PEA PROTEIN-BASED CAPSULES FOR DELIVERY OF PROBIOTICS IN ANIMAL FEED

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By

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ABSTRACT

Probiotics have been used as a prophylactic antibiotic alternative to improve health and growth performance in animals. However, the selection of probiotic strains is limited due to the lack of stability during storage, processing and passage through the upper gastrointestinal tract. In the current study, pea protein isolate-alginate based capsules (PPC) were made by extrusion and their efficacy to improve probiotic viability during storage, feed processing and delivery to the distal intestine were investigated. Encapsulation protected against loss of *Bifidobacterium adolescentis* viability during freeze-drying compared to non-encapsulated bacteria. Capsules also improved the viability of freeze-dried *B. adolescentis* during long-term (335 d) storage at -80 °C. In contrast, encapsulation did not improve viability of *B. adolescentis* or *Lactobacillus reuteri* when stored above 0 ºC. Loss of viability during short duration (up to 180 s) heat challenge (up to 90 °C) was reduced by encapsulation. Encapsulation also protected against loss of viability during heat challenge up to 90 °C with a pressure of 95 MPa for 30 s, however, encapsulation did not protect bacteria added to feed and subject to commercial pelleting. A naturally selected antibiotic resistant *L. reuteri*, was selected to facilitate strain-specific tracking of viable bacteria in the gastrointestinal tract. Supplementation of pigs with encapsulated antibiotic resistant *L. reuteri* in feed did not increase bacterial counts in upper tract compared with non-encapsulated bacteria, however, the counts of viable antibiotic resistant *L. reuteri* in the distal gastrointestinal tract and feces of pigs was increased. In conclusion, pea protein isolate-alginate based encapsulation improved bacteria viability during freeze-drying and provided limited benefit during cold storage of sensitive bacteria. Improved resistance to environmental challenge (heat and pressure) was observed, but was insufficient to protect against conditions found during

commercial feed pelleting. Encapsulation may improve the shelf life of probiotic bacteria during cold storage and this work is the first work to show improved delivery of bacteria to the distal digestive tract of the pig. However, post-pellet application technologies and improved methods of viability protection during storage above 0 ºC are required to broaden the commercial application of probiotics in the feed industry.

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DEDICATION

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LIST OF ABBREVIATIONS

PPC	Pea protein isolate-alginate capsules
AL	Alginate
PPI	Pea protein isolates
AB	Antibiotic
EU	European Union
EFSA	European Food Safety Authority
DFM	Direct Fed Microbial
AAFCO	Association of American Feed Control Officials
FDA	Food and Drug Administration
GRAS	Generally Recognized As Safe
CFIA	Canadian Food Inspection Agency
RNA	Ribonucleic acid
BA	*Bifidobacterium adolescentis*
LR	*Lactobacillus reuteri*
LRR	Antibiotic resistant *Lactobacillus reuteri*
LRW	Wild-type *Lactobacillus reuteri*
SGJ	Simulated Gastric Juice
SIJ	Simulated Intestinal Juice
M	Skim-milk
H	Cysteine-HCl
G	Glycerol
GI	Gastrointestinal
PAS	Pea protein isolate-alginate solution
SEM	Standard error of mean
RCM	Reinforced Clostridial Medium
MRS	De Man, Rogosa and Sharpe
ATP	Adenosine triphosphate
NADH	Nicotinamide adenine dinucleotide
SOD	Superoxide dismutase
DNA	Deoxyribonucleic acid
IgA	Immunoglobulin A
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
qPCR	Quantitative polymerase chain reaction
DM	Dry Matter
ANOVA	Analysis of variance
FOS	Fructooligosaccharides

1 GENERAL INTRODUCTION

Probiotic organisms, as defined originally in 1907 by Elie Metchnikoff, are microorganisms that hold the promise to promote host health upon ingestion (Metchnikoff and Mitchell, 1907). The addition of probiotic organisms to animal feeds has been reported to support a number of benefits in livestock species including improvement in animal growth performance (Cao et al., 2013), decline of pathogenic organisms in infected animals (Casey et al., 2007), and decreased incidence of diarrhea (Jood et al., 2012). Probiotic organisms have thus been commercially applied in the feed industry as growth promoters and alternatives to antibiotics across species (Khosravi et al., 2012; Suo et al., 2012; Giri et al., 2013).

One crucial requirement for applying probiotics in the feed industry is to maintain a sufficiently high viability of the probiotic organism under various processing, storage and feed delivery conditions such that sufficient numbers of live organisms reach the colon to be able to mediate beneficial effects (Bouhnik, 1993). Challenges to probiotic viability include changes in moisture concentration, heat stress, physical compression, oxygen exposure and exposure to other feed ingredients with selective antimicrobial action presented during drying, manufacturing, storage, delivery and handling (Kurtmann et al., 2009; Weinbreck et al., 2010; Borges et al., 2012). Furthermore, following ingestion, probiotic organisms may be sensitive to digestive enzymes, gastric acid, bile salts and secreted antimicrobial compounds present in the gastrointestinal tract (Brinques and Ayub, 2011; Zhang et al., 2013).

To date, the primary solution to these challenges has been the selection of thermostable, acid and oxygen tolerant strains. However, very few strains meet this requirement, which limits the application of other probiotic species that could provide added health benefits. In the current

feed market, only a few probiotic organisms (*Enterococcus feacium*, spore generating *Bacillus* spp, and *Saccharomyces cerevisiae*) have been incorporated into commercial pelleted feeds. The development and application of technologies that provide a more static environment for microbes during feed processing and delivery could broaden the selection of probiotic strains suitable for commercial application.

Recently, encapsulation methods have been employed to entrap probiotics into a coating matrix to provide additional protection against environmental changes and mediate controlled release in the distal gut. Alginate is an inexpensive, non-toxic, gel-forming material. It has been broadly used in encapsulation (Su et al., 2011; Sathyabama et al., 2014). However, alginate alone has not shown a sufficient protection of probiotics (Lee and Heo, 2000). The addition of a concentrated protein source, such as pea or whey protein has provided additional protection in combination with alginate (Klemmer et al., 2011a). Other cryoprotectants like skim milk, glycerol and cysteine-HCl may also provide significant protection during storage and freeze-drying (Pan et al., 2013).

An efficient and low-cost coating for probiotic application in animal feed is needed. Thus, the overall objective of this project was to investigate the efficacy of pea protein isolate and alginate based capsules in maintaining probiotic viability during storage, feed processing and delivery to the intestine in food animals.

2 LITERATURE REVIEW

2.1 Challenges to maintaining viability and efficacy of probiotics in feed applications

Probiotic organisms have been incorporated into the diets of animals for decades and are reported to provide several significant benefits to animal health and production (Vanbelle et al., 1990). Recent restrictions on antibiotic use in animal agriculture have increased interest in the use of probiotics as alternatives for antibiotic growth promotion and prophylaxis (Cheng et al., 2014). Antibiotic use for growth promotion in livestock was banned by the European Union (EU) in 2006 due to the risk associated with an increase in the prevalence of antibiotic-resistant genes in pathogens that can adversely affect humans (Dibner and Richards, 2005). Increased regulatory restriction on antibiotic use, combined with consumer demand for products raised without the use of antibiotics, has expanded the market for probiotics in the animal feed industry such that the probiotic industry is expected to grow annually by 7.7 % between 2016 and 2021 to an anticipated market value of $4.71 billion US (Anonymous, 2017a).

In the feed market, there are many commercial products that contain single or multiple probiotic strains. A summary of probiotics currently used in animal feed market are listed in **Table 2.1**. The summary is based on a review of products registered by the European Food Safety Authority (Additives and Products or Substances used in Animal) and is supplemented by a review of company websites known to offer probiotic products. The United States, the EU and Canada each have unique regulatory systems for identifying and regulating probiotic products. The United States uses the phrase Direct-Fed Microbial products (DFM) for probiotics in animal feed. A list of the approved DFMs that can be incorporated into animal feed can be found in the Association of American Feed Control Officials (AAFCO) (Pendleton, 1998).

Table 2.1. List of commercial direct feed microbial products used in the animal feed industry. Products are summarized by probiotic strain, product and company name, target animal(s), heat tolerance, storage conditions and shelf-life*.

