), irradiated meats are not popular among consumers because of concerns about irradiation safety (Henkel, 1998). In Canada, onions, potatoes, spices and wheat flour are approved for irradiation treatment, but

changes in regulations to include ground beef and other meat products are still undergoing review.

Irradiation has been reported to be an effective tool to eliminate *E. coli* O157:H7 under various conditions (Lopez-Gonzalez et al. 1999, Thayer and Boyd, 1993, Clavero et al. 1994, Fu et al. 1995, Thayer and Boyd, 2001). Irradiation causes damage to DNA by forming thymine dimers and producing free radicals and hydrogen peroxide within bacterial cells thus killing them (Hussein and Sakuma, 2005).

Clavero et al. (1994) studied the effect of gamma irradiation (Cobalt-60) treatment at doses between 0 to 2.52 kGy against *E. coli* O157:H7 in ground beef. They reported a D_{10} (dose required to inactivate 90% of a microbial population) value of 0.241 to 0.317 kGy for *E. coli* O157:H7 and found that fat content did not affect the inactivation of the pathogen by irradiation. However, they noted that *E. coli* O157:H7 had a significantly higher D_{10} value when irradiated at -17 to -15 ° C than when irradiated at 3 to 5 ° C.

2.9.5.3 Use of additives in ground beef

Organic acids such as lactic and citric when used alone were shown to be less effective than when used together against *E. coli* O157:H7 in ground beef (Abdul-Raouf et al. 1993). Cutter and Siragusa (1995a) were successful in inhibiting Gram-negative bacteria in broth systems by use of nisin with chelators but reported that the combination did not produce the same results in beef (Cutter and Siragusa, 1995b).

Venkitanarayanan et al. (1999) used lactoferricin B, a hydrolyzed glycoprotein derived from milk as a natural antimicrobial for inhibition of *E. coli* O157:H7 in ground beef stored at 4 and 10° C. They found that lactoferricin B at 100 µg/g inhibited only 0.8

log cfu/ml of *E. coli* O157:H7 and thereby showed limited value of this agent for elimination of the pathogen.

Kennedy et al. (2000) used the lactoperoxidase system (LPS) as a natural alternative to chemical treatment to reduce *E. coli* O157:H7 in ground beef. They found that when the LPS system was triggered by addition of LPS components such as lactoperoxidase, thiocyanate, glucose and glucose oxidase, a 6 log reduction in *E. coli* O157:H7 occurred in ground beef stored at 12° C. In a similar study combination of the lactoperoxidase system with the glycerol monoester of lauric acid (monolaurin) was shown to be ineffective in eliminating *E. coli* O157:H7 from ground beef (McLay et al. 2002). They also reported in earlier studies that the sucrose monoesters of laurate, palmitate, stearate and oleate at concentrations up to 2.44 % in ground beef were ineffective against *E. coli* O157:H7.

Yin and Cheng (2003) used four organosulfur compounds derived from garlic, namely diallyl sulfide (DAS), diallyl disulfide (DADS), s-ethyl cysteine (SEC), and n-acetyl cysteine (NAC) against *E. coli* O157:H7 in ground beef stored at 15° C. After 6 days all four organosulfur compounds used at 10μM resulted in > 6 log cfu/g reductions of *E. coli* O157:H7 compared to the control. Ceylan et al. (2000) noted that ground beef with 0.5 to 1.5 % garlic had 90% lower *E. coli* O157:H7 numbers when heated at 51 to 72° C suggesting a synergistic effect of the addition of garlic with heat treatment.

Fang and Tsai (2003) studied the effect of nisin, organic acids (namely acetic and lactic acid) with potassium sorbate and the chelators disodium ethylenediamine tetraacetic acid (EDTA) and sodium hexametaphosphate (HMP) in combination against *E. coli* O157:H7 in stored ground beef. They noted that acetic acid and potassium sorbate

when used at > 1% and 1000 ppm, respectively, inhibited *E. coli* O157:H7 in ground beef stored at 10 and 30 ° C after 4 and 1 d, whereas the chelators failed to kill *E. coli* O157:H7 at both temperatures. When the antimicrobials were immobilized in calcium alginate gels, only lactic and acetic acid resulted in inhibition of *E. coli* O157:H7 in ground beef stored at 10 and 30° C for 9 and 1 d, respectively. Nisin in combination with EDTA or acetic acid or potassium sorbate showed enhanced reductions of *E. coli* O157:H7 at 10° C.

2.9.5.4 Use of protective cultures

Moon et al. (2002) showed that use of bacteriocin-producing *Pediococcus acidilactici* at 5 log cfu/g in combination with acetic acid at 0.25% or lactic acid at 0.35% in ground beef stored at 4° C resulted in 2.8 cfu/g reductions of *E. coli* O157:H7 compared to the control.

Vold et al. (2000) inoculated adventitious organisms isolated from ground beef at 5 to 6 log cfu/g into ground beef artificially contaminated with *E. coli* O157:H7 and stored at 12° C either aerobically or anaerobically. They found that these "background" bacteria inhibited *E. coli* O157:H7 to varying extents depending on the strain of LAB used, and higher inhibition was reported when meat was stored under anaerobic conditions. They attributed inhibition to either bacteriocin production or to the competitive nature of the background flora. Tamplin (2002) also noted a decrease in the predicted maximum population of *E. coli* O157:H7 which depended on the level of background bacteria present in ground beef stored at 10° C for 12d. In contrast with these results, Saad and Franco (1999) found that when ground beef was inoculated with *E.coli,*

Pseudomonas putida or *Leuconostoc* sp. these organisms did not affect the level of *E. coli* O157:H7 when stored at 8.5 and 25 ° C and analysed after 4 and 2 d storage, respectively.

Smith et al. (2005) reported a 1.5 log reduction in *E. coli* O157:H7 numbers in ground beef stored at 5° C for 12d when LAB at 7 log cfu/g were added. When four different LAB strains were used in combination they noted a 3 log reduction in *E. coli* O157:H7 after day 5 compared to control samples. They also reported that no changes in sensory quality were found in beef inoculated with LAB.

Some work has been done using bacteriophages for control of *E. coli* O157:H7 in beef (O'Flynn et al. 2004). However, it is unlikely that bacteriophage will find use as agents for the control of *E. coli* O157:H7 in ground meats due to adsorption of phage to fat particles which reduces their effectiveness.

2.9.6 Control of *E. coli* O157:H7 in dry fermented sausages.

The various intrinsic and extrinsic factors that influence the survival of *E. coli* O157:H7 in dry fermented sausages should be taken in to consideration while planning a control strategy. These factors include the initial level of *E. coli* O157:H7 contamination; the extent of antimicrobial production (lactic acid or bacteriocins) by the starter cultures used; the temperature and duration of fermentation and drying; the concentration of various curing agents like salt, sodium nitrite and spices; temperature and length of storage; type of packaging; the internal temperature reached if a thermal processing step is included; and addition of other chemical or natural antimicrobials.

2.9.6.1 Effect of fermentation and drying of meat on *E. coli* O157:H7

Experiments have been conducted by altering the fermentation and drying conditions during sausage manufacture to achieve temperature-time combinations that

maximize *E. coli* O157:H7 lethality. However, fermentation and drying were only capable of causing a reduction of about 1-2 log units in the numbers of *E. coli* O157:H7 during the manufacture of pepperoni (Hinkens et al. 1996, Riordan et al. 1998, 2000), salami (Glass et al. 1992, Clavero and Beuchat 1996, Nickelson et al. 1996, Faith et al. 1998) and summer sausage (Caligioglu et al. 1997).

Glass et al. (1992) were the first to show that *E. coli* O157:H7 was capable of surviving the dry sausage manufacturing process. They inoculated a sausage batter containing 3.5 % salt and 156 μg $NaNO_2$ with a 5 stain cocktail of *E. coli* O157:H7 (~ 5 x 10^4 cfu/g). The sausage was fermented (after stuffing into casings) using a commercial starter culture to a pH of 4.8 and dried at 12.8° C (70% RH) to a moisture protein ratio of ≤ 1.9:1. Although *E. coli* O157:H7 did not increase in number, they survived the processes of fermentation, drying and also vacuum packed storage at 4° C for 2 months. Only a 1 $\log_{10}$ cfu/g reduction in *E. coli* O157:H7 numbers was found at the end of fermentation and drying and a greater than 1 log reduction in *E. coli* O157:H7 numbers was noted at the end of storage. In the same experiment they showed that *E. coli* O157:H7 survived a pH of 4.6 (adjusted with lactic acid) for ≥ 14 days in tryptic soy broth and suggested that the acid tolerance of these pathogens may be one of the reasons for their survival in dry fermented sausages.

Cosansu and Ayhan (2000) showed that *E. coli* O157:H7 decreased by > 3 $\log_{10}$ cfu/g after fermentation of Turkish soudjouck sausage (which contains ground beef as an ingredient) at 24±2° C (90 to 95 % RH) for 3d followed by drying at 22±2° C and 80 to 85 % RH for 5d. A further decrease of only 0.2 log cfu/g was evident after storage under vacuum at 4° C for 2 months, whereas in non-vacuum packed sausages *E. coli* O157:H7

were undetectable after the storage period. Naim and co-workers (2002) found that inoculation of *E. coli* O157:H7 in meat batter at 10^7 cfu/g and subsequent fermentation at 37 ° C or 24° C (pH < 5.0) and drying at 14° C to 0.9 or 0.8 a_w resulted in a 1.4 log cfu/g reduction in *E. coli* O157:H7 numbers. They also noted that fermentation at 37° C followed by holding at 37° C for 5d resulted in > 5 log reduction in *E. coli* 0157:H7 numbers in products dried to 0.8 a_w (Naim et al. 2003).

Tomicka et al. (1997) studied the combined effect of starter culture (10^7 cfu LAB/ml), dextrose (0.8%), NaCl (2 %), nitrite (200 ppm) and temperature (37 and 22° C) on the survival of E. *coli* O157:H7 (10^0 to 10^5 cfu/ml) in BHI broth. Two systems which modeled the American style high temperature, short time fermentation process (37° C for 1 d) and the European style low temperature, long time (22° C for 3 d) processes for sausage fermentation were studied. After fermentation, the samples were stored at 10° C. In the American type model *E. coli* O157:H7 survived >34 days in BHI with starter and dextrose and >54 days in BHI containing starter, dextrose, NaCl and sodium nitrite. In the European type model *E. coli* O157:H7 did not survive past 9 d in BHI containing starter culture and dextrose. Similarly in BHI containing starter, dextrose, NaCl and sodium nitrite, at the lower inoculation level ($\leq 10^4$ cfu/ml) the organism did not survive > 9 d, however at high inoculum levels (10^5 cfu/ml) the organism survived > 30 d. They concluded that lower temperature and longer fermentation time results in greater lethality to *E. coli* O157:H7 compared to high temperature and short fermentation time.

It is important to note that in some of the above experiments (Cosansu and Ayhan 2000, Naim et al. 2003) time/temperature combinations used during fermentation were beyond those allowed by AAFC (1992) and could allow the growth of other pathogens

like *Salmonella, Listeria, Staphylococcus* and *Yersinia* which would generate hazardous products.

2.9.6.2 Effects of curing agents and hurdle technology

The type and concentration of curing agents such as salt, nitrite, potassium sorbate, and ascorbates present were shown to affect the survival of *E. coli* O157:H7 in Chinese style sausages (Yu and Chou 1997). Riordan et al. (1998) studied the effect of pH, salt and sodium nitrate on the survival of *E. coli* O157:H7 in pepperoni and found that at 2.5% salt, 100ppm $NaNO_2$ and pH 4.8 only a 0.8 log cfu/g reduction in *E. coli* O157:H7 numbers was obtained after fermentation and drying, but at a pH of 4.4-4.6 and 3.3% salt plus 300 ppm nitrite, a 4.79 log cfu/g reduction in *E. coli* numbers was found when enumerated on TSA with Sorbitol MacConkey agar (SMAC) overlay. The reduction of *E. coli* O157:H7 was greater when enumeration was done using selective SMAC media without overlay indicating the presence of injured cells that would not be enumerated by the selective agar alone.

Casey and Condan (2000) used a fermented meat model medium to study whether elimination or reduction of nitrite concentration in fermented sausage was likely to affect the growth or survival of *E. coli* O157:H4, a verotoxin negative relative of *E. coli* O157:H7. When the pH of the medium was decreased by growth of *P. acidilactici*, survival of *E. coli* O157:H4 was dependent on nitrite levels and growth was inhibited at ≥ 100 ppm of nitrite. After 2 d in the presence of 200 ppm nitrite, the number of survivors was 1000-fold less than in the absence of nitrite. In the laboratory scale fermented sausage in the presence of 200 ppm nitrite, *E. coli* O157:H4 levels decreased 100 times faster than in its absence.

A strategy to eliminate *E. coli* O157:H7 during fermented sausage manufacture based on increasing the concentration of a single curing agent may not be successful, as the increased levels would result in a product unacceptable in organoleptic quality (Riordon et al. 2000). The latter authors suggested that multiple hurdles such as changes in product formulation to yield low pH, 3.3 % salt, 100 ppm sodium nitrite and addition of sodium lactate together with a mild heating step (40° C) could be effective and may satisfy regulatory agencies and the sensory expectations of consumers. Following such an approach, Uyttendaele et al. (2001) investigated growth of *E. coli* O157:H7 exposed to sub-optimal factors in combination (22° C, 7° C or -18° C with 0.5% NaCl, 5.0% NaCl at pH 7.0 or pH 4.5 by the addition of lactic acid) in a simulated beef gravy medium. They observed prolonged survival of *E. coli* O157:H7 during imposition of stress as a number of growth factors became sub-optimal. Therefore, they concluded that application of the hurdle concept for preservation of food might inhibit growth but could cause prolonged survival of *E. coli* O157:H7 in minimally processed foods.

2.9.6.3 Influence of storage and packaging on *E. coli* O157:H7 in dry fermented sausages

2.9.6.3.1. Storage temperature

Clavero and Beuchat (1996) inoculated *E. coli* O157:H7 (3.56 to 4.94 log cfu/g) in sliced salami which was stored at 5 and 20° C for 32 d. The numbers of *E. coli* O157:H7 were reduced by 2 and 5 log cfu/g respectively at 5 and 20° C. Extended storage of fermented sausage (pH 4.6) at 32° C reduced numbers of *E. coli* O157:H7 by at least 4 log cfu/g (Nickelson et al. 1996). Lahti et al. (2001) studied the effect of freezing on the viability of *E. coli* O157:H7 in dry sausage and found that when samples

containing low numbers (<3.2 log cfu/g) of *E. coli* O157:H7 were analyzed by enrichment after freezing (-70° C) and thawing, *E. coli* O157:H7 was most often detected with a 24 h enrichment. Even when the samples were stored in the freezer for several months, *E. coli* O157:H7 was still isolated after 24 h enrichment (< 10 cfu/g) from 20 of 23 samples. Others have also found that survival of *E. coli* O157:H7 in acid foods (dry fermented sausages, mayonnaise) stored at refrigeration temperature was prolonged compared to survival at ambient temperatures (Rocelle et al. 1996, Zhao and Doyle, 1994). Lahti et al. (2001) concluded that *E. coli* O157:H7 could survive freezing in dry sausages. The survival of *E. coli* O157:H7 at cold temperatures may be due to production of cold shock proteins (Bollman et al. 2001)

Clavero and Beuchat (1996) showed that in salami, *E. coli* O157:H7, if present at ≤ 100 cfu/g would be unlikely to survive storage at 5° C for 32 d. However, contamination of salami with *E. coli* O157:H7 at 10^4 to 10^5 cfu/g after processing would pose a health risk to consumers even after 32 d, if storage were at 5° C. In a similar study, Nissen and Holck (1998) studied the effect of storage on *E. coli* O157:H7 viability in Norwegian sausage. They inoculated raw sausages (8 cm diameter and 50 cm length) of approximately 2.7 kg with two levels of *E. coli* O157:H7 and fermented them at 27° C for 4d followed by drying at 14° C for 15d. The pathogen was not detected after 5.5 months storage at either 4 or 20° C if initially present at low levels (10^3 cfu/100g). However, at high levels of *E. coli* O157:H7 (10^5-10^7 cfu/100g), the organisms were present at 500 cfu/100g after 5.5 months storage at 4° C. In other work with dry fermented sausage it was determined that if *E. coli* O157:H7 were initially present in low numbers (< 100

cfu/g) then it would be unlikely to survive storage for 3-4 weeks at 5-7° C (Rocelle et al. 1996).

Storage at ambient temperature was also found by Faith et al. (1997, 1998) to be more effective in controlling *E. coli* O157:H7 than storage at 4° C. However, long term storage at ambient temperature may not be beneficial from a spoilage point of view. Long term storage at room temperature may increase oxidative deterioration resulting in undesirable sensory and quality changes in the sausage (Stiebing et al. 1999). Safety related to other pathogens such as *Staphylococcus aureus* should also be considered when storing at room temperature (Nissen and Holck, 1998) but this should not be an issue provided current "degree·hour" requirements for sausage manufacture (AAFC, 1992) have been followed.

2.9.6.3.2. Type of packaging

Faith et al. (1997) inoculated a pediococcal starter (10^8 cfu/g) and a 5 strain cocktail of *E. coli* O157:H7 ($\geq 2x\ 10^7$ cfu/g) into pepperoni batter and fermented the mixture at 36° C (85% RH) to pH < 4.8 and then dried the sausage at 13° C (65% RH) to a moisture:protein ratio of 1.6:1. Fermentation and drying resulted in a 2 log unit reduction. At 21° C storage, *E. coli* O157:H7 decreased to about 2 and 3.8 log cfu/g within 14 d in products stored under vacuum and in air, respectively, and a 5 log reduction was observed for both atmospheres within 28 d. Regardless of the storage atmosphere, numbers did not decrease below 3.6 or 3.7 log cfu/g after 90 d of storage at -20 or 4° C, respectively.

Yu and Chou (1998) subjected Chinese style sausage to air, vacuum or nitrogen packaging and stored them at 5 or 25° C. *E. coli* O157:H7 in sausages decreased 1.16-

1.55 log cfu/g after 40 d of storage at 5° C. Viable cells of *E. coli* O157:H7 declined more rapidly in sausage stored at 25° C than at 5° C. No viable *E. coli* O157:H7 was detected in either vacuum-packaged or nitrogen-packaged sausage after 40 d of storage at 25° C but numbers of *E. coli* O157:H7 were reduced to non-detectable levels in air packaged sausages after 20 d of storage at 25° C. Refrigerated storage and vacuum or nitrogen packaging provided conditions that retarded the decline in viability of *E. coli* O157:H7 in sausages. Cosanu and Ayhan, (2000) showed that in Turkish soudjouck, survival of *E. coli* O157:H7 was longer in vacuum packaged samples (> 2 months) than non-vacuum packed samples (> 1 mo.).

Whey protein isolate (WPI) films (pH 5.2) containing p-aminobenzoic acid (PABA) and 0.75 % or 1.0 % sorbic acid were found to decrease the *E. coli* O157:H7 population from an initial level of 6 $\log_{10}$ cfu/g to approximately 3-3.5 $\log_{10}$ cfu/g in summer sausage and bologna after 10 days of storage at 4° C (Cagri et al. 2001). PABA and sorbic acid (0.5:0.5) yielded greater reductions in summer sausage (3.6 $\log_{10}$ cfu/g) than in bologna (3.1 $\log_{10}$ cfu/g).

2.9.6.4. Thermal or irradiation treatments for inactivation of *E. coli* O157:H7 in dry sausages

Hinkens et al. (1996) inoculated a five strain mixture of *E. coli* O157:H7 (≥ 2x 10^7 cfu/g) into pepperoni batter which was stuffed in casings and fermented at 36° C (85% RH) to pH ≤ 5.0 and then dried at 13° C (65% RH) to a moisture: protein ratio of ≤1.6:1. *E. coli* O157:H7 numbers decreased by 1.2 log cfu/g after fermentation and drying. Treatment at 63° C (instantaneously) or 53° C for 60 min resulted in ≥ 5 log cfu/g decrease in *E. coli* O157:H7 without visibly affecting sausage texture and appearance.

This study formed the basis for the regulation involving validation of dry fermented sausages by heat treatment.

Calicioglu et al. (1997) fermented semi-dry beef summer sausage inoculated with *P. acidilactici* and a five strain mixture of *E. coli* O157:H7 at an initial temperature of 29° C which was increased to 41° C over approximately 13 h (80 % RH) with a final pH of 4.6 or 5.0. After fermentation the temperature was raised to 54° C at 60 % RH. Fermentation alone resulted in only a 1.39 log cfu/g reduction while fermentation to pH 4.6 and heating to an internal temperature of 54° C reduced numbers of *E. coli* O157:H7 by > 6 log cfu/g. In contrast, fermentation to pH 5.0 and heating to 54° C resulted in only a 3.2 log unit decrease of *E. coli* O157:H7. Similar results with *E. coli* O157:H7 following exposure to increased heating time or temperature when sausages were fermented to low pH were reported by others (Ellanjosyula et al. 1998, Riordan et al. 2000). Non-acid adapted cells were generally more resistant to heat than acid-adapted cells and sensory trials indicated that pepperoni heated at 58.3 ° C for 61.3 min was different from non-heat treated pepperoni, however pepperoni heated at 61° C for 17.9 min was similar to the non-heat treated product (Riordan et al. 2000).

Though post fermentation heating can yield more than a 5 log unit reduction of *E. coli* O157:H7, heating, apart from increasing the production cost will cause undesirable changes in the texture and taste of semidry and dry sausage products apart from losing the identity of the traditional products (Lahti et al. 2001, Johnson et al. 2000, Riordan et al. 1998, 2000).

Johnson et al. (2000) reported that the irradiation of meat before dry fermented sausage manufacture at doses of 1.5-3.0 kGy to resulted in a 5 log reduction of *E. coli*

O157:H7, but the sensory quality of the sausages was altered. Irradiation of meat products may have produced free radicals and initiated oxidative reactions resulting in poor sensory quality (Murano, 1995).

2.9.6.5. Selection of starter cultures to inhibit *E. coli* O157:H7

In addition to production of lactic acid, LAB starter cultures may produce bacteriocins or other low molecular mass compounds which are inhibitory to pathogenic organisms. The bacteriocins of LAB are mostly effective only against Gram-positive bacteria (Jack et al. 1995) but some low molecular mass antimicrobials are able to penetrate the outer membrane of Gram-negative organisms (Helander et al. 1997) and therefore, may have antimicrobial effects against *E. coli* O157:H7. Sickle and Sofos (1996) evaluated three LAB, *Leuconostoc mesenteroides* LM3, *Lactococcus lactis* subsp. *lactis* DL 16, and *P. acidilactici* F for potential inhibitory activity against 8 strains of *E. coli* O157:H7. *P. acidilactici* F metabolites were not inhibitory to *E. coli* O157:H7, whereas metabolites other than hydrogen peroxide produced by the other two LAB were slightly inhibitory to all 8 strains of *E. coli* O157:H7 tested.

Kang and Fung (1999) stimulated acid and bacteriocin production by *P. acidilactici* using manganese (Mn) ions at 0.0005% and oxyrase at 0.3 units/g. They found 3 log reductions of *E. coli* O157:H7 after 24 h fermentation at 40° C when Mn alone was used. Oxyrase alone yielded 2.2 log reductions, whereas the combination yielded 3.35 log reductions of *E. coli* O157:H7. Later, Kang and Fung (2000) showed that use of Mn with *P. acidilactici* also resulted in increased reductions of *Listeria(L.) monocytogenes* and *S. aureus* by an additional 1 log after fermentation at 40° C for 24h at

RH 96% compared to use of the starter culture alone. Starter culture requirements for Mn ions were also reported by others (Hagen et al. 2000).

Lahti et al. (2001), compared the effect of two starter cultures, commercial culture mixture A (*Staphylococcus xylosus* DD-34, bacteriocin producing *P. acidilactici* PA-2 and *Lb. bavaricus* MI-401) and commercial culture B (*S. carnosus* MIII and *Lb. curvatus* Lb3) on the survival of *E. coli* O157:H7 during dry sausage manufacture. Sausages were fermented in a smoke chamber at 17-23° C for 15 d and then stored at 15-17° C for 34 d. Results showed that the reduction of *E. coli* O157:H7 was greater with starter culture B than starter culture A. When starter culture A was used the numbers of *E. coli* O157:H7 in low inoculum sausages (2.30 log cfu/g) decreased below 1.0 log cfu/g in 21 d whereas with starter culture B, the same decrease was achieved in 14 d. With starter culture A, fermentation and storage for 49 d reduced the concentration of *E. coli* O157:H7 in the high inoculum sausages (5.46 to 5.68 log cfu/g) by 2.79 log cfu/g, whereas the reduction with starter culture B was 4.8 log cfu/g. They concluded that when starter culture B was used, the reduction of *E. coli* O157:H7 was two-fold greater compared to the previous studies by Glass et al. (1992), Hinkens et al. (1996), Calicioglu et al. (1997), and Faith et al. (1997, 1998). Neither of the tests used by Lahti et al. (2001) followed the current regulations of AAFC, and therefore the results of these experiments may not be of value for application by industry.

Pidcock et al. (2002) studied the possibility of using non-traditional meat starter cultures such as *Lb. acidophilus*, *Lb. paracasei*, and *Bifidobacterium lactis*. They noted that when these cultures were used in combination with the more traditional *P.*

pentosaceus starter, > 2.5 log reduction in *E. coli* O111 and *L. monocytogenes* were obtained depending on the cultures used, after 7 d fermentation.

Callewaert et al. (2000) used bacteriocin producing *Enterococcus* (E.) *faecium* and *Lb. amylovorin* strains as starter cultures for control of *Listeria* spp. during sausage fermentation and compared the results with those obtained following use of *Lb. sakei*. *E. faecium* produced the bacteriocin, enterocin, *Lb. amylovorin* produced amylovorin and *Lb. sakei* produced sakacin. *Lb. amylovorin* was outgrown by background bacteria in the sausage batter during fermentation and thus was found unsuitable for sausage manufacture. They also found *Listeria* were reduced by 3.25 log at the end of fermentation and maturation when *E. faecium* was used, whereas *Lb. sakei* yielded only a 1.5 log reduction. Earlier, Leroy and Vuyst (1999a) found that with *Lb. sakei,* the temperature and pH normally used during dry fermented sausage manufacture were suitable for the production of antilisterial bacteriocin sakacin. Other factors may have caused the relatively poor performance of *Lb. sakei* against *Listeria* in the study reported by Callewaert et al. (2000). In a similar study Benkerroum et al. (2005) showed that use of freeze dried bacteriocin producing *Lb. curvatus* and *Lactococcus lactis* subsp. *lactis* resulted in significant decrease in *L. monocytogenes* during dry fermented sausage manufacture.

Although the above two studies suggested that bacteriocin producing starter or adjunct cultures could be used as options to control pathogens in dry fermented sausages, other studies have reported that use of other bacteriocins including nisin were ineffective in meat decontamination due binding of the bacteriocin to components in the meat matrix, improper distribution, poor solubility or inactivation by meat enzymes (Kang and

Fung, 1999). The conditions during fermented sausage manufacture such as high salt and nitrite content may reduce the efficiency of bacteriocin production by starter cultures (Leroy and Vuyst, 1999b). The decrease in bacteriocin efficacy in the presence of salt may be due to interference of NaCl molecules with the receptors essential for the action of bacteriocins (Nilsen et al. 1998).

2.9.6.6 Probiotics and dry fermented sausages

Probiotic LAB having known health promoting effects in addition to having the ability to reduce pH are widely used in dairy products but not in meat products. A variety of LAB cultures including lactobacilli and bifidobacteria exert probiotic effects on health by establishing themselves as part of the natural intestinal microflora, relieving diarrhea, modulating cholesterol uptake and stimulating the immune system (Naidu et al. 1999). Very limited work has been done on the use of probiotics in dry sausage (Erkkila et al. 2000, 2001).

Lb. gasseri (Arihara et al. 1998), *Lb. paracasei* subsp. *paracasei* (Sameshima et al 1998), *Lb. rhamnosus* (Erkkilä et al. 2000), *Lb.plantarum* and *Lb. pentosus* (Klingberg et al. 2005), and *Lb. casei* and *B. lactis* (Anderson, 1998) have potential for probiotic use in fermented meats. The future for use of sausages as carriers of probiotics has been considered promising for some time now (Incze, 1998) and the use of probiotic bacteria which could also produce antimicrobials effective against *E. coli* O157:H7 was also suggested (Työppönen et al. 2003).

Sameshima et al. (1998) used the probiotic organisms *Lb. rhamnosus* and *Lb. paracasei* isolated from the human intestine for control of *S. aureus* during the manufacture of fermented sausage. When fermentation was done at 20° C for 3d, *S.*

aureus numbers increased by 2.9 log cfu/g in the control and enterotoxin was produced. However, when *Lb. rhamnosus* and *Lb. paracasei* were used, the growth of *S. aureus* remained < 3.8 log cfu/g and enterotoxin was absent from the product.

Erkkilä et al. (2000) studied three strains of probiotic *Lb. rhamnosus* for their inhibitory action against *E. coli* O157:H7 during the manufacture of dry fermented sausages. When used alone to ferment the meat, *E. coli* O157:H7 decreased by 3 log cfu/g at the end of maturing, but the numbers did not significantly differ from the control where a *P. pentosaceus* meat starter culture was used. They also found that the probiotic strains fermented the sausages as well as the control and no adverse effects on flavor profiles were noticed (Erkkilä et al. 2001). These studies show that it may be possible to use probiotic cultures as protective cultures during manufacture of dry fermented sausages.

2.9.6.7 Use of natural antimicrobials in dry fermented sausages

Natural antimicrobials such as essential oils from plant sources have been shown to be effective against *E. coli* O157:H7 in various meat and meat products (Holley and Patel, 2005). Gagné et al. (2003) used a combination of the essential oils oregano and thyme at 0.3% each, following encapsulation in 10% colloidal starch at ratio of 1:1 to inhibit *E. coli* O157:H7 during fermented sausage manufacture. They fermented the sausages according to AAFC regulations (1992) followed by drying to an a_w of 0.9 and stored the sausage at room temperature for 1 month. They found > 5 log reduction in *E. coli* O157:H7 after 4 weeks storage at room temperature, but only a 1.7 log reduction was found after the initial drying step was completed when essential oils were used. Thus essential oils have use in dry fermented sausages only if sausages can be held at room

temperatures for one additional month after product manufacture. Problems with storage at ambient temperatures were already discussed.

2. 10 *Lb. reuteri* as a protective culture

2.10.1 *Lb. reuteri* characteristics

Lb. reuteri which was earlier classified as *Lb. fermentum* was recognized and named in 1980 after the German bacteriologist G. Reuter (Kandler et al. 1986) and its classification is based on differences in mol% G +C of the DNA and presence of lysine as the diamino acid of peptidoglycan. *Lb. reuteri* is a Gram positive, facultatively anaerobic, slightly irregular rod with rounded ends and occurs either singly, in pairs or in small clusters. *Lb. reuteri* is a normal inhabitant in the gastrointestinal tract of humans, swine, poultry and other animals (Axelsson et al. 1989). They are also present in variety of foods like milk, cheese, meat and sourdough (Kandler and Weiss, 1986). There is no regulation at present that prohibits or restricts use *of Lb. reuteri* in food systems. *Lb. reuteri* is accepted as safe in food in Japan and Europe (Anonymous, 2002). A commercial product containing live *Lb. reuteri* is approved by the FDA for use in food for children above 2 years of age (FDA, 2001).

2.10.2 Production of reuterin

Growing as well as non-growing *Lb. reuteri* cells produce a non-peptide low molecular mass antimicrobial substance, β-hydroxypropionaldehyde (or reuterin), by anaerobic fermentation of glycerol. Reuterin has a broad spectrum of activity against Gram-positive and Gram-negative bacteria, including *E. coli* O157:H7 (Axelsson et al. 1989, Chung et al. 1989, Talarico and Dobrogosz, 1989). *Lb. reuteri* is a hetero-fermentative organism and during fermentation of glucose yields lactic acid, acetic acid,

ethanol and carbon dioxide. In the presence of glycerol and under anaerobic conditions *Lb. reuteri* converts glycerol into reuterin plus 1,3 propanediol and low levels of β-hydroxypropionic acid (Talarico et al. 1988). Reuterin occurs as an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of β-hydroxypropionaldehyde (Figure 2.2). Reuterin is produced by an alternate pathway during glycolysis when glycerol is present in addition to glucose (Figure 2.3). The enzyme glycerol dehydratase catalyses the convertion of glycerol to reuterin. Reuterin is used as an electron acceptor and is converted through aldehydic dismutation by glycerol oxidoreductase to form 1, 3 propanediol as long as NADH is available. When NADH is depleted due to lack of glucose, reuterin is produced and accumulated (Talarico et al. 1988). Co-fermentation of glycerol with low levels of glucose (8 mmol/L) leads to production and accumulation of reuterin by stationary phase cells of *Lb. reuteri* but the presence of glucose concentrations normally present in growth media represses reuterin synthesis. When glucose was present at 20 mmol/L with glycerol, growing cells of *Lb. reuteri* were able to produce reuterin (El-Ziney et al. 1998). Glycerol at levels > 150 mmol/L is required for reuterin production in the absence of glucose. The optimum pH and temperature for production of reuterin varies between pH 5-9 and 9-45° C, respectively, with maximum production at pH 7 and 37 ° C (El-Ziney et al. 2000). In addition to pH and temperature the production of reuterin is affected by the relative concentrations of glycerol and glucose, the type of other sugars present, the growth phase of *Lb. reuteri* and the presence of other organisms (Chung et al. 1989, Talarico et al. 1988, EL-Ziney et al. 2000, de Valdez et al. 1997). Reuterin is reported to be resistant to

heat, a wide range of pH as well as proteolytic enzymes and therefore it may act as a useful biopreservative in food (El-Ziney et al. 2000, Rasch 2002).

Fig 2.2 Reuterin occurs as an equilibrium mixture of monomeric (a), hydrated monomeric (b), and cyclic dimeric forms of β-hydroxypropionaldehyde (El-Ziney et al. 2000).

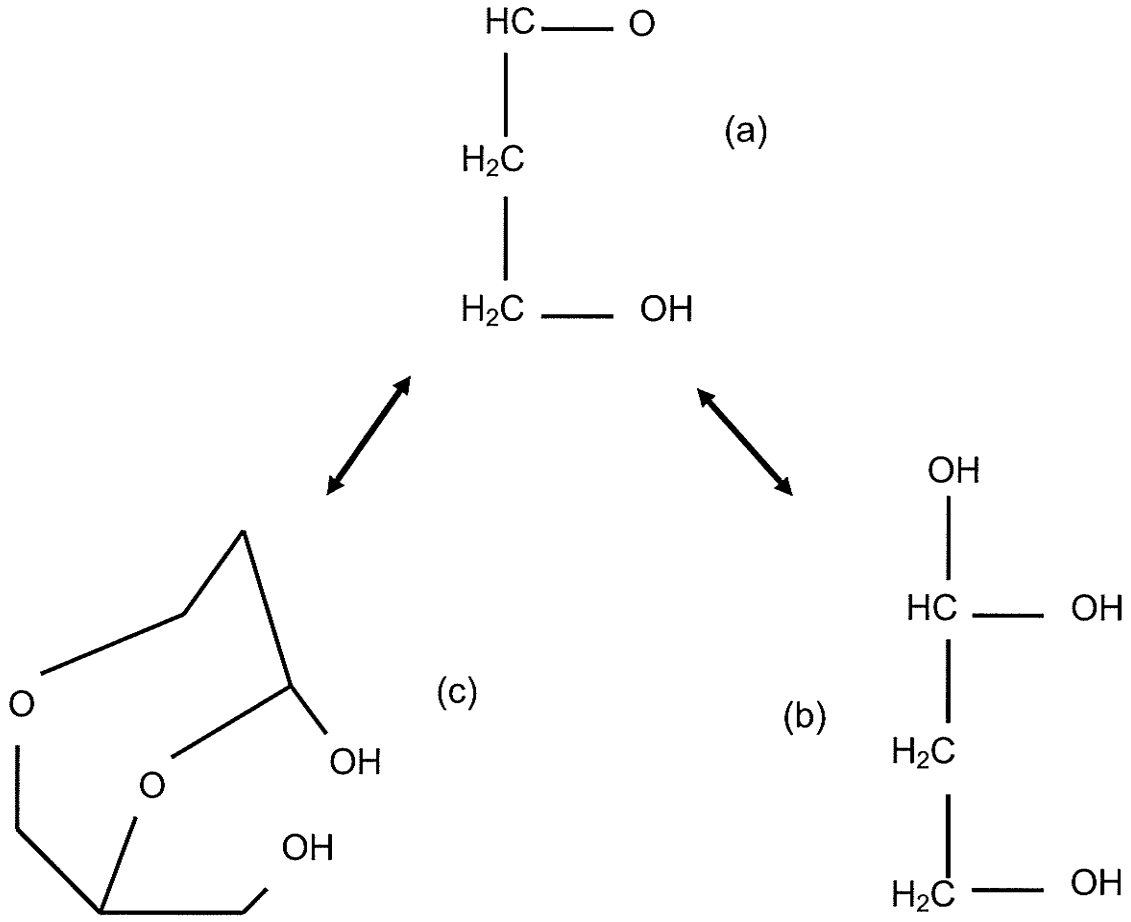
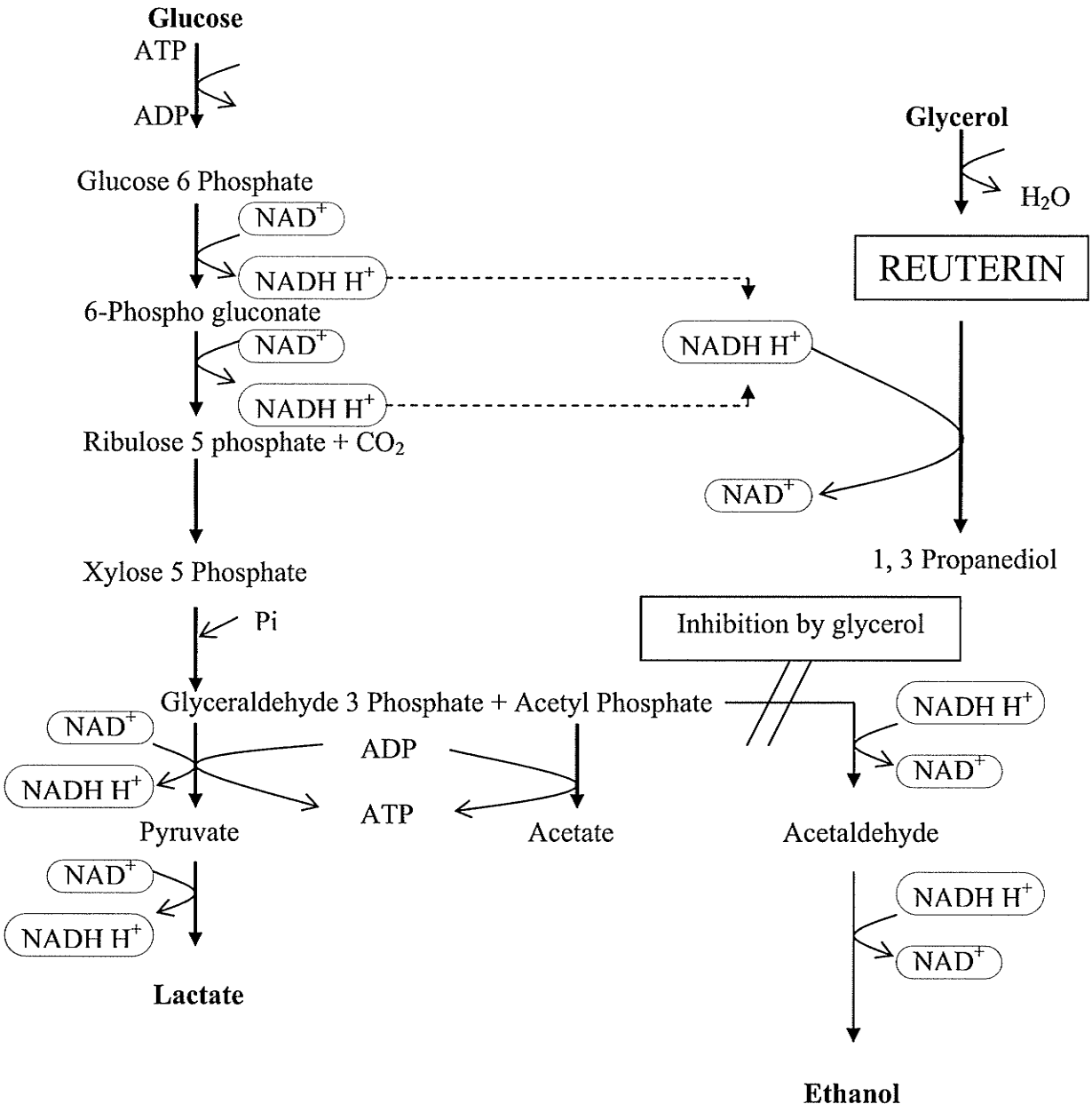


Fig 2.3 Co-fermentation of glycerol and glucose by *Lb. reuteri*



Adapted from El-Ziney et al. (2000)

2.10.3. Bactericidal effects of *Lb. reuteri* and reuterin

Dobrogosz (1988) studied the effect of reuterin on coliforms in ground beef stored at 4° C for 6 days. He noticed a reduction in viability of coliforms by 0.5 and 2 log at 50 and 100 arbritrary units (AU)/g of reuterin, whereas in the control coliforms increased by 4 cfu/g. Later Lindgren and Dobrogosz (1990) used a reuterin producing strain of *Lactobacillus reuteri* 16003 in the presence of glycerol to study its effect on shelf-life of herring fillets. The fillets were dipped into a culture containing $1x10^9$ cfu/ml of *Lb. reuteri* and 250 mmol/L glycerol and were stored at 5° C under 100% nitrogen. The level of Gram negative spoilage bacteria decreased by 3 log cfu/g by 6d and it was concluded that reuterin could improve product shelf-life.

El-Ziney et al. (1999) determined that the minimum inhibitory concentration (MIC) of reuterin for *E. coli* O157:H7 was 4 AU/ml and for *L. monocytogenes* it was 8 AU/ml. In the same study they examined the potential for reuterin to reduce viable *E. coli* O157:H7 and *L. monocytogenes* on cooked pork surfaces. They inoculated the surface of cooked pork with these two pathogens at 5 log cfu/cm^2 and challenged them with 500 AU/ml reuterin. They found reductions of 2.7 and 0.63 log cfu/cm^2, respectively, for *E. coli* O157:H7 and *L. monocytogenes*. In a similar experiment they found that when reuterin at 100 AU/ml was added to raw ground pork it yielded a 5 log cfu/g reduction in *E. coli* O157:H7 after 1 d at 7° C. However, 250 AU/ml reuterin were required to bring about a 3 log cfu/g reduction in *L. monocytogenes* after 7 d at 7° C. Lactic acid at 5% (v/v) along with 500 AU/ml reuterin acted synergistically in inhibiting *E. coli* O157:H7 and *L. monocytogenes* after 15 s at 7° C. In contrast, ethanol at 40 % (v/v) did not show any additive or synergistic effect with reuterin against these organisms.

El-Ziney and Debevere 1998 found that addition of reuterin at 50, 100 and 150 AU/g resulted in 2, 3, and 6 log reductions of *E. coli* O157:H7 in creamed cottage cheese stored at 7° C by day 7, whereas reuterin at 100, 150 and 250 AU/g resulted in reduction of *L. monocytogenes* by 2, 5, and > 5 log cfu/g, respectively, by 7d at 7° C. In the same study it was found that use of reuterin at 150 AU/ml in UHT milk stored at 7° C for 3 d resulted in > 3 log cfu/ml reductions of *L. monocytogenes*. They also noted that 0.5-3 % fat did not affect the antimicrobial activity of reuterin, however, 3% salt resulted in a synergistic effect against *L. monocytogenes*.

2.10.4 Probiotic properties of *Lb. reuteri*

Probiotics have been known for almost a century to aid in establishing a beneficial intestinal microflora and can provide a variety of health benefits. *Lb. reuteri,* a normal inhabitant of the human GI tract is considered a probiotic organism because of its stimulation of host immunity against infectious agents, its excellent ability to colonize intestinal epithelium tissue, its resistance to bile, and for its potential hypocholesterolemic effects (Taranto et al. 1998, Casas and Dobrogosz, 2000). *Lactobacillus reuteri* is resistant to bile salts and this allows the organism to survive passage through the gastrointestinal (GI) tract and colonize the GI tract in humans and exert its probiotic effects (Fuller et al. 1997). Administration of *Lb. reuteri* at the level of 10^{10}-10^{11} cfu/day to infants aged 6-36 months resulted in increased colonization and reduced duration of rotavirus-induced diarrhea in the children (Shornikova, 1997a, 1997b).

Jacobsen et al. (1999) screened 47 strains of *Lactobacillis* species for probiotic characteristics such as survival in the GI tract, colonization potential, resistance to bile

and pH, and antimicrobial activity against enteric pathogens. A strain of *Lb. reuteri* was found to survive gastric passage and showed good colonization of Caco-2 cells. This strain had strong antimicrobial activity against pathogens such as *L. monocytogenes, S. aureus, Bacillus cereus, E. coli, Salmonella typhimurium, Shigella flexneri* and *Yersinia enterocolitica*, whereas it did not show any antimicrobial activity against normal intestinal microbiota.

De Smet et al. (1998) found that when pigs were fed *Lb. reuteri* cells possessing active bile salt hydrolase (BSH) for 4 weeks, significant reductions in total and LDL – cholesterol occured indicating that *Lb. reuteri* had anticholesterolemic activity. Similar cholesterol lowering effects were also found when mice were fed with *Lb. reuteri* at 4 log cfu per day for 7 d (Taranto et al. 1998). *Lb. reuteri* is the only bacterial species known to secrete reuterin into the surrounding environments in sufficient quantity to provide antimicrobial activity against bacteria, protozoa, yeast and fungi (Talarico and Dobrogosz, 1989).

Casas et al. (1998) evaluated the probiotic potential of *Lb. reuteri* in poultry. They showed that following administration, *Lb. reuteri* colonized both the chicken and turkey GI tract, reduced incidence of stress-induced avian growth depression (AGD), stimulated the host's cell-mediated and humoral immune responses and protected host animals against bacterial, viral and protozoan challenges. There is also evidence to show that *Lb. reuteri* reduced *Cryptosporidium(C.) parvum* diarrhea in piglets and enhanced resistance to *C. parvum* infection in mice (Casas et al. 1998). *Lb. reuteri* confirms Metchinoff's proposal of the probiotic concept (Casas and Dobrogosz 2000).

2.11. Survival of probiotics

Consumer preference for probiotic-containing over standard products has resulted in the appearance of increased numbers of probiotic products on supermarket shelves in recent years. The survival of probiotic organisms in food products that serve as the carrier is essential. Various research studies have shown that probiotic organisms survive poorly in foods like yoghurt and fermented milks (Kailasapathy and Rybka, 1997, Klaver et al. 1993, Hughes and Hoover, 1995). The pH of products such as yoghurt may drop to 4.5 or even lower, making it difficult for the organisms to grow or survive in these environments. Bifidobacteria are not tolerant to oxygen and low pH (Gomes and Malcata 1999) compared to lactobacilli and there is strain to strain variation in survival in food products and at low pH (Roa et al. 1989, Shah et al. 1995). Other factors such as processing conditions, storage temperature, composition of the food matrix, competition with starter cultures or other organisms present affect the survival of probiotics in foods. Probiotics exert their action after colonization and growth in the distal ileum and colon, which means they must survive passage through the oesophagus, acidic stomach and the upper part of the intestine to be effective as probiotics. The selection of the most suitable probiotic for a particular food application must consider not only survival throughout the storage life of the food but also its ability to colonize epithelial surfaces in the lower intestine, and once established produce antimicrobial activity of value to the host. If probiotic bacteria are able to inhibit the growth of undesirable bacteria in food by production of bacteriocins or other antimicrobials, these serve as an added advantage to consumers by not only providing health promoting foods but also foods that are safe. Such organisms must not only tolerate extended storage at low pH in carrier foods (if these are fermented) but also the low pH of the stomach. Lactobacilli and bifidobacteria

are the most commonly studied probiotic organisms. Some researchers have suggested the consumption of 8 log viable cells of probiotics is required each day and others recommend that there be 6 log cfu/g of viable cells in the product before consumption (Shah, 2000, Kailasapathy and Chin, 2000). Although the minimum level of probiotic needed to provide beneficial effects is still unclear, high viability of the organism is essential. Microencapsulation of probiotics as a means of protecting probiotic bacteria against the adverse conditions in food and during passage through the GI tract is considered to have potential value.

2.12 Microencapsulation of probiotics

Microencapsulation is a technique by which bacterial cells are enclosed within a polymer membrane to prevent damage to the cells from the environment (Krasaekoopt et al. 2003). Bacterial cells are traditionally preserved by either spray or freeze drying to form a concentrated powder. When added to food products containing moisture the powder releases the bacteria into the surrounding environment thus injuring the cells. Audet et al. (1988) and Rao et al. (1999) suggested immobilization of bacterial cells within hydrocolloid beads. Thereafter interest and research on microencapsulation of probiotics increased dramatically. A variety of wall materials such as alginate, starch, κ-carrageenan-locust bean gum, xanthan-gellan, cellulose acetate, phthalate, chitosan, and gelatin have been used for microencapsulation (Audet et al. 1988, Rao et al. 1989, Sheu and Marshall, 1993, Sultana et al. 2000, Sun and Griffiths, 2000). Two methods of encapsulation involving extrusion or emulsion have often been used for encapsulation of probiotics (Kailasapathy, 2002). Microencapsulation by extrusion involves adding the cultures to a polymer solution (often sodium alginate) and extruding them through a

syringe needle into a calcium chloride solution. Microencapsulation by emulsion technology involves adding a culture-polymer mixture to vegetable oil to form an emulsion. The emulsion is broken usually by addition of $CaCl_2$ leading to formation of spheres encapsulating the intended probiotic organism. Extrusion is a popular method because it is a simple procedure, but the capsules generated are large (1 to 2 mm) compared to those generated by the emulsion method (25 to 100 μm) (Krasaekoopt et al. 2003).

2.12.1 Applications of microencapsulation technology

Microencapsulation using these polymers has been shown to protect probiotics from bacteriophages (Steenson et al. 1987), and increase survival during acid challenge (Hansen et al. 2002), freezing (Shah and Ravula, 2000), during passage through the GI tract (Lee and Heo, 2000) and storage (Kebary et al. 1998). Microencapsulation of probiotics as a means of improving the survival of these organisms has been studied in yoghurt (Sultana et al. 2000, Adikari et al. 2000), iced milk (Keraby et al. 1998, Sheu et al. 1993), nutrient enhanced chocolate bars (Siuta-Cruce and Goulet, 2001) as well as mayonnaise (Khalil and Mansour, 1998) (Table 2.3).

Table 2.3 Application of microencapsulation in different products

Wall material used	Concentration of wall material (%w/v)	Method of encapsulation	Microencapsulated organism	Product applied	Reference
Alginate	1.8	Extrusion	*Lb. delbrueckii* spp. *bulgaricus* *S. thermophilus*	Yoghurt	Prevost et al. (1985)
Alginate	1.8	Extrusion	*S. lactis* ssp *diacteylactis* *S. cremoris*	Cheese	Prevost and Divies (1987)
κ– carrageenan and locust bean gum	3 and 1.5	Emulsion	*S. thermophilus*	Yoghurt	Audet et al. (1988)
Alginate	1.5	Extrusion	*Lactococcus lactis* ssp lactis diacetylactis	Cream	Prevost and Divies (1992)
Alginate	3.6	Emulsion	*Lb. delbrueckii* spp. *bulgaricus*	Frozen dessert	Sheu et al. (1993)
Alginate	3	Emulsion	*Lb. delbrueckii* spp. *bulgaricus*	Frozen ice milk	Sheu and Marshall (1993)
Alginate	3	Extrusion	*B. bifidum* and B. *infantis*	Mayonnaise	Khalil and Mansour (1998)
Alginate	3	Emulsion	*B. bifidum*, *B. infantis*	Ice milk	Kebary et al. (1998)
Gellan-Xanthan	0.75 and 1	Extrusion	*B. infantis*	Yoghurt	Sun and Griffiths (2000)
κ– carrageenan	2	Emulsion	*B. longum*	Stirred Yoghurt	Adhikari et al. (2003)
Alginate	1	Emulsion	*Bifidobacteria* spp.	Milk	Hansen et al. (2002)
Alginate and Starch	0.6	-	*Lb. acidophilus*	Milk	Jankowski et al. (1997)

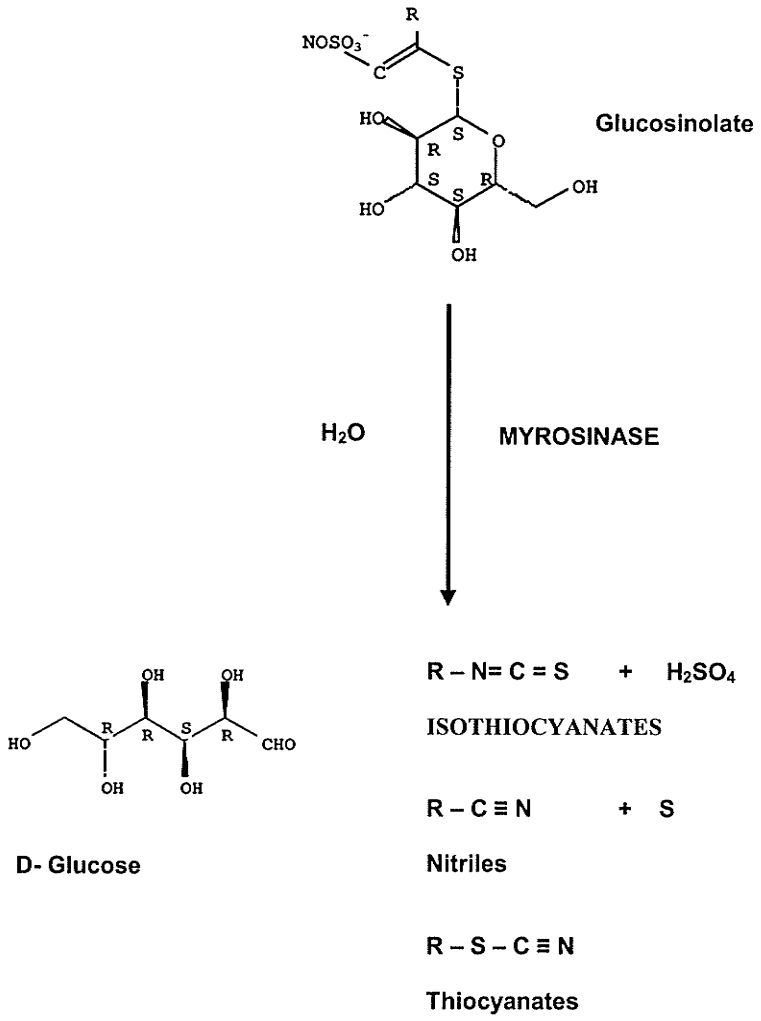
2.13 Allyl Isothiocyanate (AIT) as a natural antimicrobial

2.13.1 Sources and characteristics

Oil of mustard from brown or black mustard, horseradish, cabbage, Brussels sprouts, broccoli and other cruciferous vegetables is a rich natural source of allyl isothiocyanate (Delaquis and Mazza, 1995). AIT is present in the form of the glucosinolate, sinigrin, in these plants and is hydrolysed by the endogenous enzyme myrosinase to yield allyl and other isothiocyanates (Figure 5) (Fenwick et al. 1982). AIT (in the form of oil of mustard) is used primarily as a flavouring agent in variety of foods. AIT has been available commercially from as early as 1937 (IARC, 1985)

AIT from natural sources is approved for use in foods as a preservative in Japan (Kim et al. 2002). AIT from oil of mustard is on the FDA Generally Recognised as Safe (GRAS) list (CFR, 1999) and is regarded as a food additive permitted for direct addition to food for human consumption (FDA, 2002). According to the Environmental Protection Agency Federal Registry "AIT is exempt from the requirement of a tolerance for residues when used as a component of food grade oil of mustard in or on all agricultural commodities when applied according to approved labeling" (EPA, 1996). Therefore the use of AIT (in the format above) in food and feed is not restricted by any regulations or by any statutory boards. AIT is classified by the International Agency for Research on Cancer (IARC) as a Group 3 agent: Unclassifiable in relation to causing carcinogenicity in humans (IARC Monograph, 1999).

Fig 2.4 Hydrolysis of glucosinolates by the enzyme myrosinase



After Mazza (1984)

2.13.2 Bactericidal effects of allyl isothiocyanate

The mechanism whereby AIT is bactericidal involves destabilization of the target cell cytoplasmic membrane causing leakage of metabolites from the cytoplasm. This was evident when cells of *E. coli* K-12, which possess β-galactosidase activity located intracellularly, were exposed to AIT. Following treatment an increase in β-galactosidase activity was detected extracellularly in the culture medium (Lin et al. 2000b). AIT was also shown to cleave the disulphide bonds of intracellular enzymes and inhibit oxygen uptake, which may also contribute to its bactericidal effects (Kawakishi and Kaneko, 1987). Gram negative bacteria including *E. coli* O157:H7 were more sensitive to inhibition by AIT than Gram positive organisms and its potential for use as an antimicrobial agent for foods has been recognized (Lin et al. 2000a).

E. coli O157:H7 were inhibited on agar surfaces when exposed to gaseous AIT at 1000μg AIT/L atmosphere. Bactericidal activities increased with time of exposure, AIT concentration and temperature. Although AIT in both liquid and gaseous forms were antimicrobial, gaseous AIT had greater antimicrobial potency (Delaquis and Sholberg, 1997). High humidity was also reported to enhance the antimicrobial potency of AIT (Furuya and Isshiki, 2001).

In a study by Ward et al. (1997) it was shown that the volatile distillate extracted from horseradish root containing approximately 90 % allyl isothiocyanate completely inhibited *E. coli* O157:H7 on agar during 7 days storage at 12° C. However, concentrations 5-fold higher were required to inhibit this pathogen in roast beef.

Kim et al. (2002) studied the effect of AIT and acetic acid either alone or in combination on the shelf-life of cooked rice stored at 10, 25 or 60° C over 48 h at 12h intervals. Combination of AIT (2 μL/pack) with acetic acid (1200 μL/pack) resulted in complete inhibition of total aerobic populations at 24, 12 and 24 h, during 10, 25 and 60° C storage, respectively, but sensory differences in odor and taste were appreciable in the treated groups. They concluded that AIT was more effective at 25 ° C than 10 ° C.

In an agar disk assay, exposure of *E. coli* O157:H7 to AIT in the vapor form at 8μL (in a 950 cc jar) resulted in a 7 log reduction at 37° C, whereas in wet alfalfa seeds a dose of 50 μL was required to reduce *E. coli* O157:H7 by 2.7 log cfu/g (Park et al. 2000). They also found that 100 μL of AIT in the same size jar was insufficient to kill 2.9 log cfu/g of *E. coli* O157:H7 when present on dry alfalfa seeds.

Lin et al. (2000a) compared the effect of AIT vapors on *E. coli* O157:H7 in iceberg lettuce and apple stem scars. They found 8 log cfu/g reductions of *E. coli* O157:H7 in iceberg lettuce in 2d at 4° C when AIT was used at a level of 400 μL in a 4 L container. They found only a 3 log reduction in *E. coli* O157:H7 numbers on apple stem scars, even when AIT was used at 600 μL. They concluded that the antimicrobial effectiveness of AIT is partly dependent on the type of product treated.

2.14 Mustard flour as a source of AIT

Canada is the largest mustard exporter in the world and is one of the world's top five mustard producers. Mustard has been used as a spice or a condiment for centuries throughout the world. Mustard flours when deheated (deodorized) are used as emulsifiers, binders and bulking agents, but non-deheated mustard is used as a spice in seasonings and for flavouring in meat formulations, particularly in sausage and salami

manufacture (Cui, 1997). Brown or oriental mustard (*Brassica juncea*) and yellow or white mustard (*Sinapis alba*) contain glucosinolates, which form isothiocyanates, cyanides and thiocyanates following enzymatic action by myrosinase (Tsao et al. 2002). In deheated mustard flour for use as binder, myrosinase is inactivated by thermal treatment to prevent development of mustard flavor. Allyl isothiocyanate, one of the predominant breakdown products from glucosinolates, as noted previously, has been reported to have antimicrobial effects against bacteria including *E. coli* O157:H7 in pre-cooked roast beef, alfalfa seeds, lettuce, and 'Asazuke' (a low salt vegetable) (Ward et al. 1998, Park et al. 2000, Lin et al. 2000, Ogawa et al. 2000). Although pure AIT from natural sources can be used as a food preservative in Japan, its use in food is restricted as a flavoring agent in Canada. Moreover, use of pure AIT in food has limitations because of the strong pungent smell associated with volatile AIT which causes eye and nasal irritation and a burning sensation of the skin and tongue. Non-deheated mustard flour, being a natural source of AIT, may be used as an alternative approach in food systems to eliminate *E. coli* O157:H7. Studies in our laboratory have shown potential for use of AIT and mustard flour as an antimicrobial against *E. coli* O157:H7 in ground beef (Nadarajah et al. 2005a, 2005b).

Chapter 3

Bactericidal effects of *Lb. reuteri* and Allyl Isothiocyanate on *E. coli* O157:H7 in refrigerated ground beef

3.1 Abstract

Two naturally occurring antimicrobial agents were tested in packages of refrigerated ground beef for their ability to reduce the viability of *Escherichia coli* O157:H7 during storage. Allyl isothiocyanate (AIT) and *Lactobacillus reuteri* were tested separately and together for their action against a cocktail of 5 strains of *E. coli* O157:H7 in ground beef held at 4° C for 25 d. Ground beef prepared from whole, raw inside round beef roasts was inoculated with low (3 log cfu/g) or high (6 log cfu/g) levels of the *E. coli* O157:H7 mixture. The beef was treated with AIT (about 1300 ppm) and/or *Lactobacillus reuteri* along with 250 mM glycerol/kg meat at two levels (3 log cfu/g and 6 log cfu/g) and according to a design that yielded 8 control plus 10 different treatments. Samples were analysed for *E. coli* O157:H7 survivors, numbers of total bacteria and lactic acid bacteria on days 0 to 25 at 5d intervals. *Lactobacillus reuteri* at both input levels with glycerol killed *E. coli* O157:H7 at both inoculated levels before day 20. AIT completely eliminated *E. coli* O157:H7 when present at the low inoculum level (3 log cfu/g) and reduced viability >4.5 log cfu/g at the high inoculum level (6 log cfu/g) by the end of the storage period. Combination of *Lactobacillus reuteri* with AIT did not yield an additive effect against *E. coli* O157:H7 viability. *Lactobacillus reuteri* in the presence of glycerol was highly effective against *E. coli* O157:H7 in ground beef during refrigerated storage (4° C) in modified atmosphere packages. Sensory testing is planned to evaluate effects of treatments.

3.2 Introduction

Foodborne illness outbreaks and ground beef recalls due to *Escherichia coli* O157:H7 contaminations continue to be a major cause of concern to the public, regulatory authorities and the food industry. *E. coli* O157:H7, which can have serious human health effects particularly among children, is reported to have a very low infective dose (Buchanan and Doyle, 1997). In the US and Canada, contaminated ground beef is the most common vehicle for transmission of *E. coli* O157:H7. In spite of Food Safety and Inspection Service (FSIS) and Canadian Food Inspection Agency (CFIA) regulations and a WHO warning (WHO, 1997) predicting an increase in foodborne *E. coli* O157:H7 outbreaks, frequent reports of *E. coli* O157:H7 contamination in ground beef continue to occur. In the year 2002 alone, nearly 10.75 million Kg of ground beef were recalled because of possible *E. coli* O157:H7 contamination. This has caused significant economic loss to the industry and generated a lack of confidence regarding the safety of these products in the minds of some consumers (FSIS, 2002).