Category	Strain(s)	Name	Company	Animal	Heat Tolerance	Storage Conditions & Shelf-life	Ref.
Bacillus spp.	*B. subtilis* (C-3102)	Calsporin®	Asahi Calpis Wellness	Broilers, Hens, Swine, Horses, Dogs	Can be pelleted up to 90 °C	Stored 37 months (mo) at 25 °C under 60 % relative humidity (RH) or 6 mo at 40 °C under 75 % RH.	1
	B. subtilis (DSM 17299)	GalliPro®	Chr. Hansen	Poultry	Can be pelleted up to 84 °C (86.7 % survival)	Stored up to 35 mo at ambient temperature or for 24 mo when stored at 37 °C (82 % survival rate).	2
	B. subtilis (CBS 117162)	Animavit®	Kemin	Pig	Can be pelleted at 80 °C (80 % recovery)	Stored at 25 °C or 30 °C for 18 mo (results in 24 % reduction in viability).	3
	B. subtilis (ATCC PTA-6737)	Clostat®	Lactosan	Poultry	Can be pelleted up to 90 °C	Stored for 3 years (yr) at room temperature.	4
	B. licheniformis (DSM 5749) and *B.subtilis* (DSM 5750)	BioPlus®	Chr. Hansen	Swine, Poultry	Can be pelleted up to 95 °C	Stored for 24 mo in a cool, dry place at temperatures not exceeding 25 °C.	5
	B. cereus var. *toyoi* (NCIMB 40112/CNCM I-1012*)*	Toyocerin®	Rubinum S.A	Poultry, Pig	Can be pelleted to 90 °C	Stable when stored for 21 mo at 15-25 °C or 6 mo for 30 °C.	6
	B. amyloliquefaciens (CECT 5940)	Ecobiol®	Norel S.A. (Evonik)	Poultry	Can be pelleted up to 80 °C	Stable when stored for one year at ambient temperature in original packaging.	7

Table 2.1. List of commercial direct feed microbial products used in the animal feed industry. Products are summarized by probiotic strain, product and company name, target animal(s), heat tolerance, storage conditions and shelf-life. Continued.

Category	Strain(s)	Name	Company	Animal	Heat Tolerance	Storage Conditions & Shelf-life	Ref.
Lactic acid bacteria	*Pediococcus acidilactici* (MA18/5M)	Bactocell®	Lallemand	Laying hens /Fish/Pig	Apply through drinking system (no information on pelleting)	Stored for 24 mo at room temperature.	8
	L. farciminis (CNCM MA 67/4R)	Biacton®	Chemvet	Pig/Poultry	Can be pelleted, heat resistant at 80 ˚C for 20 min and at 95 ˚C for 2 min	Coated bacteria stored for 12 mo in dry and original packaging.	9
	L. acidophilus and *P. freudenreichii*	Bovamine® Dairy	Chr. Hansen	Rumen	Do not exceed 48.9 °C when mixing (no information on pelleting)	Stored in freezer until mixture with feed (no information on the length of storage).	10
	L. reuteri	Finelact®	Asahi Calpis Wellness	Poultry	Add to the drinking water (no information on pelleting)	Stored for two mo when refrigerated at 39 °F (4 °C) or lower and two years when refrigerated at -15 °C.	11
	L. acidophilus	PoultriMax ®	Chr. Hansen	Poultry	Post pelleting application	Stored in a cool, dry location.	12
	E. faecium (NCIMB 11181)	Lactiferm®	Chr. Hansen	Pig, Calves and Poultry	Post pelleting application	Stored refrigerated (4-5 ºC) in original, closed packaging.	13

Table 2.1. List of commercial direct feed microbial products used in the animal feed industry. Products are summarized by probiotic strain, product and company name, target animal(s), heat tolerance, storage conditions and shelf-life. Continued.

Category	Strain(s)	Name	Company	Animal	Heat Tolerance	Storage Conditions & Shelf-life	Ref.
	E. faecium NCIMB 10415	Cernivet® LBC ME20 PLUS	Cerbios-Pharma SA	Calves, Chickens and Swine	Microencapsulated can be pelleted (no pelleting temperature information)	Stored for 18 mo at 2-8 °C, enclosed in the original container.	14
	E. faecium (DSM 7134)	Bonvital®	Lactosan	Calve, Piglet and Broiler	Can be pelleted at 70-72 °C. After pelleting, 85 % of the initial activity was recovered	Stored for 12 mo at 4 °C or 20 °C or stable when stored for 6 mo at 37 °C.	15
	E. faecium (NCIMB 10415)	Cylactin®	DSM	Multispecies	Can be pelleted up to 95 °C	Stored for up to one year when refrigerated, or six mo at 2-8 °C, or for two mo at 25 °C or for 4 weeks (wk) at 37 °C.	16
	E. faecium (CECT 4515)	Fecinor®	Norel S.A. (Evonik)	Poultry, Swine	Good resistance up to 60°C. At 70 °C there was 0.4 log loss (no information on pelleting)	Stored for 12 mo at 25 °C and 40 °C (0.7 log loss).	17
	E. faecium (DSM 10663/NCIMB 10415)	Oralin®	Chevita GmBH	Calves, Piglets, Broilers, Turkeys, Dogs and Cats	Not able to pellet	Stored for 12 mo at room temperature.	18
Yeast	*S. cerevisiae* (CNCM I-1077 and I-1079)	Levucell® series Proternative ® series	Lallemand	Multiple species	Can be pelleted in Titan model (encapsulated) up to 80 °C	Stored for 24 mo in closed packaging in cool dry place.	19

Table 2.1. List of commercial direct feed microbial products used in the animal feed industry. Products are summarized by probiotic strain, product and company name, target animal(s), heat tolerance, storage conditions and shelf-life. Continued.

Category	Strain(s)	Name	Company	Animal	Heat Tolerance	Storage Conditions & Shelf-life	Ref.
	S. cerevisiae (NCYC Sc 47)	ActiSaf®	Lesaffre	Ruminants	Can be pelleted up to 80 °C	Stored for 12 mo at 30 °C (less than 0.1 log value loss).	20
	S. cerevisiae (MUCL 39885)	Biosprint®	Prosol SpA	Multiple species	Can be pelleted up to 80 °C	Stored for 24 mo in an aluminium foil package under vacuum at 25 °C.	21
	S. cerevisiae (NCYC R-625)	InteSwine®	Integro Gida SAN. Ve TIC A.S	Pig	Can be pelleted at 85 °C	Stored for 12 mo at 30 °C (less than 0.1 log value loss).	22
Others	*Cellulomonas sp.*	Spiromac-c	Geomarine Biotechnologies	Beef cattle	Not indicated	Stored for two years in a dark, cool place away from direct sunlight.	23
	Live intestinal microflora derived from Specific Pathogen Free chickens	Aviguard®	MSD Animal Heath/ Lallemand	Poultry	Spray application. no pelleting information	Stored for 18 mo in original packaging.	24
	E. faecium, P. acidilactici, B. animalis, L. reuteri	PoultryStar®	Biomin	Poultry	Can be pelleted in the microencapsulated form (no temperature information)	Stored for 12 mo in the original package and stored in cool and dry place.	25

Table 2.1. List of commercial direct feed microbial products used in the animal feed industry. Products are summarized by probiotic strain, product and company name, target animal(s), heat tolerance, storage conditions and shelf-life. Continued.

Category	Strain(s)	Name	Company	Animal	Heat Tolerance	Storage Conditions & Shelf-life	Ref.
	L. acidophilus, L. helveticus, L. bulgaricus, L. lactis, S. thermophilus and E. faecium	Lactina®	Chevita	Pig and Poultry	No information.	Stored for a year under nitrogen at 5 °C (while under oxygen lead to 2 log reduction). With storage at 37 °C, there was a loss of 2 log/g under nitrogen and 4 log/g in the presence of oxygen.	26

*References are listed in Appendix A.