In recent years, there has been an increase in studies on the role of lactic acid production by lactic acid bacteria (LAB) in the inhibition of pathogenic micro-organisms in food. In addition to lactic acid, these organisms may also produce bacteriocins, antibiotics or low molecular mass compounds that are inhibitory to pathogenic organisms. The bacteriocins of LAB are mostly effective against Gram-positive bacteria (Jack et al. 1995) but low molecular mass antimicrobials (such as organic acids) are able to act against Gram-negative organisms if they can penetrate the bacterial membrane (Helander et al. 1997). *Lactobacillus reuteri* is a normal inhabitant of the human and animal gastrointestinal tracts (Kandler et al.1980) and is present in variety of foods like

milk, cheese, meat and sourdough (Kandler and Weiss, 1986). *Lactobacillus reuteri* produces a non-peptide low molecular mass antimicrobial substance named reuterin during anaerobic metabolism of glycerol by a dehydratase reaction to yield β-hydroxypropanaldehyde (reuterin). The latter has a broad spectrum of activity against both Gram-positive and Gram-negative bacteria, including *E. coli* O157:H7 (Axelsson et al. 1989, Chung et al. 1989). *Lactobacillus reuteri* and reuterin are reported to be unaffected by a wide range of pH while reuterin is resistant to heat or proteolytic enzymes, and thereby may potentially act as an ideal bio-preservative in food (El-Ziney et al. 2000, Rasch, 2002). *Lb. reuteri* along with glycerol has been used successfully to extend the shelf life of herring, inhibit *E. coli* O157:H7 in ground beef and skim milk (El-Ziney, 1997, Lindgren and Dobrogosz, 1990). Reuterin was reported to inhibit *E. coli* O157:H7 and *Listeria monocytogenes* in vacuum packed ground ham, cottage cheese and UHT milk (El-Ziney and Debevere, 1998, El-Ziney et al. 1999).

Allyl isothiocyanate (AIT), because of its natural origin (horseradish and mustard) and its generally recognized as safe status (GRAS) (Code of Federal Regulations, 1999) is attracting more attention as a potential antimicrobial agent in food (Lin et al. 2000). Allyl isothiocyanate is present as a precursor in the form of the glucosinolate, sinigrin, in these natural sources. Sinigrin is hydrolysed by the endogenous enzyme myrosinase to yield allyl and other isothiocyanates upon physical injury to plant tissue in which it occurs (Fenwick et al. 1982). AIT has been shown to have strong antimicrobial activity against a wide variety of microorganisms including yeast and fungi (Delaquis and Sholberg, 1997, Delaquis et al. 1999, Isshiki et al. 1992, Lin et al. 2000, Park et al. 2000, Ward et al. 1998). In our laboratory AIT alone has previously been shown to reduce *E.*

coli O157:H7 by > 3.5 log cfu/g in ground beef stored at 4° C for 21 d (Nadarajah et al. 2002) and by ≥ 2.0 log cfu/g in dry sausage beaker fermentations (Holley and Santos, unpublished). So far, studies have shown that AIT use at levels which yield complete elimination of *E. coli* O157:H7 from ground beef generate a strong odor which is not likely to be acceptable to all consumers. Therefore, a combination of AIT with another antimicrobial was used as an approach which would allow reduction in the critical level of AIT for required effectiveness. AIT was reported to act by affecting protein structure through cleavage of disulfide bonds (Kawakishi and Namiki, 1969) and therefore use of bacteriocins which are protein in nature or use of other proteinaceous antimicrobials like lysozyme are unlikely to generate additive antimicrobial effects against pathogens like *E. coli* O157:H7. Interestingly, LAB were reported to be more resistant to AIT than a number of pathogenic microorganisms (Ward et al. 1998). Therefore it was hypothesized that *Lb. reuteri* and reuterin may have potential for use in combination with AIT to increase the safety of modified atmosphere packaged ground beef by controlling *E. coli* O157:H7 and extending product shelf life. This work was undertaken to determine whether there would be additive or synergistic interactions between these agents against *E. coli* O157:H7 in ground beef during refrigerated storage.

3.3 Materials and methods

3.3.1 Cultures and growth conditions

Lactobacillus reuteri 1063 (ATCC 53608), a known reuterin producer was obtained from the ATCC and maintained in MRS broth (Difco - Becton, Dickinson Co., Sparks, MD) containing 20% glycerol at -70° C. After thawing cultures were transferred

to MRS broth and incubated anaerobically at 37° C for 24-48 h. The organism was sub-cultured twice without glycerol before being inoculated into ground beef.

Five *E. coli* O157:H7 strains, # 7128, 7110 and 7236 (human isolates), plus # 7282 and 7283 (from hamburger) were provided by Dr. R. Khakria, Laboratory Center for Disease Control, Ottawa, Canada. *E. coli* O157:H7 strains were maintained in tryptic soy broth, incubated at 37° C and sub-cultured twice before use in experiments. Numbers of viable cells in bacterial cultures were standardized at OD_{600nm} by dilution in 0.1% (w/v) peptone. Dilutions in peptone were mixed to contain an equal number of cells of each strain in the inoculation cocktail.

3.3.2 Preparation of ground beef

Uncooked inside round beef roasts were obtained from a local retail supermarket. The beef was aseptically ground in an electric meat grinder with a plate having 12.7 mm diameter multiple perforations (Model: 84142, Hobart, Don Mills, ON). The five strain cocktail of *E. coli* O157: H7 was prepared in 100 ml of peptone water and 10 ml/ kg meat was added to ground beef so as to reach the desired initial inoculum level according to the experimental design (Table 3.1). In addition to the controls and treatments tabulated, fresh ground raw beef containing 250 mmol glycerol, ground beef with 250 mmol glycerol and either 3 or 6 log cfu *E.coli* O157:H7/g were also vacuum packaged and examined for changes in bacterial content and pH as noted in the next section. To obtain a uniform distribution of bacteria in the ground beef, samples containing added bacterial suspension were mixed well by hand (using sterile gloves) and re-ground in a manual meat grinder (9.5mm plate) before packaging. In treatments inoculated with both *E. coli* O157:H7 and *Lb. reuteri* (plus 250 mmol glycerol/kg meat), the meat was re-ground

between bacterial additions. Twenty five g of ground beef were weighed and sealed in Deli*1 bags (Winpak, Winnipeg, MB) after nitrogen flushing using a vacuum packaging machine (Model GM-2000, Bizerba Canada Inc, Missisauga, ON). The bags were composed of nylon/EVOH (ethylene vinyl alcohol)/polyethylene, were 75 μm thick and measured 30 x 45 cm. Oxygen transmission of the film was 2.3cc/m^2 over 24 h at 23° C, and moisture vapor transmission was 7.8 g/m^2 over 24 h at 37.8° C and 90% relative humidity. Where used, 0.7 ml of allyl isothiocyanate (94% purity) (Acros Organics, Geel, Belgium) was added to 0.3 ml of commercial corn oil and placed on a sterilized filter paper which was placed in the bag on top of the meat just before packaging.

The ground beef was stored at 4° C and 3 bags from each treatment were removed at day 0, 5, 10, 15, 20, 25 and analyzed for total bacteria, LAB and *E. coli* O157:H7 numbers as well as for AIT head space concentrations (where used) by gas liquid chromatography (GLC).

3.3.3 Enumeration of bacterial numbers and pH measurement

Eleven g of meat was diluted with 99 ml of peptone water (1:10) and homogenized in a stomacher bag for one min using a Stomacher 400 system (Seward laboratory, London, UK). Serial dilutions were prepared in peptone water and plated using a spiral plater (Autoplate 4000, Spiral Biotech,Norwood, MA). Tryptic soy agar, MRS agar and Sorbitol MacConkey agar (Difco-Becton Dickinson Co.) supplemented with cefixime-tellurite (Dynal Inc., Lake Success, NY) (CT-SMAC) were used for enumeration of total bacteria, LAB and *E. coli* O157:H7 numbers, respectively. The plates were incubated at 37° in all cases. Tryptic soy agar and CT-SMAC agars were incubated aerobically for 48 and 24 h, respectively. MRS agar was incubated for 48h

anaerobically using anaerobic jars and the BBL GasPak plus system (Becton Dickinson Co.) The surface meat pH of each sample was measured after removal of material for bacteriological examination. The pH meter used was a model IQ240 (IQ Scientific Instruments Inc., San Diego, CA) equipped with an ISFET surface probe.

3.3.4 Measurement of AIT in package head space

Modification of methods by Lim and Tung (1997) and Kim et al. (2002) were used to evaluate the AIT levels in the head space of treatments where it was used at day 0, 10, 15, 20 and 25. The AIT head space concentrations were evaluated by GLC (Varian Star 3400cx_Varian Chromatography Systems, Walnut Creek, CA). Samples from the package head space were drawn by piercing the nylon/EVOH /polyethylene bags with a gas tight 0.25 ml syringe (Precision Sampling Corp, Baton Rouge, LA) and injected into the GLC equipped with a J&W BD5MS column (30m x 0.25 mm ID., 25μm wall thickness). Column temperature was programmed to increase from 60° C to 95° C at a rate of 12.5° C per min and was held at 95° C for 45 sec. The inlet temperature and the flame ionization detector temperature were set at 250° C. Helium was used as the carrier gas. Varian Star chromatography software was used to control the GLC, identify and confirm the presence of AIT and for peak integration.

3.3.5 Statistical analysis

All data were analysed using Statistical Analysis System (version 8.1) software (SAS Institute, Cary, NC). Analysis of variance by general linear model (GLM) and Duncan's multiple range tests were used to find significant differences ($P<0.05$) among treatments at a particular time (number of days) and among different times for each treatment.

3.4 Results

3.4.1 Effect of *Lb. reuteri* and glycerol on *E. coli* O157:H7 viability

Data in Table 3.2 show the ability of *Lb. reuteri* in the presence of glycerol to eliminate *E. coli* O157:H7. *Lb. reuteri* at 6 log cfu/g reduced *E. coli* O157:H7 to undetectable levels by day 10 and day 20 when the initial *E. coli* O157:H7 levels were 3 and 6 log cfu/g, respectively. *Lb. reuteri* at 3 log cfu/g eliminated viable *E. coli* O157:H7 from the meat by day 15 and day 20 when the initial *E. coli* O157:H7 levels were 3 and 6 log cfu/g, respectively. Significant differences ($P<0.05\%$) were noticed in *E. coli* O157:H7 levels as storage time increased and between different treatments at each *E. coli* O157:H7 inoculum level.

3.4.2 Changes in meat pH

The initial surface pH of fresh ground beef with or without glycerol addition was 5.5 to 5.7. In all treatments whether glycerol or bacteria were added, lowest pH values of 4.7 to 4.8 were reached at day 10. Thereafter, from day 15 to 25, pH values of 5.1 to 5.3 were observed. Effects of the addition of high numbers (6 log cfu/g) of either *Lb. reuteri* or *E.coli* O157:H7 upon meat pH were not apparent.

3.4.3 Effect of AIT on *E. coli* O157:H7

Data in Table 3.3 show the effect of AIT alone upon *E. coli* O157:H7 viability in ground beef. Greater than a 4.5 log reduction in *E. coli* O157:H7 occurred by day 25, when the initial level of *E. coli* O157:H7 was 6 log cfu/g. Viable *E. coli* O157:H7 were reduced to an undetectable level by day 15 when the initial inoculum level of *E. coli* O157:H7 was 3 log cfu/g.

3.4.4 Combined effects of *Lb. reuteri* and AIT

Data in Table 3.4 show the combined effects of *Lb. reuteri* and AIT upon *E. coli* O157:H7 viability in ground beef stored at 4° C. After 25d in the high inoculum test groups (*E. coli* O157:H7 at 6 log cfu/g), *E. coli* O157:H7 levels dropped to 2.1 and 1.3 log cfu/g when *Lb. reuteri* was present at levels of 6 log cfu/g and 3 log cfu/g, respectively in combination with AIT. On the other hand, in experiments having initial *E. coli* O157:H7 levels of 3 log cfu/g, *Lb. reuteri* (3 log cfu/g) in combination with AIT reduced the *E. coli* O157:H7 to undetectable levels in 15 d, whereas *Lb. reuteri* at 6 log cfu/g (in combination with AIT) took 10 days to reduce *E. coli* O157:H7 below detectable levels.

3.4.5 Antimicrobial effects on total bacterial numbers in ground beef

The effect of *Lb. reuteri*, AIT and the combination of the two agents on total viable bacteria in ground beef stored at 4° C is shown in Table 3.5. In the untreated control the total bacterial numbers increased from an initial level of 5.1 log cfu/g to 6.9 log cfu/g after 25 d storage. When the control was repeated and meat containing glycerol only was included, lower numbers of bacteria were initially present. In uninoculated vacuum packed ground beef or in ground beef with glycerol added, initial numbers of total bacteria were 3.04 ± 0.13 log cfu/g. By day 10 in both treatments numbers reached 7 log cfu/g and peaked at close to 8 log cfu/g by day 15. Thereafter numbers decreased and by day 25 numbers in the glycerol added control were 7.24 ± 0.31 log cfu/g compared to 6.23 ± 0.37 log cfu/g in the unammended control meat (data not shown). These results are in contrast with all other treatments where the final total numbers after 25 d of storage were between 3.9 to 4.6 log cfu/g.

3.4.6 Antimicrobial effects on lactic acid bacteria

The effects of *Lb. reuteri*, AIT and their combination on LAB are presented in Table 3.6. In the untreated control, the LAB level increased from 3.8 to 6.1 log cfu/g from day 0 to day 25. In treatments containing AIT alone the levels of LAB decreased initially but rose again after day 10. In samples containing *Lb. reuteri* alone at 3 log cfu/g the LAB levels increased throughout, whereas in treatments containing *Lb. reuteri* at higher levels (6 log cfu/g), the number of LAB dropped slightly over time. In treatments containing *Lb. reuteri* (3 log cfu/g) and AIT, the levels of LAB increased throughout the trials, whereas in the case of *Lb. reuteri* inoculated at 6 log cfu/g, the levels of LAB dropped one log cfu/g at 5d and remained lower than in the control (C3) without AIT throughout the trial.

3.4.7 Level of AIT during storage

Changes in the level of AIT in the ground beef package head space during different days of storage at 4° C are shown in Fig 3.1. Concentrations of AIT dropped from an initial level of 1287 μg/ml to 517 μg/ml by day 25.

3.5 Discussion

From the work presented here there is clear indication that both *Lb. reuteri* and AIT have strong bactericidal effects against *E. coli* O157:H7 in ground beef stored at 4° C. *Lb. reuteri* in the presence of glycerol was found to be better than AIT in killing *E. coli* O157:H7, which should not have been surprising since the AIT was applied to a filter paper insert in the package and not mixed with the meat. Glycerol was used in combination with *Lb. reuteri* in food systems earlier and found to be a necessary precursor for reuterin production by the organism (El-Ziney, 1997, Lindgren and Dobrogosz, 1990). In our experiments *Lb. reuteri* at 3 log cfu/g was found to be as

effective as 6 log cfu/g in killing 3 or 6 log cfu *E. coli* O157:H7/g but *Lb. reuteri* at the higher level was slightly better at reducing *E. coli* O157:H7 when it was present at low levels. El- Ziney (1997) apparently found similar results when *Lb. reuteri* was used at 8 log cfu/g with 150 mmol/L glycerol to kill *E. coli* O157:H7 in ground beef, vacuum packaged and stored at 7° C.

In the latter experiments there was a ≤ 3 log reduction in viable *E. coli* O157:H7 numbers by day 14. *E. coli* O157:H7 lethality in our experiments was more pronounced and this was probably because of the higher glycerol level (250 mmol/kg) used here and thus greater production of reuterin (El-Ziney, 1997). *Lb. reuteri* did not produce any odour or colour change nor was visible gas produced. The inhibitory effect of *Lb. reuteri* was assumed to be due to production of reuterin since *Lb. reuteri* ATCC 53608 is reported to produce reuterin by anaerobic fermentation of glycerol (Axelsson et al. 1989, Chung et al. 1989). Admittedly, the inhibitory action of *Lb. reuteri* could also have been due to production of lactic acid, hydrogen peroxide, a bacteriocin named reutericin 6 (Kabuki et al. 1997, Toba et al. 1991) or a tetramic acid derivative, reutericyclin (Gänzle et al. 2000), however, it is not known whether the *Lb. reuteri* strain (ATCC 53608) used in our experiments produces reutericin 6 or reutericyclin. Nonetheless, it is clear from the data obtained that inhibition reported was not due to pH reduction from glycerol fermentation. Development of methods to quantify reuterin and to estimate the influence of other potential inhibitory substances that might be produced in beef during storage will be helpful in characterizing the dynamics of the inhibitory action of *Lb. reuteri* against *E. coli* O157:H7.

Although AIT had strong inhibitory activity against *E. coli* O157:H7, it was not able to completely eliminate the pathogen when the latter were present at artificially high levels of 6 log cfu/g. Similar results were noticed earlier in our lab (Nadarajah et al. 2002) where a >3.5 log cfu/g reduction in viable *E. coli* O157:H7 was seen by day 21 in ground beef stored at 4° C. AIT was reported to affect β-galactosidase activity and induce cellular metabolic leakage thereby inhibiting Gram negative bacteria (Lim and Tung, 1997). Other mechanisms such as bactericidal action by attacking disulfide bonds and affecting protein structures (Kawakishi and Kaneko, 1987), or affecting the respiration of cells (Kojima and Ogawa, 1971) were proposed, although the complete mechanism of action of AIT is still unknown. AIT did not induce visible colour changes in meat but a strong pungent odour was noticed when bags were opened. Although the strength of AIT smell decreased as time progressed, it was noticeable even on day 25. Combined use of AIT with reuterin was seen as an opportunity to reduce the minimum concentration of AIT needed for effectiveness against *E. coli.* In addition to drastically reducing the number of viable *E. coli* O157:H7, both *Lb. reuteri* and AIT extended the shelf life of ground beef by reducing the total and LAB numbers present (Tables 3.5 and 3.6). Although it was reported earlier that LAB are resistant to reuterin produced by *Lb. reuteri* and AIT (El-ZIney, 1997, Ward et al. 1998), our results (Table 3.6) suggest that these numbers were reduced as time progressed. Reason for the difference may include our use of a higher AIT concentration than used previously (Ward et al. 1998) or the development of a feed back inhibitory effect by reuterin on LAB as has been reported to occur with *Lb. reuteri* against itself (Rasch, 2002). Reduction in LAB numbers in the presence of high levels of *Lb. reuteri* may also have been due to substrate competitive

effects exerted against the adventitious LAB. The AIT levels in the head space were reduced as storage time advanced and this may have been due to a variety of factors including its decomposition (Kawakishi and Namiki, 1969), lowered stability at acid pH (Rhee et al. 2002) or permeation through the packaging film (Lim and Tung, 1997). It was suspected that there could have been some sample loss during needle penetration of sample bags during head space sampling for AIT measurement by gas chromatography. This loss could have affected recovery to some extent although trends toward lower concentrations of AIT in the head space were relatively consistent. The combination of *Lb. reuteri* with AIT did not yield the synergistic effects expected. It is possible that AIT inhibited *Lb. reuteri* during the storage experiments or it caused inactivation of reuterin. Alternatively, reuterin may have interfered with or inactivated AIT. At the pH values observed in this study (4.7-5.7) AIT has been reported to be stable (Rhee et al. 2002). In previous studies, the combination of allyl isothiocyanate with acetic acid (2 μL and 1200 μL respectively / pack) was reported to act synergistically and inhibit total viable aerobic bacteria in cooked rice stored at 10° C (Kim et al. 2002), although Rhee et al. (2002) found that the use of "non-deheated" mustard at 10% (which yields AIT on degradation) in combination with low levels of acetic acid did not yield additive inhibitory effects against *E. coli* O157:H7 unless the acetic acid concentration was above 1%.

Although in our experiments the *E. coli* O157:H7 numbers dropped below detectable levels (40 cfu/g) as early as 10d of storage, the possible presence of an injured or viable but non-culturable population of *E. coli* O157:H7 should be taken into account. Recovery of these injured cells by enrichment or immuno-concentration (Dynabeads) would be useful in fully characterizing the extent of the lethal effects of the treatments

used. *Lb. reuteri* with glycerol was a highly effective treatment for control of *E. coli* O157:H7 in ground beef. Combinations of AIT with acetic acid or other low molecular weight organic acids may be a useful alternative approach but its use with *Lb. reuteri* was not as effective as had been anticipated.

Table 3.1 Experimental design[a] used for challenge of *E. coli* O157:H7 by *Lb. reuteri*[b] with or without AIT[c] in ground beef stored at 4° C under N_2 for up to 25d.

Treatment	*E. coli* O157:H7 cfu/g		*Lb. reuteri* cfu/g		AIT
code	10^3	10^6	10^3	10^6	1300 ppm
C1	-	-	-	-	-
C2	-	-	+	-	-
C3	-	-	-	+	-
C4	+	-	-	-	-
C5	-	+	-	-	-
C6	-	-	-	-	+
C7	-	-	+	-	+
C8	-	-	-	+	+
T1	+	-	+	-	-
T2	+	-	-	+	-
T3	+	-	-	-	+
T4	+	-	+	-	+
T5	+	-	-	+	+
T6	-	+	+	-	-
T7	-	+	-	+	-
T8	-	+	-	-	+
T9	-	+	+	-	+
T10	-	+	-	+	+

[a]*All* control (C) and treatments (T) were replicated three times in duplicate

[b]*Lb. reuteri* was used with 250 mmol glycerol/ kg of beef

[c]AIT (0.7 ml AIT + 0.3 ml corn oil) was added to a filter paper insert.

Table 3.2 Viability of *E. coli* O157:H7 in ground beef treated with *Lb. reuteri* and stored at 4° C under nitrogen

Experimental Design code	Treatment[a]		Days Storage at 4° C					
	E. coli	*Lb. reuteri*	0	5	10	15	20	25
C4	3.0	none	3.12 ± 0.09^{Ac}	3.19 ± 0.09^{Ac}	2.89 ± 0.04^{Bc}	2.75 ± 0.09^{Cb}	2.67 ± 0.19^{Cb}	2.76 ± 0.09^{Cb}
T1	3.0	3.0	2.93 ± 0.09^{Ad}	2.87 ± 0.06^{Ad}	0.17 ± 0.41^{Bd}	0^{Bd}	0^{Bc}	0^{Bc}
T2	3.0	6.0	2.95 ± 0.03^{Ad}	2.76 ± 0.12^{Bd}	0^{Cd}	0^{Cd}	0^{Cc}	0^{Cc}
C5	6.0	none	5.99 ± 0.13^{Aa}	6.02 ± 0.07^{Aa}	5.68 ± 0.09^{Ba}	5.27 ± 0.11^{Ca}	4.99 ± 0.07^{Da}	5.17 ± 0.14^{Ca}
T6	6.0	3.0	5.83 ± 0.06^{Ab}	6.07 ± 0.19^{Aa}	4.79 ± 0.19^{Bb}	1.20 ± 1.32^{Cc}	0^{Dc}	0^{Dc}
T7	6.0	6.0	5.95 ± 0.06^{Aa}	5.63 ± 0.27^{Ab}	5.63 ± 0.09^{Aa}	0.92 ± 1.01^{Bc}	0^{Cc}	0^{Cc}

[a] $\log_{10}$ cfu inoculation levels/g ground beef

Values represent mean $\log_{10}$ cfu *E. coli* O157:H7/g ± standard deviation

Mean superscript with different capital letters in the same row and different lower case in same column differ significantly ($P<0.05$)

Table 3.3 Viability of *E. coli* O157:H7 in ground beef treated with AIT and stored at 4° C under nitrogen

Experimental Design code	Treatment		Days Storage at 4° C					
	E. coli[a]	AIT	0	5	10	15	20	25
C4	3.0	-	3.12 ± 0.09^{Ab}	3.19 ± 0.09^{Ac}	2.89 ± 0.04^{Bc}	2.75 ± 0.09^{Cc}	2.67 ± 0.19^{Cb}	2.76 ± 0.09^{Cb}
T3	3.0	+	2.76 ± 0.33^{Ac}	2.15 ± 0.10^{Bd}	0.65 ± 1.00^{Cd}	0^{Dd}	0^{Dd}	0^{Dd}
C5	6.0	-	5.99 ± 0.13^{Aa}	6.02 ± 0.07^{Aa}	5.68 ± 0.09^{Ba}	5.27 ± 0.11^{Ca}	4.99 ± 0.07^{Da}	5.17 ± 0.14^{Ca}
T8	6.0	+	5.93 ± 0.07^{Aa}	5.27 ± 0.25^{ABb}	4.76 ± 0.24^{Bb}	3.33 ± 0.41^{Cb}	1.91 ± 0.98^{Dc}	1.17 ± 0.91^{Ec}

[a]$\log_{10}$ cfu/g meat at inoculation

Values represent mean $\log_{10}$ cfu *E. coli* O157:H7/g ± standard deviation

Mean superscript with different capital letters in the same row and different lower case in same column differ significantly ($P<0.05$)

Table 3.4 Viability of *E. coli* O157:H7 in ground beef treated with *Lb. reuteri* and AIT and stored at 4° C under nitrogen

Experimental Design code	Treatment			Days Storage at 4° C					
	E. coli[a]	*Lb. reuteri*[a]	AIT	0	5	10	15	20	25
C4	3.0	-	-	3.12 ± 0.09^{Ab}	3.19 ± 0.09^{Ad}	2.89 ± 0.04^{Bc}	2.75 ± 0.09^{Cc}	2.67 ± 0.19^{Cb}	2.76 ± 0.09^{Cb}
T4	3.0	3.0	+	2.89 ± 0.06^{Ac}	2.30 ± 0.30^{Bc}	1.81 ± 0.09^{Cd}	0^{Dd}	0^{Dd}	0^{Dd}
T5	3.0	6.0	+	2.91 ± 0.01^{Ac}	2.36 ± 0.08^{Bc}	0^{Cc}	0^{Cd}	0^{Cd}	0^{Cd}
C5	6.0	-	-	5.99 ± 0.13^{Aa}	6.02 ± 0.07^{Aa}	5.68 ± 0.09^{Ba}	5.27 ± 0.11^{Ca}	4.99 ± 0.07^{Da}	5.17 ± 0.14^{Ca}
T9	6.0	3.0	+	6.02 ± 0.08^{Aa}	5.44 ± 0.13^{ABb}	5.03 ± 0.18^{Bb}	3.83 ± 0.20^{Cb}	1.82 ± 1.41^{Dc}	1.28 ± 1.00^{Dc}
T10	6.0	6.0	+	6.01 ± 0.05^{Aa}	5.06 ± 0.36^{Bc}	5.01 ± 0.15^{Bb}	4.04 ± 0.42^{Cb}	2.55 ± 0.50^{Db}	2.07 ± 1.05^{Db}

[a] $\log_{10}$ cfu/g meat at inoculation

Values represent mean $\log_{10}$ cfu *E. coli* O157:H7/g ± standard deviation

Mean superscript with different capital letters in the same row and different lower case in same column differ significantly ($P<0.05$)

Table 3.5 Changes in total number of bacteria/g of ground beef following treatment with *Lb. reuteri* and/or AIT during storage at 4° C under nitrogen

Experimental Design code	Treatment[a]		Days Storage at 4° C					
	Lb. reuteri[a]	AIT	0	5	10	15	20	25
C1	-	-	5.09 ± 0.13^{Fab}	5.61 ± 0.22^{Eab}	5.93 ± 0.16^{Da}	6.48 ± 0.13^{Ca}	6.69 ± 0.06^{Ba}	6.87 ± 0.08^{Aa}
C2	3.0	-	4.96 ± 0.18^{Bab}	5.35 ± 0.31^{Ab}	4.82 ± 0.20^{Bc}	3.98 ± 0.15^{Cd}	3.67 ± 0.14^{De}	3.94 ± 0.07^{Cd}
C3	6.0	-	5.12 ± 0.07^{Cab}	5.87 ± 0.23^{Aa}	5.36 ± 0.26^{Bb}	4.49 ± 0.21^{Db}	4.36 ± 0.22^{Dbc}	4.38 ± 0.07^{Dc}
C6	-	+	4.81 ± 0.43^{Abc}	3.75 ± 0.14^{Ce}	3.69 ± 0.92^{Cde}	4.29 ± 0.17^{Bbc}	4.31 ± 0.15^{Bdc}	4.54 ± 0.08^{Bb}
C7	3.0	+	5.17 ± 0.12^{Aa}	4.46 ± 0.19^{Bc}	3.75 ± 0.19^{Dd}	4.15 ± 0.15^{Cdc}	4.53 ± 0.26^{Bb}	4.64 ± 0.07^{Bb}
C8	6.0	+	4.63 ± 0.42^{Ac}	4.06 ± 0.22^{Cd}	3.47 ± 0.21^{De}	4.33 ± 0.21^{BCbc}	4.12 ± 0.09^{Cd}	4.55 ± 0.15^{Abb}

[a] $\log_{10}$ cfu/g meat at inoculation

Values represent mean $\log_{10}$ cfu/g ± standard deviation

Mean superscript with different capital letters in the same row and different lower case in same column differ significantly ($P<0.05$)

Table 3.6 Changes in lactic acid bacterial viability following treatment of ground beef with *Lb. reuteri* and/or AIT during storage at 4° C under nitrogen

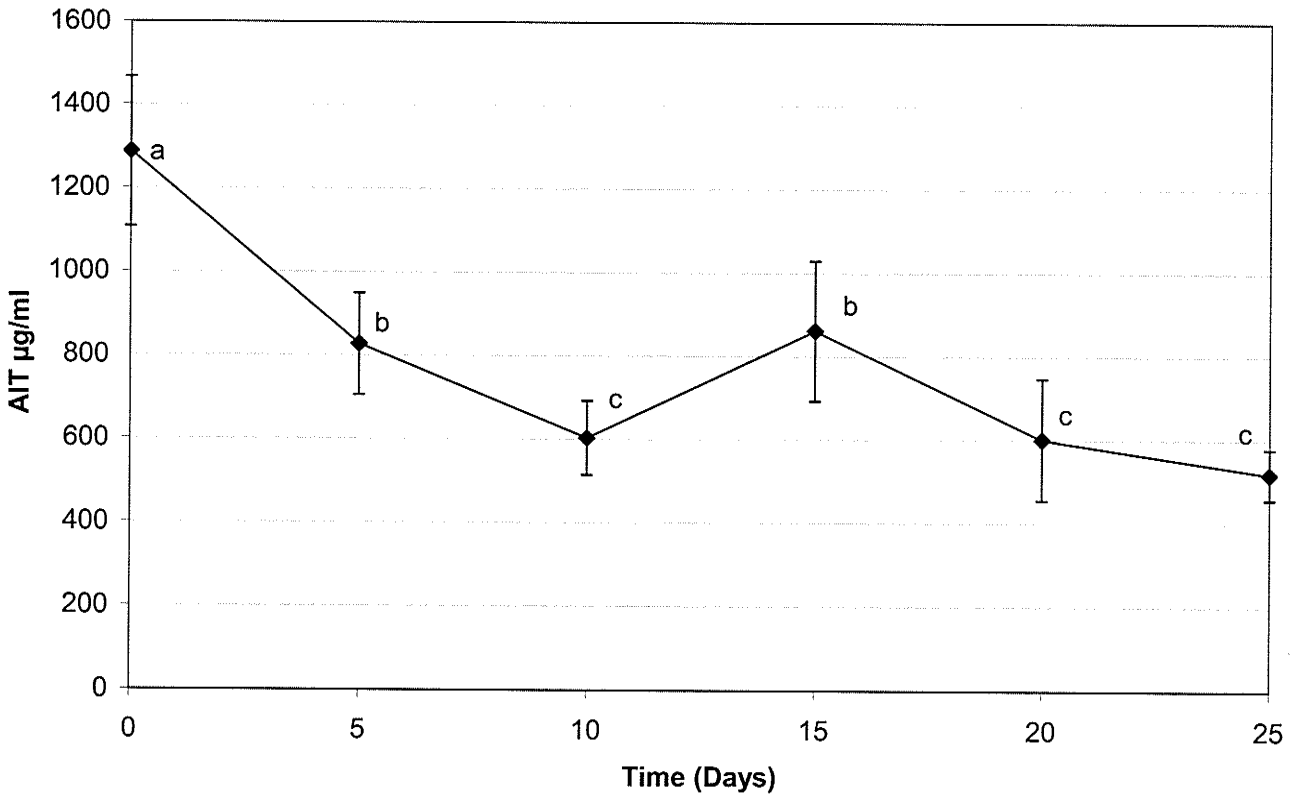
Experimental Design code	Treatment		Days Storage at 4° C					
	Lb. reuteri[a]	AIT	0	5	10	15	20	25
C1	-	-	3.76 ± 0.07^{Dd}	5.03 ± 0.19^{Cb}	5.18 ± 0.15^{Cbc}	5.62 ± 0.33^{Bb}	5.96 ± 0.28^{Aa}	6.11 ± 0.42^{Aa}
C2	3.0	-	4.77 ± 0.06^{Cb}	5.28 ± 0.23^{Bb}	5.43 ± 0.14^{ABb}	5.37 ± 0.04^{Bc}	5.49 ± 0.18^{ABb}	5.64 ± 0.29^{Ab}
C3	6.0	-	6.11 ± 0.07^{Aa}	6.09 ± 0.14^{Aa}	6.09 ± 0.21^{Aa}	5.93 ± 0.17^{Ba}	5.86 ± 0.07^{BCa}	5.71 ± 0.06^{Cb}
C6	-	+	4.29 ± 0.15^{Bc}	3.54 ± 0.15^{Cd}	4.25 ± 0.22^{Bd}	4.39 ± 0.12^{Be}	4.44 ± 0.18^{Bc}	4.69 ± 0.15^{Ad}
C7	3.0	+	4.22 ± 0.25^{Cc}	4.61 ± 0.18^{Bc}	5.01 ± 0.14^{Ac}	4.99 ± 0.46^{Ad}	4.65 ± 0.46^{ABc}	4.59 ± 0.16^{Bd}
C8	6.0	+	6.12 ± 0.24^{Aa}	5.08 ± 0.26^{Cb}	5.37 ± 0.20^{Bb}	5.35 ± 0.11^{Bc}	5.41 ± 0.17^{Bb}	5.43 ± 0.14^{Bc}

[a] $\log_{10}$ cfu/g meat at inoculation

Values represent mean $\log_{10}$ cfu/g ± standard deviation

Mean superscript with different capital letters in the same row and different lower case in same column differ significantly ($P<0.05$)

Fig 3.1 AIT levels in package head space during storage of ground beef at 4° C



Data points and error bars represent the mean and standard deviation of six values respectively

Data points marked with different letters differ significantly ($P<0.05$)

Chapter 4

Lethal effects of non-deheated (hot) mustard flour on *E. coli* O157:H7 in refrigerated ground beef

4. 1 Abstract

The antimicrobial effect of deheated and non-deheated mustard flour on *E. coli* O157:H7 in refrigerated ground beef was studied. Mustard flours were added at 10 and 20% to ground beef inoculated with 3 log cfu/g of a five strain cocktail of *E. coli* O157:H7 and stored at 4° C for ≤ 18d. The effect of pure allyl isothiocyanate (AIT) (about 1300 ppm) on *E. coli* O157:H7 was also studied to compare the effects of mustard flour with AIT. Samples were analysed in triplicate at 3 d intervals both for *E. coli* O157:H7 and total bacterial numbers. Non-deheated mustard flour at 20% completely eliminated *E. coli* O157:H7 at day 3 whereas AIT and 10 % non-deheated mustard flour required 15 and 18d, respectively, to completely eliminate *E. coli* O157:H7. Deheated mustard flour at both 10 and 20% did not eliminate *E. coli* O157:H7 by the end of storage. Total bacterial numbers were significantly reduced ($P< 0.05$) when 20% non-deheated mustard flour or AIT were used, indicating potential extension of ground beef shelf life. Isothiocyanates and probably AIT present in the mustard flour are the agent(s) responsible for the antimicrobial effects of mustard flour.