The US Food and Drug Administration (FDA) also has a program called "Generally Recognized as Safe" (GRAS), whereby ingredients on this list are exempt from regulation as additives (Bajagai et al., 2016). In contrast, EU regulations require that each probiotic product registration must provide documentation detailing product identity, safety and efficacy to be assessed by a scientific commission (Bajagai et al., 2016). In Canada, probiotics in animal feed are regulated by the Canadian Food Inspection Agency (CFIA). Four microorganisms are listed in Feed Ingredient Schedule IV part II, including three bacterial genera (*Bacillus, Enterococcus, Lactobacillus*) and yeast. Part II of Schedule IV lists feed ingredients and additive categories that have been registered based on a proprietary manufacturing process. Unfortunately, CFIA does not provide detailed information regarding registered products. Similarly, there is no list of registered commercial probiotic products provided by the FDA in the United States. Thus, the probiotic list provided in the table may not include all probiotic products on the global market. However, the table provides a comprehensive list of commonly used probiotics strains currently used in animal nutrition identified through an extensive literature search.

Information summarized in **Table 2.1** includes product category, the specific microorganism, product name, name of the manufacturer, target animal(s), heat tolerance, storage condition and shelf-life. Commercial probiotic products can be placed into three main categories based on the taxonomy of the organisms including *Bacillus* spp. (*B. subtilus*, *B. licheniformis*, *B. cereu*, *B. amyloliquefaciens*), lactic acid bacteria (*L. farciminis, L. acidophilus*, *L. reuteri*, *Enterococcus faecium*, *Pediococcus acidilactici*) and yeast (*Saccharomyces cerevisiae*). The taxonomic distribution of probiotic organisms is extremely narrow compared to the diversity of organisms colonizing the digestive tract of animals with a potential probiotic role. A major factor affecting taxonomic diversity in successfully commercialized probiotic strains is

the requirement to provide the animal with viable organisms and the susceptibility of most microorganism species to losses during processing, storage and passage through the digestive tract.

Among the three categories listed in **Table 2.1**, only spore-forming *Bacillus* spp. and yeast strains show consistent survival after pelleting (a common process used in feed milling) and can survive storage at ambient temperature for a reasonable period of time. Lactic acid bacteria products are typically applied just prior to feeding by mixing with feed, or with water, due to limited resistance to conditions associated with feed processing and storage (Kalavathy et al., 2003; Jung et al., 2008). The only exception is a microencapsulated lactic acid bacteria (*L. farciminis* (Chemvet, 2017) and *E. faecium* (Simon et al., 2005).

Ideally, bacterial strains suitable for animal feed production should be able to maintain high viability during several challenges experienced during probiotic manufacturing, feed processing and gastrointestinal delivery, as illustrated in **Figure 2.1**. Probiotic manufacturing exposes organisms to thermal, oxidative and chemical stressors during culture, drying, packaging and storage. Probiotics are also exposed to mechanical, oxidative and thermal stressors during feed processing and storage. Finally, on the delivery of formulated feed to the farm, probiotic strains are exposed to chemical and thermal stressors during storage in outside feed bins and transport to the feed bunk, as well as during passage through the upper gastrointestinal tract. Several studies have also highlighted the potential challenges probiotics face during food industry processing (Sissons, 1989; Tripathi and Giri, 2014). However, very few studies have investigated the effects of feed processing on probiotics (Kosin and Rakshit, 2006). Here, we reviewed the challenges in the feed industry associated with the commercial delivery of viable probiotic products.

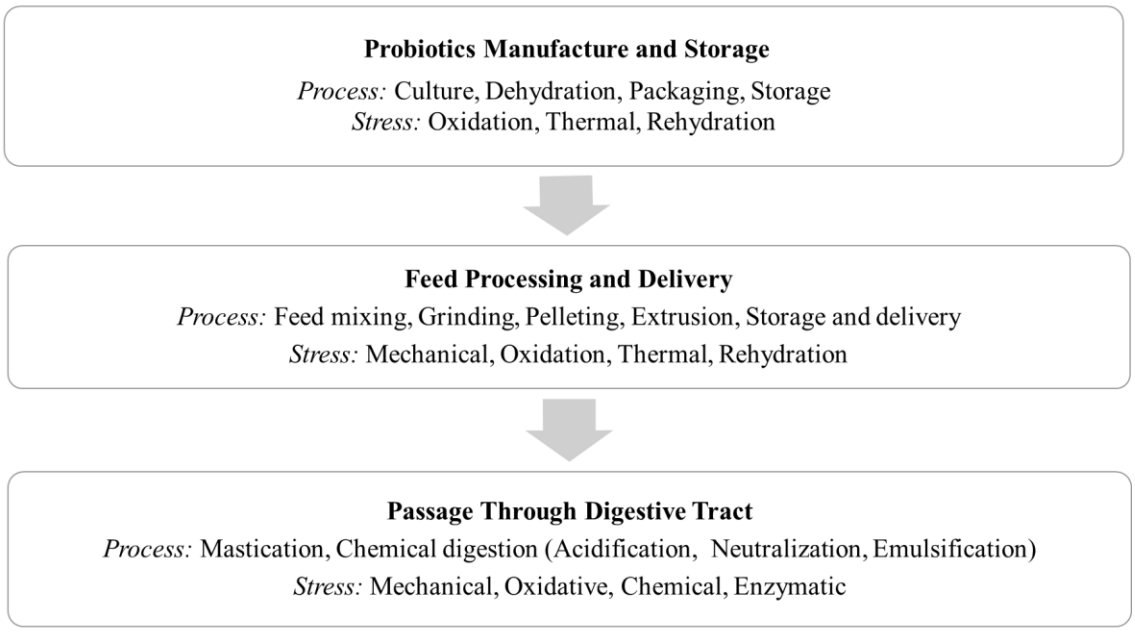


Figure 2.1. Possible challenges to probiotics used in animal feed

2.1.1 Probiotic manufacture and storage

Probiotic products are manufactured prior to being incorporated into feed. The primary process of probiotic manufacturing includes selection of strains, fermentation, dehydration and packaging. After probiotic production, the product needs to be stored properly to maintain viability for future application. Probiotic manufacturing and storage can affect probiotic properties in several ways (e.g. viability, adhesion, thermophilic, immunologic functions) (Grześkowiak et al., 2011). Strain selection (Godward et al., 2000), cultural media and methods (Elo et al., 1991), drying methods and duration (Chávez and Ledeboer, 2007), protective additives (Corcoran et al., 2004), packaging material and method (Talwalkar et al., 2004), storage condition and solution (Mortazavian et al., 2007) can all affect probiotic properties, which could alter their future performance. A detailed review of each aspect is discussed below.

2.1.1.1 Probiotic selection and fermentation

Probiotic strains developed for commercial application undergo a selection process based on their non-pathogenic and health-promoting effects, as well as their ability to survive stress experienced during probiotic processing and gastrointestinal digestion (Kosin and Rakshit, 2006). Common selection processes include: 1) isolation, taxonomic identification and biosafety check; 2) acid tolerance (e.g. mimic stomach conditions); 3) bile tolerance; 4) adherence to epithelial tissue; 5) antimicrobial ability/antagonisms to pathogens; 6) stimulation of immune system; 7) resistance to processing conditions (e.g. heat, oxygen and storage); and 8) animal growth/health performance (Rodriguez et al., 2003; De Angelis et al., 2006; Torshizi et al., 2008). Another major challenge in selecting suitable probiotic strains in the feed industry is the retention of bacterial viability during processing, and especially during pelleting. Identification of thermophilic probiotic bacterial strains, development of protective additives, and development of a coating capable of increasing resistance to stressors encountered during probiotic manufacturing and feed processing have been areas of significant research interest.

Probiotic strains selected for commercial application must be produced in large quantities typically by large-scale fermentation. Differences in fermentation time, growth medium composition and other culture conditions (e.g. temperature and pH) affect probiotic viability and function and must be controlled (Bajagai et al., 2016). The detailed information regarding the effect of fermentation conditions on probiotic viability and function has been reviewed by others (Champagne et al., 1996; Lacroix and Yildirim, 2007; Meng et al., 2008).

2.1.1.2 Dehydration

Following fermentation, spent culture medium is commonly removed by centrifugation and replaced with a dehydration medium prior to further dehydration, packaging, storage and

distribution. Dehydration is commonly used for long-term stabilization of probiotic organisms. However, survival of probiotic organisms during the dehydration process is critical and varies by dehydration method, bacterial species, bacteria cell harvest conditions, components of the dehydration medium and drying time (Golowczyc et al., 2011). Loss of probiotic viability during dehydration may be caused by leakage of intracellular components from cells into the surrounding environment due to cell membrane damage and increased cell permeability (Teixeira et al., 1995). In addition, approximately 80 % of bacterial cell reactions are related to water bound to macromolecules and organelles (i.e. bound water), instead of free water (Webb, 1960). The loss of bound water decreases the survival rate in probiotics by weakening cellular molecular bonds resulting in the loss of cell surface proteins and causing physical damage to the cell wall and membrane (Brennan et al., 1986). Interestingly, such damage may not result in immediate cell death but can cause bacteria to become sensitive to challenge compounds like oxgall (bile salts) and lysozyme encountered in the digestive tract (Brennan et al., 1986).