4.2 Introduction

The incidence of *E. coli* O157:H7 which was first reported to cause hemorrhagic colitis and hemolytic uremic syndrome two decades ago (Riley et al. 1983) has been increasing year after year. Consumption of undercooked ground beef has been a common cause of *E. coli* O157:H7 outbreaks (hamburger disease) in North America. United States

and Canada have the highest frequencies of foodborne illness caused by *E. coli* O157:H7 in the world. *E. coli* O157:H7 is a "zero-tolerated" pathogen in beef in the U.S., and as a result thousands of tons of ground beef are recalled every year for possible *E. coli* O157:H7 contamination. This has led to considerable financial loss for the meat industry. Canada is the single largest mustard exporter in the world and is one of the world's top five mustard producers. Mustard has been used as a spice or a condiment for centuries throughout the world. Mustard flours when deheated are used as emulsifiers, binders and bulking agents, but non-deheated mustard is used as a spice in seasonings and for flavouring in meat formulations, particularly in sausage and salami manufacture (Cui, 1997). Brown or oriental mustard (*Brassica juncea*) and yellow or white mustard (*Sinapis alba*) contain glucosinolates, which on enzymatic breakdown by myrosinase yield isothiocyanates, cyanides and thiocyanates (Tsao et al. 2002). The heat or flavour of the mustard is due to the presence of isothiocyanates and when used as binders the flour is treated to inactivate the myrosinase. Allyl isothiocyanate, one of the predominant breakdown products of glucosinolates, has been reported to have antimicrobial effects against bacteria including *E. coli* O157:H7 in ground beef, pre-cooked roast beef, alfalfa seeds, lettuce, and 'Asazuke' (a low salt vegetable) (Muthukumarasamy et al. 2003, Ward et al. 1998, Park et al. 2000, Lin et al. 2000, Ogawa et al. 2000). Although pure AIT from natural sources can be used as a food preservative in Japan, its use in food is restricted as a flavouring agent in Canada. Moreover, use of pure AIT in food has limitations because of the strong pungent smell associated with volatile AIT which causes eye and nasal irritation and a burning sensation of the skin and tongue. Mustard flour,

being a natural source of AIT, may be used as an alternative approach in food systems to eliminate *E. coli* O157:H7.

4.3 Objective

The objective of the research was to study the lethality of mustard flour toward *E. coli* O157:H7 in refrigerated nitrogen packed ground beef. The effect of deheated mustard, which is incapable of producing isothiocyanates due to enzyme inactivation, was compared with non-deheated mustard to understand if isothiocyanates from the flour were responsible for lethal effects on *E. coli* O157:H7. The effect of pure allyl isothiocyanate on *E. coli* O157:H7 was also studied.

4.4 Materials and methods

Five strains of *E. coli* O157:H7, 7128, 7110, 7236 (human isolates) and 7282 and 7283 (hamburger isolates) were kindly donated by Dr. R. Khakria, Laboratory Center for Disease Control, Ottawa, Canada. *E. coli* O157:H7 strains were maintained in tryptic soy broth at 37° C. Inside round beef roasts were obtained from a local supermarket and aseptically ground manually in a meat grinder (9.5 mm plate). The five strain cocktail of *E. coli* O157:H7 prepared in peptone water was inoculated in the meat to reach a level of 3 log cfu/g. Deheated and non-deheated mustard flour at 10 and 20% were added to the ground beef and re-ground to evenly mix the mustard flour. Twenty five grams of meat were placed in Deli*1 bags (Winpak, Winnipeg, Canada), a vacuum was generated, back flushed with nitrogen and the bags were sealed. Samples to study the effect of AIT were prepared by adding 1 ml of pure allyl isothiocyanate (Acros Organics, Geel, Belgium) and a commercial corn oil mixture (AIT: corn oil at a ratio of 7:3) to sterile filter paper inserts and one insert was placed in each bag containing 25g of *E. coli* O157:H7 -

inoculated beef before packaging. The packaged ground beef was stored at 4° C. Triplicate samples from each treatment were analysed for *E. coli* O157:H7 and total bacterial numbers from 0 to 18d at 3d intervals. Eleven grams of beef were mixed with 99ml of peptone water and stomached for 1 min. Serial dilutions were prepared in peptone water and plated with a spiral plater. Sorbitol MacConkey agar supplemented with cefixime-tellurite (CT-SMAC) and tryptic soy agar (TSA) were used for enumerating *E. coli* O157:H7 and total bacteria, respectively. The plates were incubated at 37° C aerobically for 24 and 48h for CT-SMAC and TSA, respectively. In order to determine if the *E. coli* O157:H7 cells were killed or injured as a result of mustard flour or AIT treatment, a resuscitation step on TSA (incubated at 37° C for 3h) was used followed by over-laying with CT-SMAC agar. Survivors on the TSA over-laid CT-SMAC plates indicated recovery of injured *E. coli* O157:H7 cells. All data were analysed by Statistical Analysis System (version 8.1) software. Analysis of variance by the General Linear models procedure and Duncan's multiple range tests were used to find significant differences ($P< 0.05$) among and between treatments.

4.5 Results and Discussion

4.5.1 Bactericidal effects on *E. coli* O157:H7

The effect of AIT, deheated and non-deheated mustard flour on the viability of *E. coli* O157:H7 in ground beef stored under nitrogen at 4° C are shown in Fig 4.1. Non-deheated mustard at 20% was most effective in killing *E. coli* O157:H7 compared to deheated mustard at both 10 and 20% or AIT at about 1300 ppm. Non-deheated mustard at 20% completely eliminated *E. coli* O157:H7 at an initial level of 3 log cfu/g from ground beef by day 3, whereas AIT at about 1300 ppm and non-deheated mustard at 10%

required 15 and 18 d, respectively, to reduce *E. coli* O157:H7 below detectable levels (40 cfu/g). Deheated mustard at both levels did not completely kill *E. coli* O157:H7 in ground beef stored under nitrogen at 4° C even after 18 days. On TSA over-laid with CT-SMAC plates, no recovery of injured cells occurred, indicating that *E. coli* O157:H7 were killed in treatments where no *E. coli* O157:H7 grew on CT-SMAC plates.

The results presented clearly show that non-deheated mustard flour at 20% had strong bactericidal effects against *E. coli* O157:H7 in ground beef stored under nitrogen at 4° C. Non-deheated mustard flour at 10% and AIT were able to completely eliminate *E. coli* O157:H7 but required significantly longer than 20% non-deheated mustard flour. The only difference between the two types of mustard flour used was the presence of active myrosinase in the non-deheated product. Lethal effects of the latter flour were attributed to myrosinase action, causing formation of isothiocyanates in the presence of moisture from the meat. Deheated mustard, with inactive myrosinase, was not significantly lethal to *E. coli* O157:H7. Allyl isothiocyanate is the main component of mustard that might be responsible for bactericidal effects (Isshiki et al. 1992). The average allyl isothiocyanate levels in mustard ranges from 7 to 13 mg/g. The results showed that non-deheated mustard flour at 20% was more effective than AIT (about 1300 ppm) in killing *E. coli* O157:H7. The increased lethality of mustard flour may be due to synergistic effects of AIT with other isothiocyanates present in mustard. The bactericidal activity of mustard flour may provide an opportunity for its use in meat and more widely in the food industry as a secondary preservative to control pathogenic microorganisms. This should provide added assurance of safer food for consumers.

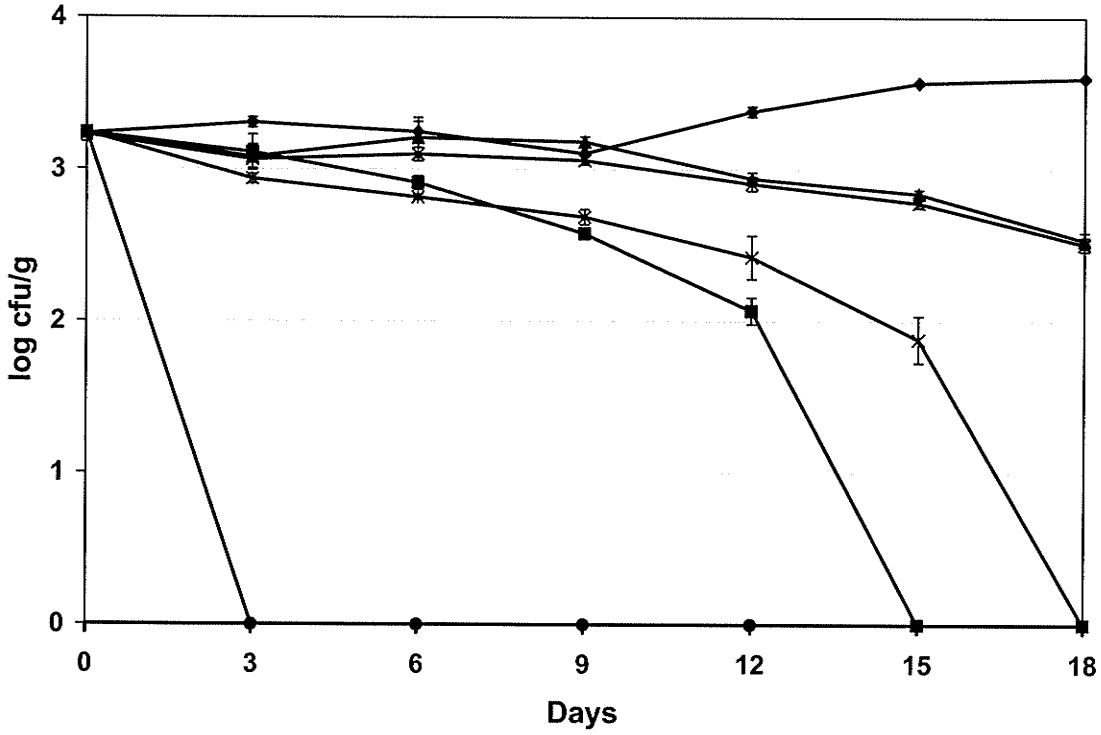
4.5.2 Effect on total bacterial numbers

The effect of AIT, deheated and non-deheated mustard flour on the total bacterial numbers in ground beef stored under nitrogen at 4° C is shown in Fig 4.2. All treatments significantly delayed the growth of total aerobic mesophiles in ground beef stored at 4° C. In the untreated control the total bacterial count was 6.88 log cfu/g by 18d, indicating that the beef could be close to spoilage if untreated with any antimicrobials. Non-deheated mustard flour at 20% and AIT were able to maintain the total bacterial numbers at < 4 log cfu/g by the end of 18d.

4.6 Conclusion

Non-deheated mustard flour at 20% was more lethal than deheated flour to *E. coli* O157:H7 in ground beef. Non-deheated mustard flour may be used as a natural antimicrobial in ground beef to eliminate *E. coli* O157:H7 and has potential for similar use in other susceptible foods where mustard flavour is compatible. The use of mustard flour may not only increase the safety of ground beef but also extend its shelf life.

Fig 4.1 Viability of *E. coli* O157:H7 in ground beef treated with AIT, deheated and non-deheated mustard flour, stored at 4° C under nitrogen packaging



Control (Beef + E. coli O157:H7)

Beef + E. coli O157:H7 + AIT

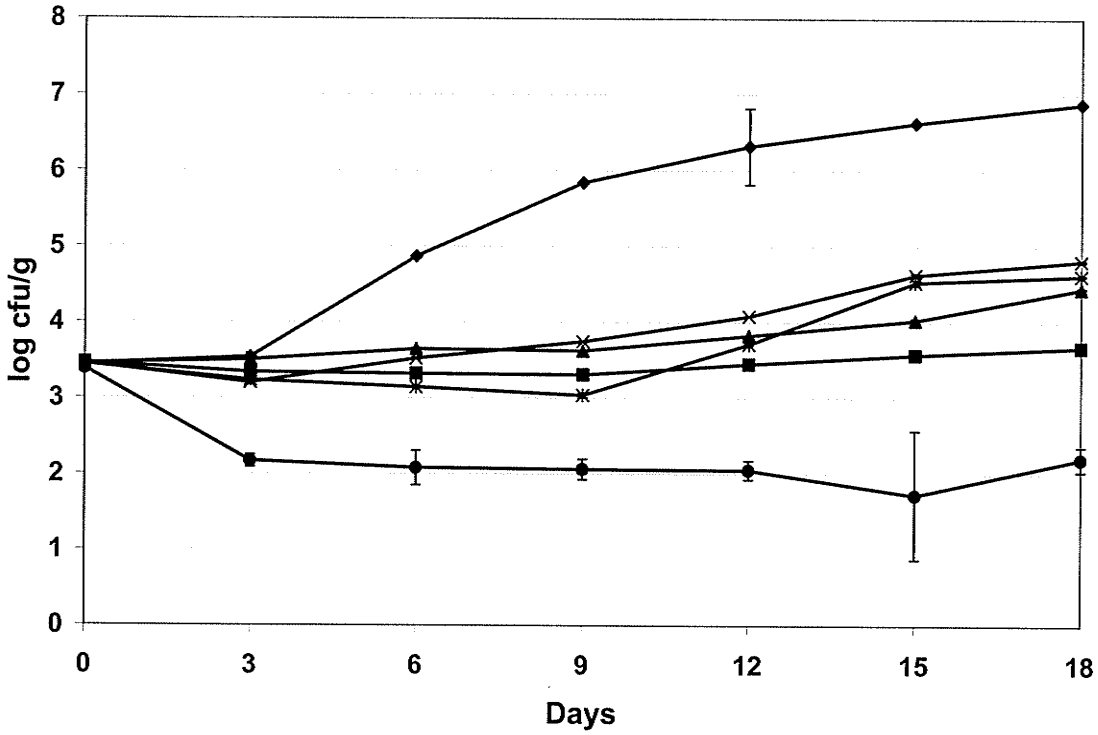
Beef + E. coli O157:H7 + 10% deheated mustard flour

Beef + E. coli O157:H7 + 20% deheated mustard flour

Beef + E. coli O157:H7 + 10% non-deheated mustard flour

Beef + E. coli O157:H7 + 20% non-deheated mustard flour

Fig 4.2 Changes in total number of viable bacteria in ground beef stored at 4° C under nitrogen packaging after treatment with AIT, deheated and non-deheated mustard flour.



Control (Beef + E. coli O157:H7)

Beef + E. coli O157:H7 + AIT

Beef + E. coli O157:H7 + 10% deheated mustard flour

Beef + E. coli O157:H7 + 20% deheated mustard flour

Beef + E. coli O157:H7 + 10% non-deheated mustard flour

Beef + E. coli O157:H7 + 20% non-deheated mustard flour

Chapter 5

Stability of *Lactobacillus reuteri* in different types of microcapsules

5.1 Abstract

This study was designed to find the most suitable method and wall material for microencapsulation of the probiotic bacterium *Lactobacillus (Lb) reuteri* to maintain cell viability during gastric challenge. Five *Lb. reuteri* strains were individually encapsulated using alginate, alginate plus starch, κ-carrageenan with locust bean gum, or xanthan with gellan by extrusion or phase separation (emulsion). The morphology of the microcapsules was studied using phase contrast and cryo-scanning electron microscopy (cryo-SEM). The resistance of these microcapsules and the viability of contained *Lb. reuteri* to simulated gastric juice were studied. The shape and size of the microcapsules produced varied with the preparation method and type of wall material. Extruded microcapsules were larger and more uniformly shaped. Survival of microencapsulated *Lb. reuteri* was significantly better than that of planktonic cells and varied with the strain, method of microencapsulation and wall material used. In general, microencapsulation using alginate and alginate with starch by both extrusion and phase separation were found to provide bacteria significantly greater protection ($p< 0.05$) against simulated gastric juice.

5.2 Introduction

Probiotics have been known for almost a century to aid in establishing a beneficial intestinal microflora and provide a variety of health benefits. Excluding natural health products, dairy and fermented foods are the main commercial sources of probiotic bacteria in the human diet. Consumer preference for probiotic-containing products has contributed to an expansion of the variety of probiotic products available at retail stores

in recent years. The survival of probiotic organisms in the foods that serve as their carriers is essential. Various studies have shown that probiotic organisms survive poorly in foods like yoghurt and fermented milks because they do not tolerate exposure to acidic environments (Kailasapathy and Rybka, 1997, Klaver et al. 1993, Hughes and Hoover, 1995). Probiotics exert their action after colonization and growth in the distal ileum and colon, which means they must survive passage through the oesophagus, acidic stomach and the alkaline conditions of the small intestine to be effective. The selection of the most suitable probiotic for a particular food application must consider not only the survival of the organism throughout the storage life of the food but also its ability to colonize epithelial surfaces in the lower intestine. In addition, once established it must exert its beneficial biological influence through antimicrobial production or other activity of value to the host such as stimulation of immunity, production of anti-cholesterol factors and anti-carcinogenic properties (Naidu et al. 1999)

Lactobacillus reuteri has been reported to possess probiotic properties as the organisms are normal inhabitants of the gastrointestinal system, possess good colonization potential, and inhibit disease causing organisms (Casas and Dobrogosz, 2000). *Lb. reuteri* produces a potent low molecular weight, non-peptide antimicrobial substance (β – hydroxypropionaldehyde or reuterin) during anaerobic metabolism of glycerol (Axelsson et al. 1989). Reuterin has a broad spectrum of activity against both Gram-positive and Gram-negative bacteria (Chung et al. 1989). *Lb. reuteri* in the presence of 250 mmol glycerol has been used successfully to eliminate 6 log cfu/g *E. coli* O157:H7 in ground beef within 20 d at 4° C (Muthukumarasamy et al. 2003). Reuterin was used to inhibit *Listeria monocytogenes* and *E. coli* O157:H7 in vacuum-packed

ground ham, cottage cheese and ultra high-temperature treated milk (El-Ziney and Debevere, 1998, El-Ziney et al. 1999). Microencapsulation has been considered as a means with good potential for protecting probiotic bacteria against adverse conditions in food and during passage through the GI tract. Microencapsulation has been shown to be capable of protecting bacterial cells by retaining them within a polymer membrane or matrix and yielding increased survival (Audet et al. 1988, Sheu and Marshall, 1993). Two widely used methods of encapsulation are extrusion and emulsification (Krasaekoopt et al. 2003). A variety of wall materials such as alginate, starch, κ- carrageenan-locust bean gum, xanthan-gellan, chitosan, and gelatin have been used for microencapsulation (Audet et al. 1988, Sheu and Marshall, 1993, Sultana and others 2000, Sun and Griffiths, 2000), but comparative studies evaluating performance of the different methods to allow choice of the best wall material for use with probiotic organisms are not available. The present study was designed to allow selection of best combination of microcapsule production method and type of wall material for encapsulation of *Lb. reuteri* to maximize bacterial viability.

5.3 Materials and methods

5.3.1 Culture and growth conditions

Lb. reuteri 1063 (ATCC 53608) was obtained from the American Type Culture Collection (Manassas, VA), and *Lb. reuteri* strains ATCC 55730 (SD2112), PTA 4659 (MM2-3), PTA 4965 (CF2-7F) and DSM 16666 (RC14) were kindly provided by Biogaia Biologics Inc. (Raleigh, NC). All strains were maintained in deMan Rogosa Sharpe (MRS) broth (BBL, Becton Dickinson, Sparks, MD) containing 20% glycerol at

-70° C. Frozen stock cultures of *Lb. reuteri* were sub-cultured twice in MRS broth incubated at 37° C before use in microencapsulation experiments. Overnight cultures were centrifuged, washed and suspended in 0.1% peptone water to achieve 8 log cfu/ml following standardization at an optical density of 600 nm.

5.3.2 Microencapsulation procedure

Sodium alginate, κ-carageenan, locust bean gum, xanthan, gellan (Sigma-Aldrich Co., St. Louis, MO) and corn starch (National Starch and Chemical Co., Bridge Water, NJ) were used as wall materials for microencapsulation of *Lb. reuteri*. Alginate (3% w/v), or 2 % (w/v) alginate plus 2 % (w/v) starch, 1.75% (w/v) κ-carageenan plus 0.75% (w/v) locust bean gum (LBG) or 1% (w/v) xanthan plus 0.5% (w/v) gellan were used for microencapsulation by both extrusion and a two phase (water/oil) emulsion system (modified from Sheu and Marshall, 1993, Sultana et al. 2000, and Sun and Griffiths, 2000). Glassware and reagents used in the experiments were sterilized before use. All wall materials in appropriate concentration were added to distilled water and were autoclaved and allowed to cool to 25° C (κ-carageenan plus LBG wall material were maintained at 40°C to prevent solidification). To this wall material 9 log cfu/ml *Lb. reuteri* cells (standardized as above in 0.1% peptone water) were added at a ratio of about 1:5 (v/v) so that the desired level of 8 log cfu/ml was reached in the microcapsules. This wall material-culture mixture was used for microencapsulation by both extrusion and emulsion methods. Extrusion was performed by expression of the wall material-culture mixture through a 21G syringe needle dropwise into 0.5M $CaCl_2$. Microcapsules were held at 25° C for 30 min to ensure complete solidification. Microcapsules were separated by filtration through Whatman #4 filter paper and stored in sterile petri dishes at 4° C

until used in challenge experiments. The two phase emulsion (phase separation) method involved adding one part wall material (with added culture) to 5 parts commercial corn oil (v/v) (continuous phase) containing 0.02% tween 80 (Fisher Scientific, Fair Lawn, NJ). The mixture was stirred at 800 rpm for 5 min using a Sorvall- Omni mixer (Ivan Sorvall Inc, Norwalk, CT) to form a uniform water-in-oil emulsion. Sterile 0.1M $CaCl_2$ was added at the sides of the beaker until the emulsion was completely broken. The beads formed were collected by vacuum filtration through Whatman # 4 filter paper and washed using 200 ml sterile 0.1% peptone water. In the case of κ-carageenan-LBG and xanthan-gellan, one part of polymer-culture mixture was added to 5 parts of commercial corn oil warmed to 40°C and then stirred using the Sorvall- Omni mixer at 800 rpm. The dispersed polymer was allowed to cool to 25° C to form microcapsules. The microcapsules were vacuum filtered and washed with an equal volume of 0.5M $CaCl_2$. All microcapsules were stored at 4° C and were used in gastric challenge experiments within a day.

5.3.3 Microcapsule morphology

The size of the microcapsules was measured using an electric digital micrometer (Marathon Watch Co., Richmond Hill, ON) or by an inverted stage phase contrast microscope (Nikon Diaphot TMD, Kanagawa, Japan) equipped with a TV camera (Panasonic WV1550, Matsushita Electric Industrial Co. Ltd., Osaka, Japan) and a stage micrometer, depending on the size of the capsules. The shape of the microcapsules was observed both visually and with the phase contrast microscope. Planktonic *Lb. reuteri* strains were fixed in 0.1 M cacodylate buffered (pH 6.8) 2.8% glutaraldehyde solution and washed with a series of 20 to 100% ethanol concentrations and examined through a

Philips XL30 environmental scanning electron microscope (ESEM) operated at 7.5 kV accelerating voltage. A Quanta 200 ESEM (FEI Company, Hillsboro, OR) fitted with cryo-stage attachment (CT 1500 cryotransfer system, Gatan, Abingdon, UK) was used in conventional mode at an accelerating voltage of 15 to 20kV to examine the external and internal appearance of the microcapsules. Larger capsules prepared by the extrusion method were placed on rivet holders, whereas smaller capsules made by emulsion were placed on Balzers holders (Gatan, UK). Holders were plunged into a liquid nitrogen slush, and placed in the cryo-chamber of the cold stage. Samples were observed unfractured and fractured. Microcapsules were mounted on rivets with the adhesive Tissue Tek O. CT. (BDH Laboratory Supplies, Poole, England), another rivet was placed on top and sealed with Tissue Tek. The assembly was plunge-frozen, and the rivets were knocked apart with a cooled scalpel to create a fracture. When Baltzers holders were used they were split apart under liquid nitrogen and loaded into the specimen carrier for cryo-stage observation. Samples were sputter-coated with gold and viewed through the cryo-SEM. Images were captured as grayscale digital images in TIFF format.

5.3.4 Survival of *Lb. reuteri* in simulated gastric juice

Non-encapsulated cells of *Lb. reuteri* were tested at a level of 8 log cfu/ml in simulated gastric juice (0.08 M HCl containing 0.2% NaCl) at pH 1.5 (after addition of capsules) for survival of *Lb. reuteri* over 2 h at 30 min intervals (Hansen et al. 2002). To study the effects of microencapsulation on *Lb. reuteri* survival in simulated gastric juice, one ml (measured by displacement of water) of microcapsules was added in triplicate to 9 ml of the simulated gastric juice pre-warmed to 37° C, and viability of *Lb. reuteri* was followed as noted above (Hansen et al. 2002, Rao et al. 1989). Capsules were harvested

every 30 min by filtration, added to 9 ml of 0.5 M phosphate buffer and ground using a sterile mortar and pestle. Serial dilutions were prepared in peptone water and plated on MRS agar with a spiral plater (Autoplate 4000, Spiral Biotech, Norwood, MA). Plates were incubated anaerobically for 24-48h at 37° C using the BBL GasPak plus system. Experiments were done in duplicate and repeated three times.

5.3.5 Survival of *Lb. reuteri* in simulated bile juice

Planktonic *Lb. reuteri* cells were tested at a level of 8 log cfu/ml in simulated bile juice made with MRS broth containing 1.2 % (w/v) bile salts (Sigma-Aldrich Co.) pre-warmed at 37° C, and survival of *Lb. reuteri* monitored over 6h at 37° C (Song et al. 2003). Aliquots were taken at 1h intervals and plated on MRS agar as described above.

5.3.6 Statistical analysis

All data were analyzed by Statistical Analysis System (version 8.1) software (SAS Institute, Cary, N.C.). Analysis of variance was done by the General Linear Models procedure and Duncan's multiple range tests were used to find significant differences ($p < 0.05$) among treatments at each sampling time and among different times for each treatment.

5.4 Results

5.4.1 Microcapsule morphology

Planktonic *Lb. reuteri* cells appeared as regular rods about 2μm long with some shorter forms and an occasional elongated cell present (Fig 5.1). The average size of the capsules was 2 to 4 mm by the extrusion method and 20μm to 1mm by phase separation (Table 5.1). The shape of the microcapsules produced varied with the method and type of wall material. On extrusion, alginate (Fig 5.2a, b) and alginate with starch formed regular

spherical capsules whereas the other two wall materials yielded imperfect spheres (e.g Fig 5.2c). The shape of the capsules formed by phase separation was also irregular (Fig 5.3a, b). *Lb. reuteri* cells were not visible at the surface of the capsules even after freeze-fracture and visualization at high magnification (≤ 15000 X). No cavities were noticed on fractured surfaces of the microcapsules. However, when microcapsules were ground with a mortar and pestle and plated on MRS agar, bacterial colonies developed indicating that viable cells were present in the encapsulating matrix.

5.4.2 Survival of *Lb. reuteri* in simulated gastric juice

When planktonic cells of *Lb. reuteri* were subjected to simulated gastric juice challenge at pH 1.5 for 2h, *Lb. reuteri* PTA 4659 survived better than the other strains. Viable free cells of *Lb. reuteri* ATCC 55730 and DSM 16666 were eliminated after 90 min when the initial level was 8 log cfu/ml. Although *Lb reuteri* strains PTA 4965 and ATCC 53608 survived gastric challenge, a 7 log cfu/ml reduction in viable numbers was noted after 2h. The survival of *Lb. reuteri* cells treated with simulated gastric juice was significantly better and overall results depended on the method of microencapsulation and wall material used (Fig 5.4-5.8). The performance of encapsulation methods and the wall materials yielding best survival in gastric juice are shown in Table 5.2. In general, microencapsulation using alginate and alginate with starch by extrusion or emulsion provided greater protection against gastric juice for all five strains of *Lb. reuteri,* and of these methods extrusion was better able to protect cells. In contrast, survival of organisms microencapsulated with xanthan plus gellan or κ-carrageenan plus locust bean gum and challenged with simulated gastric juice was poor, although survival in microcapsules prepared by emulsion formation was better ($p< 0.05$) than by extrusion (Table 5.3).

5.4.3 Survival of Lb. reuteri in bile juice

All five strains of *Lb. reuteri* survived well in bile juice (1.2 % bile salt) for 6h. No significant differences in *Lb. reuteri* numbers ($p > 0.05$) were noticed even after 6h indicating that *Lb. reuteri* cells were resistant to bile salt. Therefore microencapsulated *Lb. reuteri* were not subjected to bile juice challenge.