Residual water percentage is a parameter typically used to confirm adequate drying because the viability of probiotics is very sensitive to percent residual water (Laulund, 1994). The percent of residual water can be affected by the drying medium, storage conditions, and species of probiotic (Scott, 1958; Webb, 1960; Fry, 1966). The presence of water in the final product provides a solution for chemical reactions, leads to sharp crystal formation during subsequent freezing and accelerates the oxidation of lipids (Karel, 1980). Generally, residual water of less than 4 % is considered desirable for long-term storage of dry products (Gardiner et al., 2000), whereas residual water higher than 10 % is not suitable for storage (Desmond et al., 2002). Zayed and Roos (2004) compared 4 levels of residual water content (0 %, 2.8 %, 5.6 % and 8.8 %) and found 2.8 % or 5.6 % as optimal for freeze-dried *L. salivarius* viability during

storage. Some reports have noted that a residual water of less than 4 % contributes to dehydration damage and a lower survival rate (Fry, 1966; Heidebach et al., 2010).

Spray drying and freeze-drying are the most commonly used drying methods for probiotic products. During spray drying, a bacterial solution is forced under pressure through a nozzle to form small droplets in a heated chamber allowing for rapid evaporation of water (Gardiner et al., 2000). In freeze-drying, after initial water removal by centrifugation or filtering, water is removed after rapidly freezing bacteria by sublimation under a high vacuum (Meng et al., 2008). Thus, the stresses associated with spray drying are dehydration and thermal inactivation, whereas freeze-drying stresses include dehydration and freezing.

Of the two common drying methods, spray drying is considered more cost-effective for large-scale probiotic production (Simpson et al., 2005; Santivarangkna et al., 2007). There are many studies examining the performance of different bacteria strains during spray drying (Lian et al., 2002; Simpson et al., 2005; Gardiner et al., 2000). Bacteria strain, outlet temperature and the inclusion of protective additives are critical factors affecting bacterial viability during spray drying. For example, the survival rate of *B. infantis* and *B. longum* differ by 0.08 to 82.59 % after spray drying, respectively (Lian et al., 2002). Similarly, after spray drying, Ranadheera et al., (2015) observed species differences among the survival rate of *L. acidophilus*, *B. animalis* and *Propionibacterium jensenii* (1 to 2 log reduction colony formatting unit [cfu]/g), maintained under the same conditions (Ranadheera et al., 2015). Furthermore, several studies have noted decreased survival rate with increased outlet temperature (Outlet temperature can exceed 100 °C; Golowczyc et al., 2011; Lian et al., 2002). Although convenient and economical, spray drying is not suitable for sensitive bacteria because of the high temperatures and harsh processes that probiotics are exposed to during this drying method. However, outlet temperature reduction

(Ananta et al., 2005), environmental adaption (Desmond et al., 2001), adding protective materials (Avila-Reyes et al., 2014) and encapsulation (Desmond et al., 2002) have been successfully applied during spray drying to improve bacteria survival.

Freeze-drying is an alternative method to spray drying with comparatively higher survival rates, particularly for thermo-sensitive bacteria such as some *Lactobacillus* and *Bifidobacterium* species (Zayed and Roos, 2004; Saarela et al., 2006; Santivarangkna et al., 2007; Bolla et al., 2011). An improved survival rate (>70 %) was observed with freeze-drying compared to spray drying (< 1 %) at 75 and 85 °C for *Bifidobacterium pseudocatenulatum*, with skim milk as a carrier. A number of factors affect viability during freeze-drying including freezing speed, cold stress and cell surface area. The speed of freezing is important and can affect survival rate. Slow freezing increases the solute concentration in the unfrozen portions leading to intracellular ice crystal formation (Zhao and Zhang, 2005). Therefore, a fast freezing process is more desirable. Cold stress (i.e. freezing) can also lead to stabilization of the secondary structure of RNA resulting in a reduced efficiency of gene expression that may contribute to bacterial inactivation (van de Guchte et al., 2002).

Furthermore, cell surface area has been associated with crystal formation during freezing and affects the survival of probiotics. For example, during freeze-drying, studies have noted that enterococci with smaller spherical cells have a higher survival rate than lactobacilli with larger rod-shaped cells (Fonseca et al., 2000). However, it is noteworthy, that Chandralekha et al. (2016) reported that although freeze-drying improved survival rates compared with spray drying the loss of viability during storage was more rapid for freeze-dried products possibly negating some of the benefits of this method. Survival rate was higher in *L. helveticus* after freeze-drying compared to spray drying at a high (120 °C) or low (82 °C) temperature. However, bacteria dried

with low temperature spray drying had higher functionality, as demonstrated by lactic acid production and enzyme activity (Johnson and Etzel, 1995). Therefore, the selection of freeze-drying or spray drying should be based on bacteria strain properties and the downstream production purposes.

2.1.1.3 Storage

There are many factors that may affect the viability of dry powdered probiotics during storage including temperature, rehydration and oxygen exposure. The degree of impact varies by probiotic strain. Optimizing storage conditions, packaging method and protective additives are all important considerations in maintaining viability and extending the shelf-life of dehydrated probiotic products prior to incorporation into feed.

A long shelf-life and resistance to fluctuating storage temperatures are desired for probiotic products for user convenience and cost reduction. It is recommended that probiotic organisms be stored at 4 to 5 °C (Mortazavian et al., 2007). Increasing storage temperatures above 4 °C accelerates the loss of probiotic viability even though an ambient storage temperature is preferred in the industry to reduce storage costs. For example, a comparison of the viability of spray dried *L. paracasei* and *L. salivarius*, after 2 mo storage, drying with skim powder and stored at 4, 15 and 30 °C, indicated that the best survival rate of both strains was 4 °C with a survival rate of 92 % and 13 %, respectively. Storage at 15 °C decreased survival rate to 11 and 2 %, respectively (Gardiner et al., 2000). Therefore, a storage temperature below 4 °C was recommended for sensitive strains. Furthermore, Bruno and Shah (2003) found that -18 °C was the best temperature for storing encapsulated *B. longum*, which had a minimal 1.6 log cfu/mL loss after 20 mo of storage. In the same study, storage at 4 and 20 °C resulted in greater than 5

log cfu/mL reduction and a bacteria count below detection for encapsulated bacteria after 5 mo storage, respectively (Bruno and Shah, 2003).

Relative humidity (RH) and water activity should also be considered during storage. High RH leads to water reabsorption in dried products and a loss of viable cells during long-term storage. Under a RH of 45 % compared to 12 %, the loss of whey protein encapsulated *L. acidophilus* was approximately 2 log cfu/mL higher after 180 d at 22 °C (Rodrigues et al., 2011). A high water residual also leads to the loss of probiotic viability during storage. Controlling water residual to less than 4 % for dry products has been observed to reduce viability loss during long-term storage (Vesterlund et al., 2012).

Besides temperature and moisture, oxygen is another critical challenge probiotics face during storage. Molecular oxygen is toxic to probiotics and is especially lethal for strict anaerobic strains. In order to survive under aerobic conditions, anaerobic strains have to convert reactive oxygen to nontoxic molecules to lower the risk of death from oxidative damage (Talwalkar and Kailasapathy, 2004). In anaerobes, the oxidative and reductive reactions are regulated by pyridine nucleotides, such as Nicotinamide Adenine Dinucleotide (NADH). Generally, the activation of NADH oxidase (NADH-H_2O_2 and NADH-H_2O), which are commonly found in lactic acid bacteria, can reduce molecular oxygen (Condon, 1987). The incomplete reduction of O_2 from NADH oxidase is followed by its removal through superoxide dismutase (SOD) (Sanders et al., 1995) and NADH peroxidase (Thomas and Pera, 1983). Bacterial oxygen tolerance is related to its ability to scavenge molecular oxygen. Oxygen tolerance in anaerobic bacteria generally increases with higher levels of NADH oxidase and NADH-peroxidase (Talwalkar and Kailasapathy, 2003).