5.5 Discussion

Freeze- or spray-drying as methods for preserving probiotics that are added to food are not considered the best options for probiotic amendment because the latter come in direct contact with the product and experience decreased viability which increases the number of organisms needed to achieve desired beneficial effects. Encapsulation using polymers such as alginate, starch, gums and carrageenan is an attractive alternative to overcome this problem. Microencapsulation using these polymers has been shown to protect probiotics from bacteriophages (Steenson et al. 1987), to increase bacterial survival during acid challenge (Hansen et al. 2002), and increase probiotic survival in yoghurt, ice milk, frozen dairy desserts and mayonnaise (Adhikari et al. 2000, 2003, Sheu and Marshall, 1993, Shah and Ravula, 2000, Khalil and Mansour, 1998, Kebary et al.. 1998). In addition, microencapsulation of *Lb. reuteri* with the substrate (glycerol) required for reuterin production, along with a heterologous organism such as *Bifidobacterium* may provide opportunity to increase and sustain reuterin production in foods. Reuterin production responds through quorum sensing to the presence of different bacterial genera (El-Ziney et al. 2000).

The size and shape of the microcapsules formed in the present work were similar to those of capsules prepared in other studies (Sheu and Marshall, 1993, Sultana et al.

2000, Sun and Griffiths, 2000). *Lb. reuteri* cells were not visualized on the fractured surfaces of microcapsules. This may have been due to the opaque character of water in cryo-SEM images. Since some fractured surfaces were "etched" under vacuum to address this issue but was unsuccessful, it is also possible that bacterial exopolysaccharide, which would also be opaque in the cryo-SEM, shielded bacteria from view.

Non-encapsulated (planktonic) cells of all 5 strains of *Lb. reuteri* were resistant to simulated bile juice challenge for 6h. This is consistent with observations by others regarding the alkali tolerance of these organisms (El-Ziney et al. 2000). It is possible that acid exposure of microencapsulated *Lb. reuteri* might sensitize the cells to bile treatment, however, this was not tested in these experiments.

Although alginate and alginate-starch provided greater protection against gastric juice, the wall material and encapsulation method providing the greatest viability varied somewhat with the strain of bacteria. The strain to strain variation in sensitivity to gastric juice after microencapsulation may have been due to different acid resistance properties of these strains. In contrast with results obtained here at pH 1.5, O'Riordan et al. (2001) reported that modified starch-encapsulated bifidobacteria prepared by spray-drying were not protected against acid challenge at pH 2.8. However, in work by Wang et al. (1999), it was shown that starch granules could protect bifidobacteria strains during passage through the mouse intestinal tract. Various factors such as the diameter of the needle used for extrusion, the distance of free fall during extrusion of polymers, the concentration of $CaCl_2$ as well as the polymers themselves, the speed of agitation, and the type of emulsifier used were reported to influence the size and shape of capsules and also the viability of encapsulated organisms (Krasaekoopt et al. 2003).

Other work has shown that larger microcapsules are better able to protect bacteria inside. Lee and Heo (2000) reported that large alginate capsules (2.63 mm) offered more protection to *Bifidobacterium longum* cells against acid challenge than smaller capsules (1.03 mm). The present results with alginate containing capsules are in agreement with this observation, as it was found that the larger (2-4 mm) extruded capsules protected the cells better than the smaller (20μm – 1mm) emulsified capsules. The larger size of these capsules may have afforded additional physical protection simply by increasing the distance between encapsulated cells and the acid. However, it should be noted that *Lb. reuteri* was better protected from acid in alginate-containing microcapsules prepared by emulsification than similarly prepared microcapsules made of the xanthan or κ-carageenan-containing polymers, even though the alginate-containing capsules were 1/10 their diameter. The increased resistance of organisms in alginate and alginate-starch microcapsules to acid challenge may have been in part due to the protective nature of the polymer networks generated during capsule formation.

In contrast with alginate-containing capsules where bacterial survival was highest and where extrusion yielded the best results of all tested systems, survival of *Lb. reuteri* in xanthan-gellan and κ-carageenan-LBG microcapsules was significantly higher in capsules prepared by emulsification. With the latter polymers the size of emulsified microcapsules ($\leq$ 1 mm) was smaller than when the same polymers were extruded ($<$ 4 mm). Therefore, as with alginate not only the nature of the polymer used but also the type of network formed influenced bacterial survival. Microcapsule size was less important in providing protection against acid challenge when xanthan-gellan or κ-carageenan-LBG

were used. However, the overall survival of *Lb. reuteri* in xanthan-gellan or κ-carageenan-LBG was unsatisfactory.

Preparation of microcapsules by extrusion was found to be less laborious compared to emulsification and the size of the capsules generated by the former method were more uniform in shape and size, allowing better assessment of microencapsulation performance. However, the effect of these larger capsules on the sensory quality of food will need to be assessed before they can be recommended for food applications.

5.6 Conclusion

Microcapsule formation by extrusion of alginate or alginate plus starch wall materials provided better protection of *Lb. reuteri* cells from acid challenge than when microencapsulation was done by emulsification. κ-carageenan plus locust bean gum or xanthan plus gellan wall materials were less effective in protecting the cells from the effects of low pH.

Table 5.1 Diameter of microcapsules containing *Lb. reuteri.*

Method	Wall material	Size range[a]	Average diameter
Extrusion[b]	Alginate	2-3 mm	2.37 mm
	Alginate + Starch	2-3 mm	2.48 mm
	κ – carr[c] + LBG[d]	3-4 mm	3.72 mm
	Xanthan + gellan	2-3 mm	2.14 mm
Two phase emulsion[e]	Alginate	20 -100 μm	38 μm
	Alginate + Starch	20-100 μm	43 μm
	κ – carr + LBG	40 μm – 1 mm	90 μm
	Xanthan + gellan	100 μm-1 mm	323 μm

[a] n=50
[b] measured with a digital micrometer
[c] κ – carrageenan
[d] locust bean gum
[e] measured with an ocular micrometer-equipped phase contrast microscope

Table 5.2 Effect of selected[a] wall matrix materials and method of microcapsule formation upon survival[b] of microencapsulated *Lb. reuteri* in simulated gastric juice after 2h at 37° C.

Lb. reuteri strains	Microencapsulation method			
	Extrusion		Emulsion	
	Wall matrix	Survival	Wall matrix	Survival
PTA 4659	alginate	7.78 ± 0.17	alginate + starch	7.12 ± 0.18
PTA 4965	alginate + starch	7.60 ± 0.15	alginate	6.92 ± 0.12
ATCC 55730	alginate	7.81 ± 0.18	alginate + starch	6.43 ± 0.20
DSM 16666	alginate	6.83 ± 0.16	alginate + starch	6.12 ± 0.19
ATCC 53608	alginate	7.65 ± 0.20	alginate	7.02 ± 0.19

[a] Wall matrix materials providing the best survival

[b] Cells were recovered from mechanically ruptured microcapsules and grown on MRS agar. Results are expressed as $\log_{10}$ cfu/ml. The initial number of *Lb. reuteri* cells was $\log_{10}$ 8.09±0.12

Table 5.3 Survival of microencapsulated[a] *Lb. reuteri* in simulated gastric juice after 2h at 37° C

Wall matrix	Method	Survival of *Lb. reuteri* strains				
		PTA 4659	PTA 4965	ATCC 55730	DSM 16666	ATCC 53608
xanthan:gellan	Extrusion	3.07 ± 0.21	2.88 ± 0.17	3.34 ± 0.28	0[b]	1.02 ± 0.15
	Emulsion	5.15 ± 0.18	5.01 ± 0.20	4.19 ± 0.19	3.30 ± 0.23	4.96 ± 0.16
κ – carr: LBG	Extrusion	3.42 ± 0.11	2.05 ± 0.16	1.02 ± 0.16	2.61 ± 0.15	1.31 ± 0.25
	Emulsion	5.59 ± 0.17	5.17 ± 0.23	4.78 ± 0.27	4.26 ± 0.22	3.76 ± 0.19

[a]Microencapsulated by xanthan plus gellan and κ – carrageenan plus locust bean gum (κ – carr: LBG)
[b] lowest limit of detection was ≤ 20 cfu/ml
Results are expressed as $\log_{10}$ cfu/ml and are the mean of three values. The initial number of *Lb. reuteri* cells was $\log_{10}$ 8.13 ± .01

Fig 5.1 SEM image of *Lb. reuteri* ATCC 53608 stationary phase cells, bar indicates 2 μm.

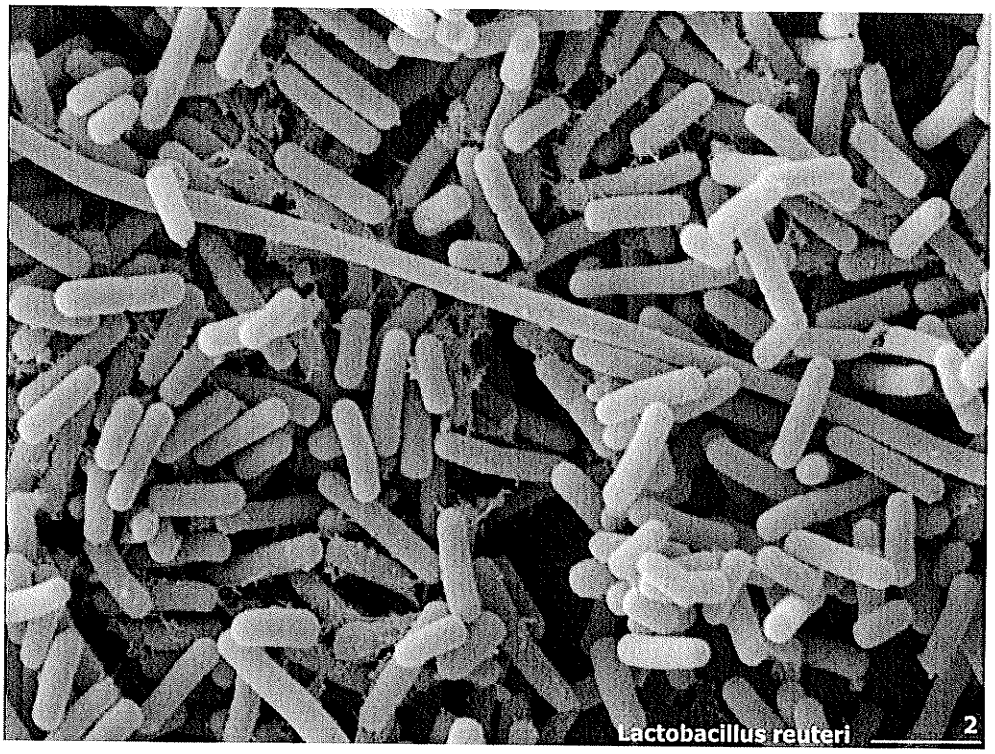


Fig 5.2a Cryo-SEM images of microcapsules prepared by extrusion (a) alginate. Microcapsules made with alginate plus starch were similar in appearance to (a)

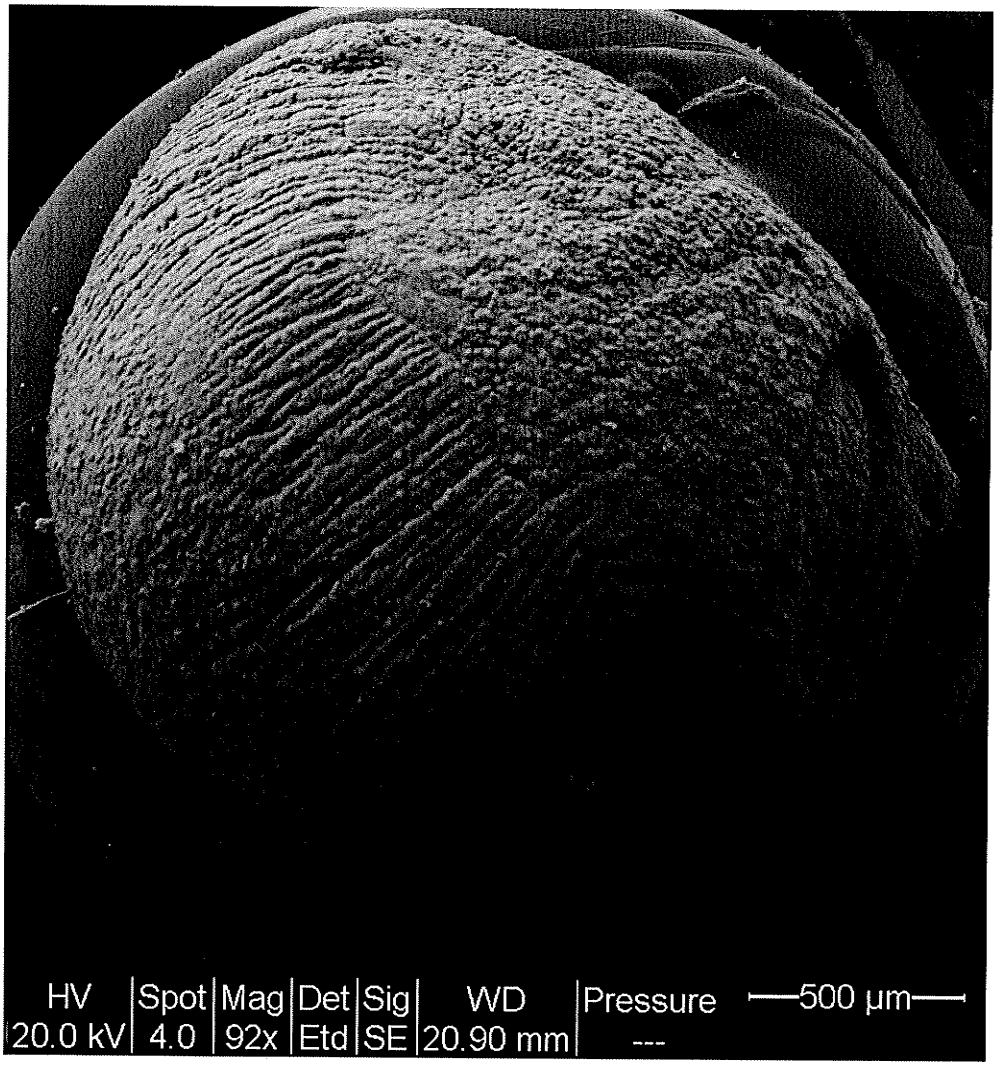


Fig 5.2b alginate –note the absence of internal cavities in the fractured surfaces.

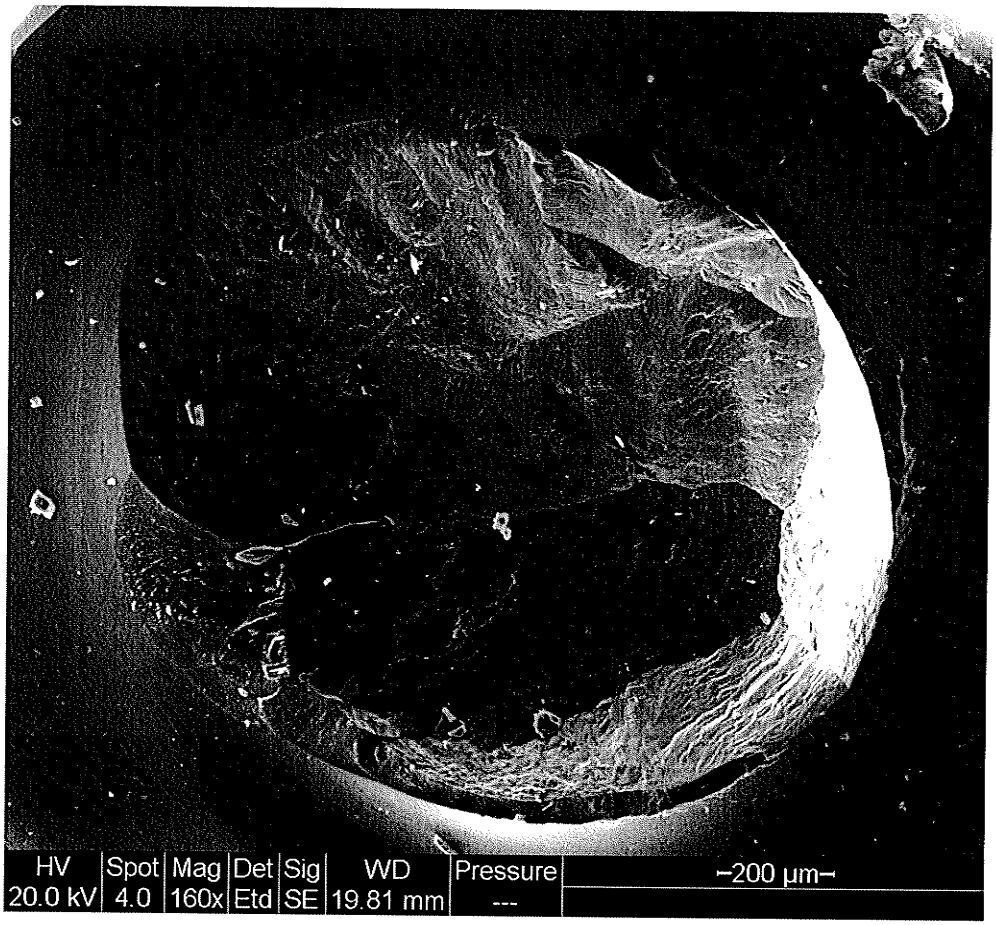


Fig 5.2c κ-carrageenan plus locust bean gum prepared by extrusion. Xanthan plus gellan microcapsules were also irregular

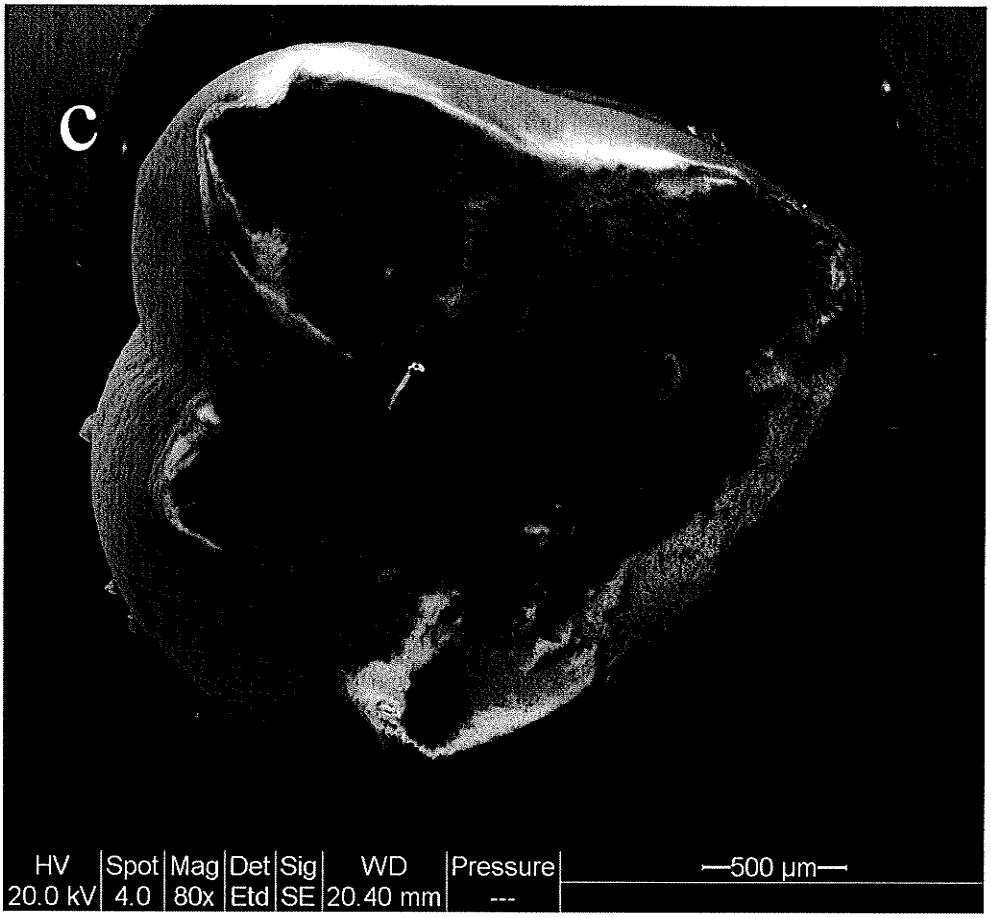
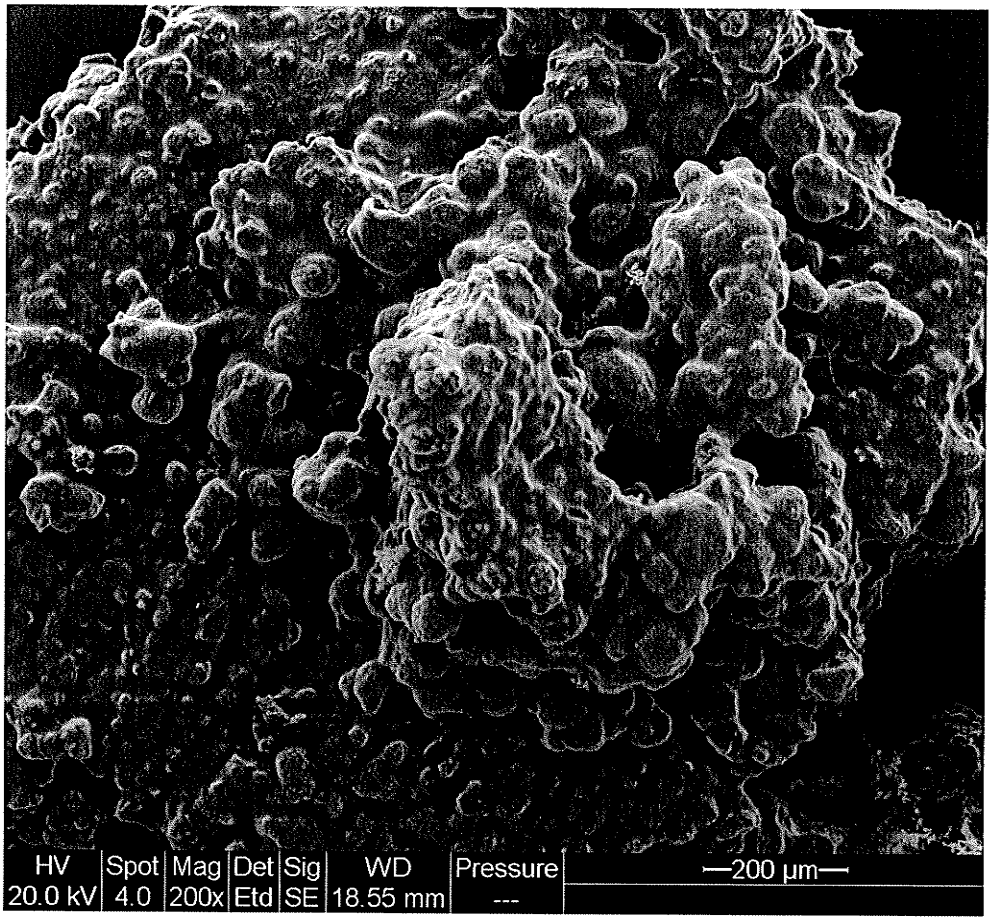


Fig 5.3a Cryo-SEM images of microcapsules prepared from emulsions (a) alginate. Microcapsules made with alginate plus starch were similar in appearance to (a)



5.3b κ-carrageenan plus locust bean gum prepared from emulsions. Microcapsules made with xanthan plus gellan were similar in appearance to (b)

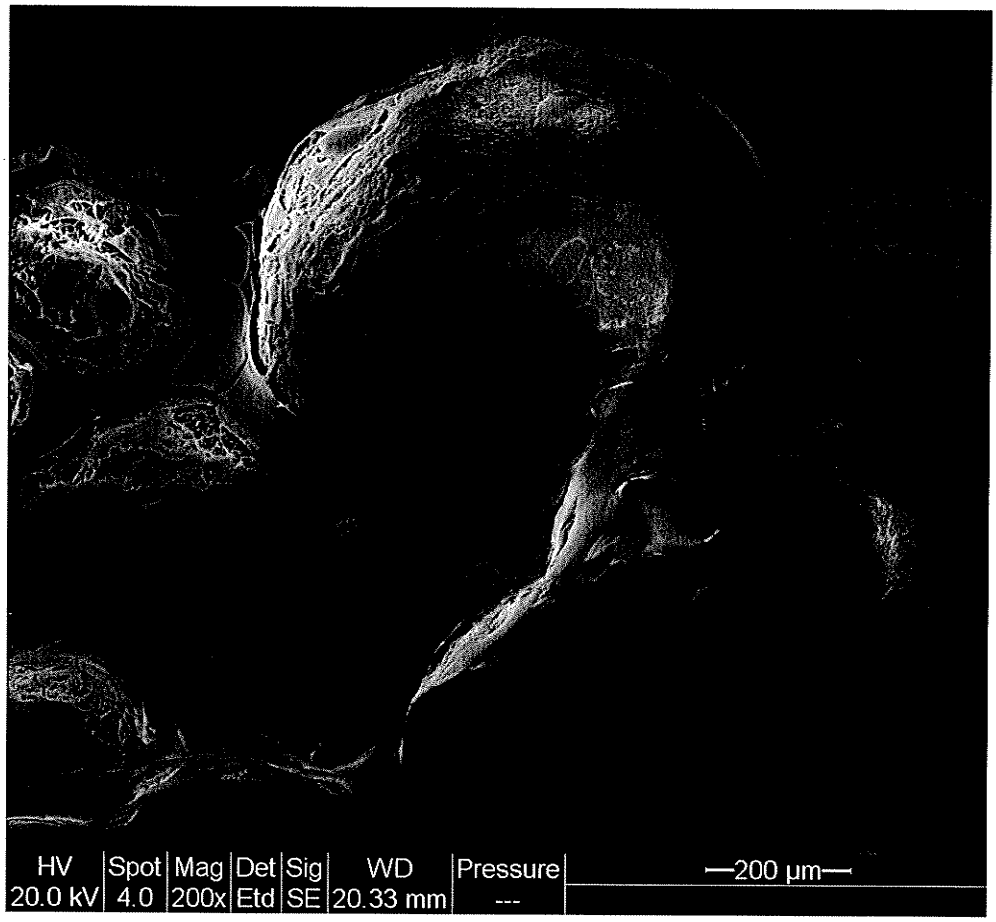


Fig 5.4 Survival of planktonic *Lactobacillus reuteri* strains in simulated gastric juice at pH 1.5 and 37° C. The error bars indicate standard deviation of the mean of three values. Symbols ◆ - *Lb. reuteri* PTA 4659 (MM2-3), ■ - *Lb. reuteri* PTA 4965 (CF2-7F), ▲ - *Lb. reuteri* ATCC 55730 (SD2112), □ - *Lb. reuteri* DSM 16666 (RC14), ● - *Lb. reuteri* ATCC 53608.

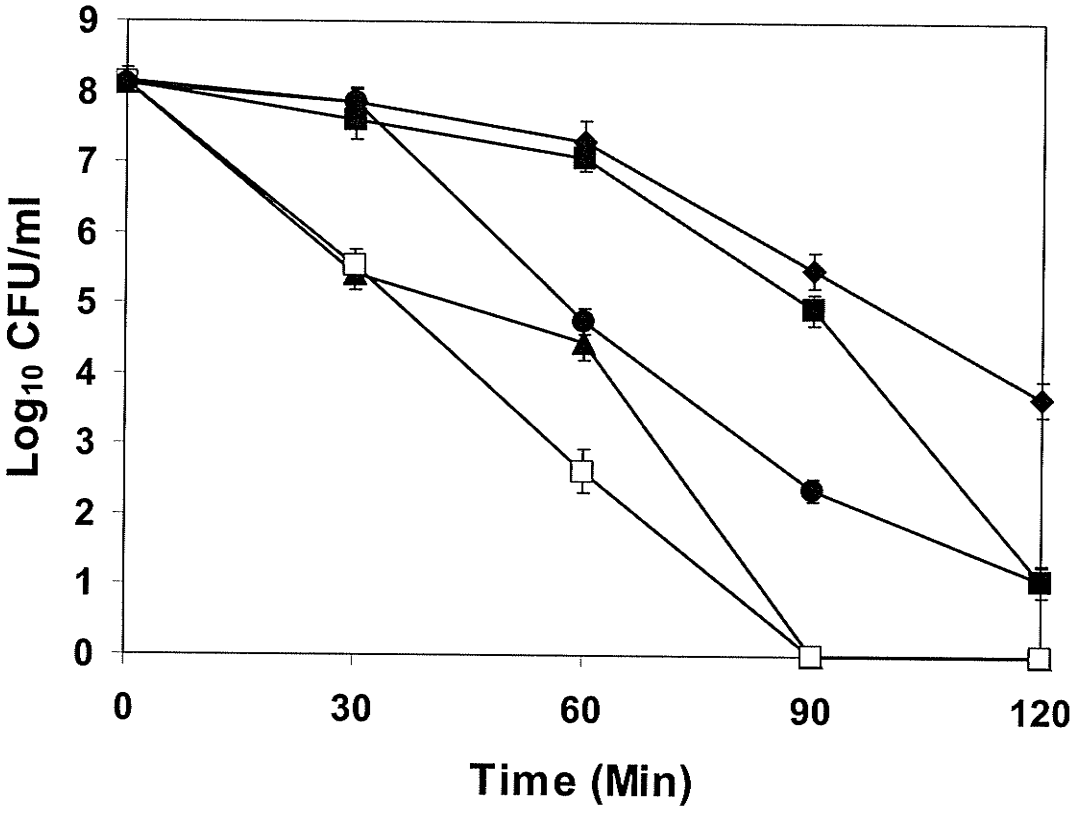


Fig 5.5 Survival of *Lb. reuteri* strains in alginate microcapsules prepared by extrusion and held ≤ 2h at 37° C in simulated gastric juice. Symbols are as in Fig 5.4.

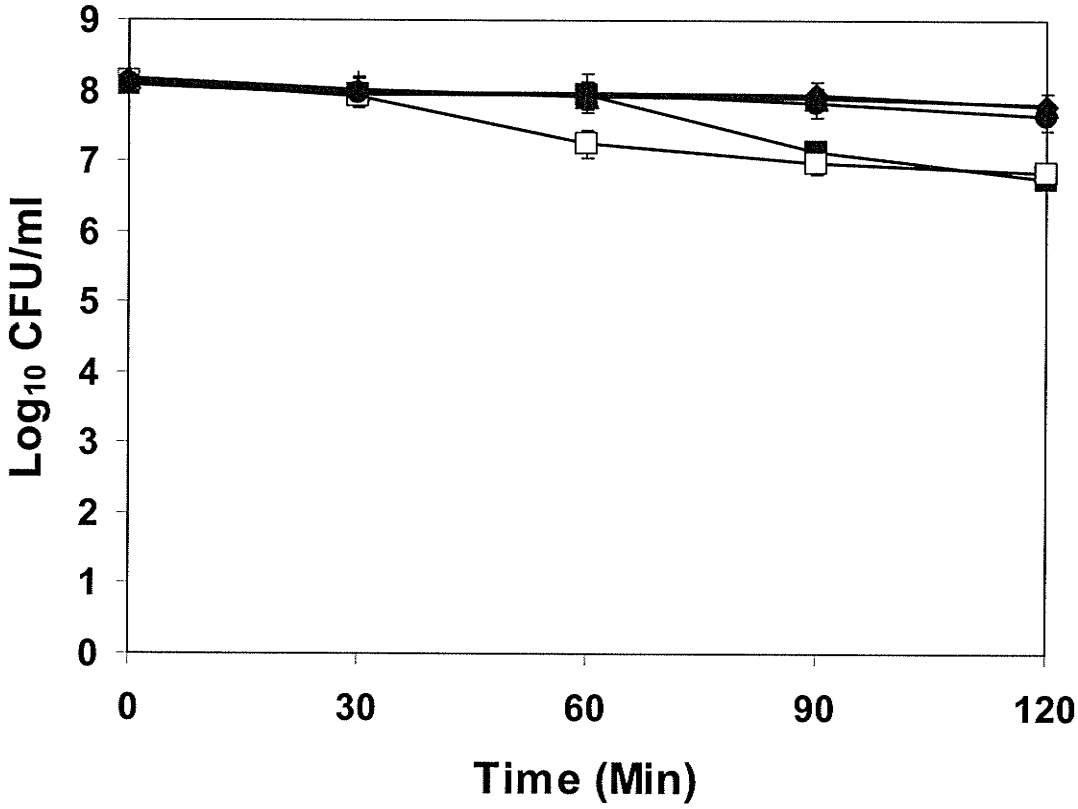


Fig 5.6 Survival of *Lb. reuteri* strains in alginate plus starch microcapsules prepared by extrusion and held ≤ 2h at 37° C in simulated gastric juice. Symbols are as in Fig 5.4.