Oxygen tolerance in probiotics is species specific. For example, *L. acidophilus* is generally more tolerant than *Bifidobacterium* spp. during oxidative stress (Talwalkar and Kailasapathy, 2003; Vasiljevic and Shah, 2008). At the same oxygen challenge level (21 %), the NADH oxidase and NADH peroxidase were consistently lower in *Bifidobacterium* spp. when compared to *L. acidophilus* strains. Oxygen tolerance of bacteria can also differ among the same genus; in *Bifidobacterium* spp., levels of NADH oxidase (ranging from 4.66 to 18.91 per mg of total protein of the cell free extract) and NADH peroxidase (ranging from 7.56 to 16.86 per mg of total protein of the cell free extract) have been observed to vary widely (Talwalkar and Kailasapathy, 2003). Differences in NADH oxidase and peroxidase in *Bifidobacterium* spp. may have also explained differences in among-species survival during incubation in ambient air; after 48 h incubation in ambient air, *B. adolescentis* did not survive, whereas the survival percentage of *B. bifidum*, *B. breve* and *B. longum* species was above 78 % (Andriantsoanirina et al., 2013).

Probiotic viability during storage may also differ by strain due to variation in their resistance level for exposure to oxygen, heat and cold stressors. For example, the viability of *L. acidophilus* was highest when stored at 2 °C, whereas the viability of *B. lactis* was highest when stored at 8 °C in yoghurt after 20 d (Mortazavian et al., 2007). In contrast to sensitive lactic acid bacteria strains, spore-forming strains and yeast are more stable during storage. The difference in storage stability among different probiotic strains is evident in the list of commercial products provided in **Table 2.1**. Whereas, *Bacillus* spp. and yeast products have a minimum one-year shelf-life when stored at ambient temperature, non-coated *Lactobacillus* spp. require storage at a lower temperature (- 4 °C or below 0 °C) to maintain their shelf-life.

Packaging is one of the solutions to reduce environmental-induced stress in probiotic organisms during storage. The type of packaging material, the permeability of packaging (e.g.

oxygen and light), packaging technique (e.g. vacuum vs. non-vacuum) all affect probiotic viability (Dianawati et al., 2016). Product packages provide a barrier between the probiotic organism and external atmosphere (e.g. oxygen and humidity). During storage of probiotic products, viability is generally higher with a lower percent oxygen in the package. For example, glass packaging is more protective than plastic barriers because less oxygen can permeate into the packaging (Dave and Shah, 1997; Jayamanne and Adams, 2004). However, due to the high cost and difficulties in handling glass packaging, plastic film packaging is more commonly used (da Cruz et al., 2007). Applying the proper packaging method can also improve bacteria viability during storage. Vacuum packaging is commonly utilized in the food industry to reduce oxygen levels in the final product and improve the shelf-life of meat and dairy products (Schillinger and Lücke, 1987; Kasımoğlu et al., 2004; Talwalkar et al., 2004). It is recommended that freeze-dried bacteria cultures are stored under vacuum or under nitrogen gas at low temperatures (El-Sadek, 1975; Champagne et al., 1991). For example, Yang and Sandine (1979) observed that vacuum packaging was the most suitable storage package for freeze-dried lactic acid bacteria stored at 25 °C, when compared to storage under gas (e.g. nitrogen, carbon dioxide and argon). Similarly, vacuum packaged *Pantoea agglomerans* maintained its viability of 10^9 cfu/mL during storage for 90 d at 4 °C, whereas a 2.5 log cfu/g reduction was observed when it was stored under nitrogen gas (Costa et al., 2002).

During probiotic manufacturing, protective materials are commonly applied to probiotics to limit damage from freezing, drying, and oxidation. Cryoprotectants are chemical compounds used during the freezing process to protect against cell damage by increasing solute concentration. Cryoprotectants can act intracellularly or extracellularly depending on their chemical properties. Penetrating cryoprotectants bind to water inside the cell to prevent

hyperosmotic injury and promote the formation of fine ice crystals over large, damaging ice crystals. Non-penetrating cryoprotectants form a viscous layer on the cell surface causing water to partially efflux from the cell and decreasing the generation of ice crystals inside the cell (Hubálek, 2003). The most commonly used cryoprotectants for bacteria are glycerol, saccharides and skim milk. Glycerol decreases the freezing point of the cellular fluid. Carbohydrate hydroxyl groups stabilize cell membrane by inhibiting oxidative cell damage (Smirnoff and Cumbes, 1989). Saccharides protect proteins against denaturation, limit intracellular dehydration and stabilize membrane function and structure during freezing (Storey et al., 1981; Rudolph and Crowe, 1985; Hino et al., 2007). Skim milk stabilizes cell membranes and milk proteins form a protective coating on cells (Abadias et al., 2001). Several investigators have observed that glycerol (2-55 %), saccharides (1-68 %) and skim milk (1-10 %) provide significant protection to bacteria viability during freeze-drying and/or with further storage (Hubálek, 2003; Capela et al., 2006; Kanmani et al., 2011a).

Oxygen reducing agents are used to extend probiotic shelf-life. L-cysteine-HCl, a sulphur-containing amino acid, is commonly employed in anaerobic bacteria media to reduce the oxygen concentration (Kataoka et al., 1997; Rymovicz et al., 2011) and as a donor of amino nitrogen that favours the growth of *Bifidobacterium* spp. (Güler-Akın and Akın, 2007). However, the effects of cysteine-HCl differ by bacterial strains and storage temperature. The addition of cysteine-HCl in encapsulated *B. animalis* improved protection during storage at -80 °C, but not under exposure to other temperatures (21, 4, or -20 °C) and not for the bacteria strains *L. caesi, L. paracasi*, or *L. acidophilus* (Sousa et al., 2012).

2.1.2 Feed processing and delivery

Compound animal feed is formulated using selected ingredients to meet nutritional requirements for growth and health with minimal cost. The most commonly used operations to produce compound animal feeds are grinding, mixing, pelleting or extruding. These methods are designed to improve feed nutritional value, adjust moisture content and modify nutrient density. However, these processes must be tightly controlled as the heat, pressure and moisture from each operation can be applied in excess, negatively affecting nutritional value and/or lowering the bioactive and thermally-sensitive feed additives (e.g. vitamins, enzymes and probiotic organisms; Pickford, 1992; Herrero-Vanrell et al., 2000; Simon et al., 2005). In the case of probiotic organisms, viability must be maintained in the range of 10^6 cfu/g of feed in order to achieve health and/or performance benefits (Herrero-Vanrell et al., 2000). Maintaining viability during feed processing is a major challenge for the probiotic industry; feed processing can markedly affect the ease of commercial application of probiotic supplements in food producing animals. This section provides a review of the conventional feed processing operations and the potential stressors associated with each.

2.1.2.1 Grinding and mixing

Grinding reduces the feed partial size thus increasing the surface area for wetting, as well as chemical and enzymatic digestion. The most commonly used methods for grinding are hammer and roller mills. A hammer mill consists of a set of hammers and a defined screen size in a grinding chamber. Feed ingredients enter the grinding chamber and are impacted by rapidly moving hammers until the feed can pass through a screen selected to achieve the desired particle size (Amerah et al., 2007). A roller mill has several sets of rollers that feed ingredients pass

through compressing the feed components to achieve the desired particle size. The distance between the rollers controls the particle size (Amerah et al., 2007).

Mixing of ingredients may occur before or after grinding of ingredients, to generate a complete diet. There are several different types of mixing machines. Mixing occurs in a chamber with single or multiple rotating paddles or screws (in a horizontal or vertical orientation) that act to blend feed ingredients to achieve uniformity. Limited information is available regarding the interaction of feed ingredients and probiotic additives. However, probiotic additives approved in the EU have included a viability test to establish the stability of the probiotic with feed premixes (FEEDAP Panel, 2006 and 2010).

The addition of probiotic additives before grinding is not common but rather, feed additives are more commonly added during mixing. Currently, there is no direct assessment of the effect of feed grinding on probiotic viability in the scientific literature. However, grinding may pose a potential hazard because the direct pressure and compression used to break down feed ingredients may be harmful to bacteria.

The pressure during grinding varies between machines and by feed ingredient, and there is little information on the exact pressure applied during this process. The pressure can be as high as 486 MPa, when a hammer mill is used to crush stones (Isnugroho et al., 2016), compared to a maximum 50 MPa in roller mills (Martens and Pingel, 2016). A pressure of more than 20 MPa was reported to have adverse effects on bacterial cell integrity, enzymatic reactions and gene expression, all of which could impact viability (Follonier et al., 2012). For example, in the case of dried *L. acidophilus*, only one-third of bacteria survived after exposure to 180 MPa (Chan and Zhang, 2002). Generally, most vegetative cells will inactivate at a range between 200 to 400 MPa, while spores inactivate around 800 MPa with exposure to a temperature of up to 120 °C

(Cheftel, 1995; Hogan et al., 2005). Similarly, Sheehan (2007) noted a reduction in bacterial viability ranging from 5.9 to 7.9 log cfu/g for *L. salivarius*, *B. lactis*, *L. casei* and *L. rhamnosus*, after a pressure challenge at 400 MPa for 5 min. However, viability was not reduced with cocci and bacilli strains hand ground in a mortar and pestle with sand (Gould et al., 1975). The effect of pressure on bacteria is dose dependent, whereas additional research on the effect of grinding on bacteria is needed to better inform feed processing; particularly with respect to potential interactions with other feed ingredients.

2.1.2.2 Pelleting and extruding

Pelleting is a commonly used feed processing operation defined which combines moisture, heat and pressure to form small particles into pellets (Falk, 1985). Pelleting of feeds improves animal performance and feed conversion rate that in turn improves feed palatability, reduces foodborne pathogens, decreases feed wastage and thermal modification of starch, binder activation, and also reduces selective feeding (Behnke, 2001; Medel et al., 2004). However, the heat and pressure used (or generated) during the pelleting process is perhaps the greatest challenge to probiotic viability during feed processing, reflected in part by the recognition that this process can aid in foodborne pathogen control (Considine et al., 2008).

In a pelleting machine, mashed feed first passes through the conditioner apparatus where the feed is exposed to dry or steam heat prior to entering the pelleting chamber (Abdollahi et al., 2013a). The conditioner process is used to gelatinize starch and improve pellet quality but can also reduce microbe and pathogen viability (Skoch et al., 1981). Conditioned feed enters the pelleting chamber and passes through a metal die by mechanical force. Pellet size is determined by the size of perforations in the die. The conditioner temperature, retention time and die hole size significantly affect feed pellet quality and nutrition value (Briggs et al., 1999; Abdollahi et

al., 2010, 2013b). Generally, steam pressure used during conditioning ranges from 102 to 442 kPa, with a feed residency time of fewer than 2 min (Thomas et al., 1997). For monogastric animal feed, the conditioner temperature ranges from 70 to 90 °C (Doyle and Erickson, 2006). A higher temperature can be used to control feed-borne pathogens, and a lower temperature can be used to aid the incorporation of sensitive enzymes or select probiotics (Spring et al., 1996; Jones and Richardson, 2004; Doyle and Erickson, 2006). The metal die size ranges from 0.5 to 10 mm in diameter across animal species, depending on feed application (Bertipaglia et al., 2010; Gopal et al., 2010; Abdollahi et al., 2013c).

The challenges for adding dried probiotic additives before pelleting are: 1) high temperature (up to 90 °C for 2 min) and rehydration (by steam addition) in the conditioner and 2) pressure and secondary heating generated by mechanical friction in the die chamber. There is limited research on probiotic survival because of the harsh conditions created during pelleting. Current research is mainly limited to heat tolerant species such as *Bacillus* spores and yeast. Less research has been focused on lactic acid bacteria due to their susceptibility to heat exposure. For example, in the case of *Bacillus* spp., Amerah et al. (2013) found that the survival rate of *B. subtilis* was more than 90 % after pelleting at 75, 85 and 90 °C using steam. Moreover, the recovery of *B. cereus* after pelleting at 75 °C was 90 % (Jadamus et al., 2001). In the case of yeast, there was a 10-fold (1 log cfu/g) loss of viable *S. cerevisiae* after pelleting with steam for 12 s at 82 °C (Aguirre-Guzmán et al., 2002). Unlike *Bacillus* spp., spores and yeast, the survival of lactic acid bacteria were low even at reduced pelleting temperatures. Among lactic acid bacteria, *E. faecium* showed an improved resistance to pelleting. The survival rate of *E. faecium* was 100, 92, 50 and 35 % after pelleting in feed at 50, 60, 70, 80 °C, respectively (Simon et al., 2005). In contrast, survival was minimal with *L. reuteri*, *L. mucosae*, *L. plantarum* and *L. rossiae*

(only about 10 % survival) after pelleting at a very low temperature of 60 °C for 40 s (De Angelis et al., 2006). A number of studies have reported survival of probiotic organisms following pelleting, although the rate of survival and pelleting conditions were not provided. Examples include studies examining *B. subtilis*, *B. licheniformis* and *Pediococcus* spp. (Ng et al., 2014); *L. acidophilus* and *L. casei* and *Scytalidium acidophilum* (Huang et al., 2004) and *B. licheniformis* (El-Haroun et al., 2006).

Extrusion is a feed process often used for aquaculture and pet feed manufacturing. Like pelleting, mash feed materials may also be conditioned (e.g. steamed or dried) prior to extrusion. The conditioned feed is then pushed through a die by screws. Compared to pelleting, the level of heat (up to 200 °C), moisture and pressure applied in extrusion are usually higher (Hilton et al., 1981). There are even fewer studies on the effect of extrusion on probiotic survival rate compared to pelleting, which could be due to the harsh and highly lethal conditions created during extrusion. Aguirre-Guzmán et al. (2002) applied low temperature, cold extrusion to shrimp feed containing *S. cerevisiae* at 72 °C using a lab meat grinder and reported a 105-fold decrease in viability. Studies have reported the inclusion of *Bacillus* spp. in extruded fish diets (Ye et al., 2011; Cha et al., 2013) but details on extrusion conditions or bacterial survival rate were not disclosed.

During hydrothermal processes in pelleting and extrusion, the biggest challenges for probiotic incorporation are the synergetic effects of heat, moisture and pressure. Processing temperatures are much higher than the temperatures reached during storage (reviewed in section 2.1.1.3). It has been reported that any temperature higher than 45 to 50 °C during processing can be harmful to probiotic viability (Kang et al., 2012; Tripathi and Giri, 2014). The pressure during pelleting and extrusion is material and method dependent. Pressure and temperature can reach up

to 123 MPa and 200 °C during feed processing, respectively (Hilton et al., 1981; Thomas et al., 1997). As mentioned above, any pressure above 20 MPa is harmful to bacteria. High pressure and heat can be lethal to bacteria when applied alone, and a dramatic reduction also occurs when heat and pressure are applied in combination (Considine et al., 2008). The combination of heat and pressure are commonly used in the food industry for sterilizing food products (Patterson and Kilpatrick, 1998). Similar synergistic effects were observed with a combination of heat and moisture (Marshall et al., 1963). Therefore, the combination of all three challenges (e.g. heat, moisture and pressure) during feed production poses a significant stress on the incorporation of probiotic additives during processing.

2.1.2.3 Storage after feed processing

Storage conditions of feed following processing are highly variable and are an additional challenge to probiotic organism survival. Feeds, once prepared, are stored and transported using auger and/or pneumatic systems to move feeds. Feed is typically stored at ambient outdoor temperatures that vary by season, and storage duration can range from days to months. Once feed is delivered into animal husbandry facilities and prepared for animal consumption, probiotics in feed are also subject to the conditions in the barn, such as temperature and moisture changes. Similar to storage prior to feed incorporation and processing, once probiotics are mixed with feed, storage under 4 °C appears optimal for viability. For example, *L. brevis*, *L. plantarum* and *Pediococcus pentosaceus* were sprayed on feed and their viability examined after storage at -22, 0, 4 and 8 °C for 25 d. The best storage temperature was 4 °C in contrast to significant viability loss after 15 d of storage at room temperature (Owunmi et al., 2016). Similarly, Aly et al. (2008) observed improved viability of *B. subtilis* and *L. acidophilus* mixed in feed over 4 weeks and stored at 4 °C, compared with storage at 25 °C. Guerra (2007) observed reduced viability of

Pediococcus acidilactici, *Lactococcus lactis* subsp. *lactis*, *L. casei* subsp. *casei* and *E. faecium* (in the range of 2.35 to 2.57 log cfu/g) mixed in pig diets and stored at room temperature for 8 d. In another study, the recovery of *B. cereus* toyoi after 8 weeks in feed was 92 % compared to *E. faecium* with a recovery of only approximately 50 % (no storage temperature indicated) (Simon et al., 2005). Feed storage remains a significant challenge to the commercial application of probiotic organisms given the range of environmental temperatures (e.g. - 40 to + 40 °C) under which feed is stored, dependant on the geographic region.