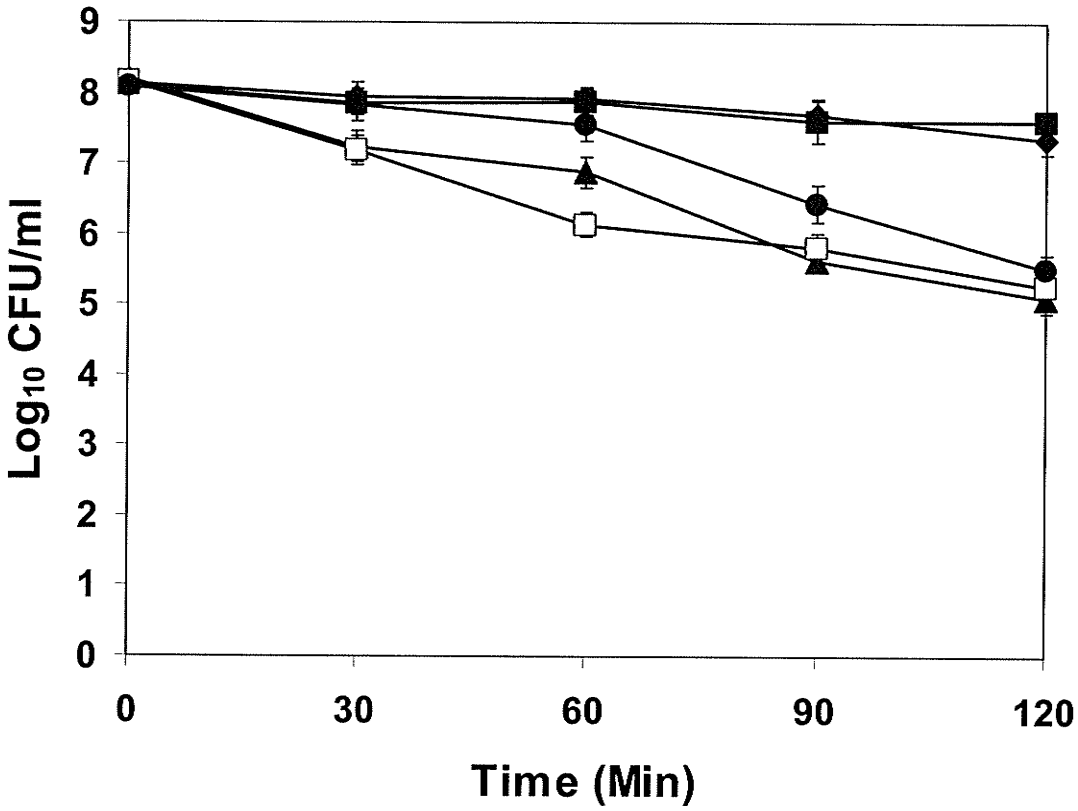


Fig 5.7 Survival of *Lb. reuteri* strains in alginate microcapsules prepared by emulsification and held ≤ 2h at 37° C in simulated gastric juice. Symbols are as in Fig 5.4.

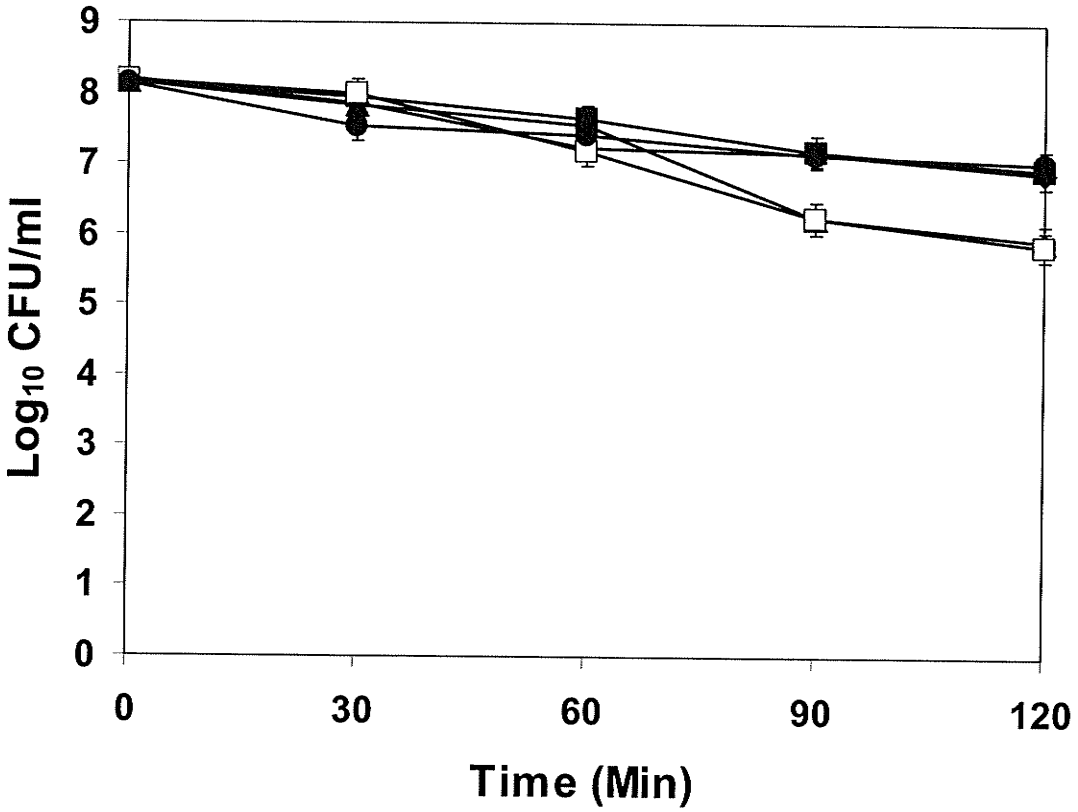
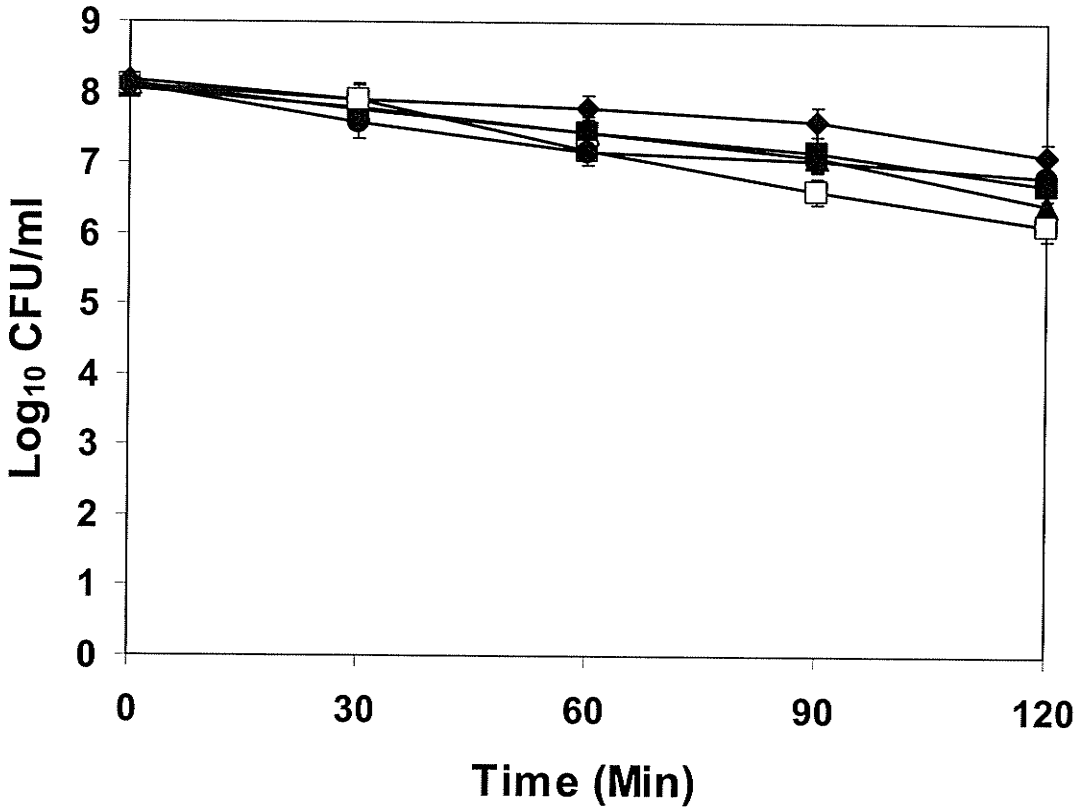


Fig 5.8 Survival of *Lb. reuteri* strains in alginate plus starch microcapsules prepared by emulsification and held ≤ 2h at 37° C in simulated gastric juice. Symbols are as in Fig 5.4.



Chapter 6

Microbiological and sensory quality of dry fermented sausages containing alginate-microencapsulated *Lactobacillus reuteri*

6.1 Abstract

Lactobacillus (*Lb.*) *reuteri* is a probiotic organism that is used in fermented dairy products as well as in dietary supplements to promote human health. In this study *Lb. reuteri* cells were added to dry sausage batter directly or microencapsulated first in alginate using either extrusion or emulsion technology and cell survival was followed during sausage manufacture. *Pediococcus* (*P.*) *pentosaceus* and *Staphylococcus* (*S.*) *carnosus* from commercial preparations were added at 7 log cfu/g as starter cultures for fermentation. The sausage batter was stuffed in 55 mm fibrous casings and fermented with smoking at $\leq$ 26° C and 88% relative humidity (RH) for 72 h according to the degree.hour guidelines of Agriculture and Agri-Food Canada. Thereafter, the sausage was dried at 75 % RH and 13° C for 25 d. The pH and water activity (a_w) of the sausage was measured during fermentation and drying. The levels of *Lb. reuteri*, *P. pentosaceus* and *S. carnosus* present during fermentation and drying were monitored by plating on a newly developed selective MRS agar supplemented with 10 mg/L penicillin G, MRS plus 0.5g/L polymyxin or mannitol salt agar, respectively. A consumer taste panel study with 72 volunteers was conducted to determine if addition of *Lb. reuteri* as planktonic cells or in microcapsules added at 1% (w/w) affected the sensory quality of the dry fermented sausages. Changes in sausage pH initially from 5.8 to 4.8 and in a_w from 0.97 to 0.89 at the end of drying, respectively, for both control and microcapsule-containing sausages were within the normal ranges of commercial products. *P. pentosaceus* numbers

increased one log during fermentation but remained at 7 log cfu/g until the end of drying. *S. carnosus* numbers decreased by 2 log cfu/g by the end of drying in all treatments as expected. No significant differences were found among treatments in terms of pH, a_w and starter culture numbers. Free *Lb. reuteri* cells dropped initially from 7.1 log cfu/g to 4.5 whereas *Lb. reuteri* cells microencapsulated by either method were only reduced by ≤ 0.5 log at the end of drying. The mean sensory score for all sausage treatments varied between 7.1-7.3 (like moderately) on a 9 point Hedonic scale for overall acceptability, appearance, flavour and texture. No significant difference in sensory quality was found between control and sausages containing either planktonic or microencapsulated *Lb. reuteri.* Thus microencapsulation of probiotics like *Lb. reuteri* may be an option to provide fermented meat products with viable heath-promoting bacteria.

6.2 Introduction

Dry fermented sausages are formulated with spices and curing salts (nitrates and nitrites) and fermented with or without smoke using starter cultures. Subsequently they are dried for about a month and are consumed uncooked (Lücke, 1998).

Foods which have health benefits beyond their nutritional content (functional foods) and particularly foods containing probiotics are products that are growing in popularity. Interest in these products is driven by consumer awareness of healthy food and the role probiotic organisms may play in promoting health (Sloan, 2004). Probiotics are available as dietary supplements or they may be incorporated directly into foods. Dairy products like yogurt, kefir and acidophilus milks are common vehicles for probiotic delivery although recently orange juice and ice cream supplemented with probiotics have become available.

Lb. reuteri, a normal inhabitant of the human GI tract is considered a probiotic organism because of its stimulation of host immunity against infectious agents, its excellent ability to colonize intestinal epithelium tissue, its resistance to acid and bile, and for its potential hypocholesterolemic effects (Taranto et al. 1998, Casas and Dobrogosz, 2000). *Lb. reuteri* produces a non-peptide low molecular mass antimicrobial substance from glycerol which is termed reuterin. This compound (β – hydroxy propionaldehyde) has a broad spectrum of activity against Gram-positive and Gram-negative bacteria, including *E. coli* O157:H7 (Axelsson et al. 1989, Muthukumarasamy et al. 2003).

Though the future for use of probiotics in dry fermented sausages was termed "promising" (Incze, 1998), research reports related to inclusion of probiotics in dry fermented sausages are few in number (Anderson 1998, Erkkilä et al. 2000, Erkkilä et al. 2001, Jahreis et al. 2002) compared to those on the use of probiotics in dairy products like yoghurt and fermented milk (Heller, 2001). Most research has shown that probiotic organisms survive poorly in fermented foods such as yogurt and fermented milks (Shah et al. 1995, Kailasapathy and Rybka 1997, Shah and Ravula, 2000). In Germany, supplementation of dry fermented sausages with bifidobacteria was attempted, but the strain used survived poorly during sausage processing (Lücke, 2000). Although the minimum level of probiotic organisms required for exerting beneficial effects in humans is still unclear, indications are that at least 6 log cfu/g are required in the final product (Krasaekoopt et al. 2003, Lücke, 2000). Dry fermented sausages with their low a_w and pH, the presence of curing salts and competing organisms, present a challenging environment for survival of probiotics during processing.

Microencapsulation has been shown to be capable of improving viability of bacterial cells by retaining them within a protective polymer membrane or matrix (Audet et al. 1988, Sheu and Marshall, 1993). Two widely used methods of encapsulation are extrusion and emulsification (Krasaekoopt et al. 2003). The microcapsules produced by extrusion methods are larger (2.4 mm) than those prepared by emulsion technology (.038 mm, Muthukumarasamy, Allan-Wojtas and Holley, unpublished). Larger capsules have the potential to protect encapsulated bacteria but porosity of the wall material must be considered. Their possible use in dry fermented sausages depends on their ability to preserve bacterial viability as well as their negligible contribution toward the sensory quality of the sausages.

Microencapsulation of probiotics for dry fermented sausage may improve not only the image of these products as a functional food but also improve their safety if the probiotic bacteria are capable of producing potent antimicrobials such as reuterin. This work reports the feasibility of using the probiotic *Lb. reuteri* in dry fermented sausages either alone or after microencapsulation to generate a consumer acceptable product.

6.3 Materials and methods

6.3.1 Selection of cultures

Twenty one bacterial strains isolated from commercial starter cultures (Holley and Millard, 1988, Holley and Blaszyk, 1998) including *Lb. plantarum*, *Lb. curvatus*, *P. acidilactici*, *P. pentosaceus*, *S. carnosus* and four strains of *Lb. reuteri* from Biogaia Biologics Inc., Raleigh NC, plus a fifth strain (ATCC 53608) from the American Type Culture Collection (Manassas, VA) were tested for their fermentation ability. Unfermented dry sausage batter was obtained frozen from a commercial sausage

manufacturer (Piller's, Waterloo, ON) without addition of starter cultures. The 21 starter cultures and 5 strains of *Lb. reuteri* were added individually in triplicate at a level of 7 log cfu/g to 100 g sausage batter at 4° C and vacuum packed using a vacuum packaging machine (model GM-2000, Bizerba Canada Inc., Mississauga, ON) in low oxygen permeable Deli*1 bags (Winpak, Winnipeg, MB) and incubated at 26° C for 64 h according to AAFC (1992) guidelines in order to simulate commercial sausage fermentation. A new sealed bag was opened at 12h intervals and the pH of the salami batter was monitored using a Sentron Titan pH meter equipped with a Lancefet probe (Sentron Europe BV, Roden, Netherlands) to evaluate culture performance.

6.3.2 Culture and growth conditions

Lb. reuteri strain ATCC 55730 (SD2112) which was the best reuterin producer as determined by a colorimetric method (Circle et al. 1945, Vollenweider et al. 2003) was selected as the probiotic organism for use in dry sausages. *P. pentosaceus* (UM 116P) and *S. carnosus* (UM 110M) were strains selected from commercial starter culture isolates for the sensory experiments. *Lb. reuteri* and *P. pentosaceus* strains were maintained in deMan Rogosa Sharpe (MRS) broth (BBL, Becton Dickinson, Sparks, MD) containing 20% glycerol at -70° C. *S. carnosus* was maintained in tryptic soy broth (TSA, BBL, Becton Dickinson) containing 20% glycerol at -70° C. Frozen stock cultures of *Lb. reuteri* and *P. pentosaceus* were sub-cultured twice in MRS (TSA for *S. carnosus*) broth incubated at 37° C before use in the experiments. Overnight cultures were centrifuged at 10,000 rpm for 10 min, washed in 0.1% peptone water and suspended in 0.1% peptone water to achieve 9 log cfu/ml by standardization at an optical density of 600 nm.

6.3.3 Microencapsulation procedure

Sodium alginate (Sigma-Aldrich Co., St. Louis, MO) was used as a wall material for microencapsulation of *Lb. reuteri* by both extrusion and a two phase (water/oil) emulsion system (modified from Sheu and Marshall, 1993). Glassware and reagents used in the experiments were sterilized before use. Alginate (3% w/v) was added to distilled water, autoclaved and allowed to cool to 25° C. To this wall material 10 log cfu/ml *Lb. reuteri* cells (standardized as above in 0.1% peptone water) were added at a ratio of about 1:5 (v/v) so that 9 log cfu/ml was reached in the microcapsules. This wall material-culture mixture was used for microencapsulation by both extrusion and emulsion methods. Extrusion was performed by expression of the wall material-culture mixture through a 21G syringe needle drop-wise into 0.5M $CaCl_2$. Microcapsules were held at 25° C for 30 min to ensure complete solidification. Microcapsules were separated by filtration through Whatman #4 filter paper and stored in sterile petri dishes at 4° C until used in sausage experiments. The two phase emulsion (phase separation) method involved adding one part of 3% (w/v) sodium alginate containing culture to 5 parts of commercial corn oil (v/v) (continuous phase) containing 0.02% tween 80 (Fisher Scientific, Fair Lawn, NJ). The mixture was stirred at 800 rpm for 5 min using a Sorvall Omni mixer (Ivan Sorvall Inc, Norwalk, CT) to form a uniform water-in-oil emulsion. Sterile 0.1M $CaCl_2$ was added at the sides of the beaker until the emulsion was completely broken. The beads formed were collected by vacuum filtration through Whatman # 4 filter paper and washed using 200 ml sterile 0.1% peptone water. The microcapsules were vacuum filtered and washed with an equal volume of 0.5M $CaCl_2$. All microcapsules were stored at 4° C and were used in sausage experiments within a day of preparation.

6.3.4 Sausage manufacture for sensory evaluation

The formulation used for making dry sausage is given in Table 1. Sausage batter was prepared by chopping pork fat, pork and beef purchased from a local wholesaler in a Titane 40 rotating bowl meat cutter (Dadaux, Bersaillin, France). *P. pentosaceus* and *S. carnosus* were added to reach a final inoculation level of 7 log cfu/g. To this batter cervelat spice mix (Hermann Laue Spice Co. Inc, Uxbridge, ON), Rapidur (a proprietary mixture of corn syrup solids and dextrose), pickle cure concentrate (6.25 % $NaNO_3$, 1% $NaHCO_3$) and salt (Canada Compound Corporation, Winnipeg, MB) were added and chopped thoroughly for 5 min. Planktonic *Lb. reuteri* cells (without alginate supplementation) were added to yield log 7 cfu/g meat batter and alginate microcapsules containing *Lb. reuteri* prepared by either emulsion technology or extrusion were added to the salami batter at 1% (w/w). Control salami batter did not have any alginate or *Lb. reuteri* added. Fibrous casings (55 mm diameter, Kalle GmbH & Co. Wiesbaden, Germany) were pre-soaked in lukewarm water (40° C for 30 min) before stuffing. Sausage batter was stuffed with a piston stuffer (Mainca Model EM30, Equipamienentos Carnicos, S. L. Barcelona, Spain) in the casings to achieve a final weight of about 500 g. Sausages were transferred to a smoke house (Allroundsystem Rondette with Titan controller, Maurer AG, Reichenau, Germany) and processed by fermentation at 26° C and 88% RH for 24 h to reach a pH of < 5.3. Thereafter fermentation temperature and RH were decreased step-wise (6x12h intervals where temperature and RH were reduced by 2° C and 2.2 % RH) from 26 to 14° C and 88 % to 75% RH over 72 h with intermittent smoking for 30 min at 48 and 60 and 72h. The degree.hour guidelines of Agriculture and Agri-Food Canada (AAFC, 1992) were followed when calculating the permitted time and

temperature combinations during fermentation. Fermentation was followed by drying at 13° C and 75% RH for 25 d.

6.3.5 Selective media for *P. pentosaceus* and *Lb. reuteri*

P. pentosaceus (UM 116P) and *Lb reuteri* SD 2112 were subjected to a Kirby-Bauer antibiotic sensitivity test (Bauer et al. 1966) using commercial antibiotic disks (BBL, Becton Dickinson, Sparks, MD) containing ampicillin, amoxicillin, bacitracin, cephalothin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, nalidixic acid, neomycin, oxytetracycline, penicillin G, polymyxin B, tetracycline, streptomycin or vancomycin. Test organisms were spread-plated on MRS agar, dried for 15 min, and different antibiotic-impregnated disks were placed on the agar surface using a dispenser (Sensi-Disc dispensor, BBL, Becton Dickinson). Plates were incubated anaerobically at 37° C for 24-48 h. Zones of inhibition denoting sensitivity or resistance to the antibiotics tested were recorded. Antibiotics to which *P. pentosaceus* was sensitive but *Lb. reuteri* was resistant and vice-versa were selected, incorporated in MRS agar and also tested against organisms that commonly occur in the meat environment such as *Pseudomonas* spp., *Shewanella* spp., *Brochothrix* spp., *Enterococcus* spp., *Lactobacillus* spp., *Staphylococcus* spp. and other *Pediococcus* spp. At least one strain of each of the previous organisms from the Department of Food Science, University of Manitoba culture collection was screened using this procedure.The developed media were also used to recover organisms from salami batter inoculated with 7 log cfu/g of *Lb. reuteri* and *P. pentosaceus* and fermented for 64h at 26° C.

6.3.6 Sampling of sausages

Samples were taken every 12h during fermentation and every 3 d during ripening for pH, a_w, *Lb. reuteri, P. pentosaceus* and *S. carnosus* numbers. For bacteriological analysis 25 g of sausage batter was mixed with 225 ml of 0.1% peptone water and homogenized in a stomacher bag for 10 min with a Stomacher 400 system (Seward Laboratory, London, UK). Samples were serially diluted and plated using a spiral plater (Autoplate 4000,Spiral Biotech, Norwood, Mass.) on newly developed selective MRS agar + penicillin G (10mg/L) or MRS + polymyxin B (0.5g/L) for specific enumeration of *Lb. reuteri* or *P. pentosaceus*, respectively. Mannitol salt agar was used for enumeration of *S. carnosus*. All plates were incubated at 37° C for 24-48h aerobically except *Lb. reuteri* plates which were incubated anaerobically using the BBL Gaspak system.

The pH of triplicate samples was recorded using a pH meter equipped with a lancefet probe (Sentron Titan pH meter).Water activity was analysed in triplicate (Novasina AW Sprint TH 500 a_w measuring unit, Axair AG, Pfäffikon, Switzerland).

6.3.7 Sensory evaluation

A consumer taste panel was used to compare the sensory quality of sausage containing *Lb. reuteri* with that of control salami. A group of 72 untrained volunteers who consumed fermented sausages at least 4 times/year was selected for the sensory panel. The sensory analysis facility in the Department of Food Science was used and panelists made their evaluations in private sensory booths. Panelists were given four slices of numerically identified blind-coded sausage samples from the different treatments (control, planktonic *Lb. reuteri*, *Lb. reuteri* encapsulated by extrusion and by emulsion) and asked to evaluate them for overall acceptability, appearance, flavour and texture on a 9 point Hedonic scale with 9 being extremely good and 1 being extremely

poor. On the questionnaire, panelists were asked if the presence of probiotics in dry fermented sausages would influence their purchasing decision.

6.3.8 Statistical analysis

All data were analysed using Statistical Analysis System (version 8.1) software (SAS Institute, Cary, NC). Analysis of variance by general linear model (GLM) and Duncan's multiple range tests were used to find significant differences ($P<0.05$) between treatments.

6.4 Results

6.4.1 Selection of cultures

All 11 pediococci and 3 lactobacilli strains tested decreased the pH of sausage batter from 6.2 ± 0.1 initially to 4.8 ± 0.1 by the end of fermentation. With the 5 strains of *Lb. reuteri* the pH of the sausage batter only decreased to 5.4 ± 0.04, indicating that the *Lb. reuteri* strains were incapable of sausage fermentation when used alone. *Lb. reuteri* SD2112 was selected for further study as it produced the largest amounts of reuterin during preliminary study. *P. pentosaceus* UM 116P was also selected because it dependably and rapidly produced acid in the sausage batter during fermentation. The choice of *S. carnosus* UM 110M was based on the vigour of the culture as well as the knowledge that it is paired with UM 116P in the lyophilized dry sausage starter culture mixture Trumark LT II (Rector Foods Ltd., Mississauga, ON) in commercial use.

6.4.2 Selective media for *P. pentosaceus* and *Lb. reuteri*

P. pentosaceus UM 116P was found to be resistant to polymyxin B but was susceptible to penicillin G. In contrast *Lb. reuteri* SD 2112 was sensitive to polymyxin B but resistant to penicillin G by the Kirby-Bauer antibiotic susceptibility test. When

polymyxin at 0.5 g/L or penicillin G at 10 mg/L were added separately to MRS agar, recovery of *P. pentosaceus* UM 116P or *Lb. reuteri* SD 2112, respectively, was possible while the growth of other genera of organisms tested was inhibited. In sausage batter inoculated with *P. pentosaceus* or *Lb. reuteri,* and held at 26° C for 64h test organisms were recovered on the respective MRS selective medium at the same level as on MRS without antibiotic addition. This indicated that MRS with polymyxin B could be used to enumerate *P. pentosaceus* and that MRS with penicillin G could be used to enumerate *Lb. reuteri* from mixed cultures of the two biochemically similar organisms.

6.4.3 Changes in sausage a_w and pH

The pH of the sausage dropped initially from 5.79 ± 0.01 to 5.31 ± 0.04 within 24 h, reaching 4.83 ± 0.02 at 72 h (the end of fermentation) and 4.74 ± 0.06 at 27d (end of drying) in control sausage (Fig 6.1). The water activity of control sausage dropped from 0.97 ± 0.01 at the beginning of fermentation to 0.89 ± 0.01 by the end of 25 d drying (Fig 6.2). No significant differences in pH or a_w were found among the control salami and the three treatments containing *Lb. reuteri* either as planktonic cells, in extruded microcapsules or microcapsules prepared by emulsion.

6.4.4 Bacteriological analysis of sausage

The level of *P. pentosaceus* increased by one log during fermentation but slowly decreased thereafter and reached 7 log cfu/g near the end of drying (Fig 6.3). *S. carnosus* numbers decreased ≤ 2 log cfu/g at the end of drying in all treatments (Fig 6.3). No significant differences were found in starter culture numbers among control, planktonic *Lb. reuteri* and microencapsulated *Lb. reuteri* treatments.

Planktonic *Lb. reuteri* dropped initially from 7.12 ± 0.12 log cfu/g to 4.54 ± 0.18 during sausage fermentation (Fig 6.4). In microencapsulated treatments *Lb. reuteri* were only reduced by ≤ 0.5 log at the end of drying. These differences in *Lb. reuteri* viability between treatments where the organisms were encapsulated or added as planktonic cells were significant ($P < 0.05$). Although *Lb. reuteri* survived slightly better when encapsulated by extrusion than in capsules prepared by emulsion, differences were not microbiologically significant.

6.4.5 Sensory evaluation

The mean score for all sausage treatments varied between 7.04 to 7.34 (where 7 = "like moderately") for overall acceptability, appearance, flavour and texture (Fig 6.5). No significant differences were found between the control and sausages prepared with either planktonic or microencapsulated *Lb. reuteri*. More than 80 % (56 of 72) of the panellists reported that the presence of probiotic organisms in dry sausages would positively influence their purchase decision.

6.5 Discussion

Lactic acid bacteria (LAB) have been used for centuries in the fermentation of dairy and meat products but pure starter cultures were only introduced for the manufacture of fermented meat < 60 years ago (Incze, 1998). Many LAB cultures have been reported to exert a positive or probiotic effect on health by re-establishing the natural balance of the intestinal microflora. In addition to these effects some organisms such as *Lb. reuteri* function as bioprotective cultures as a result of production of bacteriocins and other low molecular mass antimicrobials that are inhibitory to undesirable organisms (Axelsson et al. 1989). The potential for dry fermented sausages to

serve as a vehicle for probiotic organisms was comprehensively reviewed recently (Työppönen et al. 2003). The only human clinical study found where probiotic sausages were evaluated was separately reported by Jahreis et al. (2002). These workers noted a positive modulation in host immunity when 50 grams of probiotic fermented sausage containing *Lb. paracasei* were consumed daily for 5 weeks. Erkkilä et al. (2001) used *Lb. rhamnosus* as a probiotic organism in fermented sausages and found no significant sensory differences as a result of the presence of these organisms. Arihara et al. (1998) used a probiotic strain of *Lb. gasseri* whereas Sameshima et al. (1998) used *Lb. rhamnosus* and Lb. *paracasei* subsp. *paracasei* in meat fermentations. Most of these studies relied on the fermentation ability of the probiotic organisms and the selection of probiotic cultures for dry fermented sausages was limited to organisms that were capable of fermenting carbohydrate in meat. It is noteworthy that not all LAB, including *Lb. rhamnosus* GG can utilize lactose (Työppönen et al. 2003). Our studies indicated that when *Lb. reuteri* was used alone the organism was incapable of suitably fermenting meat batter because of limited production of lactic acid. Therefore, if probiotic organisms desired are weak lactic acid producers like *Lb. reuteri* or *Bifidobacterium,* their value in dry fermented sausages depends on their ability to survive during sausage processing. Benkerroum et al. (2005) used lyophilized *Lb. curvatus* and *Lactococcus (Lc.) lactis* subsp. *lactis* as bio-protective cultures to inhibit *Listeria* in dry fermented sausages. Addition of lyophilized cultures was reported to delay fermentation and allowed release of the probiotic organisms into the meat matrix thus reducing their viability in the final product. Another approach for selecting probiotics for use in dry fermented sausages involves isolation of desirable LAB which possess acid and bile resistance from finished

products (Papamanoli et al. 2003, Pennacchia et al. 2004). This approach requires extensive study of the isolates for other probiotic properties such as colonization potential and inhibitory activity against pathogenic bacteria.

The approach used in this study was to use microencapsulation technology as a means to protect a recognized probiotic organism from the harsh environment during sausage processing. Microencapsulation of probiotics as a means of improving their survival has been studied in yoghurt (Sultana et al. 2000, Adikari et al. 2003), ice milk (Kebary et al. 1998), and mayonnaise (Khalil and Mansour, 1998). Several wall materials including alginate, starch, alginate-starch, κ – carageenan, κ – carageenan/locust bean gum, xanthan- gellan, gelatin, and whey proteins have been used for successful microencapsulation of probiotics (Doleyres and Lacroix, 2005). Alginate wall materials were chosen in the present study as they were found to provide the best protection against simulated gastric acid challenge compared to other wall materials, regardless of the technique used for microcapsule formation (Muthukumarasamy, Allan-Wojtas and Holley, unpublished).

In a study by Kebary and Hussein (1999) using yoghurt, it was reported that *Bifidobacterium* (*B.) bifidum* and *B. infantis* after microencapsulation in calcium alginate produced antimicrobial substances that were inhibitory to *E. coli* and *S. aureus*. Encapsulated bifidobacteria rapidly retarded the growth of *E. coli* which became undetectable at 5 d of storage, while 7 d was required to achieve the same effect with non-encapsulated bifidobacteria.

In the present work, the pH of the salami dropped to 5.3 within 24 h indicating the fermentation process adhered to the degree.hour recommendations of AAFC to prevent

growth of salmonellae and *Staphylococcus aureus*. The a_w of the salami reached 0.88 after drying which is a desired level for dry salami. Brief growth and acid production by *P. pentosaceus* during fermentation were adequate to allow protein gelation and desirable texture development in the sausage. *S. carnosus* was used in the starter culture mixture to ensure proper colour development due to its reduction of nitrate to nitrite and hydrogen-peroxide destruction by catalase production. These organism also desirably affect flavour development through lipolysis (Incze, 1998). Microencapsulation by both emulsion and extrusion methods were shown to maintain the viability of *Lb. reuteri* contained within them during salami processing. Planktonic *Lb. reuteri* were reduced by > 2.5 log cfu/g whereas microencapsulated *Lb. reuteri* were reduced by only $\leq$ 0.5 log cfu/g at the end of drying. Addition of microcapsules produced by both extrusion and emulsion did not affect the overall sensory or microbiological quality of dry fermented sausages.

To our knowledge the present work is the first study investigating the potential use of microencapsulation technology to protect probiotics in meat products. Results showed that microencapsulated *Lb. reuteri* can be used in dry fermented sausages to ensure that a desired level of probiotic organisms is maintained in the final product at consumption (Lücke, 2000) without altering the quality of these traditional products.

Table 6.1 Formulation used for manufacture of dry fermented sausage

Ingredients	**Composition (% w/w)**
Beef fronts (85% lean)	17.59
Pork (90%) lean	60.67
Pork fat	17.59
Spice mix	0.44
Salt	2.90
[a]Dextrose + corn syrup solids (Rapidur)	0.69
[a]Pickle cure concentrate	0.31

[a] Canada Compound Corporation, Winnipeg, MB

Fig 6.1 Changes in pH value during fermentation of dry fermented sausages (◆ - control salami, ● - salami with planktonic *Lb. reuteri* ■ - salami with *Lb. reuteri* microencapsulated by extrusion, ▲ - salami with *Lb. reuteri* microencapsulated by emulsion).

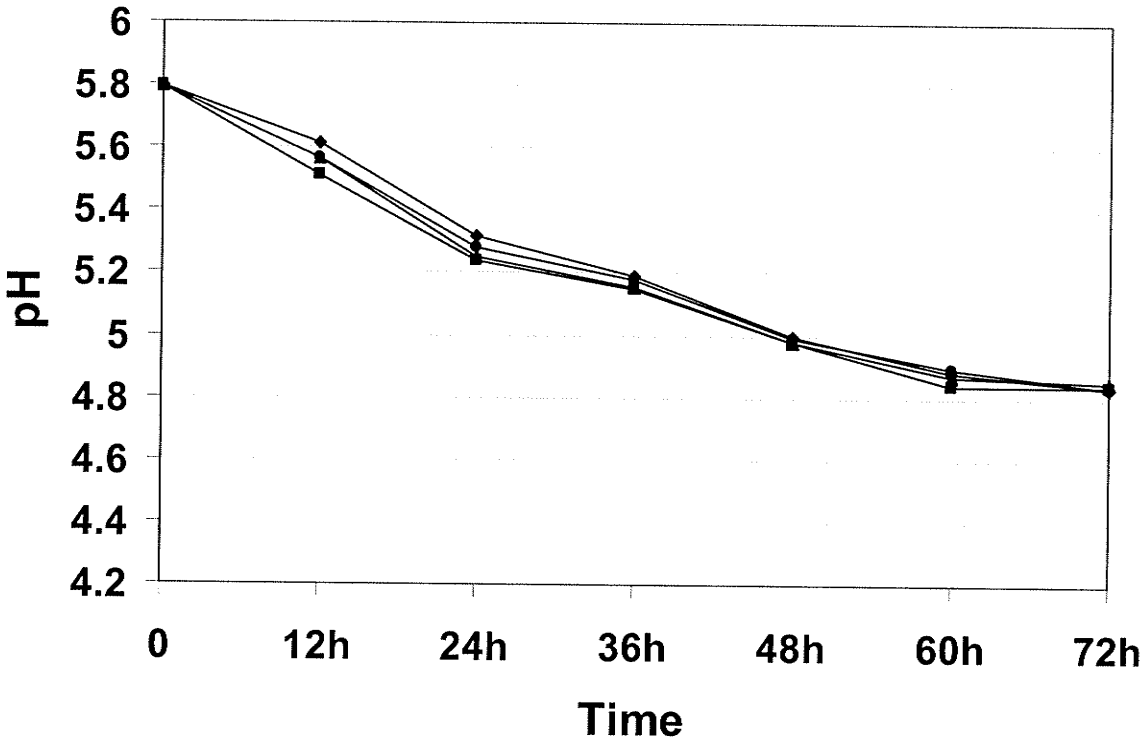


Fig 6.2 Changes in a_w value during manufacture of dry fermented sausages (◆ - control salami, ● - salami with planktonic *Lb. reuteri* ■ - salami with *Lb. reuteri* microencapsulated by extrusion, ▲ - salami with *Lb. reuteri* microencapsulated by emulsion).

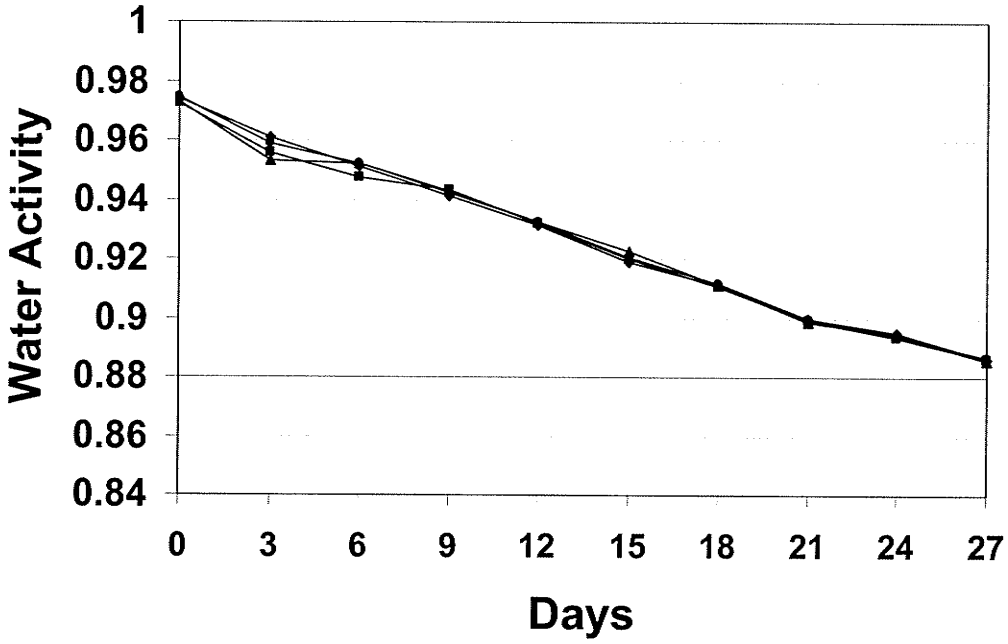


Fig 6.3 Survival of *P. pentosaceus* (straight line) and S. carnosus (dotted line) during manufacture of dry fermented sausages (◆ - control salami, ● - salami with planktonic *Lb. reuteri* ■ - salami with *Lb. reuteri* microencapsulated by extrusion, ▲ - salami with *Lb. reuteri* microencapsulated by emulsion).

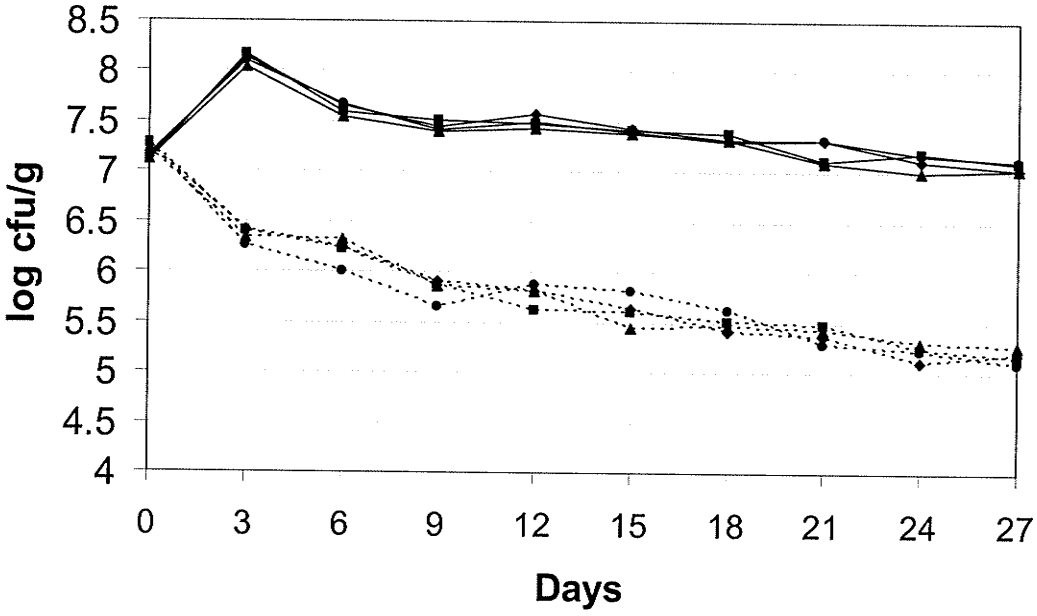


Fig 6.4 Survival of *Lb. reuteri* during manufacture of dry fermented sausages (● - planktonic *Lb. reuteri* ■ - *Lb. reuteri* microencapsulated by extrusion, ▲ - *Lb. reuteri* microencapsulated by emulsion)

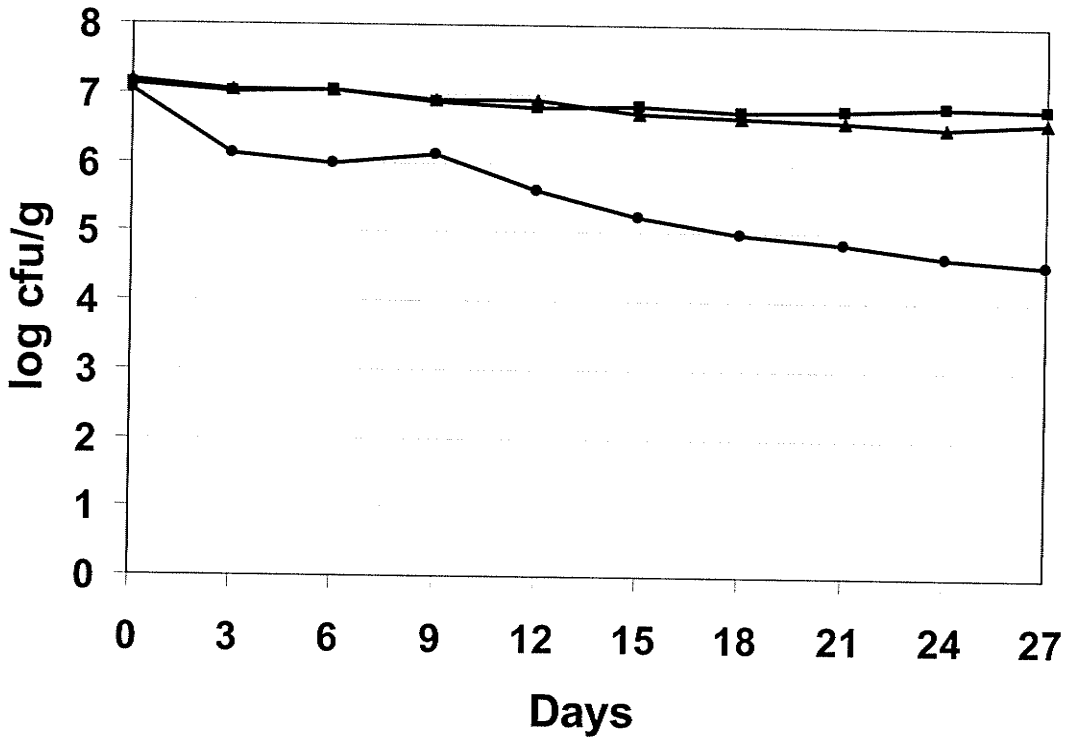
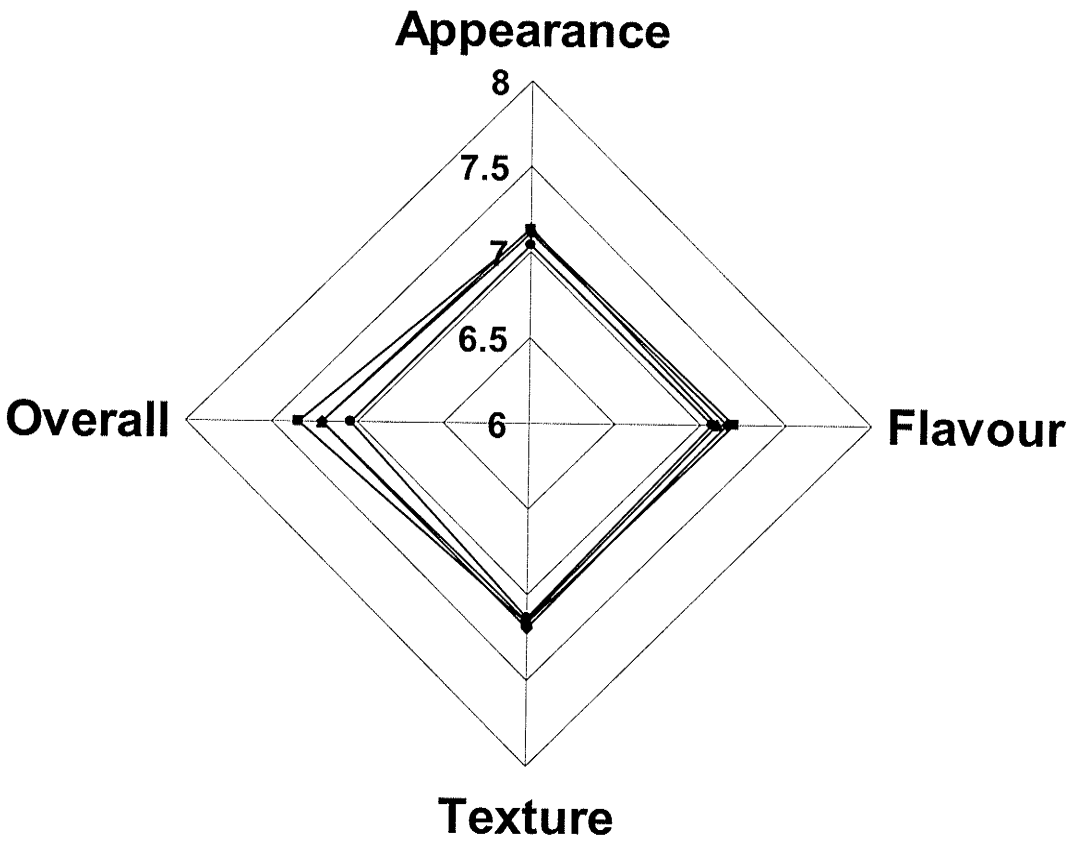


Fig 6.5 Sensory analysis of dry fermented sausages at the end of ripening (nine point Hedonic scale where 9 and 1 represent "extremely good" and "extremely poor" respectively. A score of 7 represents "like moderately"). (◆ - control salami, ● - salami with planktonic *Lb. reuteri* ■ - salami with *Lb. reuteri* microencapsulated by extrusion, ▲ - salami with *Lb. reuteri* microencapsulated by emulsion).



Chapter 7

Survival of *Escherichia coli* O157:H7 in dry fermented sausages containing microencapsulated probiotic lactic acid bacteria

7.1 Abstract

Escherichia (*E.*) *coli* O157:H7 is capable of surviving the rigorous processing steps during the manufacture of dry fermented sausages. The effect of adding two probiotic organisms, *Lactobacillus* (*Lb.*) *reuteri* and *Bifidobacterium* (*B.*) *longum* as co-cultures with the meat starter cultures *Pediococcus* (*P.*) *pentosaceus* and *Staphylococcus* (*S.*) *carnosus* on the viability of *E. coli* O157:H7 in dry fermented sausages was studied. A 5 strain cocktail of *E. coli* O157:H7 was added at 7.4 log cfu/g to the sausage batter and challenged with either or both *Lb. reuteri* or *B. longum* before or after they were microencapsulated. Sausages were fermented at ≤ 26° C and 88% relative humidity (RH) followed by drying at 75 % RH and 13° C for 25 d. The pH, water activity (a_w), protein, moisture, and numbers of all inoculated organisms were monitored during processing. The pH and a_w decreased from 5.7 and 0.98 to 4.9 and 0.88 at the end of fermentation and drying, respectively. These processes reduced *E. coli* O157:H7 by 1.0 and 0.7 log cfu/g at the end of fermentation and drying, respectively. Planktonic *Lb. reuteri* with or without *B. longum* was most effective in reducing *E. coli* O157:H7 (3.0 log cfu/g) and *B. longum* caused a 1.9 log cfu/g reduction. While microencapsulation increased survival of *Lb. reuteri* and *B. longum*, it reduced their inhibitory action against *E. coli* O157:H7.

Key Words: *E. coli* O157:H7, dry fermented sausages, reuterin, bifidobacteria, microencapsulation, probiotics.

7.2 Introduction

E. coli O157:H7 is a serious human health hazard particularly in foods that are consumed uncooked or improperly cooked (Doyle, 1991). Dry fermented sausages are traditionally consumed without cooking after manufacture by lactic acid bacterial starter culture fermentation of meat batter followed by drying at low temperature and humidity for about 30d (Lücke, 1998). Provided guidelines regarding the maximum time spent at temperatures > 15.6° C during processing before the meat reaches pH 5.3 are followed, products are regarded safe from *Salmonella* and *S. aureus* (AAFC, 1992, CFIA, 1999). The presence of salt, nitrate, nitrite, lactic acid produced by the starter cultures and low a_w at the end of drying ensure elimination of these pathogens. However, *E. coli* O157:H7 which were reported to be acid tolerant were able to survive the processing of dry fermented sausages (Glass et al. 1992). Subsequent to a series of outbreaks of *E. coli* O157:H7 related to dry sausage consumption and product recalls (CDC, 1995a, CDC 1995b, Pond et al. 2001, MacDonald et al. 2001), new guidelines governing manufacture of these products were introduced. These guidelines required validation of the process for *E. coli* O157:H7 elimination by using one of the following options: employ a heat process – eg. 63° C for 4 min; conduct "hold and test" of finished product; initiate a Hazard Analysis and Critical Control Point (HACCP) program to include raw ingredient testing plus use of a process yielding a 2 $\log_{10}$ reduction of *E. coli* O157:H7; or demonstrate a 5 $\log_{10}$ reduction of *E. coli* O157:H7 numbers (Reed, 1995, Health Canada, 2000). The "hold and test" and HACCP options are expensive due to the extensive sampling of ingredients and finished product for *E. coli* O157:H7 required to satisfy the regulation. Among the options proposed, only heat treatment at 63° C for 4 min can provide the required level of safety through a 5 log unit reduction of *E. coli* O157:H7

(Hinkens et al. 1996, Nicholson et al. 1996). However, heating of dry fermented sausages melts fat, denatures proteins and yields products with significantly different sensory quality compared to the traditional uncooked products (Johnson et al. 2000, Lahti et al. 2001).

Fermentation of sausages using lactic acid bacteria (LAB) followed by drying was shown to reduce viability of *E. coli* O157:H7 by 1-2 log units in pepperoni, salami and summer sausage (Hinkens et al. 1996, Riordan et al. 1998, 2000, Glass et al. 1992, Faith et al. 1998, Calicioglu et al. 1997). The pathogen reductions by LAB starter cultures occur mainly as a result of acidulation due to lactic acid production in combination with salt, nitrite and low a_w. Although some starter cultures may produce bacteriocins, the latter are mainly effective against Gram positive bacteria and they may be ineffective in meat environments due to inactivation by meat enzymes (Jack et al. 1995, Leroy and Vuyst 1999, Lücke, 2000). A variety of LAB and related cultures including *Lb. reuteri* and *B. longum* exert positive or probiotic effects on health by re-establishing the natural intestinal microbiota, relieving diarrhea, modulating cholesterol and improving digestion (Naidu et al. 1999). Although fermented meats have the potential to carry probiotic bacteria (Incze, 1998) very limited work has been done with them and most work has been focused on product quality (Anderson, 1998, Erkkilä et al. 2000, 2001), although there is one clinical study on blood lipid levels (Jahreis et al. 2002).

Lb. reuteri occurs naturally as a large component of the microbiota in the alimentary tract of humans and animals (Casas and Dobrogosz, 2000). *Lb. reuteri* produces a non-protein, low molecular weight antimicrobial called reuterin (β – hydroxy propionaldehyde) by anaerobic fermentation of glycerol (Axelsson et al. 1989, Chung et

al. 1989). Growth and production of reuterin by *Lb. reuteri* are optimal at pH 4.6-5.0 and 37° C and because of reuterin's stability under these pH conditions at lower temperatures, it is ideally suited for use as preservative, particularly in fermented foods (El-Ziney et al. 2000, Rasch, 2002).

Survival of probiotic bacteria is likely to be poor under conditions of low pH, a_w, and sub-optimal temperature in the presence of salt and nitrite which are characteristic of fermented sausages. Microencapsulation has been effective in protecting sensitive bacteria from harsh environments where they may not normally survive. The technique provides an excellent opportunity to expand probiotic applications from the current successful use of bifidobacteria in challenging food environments such as in yogurt, ice milk and mayonnaise (Sultana et al. 2000, Sheu et al. 1993, Khalil and Mansour, 1998) to other foods where probiotics would not otherwise survive long. The effect of microencapsulated antagonistic bacteria on pathogens such as *E. coli* O157:H7 has not been extensively studied in foods although Kebary and Hussein (1999) reported the inhibition of *E. coli* in yogurt by encapsulated bifidobacteria.

Reuterin production by *Lb. reuteri* in culture media was stimulated by the presence of heterologous bacteria such as *E. coli* and this was believed due to quorum sensing by *Lb. reuteri* (El-Ziney et al. 2000). To take into account the effects of quorum sensing, which could stimulate reuterin production or act as a form of feed-back inhibition (in the absence of heterologous cells) to reduce reuterin production, tests undertaken here included *B. longum* in microcapsules with *Lb. reuteri*. Thus the work reported describes the effect of microencapsulation upon the survival of *Lb. reuteri* and

B. longum in fermented sausages and the effects of their presence upon the survival of *E. coli* O157:H7 during dry sausage manufacture.

7.3 Materials and Methods

7.3.1 Bacterial strain preparation

Strains of *P. pentosaceus* (UM 116P) and *S. carnosus* (UM 110M) used for sausage fermentation were isolated from lyophilized commercial meat starter cultures, Trumark LTII M and LTII, respectively (Holley and Blaszyk, 1998). *Lb. reuteri* ATCC 55730 (SD2112) which was the best reuterin producer as determined by a colorimetric method (Vollenweider et al. 2003, Circle et al. 1945) was provided by Biogaia Biologics Inc., Raleigh NC. *B. longum* ATCC 15708 was obtained from the American Type Culture Collection (AATC, Manassas, VA). Five human isolates of *E. coli* O157:H7 which were non-pathogenic (verotoxigenic negative) (3581, 0304, 0627, 0628 and a non-motile strain 1840) were provided by Rafiq Ahmed, National Microbiology Laboratory, Public Health Agency, Canadian Science Centre for Human and Animal Health, Winnipeg, MB.

P. pentosaceus, Lb. reuteri and B. longum strains were maintained in deMan Rogosa Sharpe (MRS) broth (BBL, Becton Dickinson, Sparks, MD) containing 20% glycerol at -70° C. *S. carnosus* and *E. coli* O157:H7 strains were maintained in tryptic soy broth (TSA, BBL, Becton Dickinson) containing 20% glycerol at -70° C. All organisms were sub-cultured at least twice in their respective broths at 37° C before use in the experiments. Overnight cultures were centrifuged at 10,000 rpm (7800 xg) for 10 min (Sorvall RC-5, Du Pont, Newtown, CT), washed in 0.1% (w/v) peptone water and suspended in 0.1% peptone water to achieve the required inoculation level by standardization at an optical density of 600 nm using a spectrophotometer (Ultrospec

2000, Pharmacia Biotech, Baie d'Urfe, QC). The five strains of *E. coli* O157:H7 were mixed to obtain an equal number of cells of each strain in the inoculation cocktail.

7.3.2 Microencapsulation procedure

Sodium alginate at 3% (w/v) (Sigma-Aldrich Co., St. Louis, MO) was used as the wall material for microencapsulation of probiotics (Sheu and Marshall, 1993). All glassware and reagents used in the experiments were sterilized before use. Alginate was added to distilled water and mixed using a magnetic stirrer with heat until completely dissolved, autoclaved and allowed to cool to 25° C. To this wall material 10 log cfu/ml *Lb. reuteri* cells or *B. longum* cells or both (standardized as above in 0.1% peptone water) were added at a ratio of about 1:5 (v/v) so that 9 log cfu/ml was reached in the microcapsules. Glycerol (250 mmol/L) was filter-sterilized and added to the wall material-culture mixture that contained *Lb. reuteri* alone or combined with *B. longum*. The mixture was extruded through a 21G syringe needle drop-wise into 0.5M $CaCl_2$. Microcapsules were held at 25° C for 30 min to ensure complete solidification. They were separated by filtration through Whatman #4 filter paper and stored in sterile petri dishes at 4° C for less than a day until used in sausage experiments.

7.3.3 Reuterin production by free and microencapsulated cells

Reuterin production by free (planktonic) and microencapsulated *Lb. reuteri* cells in the presence/absence of *B. longum* was determined after Vollenweider et al. (2003) and Circle et al. (1945). *Lb. reuteri* cells were harvested from overnight cultures (stationary phase) by centrifugation at 10,000 rpm (7800 xg) for 10 min. To this pellet containing approximately 10^9 cfu, 10 ml of sterile water containing 250 mmol/L glycerol was added and incubated anaerobically for 3-24h (with or without 10^9 cfu *B. longum* cells

harvested similarly). After incubation, the cells were centrifuged at 10,000 rpm (7800 xg) for 10 min. One ml of the supernatant water-glycerol solution containing reuterin or pure acrolein (Sigma Aldrich, St. Louis, MO) as a standard at 0-200 μg/ml in 1 ml of sterile distilled water were placed in a screw cap vial. To this solution, 0.75 ml of 0.01M DL-tryptophan solution (Fisher Scientific, Ottawa, ON) in 0.05 M HCl were added followed by addition of 3 ml 12N HCl. The vials were incubated at 26° C for 30 min and absorbance values at 560 nm were measured. The absorbance values of reuterin-containing supernatants were compared with the acrolein standard curve and expressed as μg /ml of acrolein. Similarly, one g of microcapsules containing 10^9 cfu/g of *Lb. reuteri* with 250 mmol/kg glycerol (with or without *B. longum*) were added to sterile water and incubated at 26° C for 3-24h. The reuterin production by microencapsulated *Lb. reuteri* was monitored by assaying the suspending water and reuterin was calculated by the above colorimetric method.

7.3.4 Manufacture of dry fermented sausages for E. coli O157:H7 challenge

About 10 kg of sausage batter was prepared for each treatment by chopping pork fat, 90% lean pork and 85% lean beef at 17.59, 60.67 and 17.59 % (w/w), respectively in a Titane 40 rotating bowl meat cutter (Dadaux, Bersaillin, France) which was pre-cooled to around 1-2° C by addition of ice. *P. pentosaceus* and *S. carnosus* were added to reach a final level of 7 log cfu/g in the batter. The five strain cocktail of *E. coli* O157:H7 was added at an inoculation level of 7.4 log cfu/g to all treatments except the control. To all batters 0.44% (w/w) cervelat spice mix (Hermann Laue Spice Co. Inc, Uxbridge, ON), 0.69% (w/w) Rapidur (a proprietary mixture of corn syrup solids and dextrose), 0.31% (w/w) pickle cure concentrate containing 6.25 % $NaNO_2$ and 1% $NaHCO_3$ (Canada

Compound Corp. Winnipeg, MB) and 2.9 % (w/w) NaCl were added and chopped thoroughly for 5 min. Free cells of *Lb. reuteri* or *B. longum* or a combination of both were added to yield 7 log cfu/g meat batter, and alginate microcapsules containing *Lb. reuteri* or *B. longum* or both were added to the salami batter at 1% (w/w) according to the experimental design (Table 7.1). Glycerol at 250 mmol/kg was added to treatments that contained free *Lb. reuteri* cells (treatment 2 and 4). Fibrous casings (55 mm diameter, Kalle GmbH & Co. Wiesbaden, Germany) were pre-soaked in water (40° C for 30 min) before stuffing. Sausage batter was stuffed with a piston stuffer (Mainca Model EM30, Equipamienentos Carnicos, S. L. Barcelona, Spain) into the casings to achieve a final weight of about 500 g. Sausages were transferred to a smoke house (Allroundsystem Rondette with Titan controller, Maurer AG, Reichenau, Germany) and processed by fermentation at 26° C and 88% RH for 24 h, followed by step-wise decreases in fermentation temperature and RH (6x12h intervals where temperature and RH were reduced by 2° C and 2.2 % RH) from 26 to 14° C and 88 % to 75% RH over 72 h with intermittent smoking for 30 min at 48 and 60 and 72h. The degree.hour guidelines of Agriculture and Agri-Food Canada (AAFC, 1992) were followed when time and temperature combinations were used during fermentation. Fermentation was followed by drying at 13° C and 75% RH for 25 d.

7.3.5 Physicochemical analysis of dry fermented sausages

The pH of triplicate samples was recorded using a pH meter with Lancefet probe (Sentron Titan pH meter).Water activity was analysed in triplicate (Novasina AW Sprint TH 500, Axair AG, Pfäffikon, Switzerland). Fat, protein and moisture content of the dry fermented sausages were determined according to AOAC procedures (AOAC, 1975).

7.3.6 Microbial analysis of dry fermented sausages

Samples were taken after stuffing (0 d), at the end of fermentation (72h) and thereafter at 3d intervals during drying for pH, a_w, numbers of *E. coli O157:H7*, *P. pentosaceus, S. carnosus,* and *Lb. reuteri* or *B. longum* or both according to the treatment. After drying, sausages were stored at 4, 13, 25 or 37° C for an additional 2 weeks and analysed for *E. coli* O157:H7 at the end of storage. For bacteriological analysis, 11 g of sausage batter was mixed with 99 ml of 0.1% peptone water and homogenized in a stomacher bag (Filtra-bag, VWR International, Edmonton, AB) for 10 min with a Stomacher 400 (Seward Laboratory, London, UK). Samples were serially diluted and plated using a spiral plater (Autoplate 4000, Spiral Biotech, Norwood, Mass.) on MRS agar + penicillin G (10mg/L) or MRS + polymyxin B (0.5g/L) for specific enumeration of *Lb. reuteri* or *P. pentosaceus*, respectively (Muthukumarasamy and Holley, *unpublished*). Mannitol salt agar was used for enumeration of *S. carnosus*. *B. longum* were selectively enumerated on reinforced clostridial agar supplemented with nalidixic acid (50mg/L) after subtracting *P. pentosaceus* numbers, as both these organisms grew on this medium. *E. coli* O157:H7 were enumerated using sorbitol MacConkey agar (BBL) supplemented with cefixime and tellurite (Oxoid, Hampshire, England) to yield CT-SMAC (Zadik et al. 1993). At the end of fermentation and drying samples were also plated on TSA over-laid with CT-SMAC to detect the presence of injured cells. All plates were incubated at 37° C for 24-48h aerobically, except for *Lb. reuteri* and *B. longum* plates which were incubated anaerobically using the BBL Gaspak system.