In summary, feed processing combines numerous stressors including heat, moisture and pressure that act synergistically to produce a lethal environment for probiotic organisms. Indeed, the use of specific feed processing techniques (such as pelleting and extrusion) to control the dissemination of pathogens is in contradiction to the survival of probiotic organisms. Solutions such as the selection of more resistant microbes (Gardiner et al., 2000), reducing processing temperatures (De Angelis et al., 2006), developing post-processing probiotic application methods such as spraying and vacuum coating (Cavadini et al., 1999; Kirejevas, 2007; Pascher et al., 2008), and developing protective materials or encapsulation (Chávez et al., 2007; Rokka and Rantamäki, 2010) will be important in expanding the commercial application of probiotics in the food animal industry.

2.1.3 Transit during digestive tract delivery

Viable probiotic organisms that have survived manufacturing, processing and/or storage, must also survive the proximal gastrointestinal tract following consumption in order to be metabolically active in the distal gut to provide a health benefit. The gastrointestinal tract is a complex environment where conditions are established to affect nutrient digestion by physical, chemical, and enzymatic processes mediated through both host secretions and microbial activity

(Salminen et al., 1998). Maintaining probiotic viability at a sufficient level during passage through digestive processes of the upper gastrointestinal tract, as well as avoiding immune secretions designed to protect against pathogens, is challenging. Specific stressors encountered in the upper digestive tract include: mechanical force, low pH, bile acids, pancreatic enzymes, antimicrobial peptides, secreted IgA and other antimicrobial compounds of host origin and microbial origin arising from communities already colonized in these spaces. Mechanisms of competition with indigenous bacteria for probiotic colonization has been well reviewed (Walker and Duffy, 1998; Lee and Salminen, 2009) and will not be examined here. In this section, the environmental challenges present in the upper gastrointestinal tract of monogastric animals associated with mechanical, chemical and enzymatic processes during feed digestion will be examined. Focus will be on swine and poultry as major food producing monogastric species.

2.1.3.1 Overview of monogastric animal digestive tract physiology

Although the upper gastrointestinal tract of swine and poultry species includes anatomical structures unique to each species, the fundamental physiological processes of digestion are similar. In swine, the mouth serves in feed mastication and lubrication, the stomach localizes acidification, and the upper small intestine neutralizes stomach contents followed by enzymatic digestion and emulsification. In the mouth, feed is ground using teeth to reduce particle size and lubricated with serous and mucoid saliva (enriched in either bicarbonate or mucopolysaccharides, respectively), to facilitate swallowing and transit of the food bolus to the stomach, buffer against pH change and initiate wetting. Saliva also contains antimicrobial factors such as lysozyme and antimicrobial peptides.

In the stomach, gastric acid and pepsinogen are the primary secretions that reduce pH from 6 to 2 and initiate protein hydrolysis, respectively (Ange et al., 2000). The lower pH,

resulting from gastric acid, is a major challenge to many acid-sensitive bacteria and the antimicrobial properties associated with low gastric pH is considered a major line of defence against enteric pathogens (Giannella et al., 1972). Feed resides in the stomach from 1.4 to 6 h (Davis et al., 2001) prior to entry into the upper small intestine where liver and pancreatic secretions neutralize stomach acids, and deliver a variety of digestive enzymes and bile salts to facilitate enzymatic digestion and lipid emulsification.

Digestion and absorption of nutrients occur as feed passes through the rest of the small intestine with a transit time of 3 to 12 h (Latymer et al., 1990). On exit of the small intestine the remaining feed material enters the large intestine and caecum, where the primary function is water and electrolyte absorption. The transit time is much slower in the large intestine reaching up to 50 h in transit to the colon (Latymer et al., 1990). The undigested feed components, slow transit time and limited host secretions contribute to a high density of microbial colonization in this location, reaching 10^{11} cfu/g contents.

Poultry demonstrates some unique digestive tract physiology compared to swine. First, the crop is a non-glandular outpouching of the esophagus used to store and wet feed. In birds, the proventriculus is the site of acid secretion, which differs from the stomach of the pig in that residence time is much shorter because the crop serves as the primary postprandial storage site in the case of meal feeding. Furthermore, poultry possesses a unique organ known as the gizzard, which replaces in part the mastication function of the teeth in mammals such as swine. The gizzard is extremely efficient at feed particle size reduction, with an average particle size leaving the gizzard reported to range from 115 to 211 μm (Hetland et al., 2002). Feed passes directly from the proventriculus to the gizzard such that the pH in both locations is similar, ranging from 2.7 to 4.1 (Jiménez-Moreno et al., 2009). The small intestine of poultry functions in the same

way as the pig, although it is markedly shorter on body weight basis. The colon in birds is extremely short such that the major site of microbial fermentation is the paired ceca. The passage time in chickens is very short; in the chicken transit time is consistent between the short small intestine and colon and can be as little as 2 to 3 h (Lentle et al., 2006).

The amount of indigenous bacteria and variety of species are a reflection of the characteristics of each location of the pig digestive tract. In the stomach and upper GI tract, the number of indigenous bacteria is 10^3 to 10^5 cfu/g and consists predominantly of acid-resistant lactobacillus and streptococci due to the low pH and rapid transit time. This number increases to 10^8 cfu/g in the distal small intestine due to the slower passive time and neutralized pH, when compared to the stomach. The hindgut (cecum and colon) has a relatively slow transit time, and significant undigested substrate for fermentation, supporting a large number of bacteria (e.g. 10^{10} to 10^{12} cfu/g), diverse species, low redox potential and high short chain fatty acid content (Fooks et al., 1999). Commonly, probiotic additives are targeted for delivery to the distal small intestine and hind gut as locations where the densest populations of microorganisms and highest fermentative activity occurs.

2.1.3.2 Challenges of probiotic application in mouth and stomach

The main challenges facing probiotic additives during transit in mouth and stomach include mechanical force, enzymatic action (e.g. lysozyme, pepsin) and gastric acid. Pressures higher than 20 MPa may result in negative effects on bacterial viability (see Section 2.1.2.1; Follonier et al., 2012). The pressure in the jaw when feeding is between 0.56 to 3.39 kPa in Hanford and Sinclair minipigs (Dutra et al., 2010) and up to 0.7 MPa in humans (Kohyama et al., 2004). Therefore, although the exact effect of mechanical force by mastication on probiotic additives is unknown it is unlikely to be a primary factor affecting viability.

Lysozyme is an antimicrobial enzyme secreted by host mammals and birds and is commonly found in tears, saliva, mucus, and egg white (Jollès and Jollès, 1984). Lysozyme hydrolyzes bonds in peptidoglycans (specifically, a glycoside hydrolase that targets the 1, 4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues), the major component of the Gram-positive bacterial cell wall, causing loss of cell integrity and cell death (Rada et al., 2010). Lysozyme can also affect Gram-negative bacteria when the inner peptidoglycan region is exposed after the outer cell wall is disrupted (Saurabh and Sahoo, 2008). Lysozyme can be used in the food industry as a preservative due to its antimicrobial activity (Cardarelli et al., 2007), and has been considered as an antibiotic alternative feed additive in the animal feed industry (May et al., 2012; Oliver and Wells, 2015).