7.3.7 Statistical analysis

All data were analysed using Statistical Analysis System (version 8.1) software (SAS Institute, Cary, NC). Analysis of variance by the general linear model (GLM) and Duncan's multiple range tests were used to find significant differences ($P<0.05$) between treatments.

7.4 Results

7.4.1 Reuterin production by planktonic and microencapsulated *Lb. reuteri* cells

In a glycerol water system, the level of reuterin production decreased with time in the case of planktonic *Lb. reuteri* cells, whereas with microencapsulated *Lb. reuteri*, reuterin production increased slightly during 24h incubation (Fig 7.1). When *B. longum* was present as either free cells or when microencapsulated along with *Lb. reuteri* reuterin production was increased, but differences were not statistically significant ($P > 0.05$).

7.4.2 Physicochemical quality of dry sausages

The pH of the dry fermented sausages dropped from initial values between 5.7-5.8 to < 5.3 within 24 h, and by 72h pH values were stable at 4.9. The a_w of dry fermented sausage dropped from 0.983-0.986 at the beginning of fermentation and ranged between 0.868-0.877 by the end of 27 d drying. Significant differences in pH or a_w were not found among the control salami and the treatments containing *Lb. reuteri or B. longum* either as planktonic cells or in microcapsules.

The moisture content of sausages after 27d drying was between 37.7-39.1 %. The fat and protein content of the sausages ranged between 33.5-34.4 and 23.5-24.2%, respectively, on a wet matter basis.

7.4.3 Viability of starter and probiotic cultures during sausage processing

P. pentosaceus numbers increased by about a log by the end of fermentation, but later decreased slightly and remained between 6.77-7.02 log cfu/g by the end of 27d drying. *S. carnosus* numbers had decreased ≤ 3.0 log cfu/g by the end of drying in all treatments (results not shown).

Planktonic *Lb. reuteri* numbers dropped initially from 7.24 to 4.66 log cfu/g after sausage maturation for 27d in treatment 2 (Fig 7.2). In the presence of planktonic *B. longum*, *Lb. reuteri* levels dropped to 4.42 log cfu/g after drying. In treatments containing microcapsules with or without *B.longum*, *Lb. reuteri* were only reduced by ≤ 0.92 log cfu/g at the end of 27d drying. Thus, microencapsulation provided substantial protection to *Lb. reuteri* during sausage manufacture. Although *Lb. reuteri* numbers were slightly (not significantly) lower in the presence of *B. longum* when they were added together without microencapsulation, when both were present in microcapsules there was little effect of *B. longum* on *Lb. reuteri* viability (Fig 7.2).

Planktonic *B. longum* were reduced from an initial level of 7.31 to 5.53 log cfu/g during normal sausage manufacture in the absence of *Lb. reuteri* (Fig 7.3). Addition of planktonic *Lb. reuteri* caused a further (0.5 log) reduction in *B. longum* numbers but the difference was not significant. Significantly improved recovery of *B. longum* occurred during fermentation and drying following microencapsulation, and when *Lb. reuteri* was included in the microcapsules, a slight but significant reduction of *B. longum* numbers was found.

7.4.4 Viability of *E. coli* O157:H7 during sausage processing

When only the *P. pentosaceus* and *S. carnosus* starter cultures were used, *E. coli* O157:H7 numbers were decreased by one log during the fermentation step and by a

further 0.7 log at the end of drying (Fig 7.4). In the treatment containing planktonic *Lb. reuteri,* a 3 log cfu/g reduction in *E. coli* O157:H7 numbers was found at the end of drying. When samples were plated on TSA overlaid with CT-SMAC, similar results were observed indicating that the cells were not injured. In the treatment containing planktonic *B. longum, E. coli* O157:H7 numbers were only reduced by 1.9 log cfu/g after drying. When planktonic *Lb. reuteri* and *B. longum* were present together a 2.8 log reduction in *E. coli* O157:H7 numbers was observed, which was not different from when *Lb. reuteri* was used alone. The use of microencapsulated *Lb. reuteri* and *B. longum* either alone or in combination did not improve the reduction of *E. coli* O157:H7 numbers found when *P. pentosaceus* and *S. carnosus* cultures were used without additional treatments ($P > 0.05$). Storage of sausages for an additional 2 weeks at 4 or 13° C decreased viable *E. coli* O157:H7 by a further < 0.5 log cfu/g. However, storage at 25 or 37° C resulted in complete elimination of *E. coli* O157:H7 from all treatments.

7.5 Discussion

The pH and a_w of sausages decreased to < 4.9 and < 0.88 by end of drying, respectively, and products had a moisture/protein ratio of 1.6-1.7, which is similar to products studied by Glass et al. (1992) but slightly lower than the 1.9 targeted for commercial dry sausage products (FSIS, 2005, Faith et al. 1997). The levels of *P. pentosaceus* remained around 7 log cfu/g throughout fermentation and drying while *S. carnosus* numbers decreased by 3.0 log cfu/g which is similar to findings by Erkkilä et al. (2000). *E. coli* O157:H7 numbers decreased by 1.7 log cfu/g in total at the end of 27d of drying. This is in agreement with other studies which also reported 1-2 log reductions in *E. coli* O157:H7 at the end of fermentation (Glass et al. 1992, Clavero and Beuchat, 1996, Nickelson et al.

1996). Storage at > 20° C was reported to be far more effective in eliminating *E. coli* O157:H7 than storage at 4° C (Faith et al. 1998, Nissen and Holck, 1998). However storage at higher temperature may accelerate oxidative rancidity and yield shortened product shelf-life (Stiebing et al. 1999).

Erkkilä et al. (2000) attempted use of the probiotic *Lb. rhamnosus* to inhibit *E. coli* O157:H7 during dry fermented sausage manufacture. They did not find a significant decrease in *E. coli* O157:H7 numbers compared to sausages fermented with a commercial *P. pentosaceus* starter culture. Pidcock et al. (2002), using non-traditional meat starter cultures including *Lb. acidophilus*, *Lb. paracasei* or *Bifidobacterium lactis* in combination with *P. pentosaceus* found > 2.5 log reduction in *E. coli* O111 numbers after 7 d fermentation at 25° C, with some of the cultures being more effective than others. In the present study it was shown that addition of planktonic *Lb. reuteri* as a co-culture to meat starter cultures was able to significantly reduce the *E. coli* O157:H7 population in dry fermented sausages by a further 1.3 log cfu/g. It was also shown earlier that addition of *Lb. reuteri* at 3 or 6 log cfu/g with glycerol resulted in elimination of 3 or 6 log cfu/g *E. coli* O157:H7 from ground beef within 10-20d when stored at 4° C (Muthukumarasamy et al. 2003). Reuterin with 3% salt plus 5% lactic acid were shown to interact synergistically to reduce *L. monocytogenes* and *E. coli* O157:H7 populations in skim milk and cooked pork, respectively (El-Ziney and Debevere 1998, El-Ziney et al. 1999). Since dry fermented sausages contain both salt (as an ingredient) and lactic acid as a result of lactic fermentation it was hoped a similar interaction with reuterin would occur during dry sausage manufacture. When *Lb. reuteri* was added with glycerol to salami batter, fermented and dried, neither the elimination of *E. coli* O157:H7 nor synergistic

interactions were found. Reductions of the pathogen were substantially less than those found in ground beef. The reasons for less pronounced inhibition of *E. coli* O157:H7 during processing of dry fermented sausages than in ground beef may be due to induction of cross-resistance to reuterin in *E. coli* O157:H7 by exposure to stress caused by lactic acid, a_w and nitrite. Sausage batter also differed from ground beef because the former initially contained glucose to facilitate fermentation. El-Ziney et al. (2000) reported glucose repression of reuterin production by *Lb. reuteri* when glucose concentrations were > 8 mmol/L. The presence of glucose in the dry fermented sausage batter during formulation at $\leq$ 0.3% (w/w) may have initially repressed reuterin production by *Lb. reuteri* cells, but depletion of glucose by the end of fermentation would have reversed any repression.

The viability of *Lb. reuteri* and *B. longum* was improved during dry sausage fermentation and drying when these organisms were microencapsulated. Physical entrapment has been shown previously to protect bacterial cells against the harsh environments characteristic of yogurt, mayonnaise, frozen milk dessert and cream (Adhikari et al. 2003, Khalil and Mansour, 1998, Sheu and Marshall, 1993, Prevost and Divies, 1992). It has been shown that addition of microcapsules used in the present experiments containing *Lb. reuteri* did not significantly affect the sensory quality of fermented sausages (Muthukumarasamy and Holley, *unpublished*). In the present work, addition of *Lb. reuteri* or *B. longum* either as free cells or when microencapsulated did not significantly affect the pH or a_w of the sausages. When planktonic *B. longum* were used in the sausages without addition of *Lb. reuteri*, the reduction in numbers of *E. coli*

O157:H7 beyond that caused by meat starter cultures was only 0.25 log cfu/g. Microencapsulation of *B. longum* slightly reduced its activity against *E. coli* O157:H7.

Adhikari et al. (2000) found significantly less lactic and acetic acids in yogurt stored at 4° C for 30d when inoculated with microencapsulated *B. longum* compared to treatments containing non-encapsulated *B. longum*. They attributed this to reduced metabolic activity of the encapsulated cells compared to free cells. Microencapsulated *Lb. reuteri* either alone or in combination with *B. longum* did not yield significant reductions in numbers of *E. coli* O157:H7. It is possible that the lower antimicrobial activity shown by microencapsulated *Lb. reuteri* (± *B. longum*) was due to feed-back inhibition caused by accumulation of reuterin in the microcapsules (El-Ziney et al. 2000). This may have been caused by reductions in the normal rate of diffusion of reuterin away from the cells by the alginate capsule wall. In preliminary work it was found that reuterin was able to diffuse through the microcapsule wall but rates were not measured. Although reuterin production by *Lb. reuteri* was stimulated by *B. longum* (± microencapsulation) in aqueous media containing glycerol during preliminary work, evidence of the influence of quorum sensing (Chung et al. 1989) was not found in the present study.

Lb. reuteri may be added as a co-culture for inhibition of *E. coli* O157:H7 during dry fermented sausage manufacture, however *Lb. reuteri* did not achieve the 5 log reduction in *E. coli* O157:H7 within the 30d manufacturing cycle followed in this study. Results from the present work showed that microencapsulation of the probiotic organisms *Lb. reuteri* and *B. longum* protects these organisms during dry fermented sausage manufacture. Thus, these products may be considered reasonable vehicles for the delivery of viable probiotics.

Table 7.1 Experimental design for challenge studies against *E. coli* O157:H7 using *Lb. reuteri* and *B. longum* in dry fermented sausages[a]

Treatments	*E. coli* O157:H7 cocktail (7.4log cfu/g)[b]	*Lb. reuteri* (7 log cfu/g)[b]	*B.longum* (7 log cfu/g)[b]	Microencapsulated bacterial cells[c]
Control	-	-	-	-
Treatment 1	+	-	-	-
Treatment 2[d]	+	+	-	-
Treatment 3	+	-	+	-
Treatment 4[d]	+	+	+	-
Treatment 5	+	+	-	+[e]
Treatment 6	+	-	+	+
Treatment 7	+	+	+	+[e]

[a] *P. pentosaceus* and *S. carnosus* at 7 log cfu/g each were added to all treatments.
[b]Final inoculation level in sausage batter
[c] Microencapsulated using 3% alginate
[d] Glycerol at 250 mmol/kg added to sausage batter.
[e] Glycerol at 250 mmol/L added to wall material

Fig 7.1 Reuterin production by free and microencapsulated *Lb. reuteri* with or without *B. longum* in glycerol-water medium. Bars show planktonic *Lb. reuteri* (■), planktonic *Lb. reuteri* with *B. longum* (▨), microencapsulated *Lb. reuteri* (▥),and microencapsulated *Lb. reuteri* plus *B. longum* (⊡). Error bars indicate standard deviation of 3 values. Columns with different letters at each time are significantly different. ($P < 0.05$).

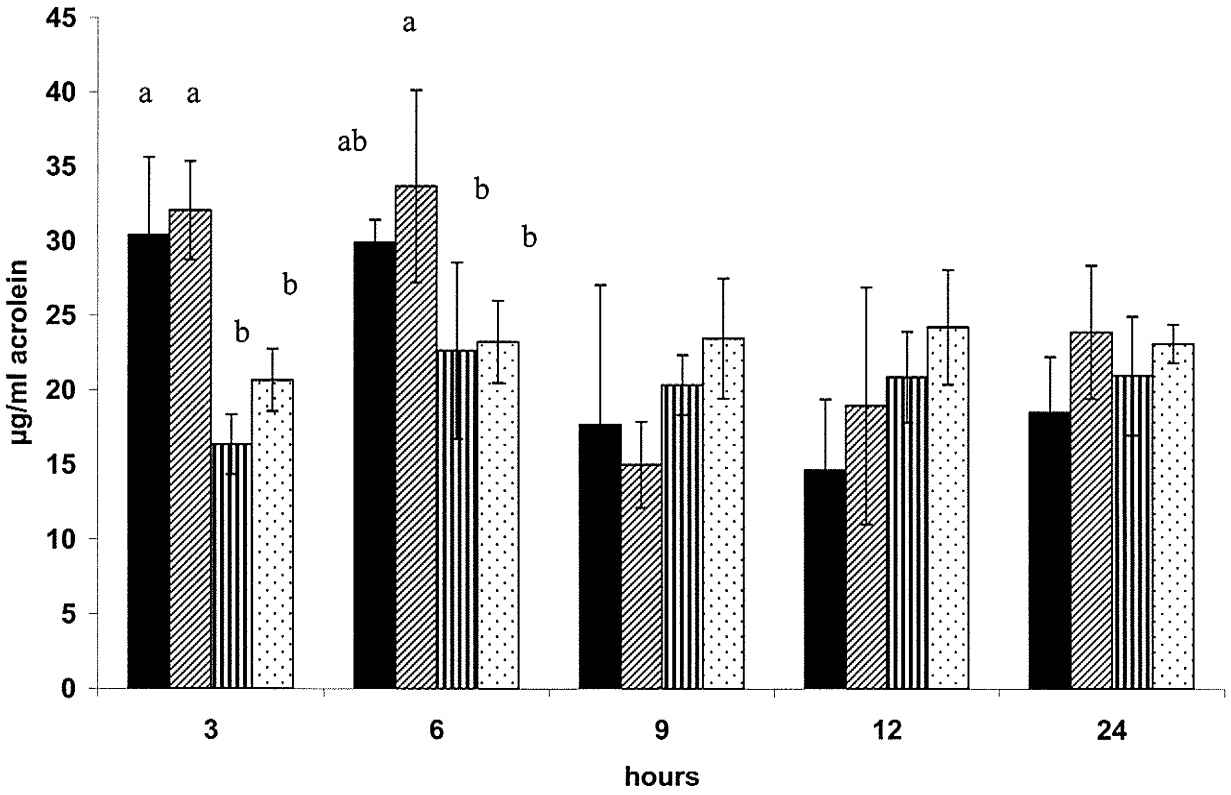


Fig 7.2 Survival of planktonic and microencapsulated *Lb. reuteri* in the presence/absence of *B. longum* during processing of dry fermented sausages. Planktonic *Lb. reuteri* (■) microencapsulated *Lb. reuteri* (□), planktonic *Lb. reuteri* with *B. longum* (▲), microencapsulated *Lb. reuteri* plus *B. longum* (Δ). Error bars indicate standard deviation of 6 observations.

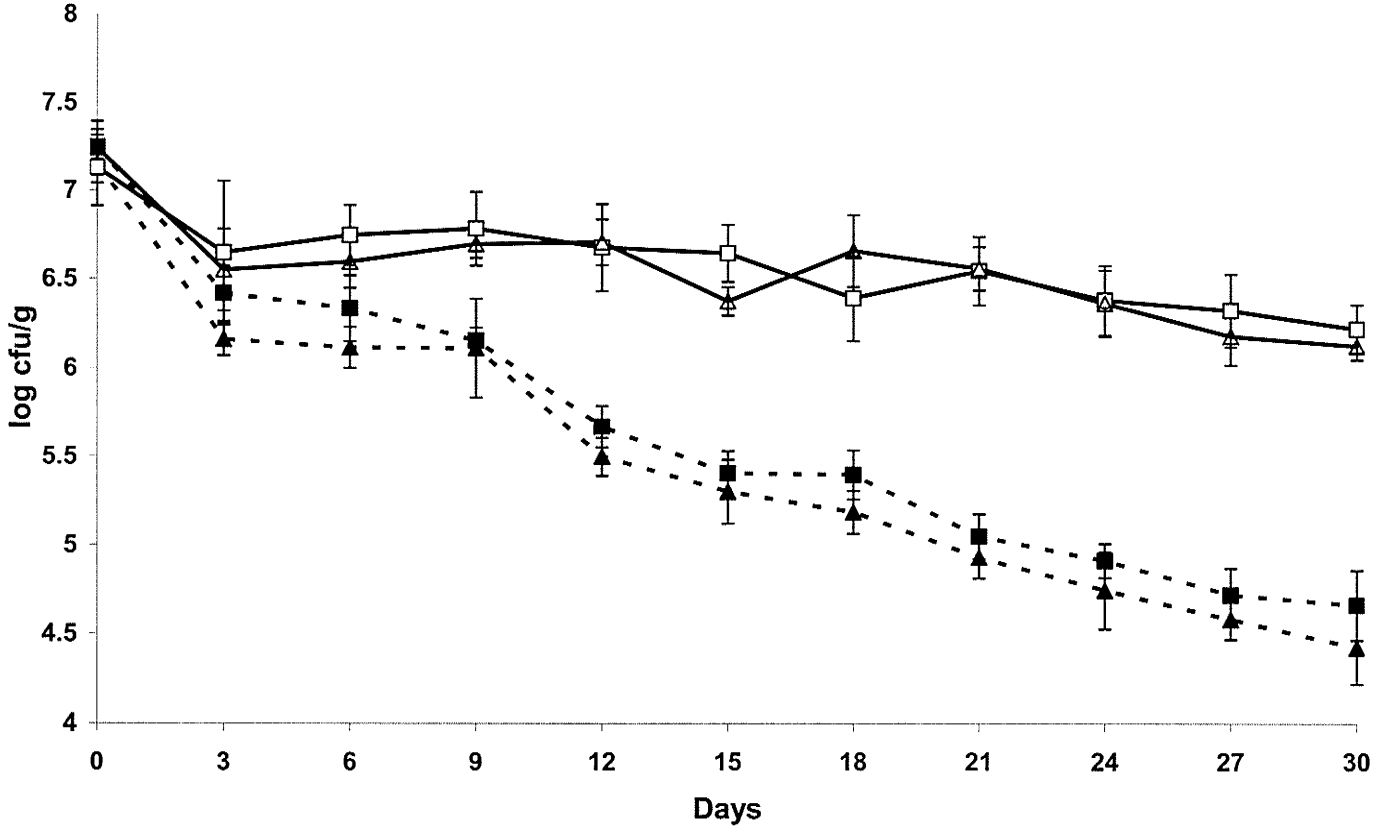


Fig 7.3 Survival of planktonic and microencapsulated *B. longum* in the presence/absence of *Lb. reuteri* during processing of dry fermented sausages. Planktonic *B. longum* (◆), Microencapsulated *B. longum* (◇), planktonic *B. longum* plus *Lb. reuteri* (▲), microencapsulated *B. longum* plus *Lb. reuteri* (Δ). Error bars indicate standard deviation of 6 observations.

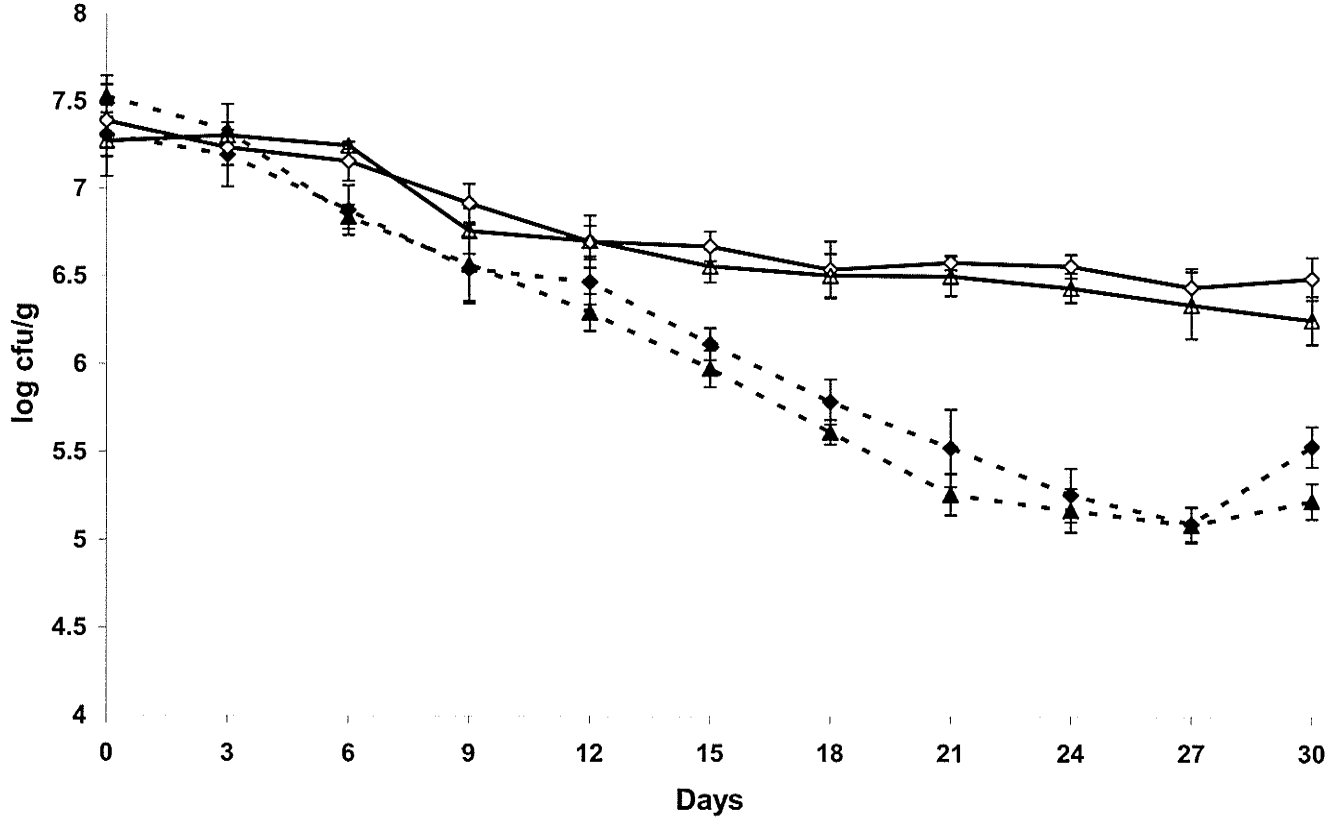
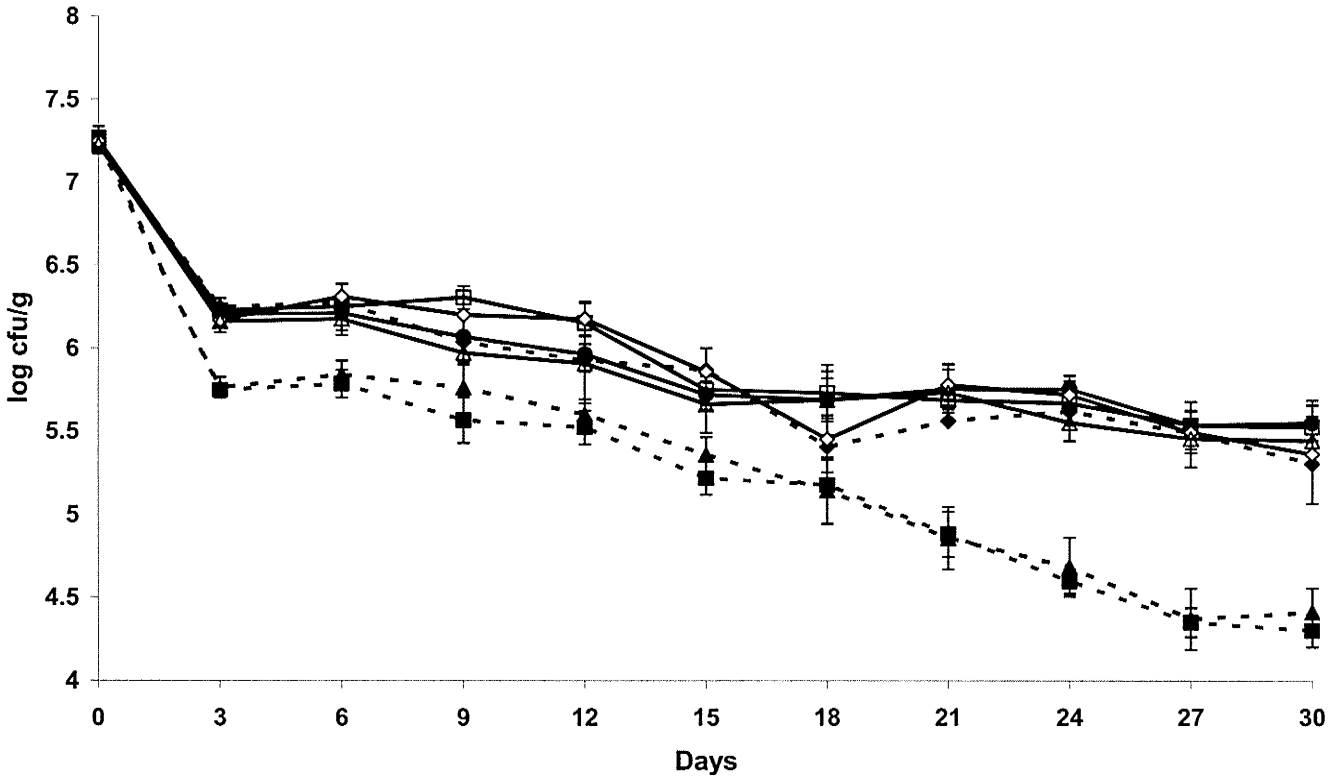


Fig 7.4 Survival of *E. coli* O157:H7 during dry sausage processing when challenged with planktonic or microencapsulated *Lb. reuteri* in the presence/absence of *B. longum*. *E. coli* O157:H7 with: *P. pentosaceus* and *S. carnosus* starter cultures only (●); planktonic *Lb. reuteri* (■); microencapsulated *Lb. reuteri* (□); planktonic *B. longum* (◆); microencapsulated *B. longum* (◇); planktonic *Lb. reuteri* and *B. longum* (▲); microencapsulated *Lb. reuteri* and *B. longum* (Δ). Error bars indicate standard deviation of 6 observations.



Chapter 8

Overall Conclusion

In Chapter 3 it is reported that the use of two natural antimicrobials, reuterin produced by *Lb. reuteri* and AIT from cruciferous plants were effective in inhibiting *E. coli* O157:H7 in ground beef. *Lb. reuteri,* when used at 3 or 6 log cfu/g in the presence of glycerol was shown to eliminate *E. coli* O157:H7 when present in ground beef at 3 or 6 log cfu/g within 20d at 4° C. AIT, when used at 1300 ppm resulted in the elimination of 3 log cfu/g of *E. coli* O157:H7. When *E. coli* O157:H7 was present at 6 log cfu/g, AIT failed to eliminate the pathogen, however, a > 4.5 log cfu/g reduction in *E. coli* O157:H7 was found at the end of 25 d storage at 4°C. It was also shown that when non-deheated mustard flour was used at 10 %, 3 log cfu/g *E. coli* O157:H7 was eliminated in 18d at 4° C. Other research (Nadarajah et al. 2005b) showed that 10 % (w/w) non-deheated mustard flour in ground beef resulted in a 3 log reduction in *E. coli* O157:H7 numbers at the end of 21d when stored at 4° C. AIT < 500 ppm or mustard flour < 10%, in dry fermented sausages and ground beef, respectively, were considered acceptable by consumer taste panel studies. (Chacon, Muthukumarasamy and Holley, 2005 unpublished, Nadarajah et al. 2005b). AIT or mustard flour levels of < 1000 ppm or 5 %, respectively were not effective against *E. coli* O157:H7 in chopped or ground beef (Chacon et al. 2005, unpublished). Based on the above findings, it is evident that the levels of AIT or mustard flour (1300 ppm or 10% respectively) used in the present experiments were sufficient to eliminate *E. coli* O157:H7 when present at 3 log cfu/g, whereas higher levels of the antimicrobials or longer storage may be required to eliminate

E. coli O157:H7 if present at 6 log cfu/g. Normally, *E. coli* O157:H7 is present in ground beef only at levels of 0-40 cfu/g.

In the present work it was found that microencapsulation of *Lb. reuteri* protected the organisms against simulated gastric juice and their survival was related to strain type, method of microencapsulation and the wall material used (Chapter 5). Use of alginate wall material and extrusion were found to be the most suitable combination for microencapsulation of *Lb. reuteri* cells. Although various studies have shown that microencapsulation protects probiotic organisms against challenging environments, the present work is the first report comparing different wall materials, and microencapsulation methods with different strains of *Lb. reuteri* to establish the optimum wall material and method for encapsulation of *Lb. reuteri*.

Lb. reuteri microencapsulated with 3% (w/v) alginate by both extrusion and emulsion were shown to protect the viability of *Lb. reuteri* cells in dry fermented sausages manufactured with *P. pentosaceus* and *S. carnosus* starter cultures (Chapter 6). When 1% (w/w) microcapsules were added to sausage batter, the physico-chemical properties and sensory quality of the sausages were found to be similar to that of control sausages that did not contain any microcapsules. Although microcapsules containing probiotics have been added to a variety of dairy products such as cheese and yogurt, this is the first study that showed dry fermented sausages could be used as a vehicle for probiotics provided these are microencapsulated by one of the methods described. The description of dry fermented sausages as high protein and energy-containing products with beneficial effects on human health, by virtue of the presence of viable probiotic bacteria, should increase the demand for these products.

When microcapsules containing *Lb. reuteri* and glycerol were added to sausage batter containing 7 log cfu/g of *E. coli* O157:H7 (Chapter 7), no significant reductions in *E. coli* O157:H7 were found compared to the control. The presence of *B. longum* along with *Lb. reuteri* was found to stimulate reuterin production by *Lb. reuteri* in water-glycerol media, but did not result in additional reductions of *E. coli* O157:H7 in dry fermented sausages. This may have been due to reduced diffusion of reuterin through the walls of the microcapsules or have been caused by to feed-back inhibition of reuterin production. Increasing the porosity of the microcapsules or generating smaller size microcapsules might result in increased penetration of reuterin through wall of the capsules to the sausage environment. Planktonic *Lb. reuteri*, when used as adjunct cultures at 7 log cfu/g resulted in 3 log cfu/g reduction in *E. coli* O157:H7 at the end processing, which meant that an additional 1.3 log reduction was generated compared to the control. *Lb. reuteri* was shown to successfully eliminate *E. coli* O157:H7 even when the latter were present at 6 log cfu/g in ground beef (Chapter 3), whereas *Lb. reuteri* was significantly less effective in dry fermented sausages. The inability of *Lb. reuteri* to cause substantial inhibition of *E. coli* O157:H7 dry fermented sausages may be attributed to factors such as the presence of salt, competing organisms, low pH, a_w and the presence of glucose. Exposure of *E. coli* O157:H7 to some of these factors in the sausages may have stimulated cross resistance to reuterin. *B. longum* was also found to be incapable of eliminating *E. coli* O157:H7 from dry fermented sausages, however, the organism might be added to sausages to serve as probiotics. Thus findings of this thesis will pave the way for use of probiotic bacteria in dry fermented sausages, however, neither of the organisms tested was effective for control of *E. coli* O157:H7 in these products.

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