Lysozyme sensitivity is both species- and strain-dependent. For example, sensitivity differed by species and strain for bacteria in the *Bifidobacterium* genus with exposure to 400 µg/mL lysozyme (Rada et al., 2010). Also, the growth percentage of different *L. delbrueckii* strains ranged from 6 to 100 % after challenge in 1 µg/mL lysozyme (Guglielmotti et al., 2007). Since the lysozyme level in the saliva of humans can reach up to 76.7 µg/mL (Tenovuo et al., 1986; Perera et al., 1997), resistance to lysozyme has become one of the primary criteria for probiotic selection in many studies in the food industry, especially for *Lactobacillus* and *Bifidobacterium* spp. (Neviani et al., 1992; Guglielmotti et al., 2007; Rada et al., 2010). There is little information on the activity of lysozyme in the intestine of domestic animals, although it has been reported that a much higher lysozyme level is found in the stomach of ruminant animals relative to other mammals (Dobson et al., 1984). Further work on the implications of lysozyme mediated effects on probiotic viability relative to food and/or companion animals may be required.

One of the biggest challenges for maintaining probiotic viability in the gastrointestinal tract is the low pH in the stomach due to gastric acid. Indeed, the gastric environment is considered a significant barrier to entry of pathogens to the small intestine (Giannella et al., 1972; Herrero-Vanrell et al., 2000). Acid conditions increase diffusion of H^+ into bacterial cells leading to increased energy expenditure (e.g. H^+ efflux) to maintain cytosolic pH and reduce the inactivation of sensitive enzymes and damage to proteins and DNA (Presser et al., 1997). For instance, the survival of *B. adolescentis* declined quickly from 9 to 6 log cfu/mL in the first 5 min of challenge and another 3 log cfu/mL for the following 10 min at pH 2 (Klemmer et al., 2011b). The viability of several species of lactic acid bacteria, including *L. rhamnosus, B. longum, L. salivarius, L. plantarum, L. acidophilus, L. paracasei,* and *B. lactis,* was also decreased by 6.01 to 7.05 log cfu/mL after challenge at pH 2 for 2 h (Ding and Shah, 2007).

Similar to other stressors, different strains showed varied resistance to low pH. Spore-forming *Bacillus subtilis* were fully resistant after challenge at pH 2 for 3 h (Guo et al., 2006). However, no bacteria survived with challenge of pH 2 for 2 h for several other spore-forming bacteria (e.g. *B. coagulans, B. laevolacticus, B. racemilacticus, Sporolactobacillus inulinus*). Only one strain of *Bifidobacterium* (*B. laevolacticus* DSM6475) showed 86 % survival after challenge in pH 2.5 for 2 h (Hyronimus et al., 2000). The differences in acid tolerance between strains may be due to the activity of H^+ - ATPase, which serves to pump out H^+ from the cell to maintain homeostasis such that species with higher enzyme activity are more tolerant (Matsumoto et al., 2004).

2.1.3.3 Challenges of probiotic application in small intestine

Another significant challenge for probiotic delivery in the intestinal tract is bile. Bile is secreted into the duodenum after feed ingestion to aid in the digestion of lipids and to neutralize

gastric acids. Bile acids are a main component of bile that are conjugated with the amino acids glycine or taurine to form bile salts. Bile salts are potent detergents important in the emulsification of lipids, thereby facilitating lipid enzymatic digestion (Hofmann and Mysels, 1992). Bile not only aids the digestion of lipids but also has antimicrobial functions (Begley et al., 2006). As a strong detergent for lipids, bile can alter cell membrane integrity and permeability by dissolving membrane lipids leading to cell leakage and death (Begley et al., 2005). Moreover, bile acids can cause cell damage on the molecular level, such as RNA and DNA damage, oxidative stress and protein misfolding (Bernstein et al., 1999; Powell et al., 2001). Interestingly, bile can also act indirectly via its antimicrobial effects by altering host gene expression through the small intestine bile acid receptor (Inagaki et al., 2006).

Survival during challenge in bile is an important selection criteria for probiotic bacteria. Despite the different concentrations, forms of bile, and culture medium used to investigate the effects of bile on bacterial viability, studies have consistently observed that bile reduces probiotic viability. However, the degree of negative effect of bile varies by species and strains. For example, after incubation in 3 % oxgall (a commercial purified animal bile), there was a reduction of about 4 log cfu/mL in viability of all strains including *L. rhamnosus*, *B. longum*, *L. salivarius*, *L. plantarum*, *L. acidophilus*, *L. paracasei* and *B. lactis*, after 4 h incubation and a further 2 log cfu/mL after 8 h incubation (Ding and Shah, 2007). *B. lactis* was the most susceptible strain with a reduction of 7.41 log cfu/mL. When *B. lactis* was exposed to 0.25 % of bile salt, there was no recovery of the bacteria after 10 min challenge, whereas *B. adolescentis* was inactivated with exposure to 0.1 % bile salt (Amor et al., 2002). In another study, tolerance was highest in spore-forming bacteria *B. subtilis* exposed to 1.2 % conjugated bile salt (minimum inhibitory concentration of 195 mM) relative to *B. cereus* (98 mM) and *B. clausii* (24 mM;

Spinosa et al., 2000). Bile not only affects spore-forming bacteria viability but spore germination rate. There was an approximately 20 % reduction in germination of *B. subtilis* after challenge for 20-25 min to 1.2 % conjugated bile salts and 1 % non-conjugated bile salts (Spinosa et al., 2000).

A combination of bile and pancreatic enzymes are commonly used in selection methods for probiotics to mimic upper intestinal conditions (Pitino et al., 2010). Pancreatic enzymes secreted by the pancreas include lipase, a variety of proteases, amylase and nucleases and have been shown to have negative effects on the viability of some sensitive bacteria strains. After challenges in intestinal juice (Pancreatin UPS, pH = 8.0) for 4 h, the viable number for *B. adolescenstis* and *B. breve* strains were below detection limit (Charteris et al., 1998). However, pancreatic enzymes did not reduce cell numbers for other resistant strains, including *L. acidophilus*, *L. rhamnosus*, *L. reuteri* and *L. plantarum* (Champagne and Gardner, 2008) and *L. casei*, *B. bifidum* and *B. animalis* (Charteris et al., 1998). Those enzymes can not only directly affect the viability of probiotics but can also reduce mucosal adhesion ability, a trait considered important for many probiotic strains. All of the bacterial strains (e.g. *L. rhamnosus*, *L. casei*, *L. johnsonii*, *L. rhamnosus* and *B. lactis*) tested by Ouwehand (2001) showed reduced adhesion to intestinal mucus after pre-treatment with pepsin, amylase, lipase, chymotrypsin, trypsin and pancreatin (Ouwehand et al., 2001).

In the small intestine, several other antimicrobial products produced and secreted into the lumen by host animals can affect probiotic efficacy. These include products of innate and acquired immunity such as antimicrobial peptides and secretory IgA (Immunoglobulin A) (Hofmann and Eckmann, 2006). These factors have proved effective in defending against pathogenic microbes (Fubara and Freter, 1973; Honorio-França et al., 1997; Ayabe et al., 2000) and may also be important in mediating the composition of commensal bacteria, including

probiotic bacteria. Indeed, secretory IgA bind commensal bacteria and are known to stimulate secretion of antimicrobial peptides (Fukushima et al., 1998; Möndel et al., 2009). However, the direct effect of intestinal IgA and antimicrobial peptides on the viability of probiotic additives is not clear.

In summary, maintaining probiotic viability from probiotic ingestion to delivery to the distal digestive tract is a complex process that involves mechanical, chemical and enzymatic processes associated with digestion. Monogastric animals (pig and poultry) possess unique digestive physiologies that may impart unique challenges in each probiotic species. Sensitivity to challenge conditions is species and strain dependent and may not only reflect viability but also probiotic function (e.g. mucus adherence). The challenges found in the stomach (associated with acid secretions) and in the small intestines (associated with bile salts and pancreatic enzymes) are the most significant. The application of additional protection materials to sensitive probiotics in animal feed may be beneficial in the delivery of viable and active probiotics to the distal digestive tract.

2.2 Encapsulation

Probiotic manufacturing, feed processing and gastrointestinal tract delivery can all contribute to added stress on probiotic bacteria. Applying additional protection against these stressors using encapsulation might help to maintain probiotic viability and/or may provide direct benefit to the target animal. Encapsulation is the process of covering or entrapping core materials in a wall-material matrix to offer additional protection to various “payloads” contained within (Risch, 1995). Encapsulation has been shown to provide protection against environmental changes including: dehydration stress during drying (Abd-Talib et al., 2013), heat challenge (Teoh et al., 2011), storage (Sousa et al., 2012), acidic challenge in simulated gastric juice

(Sultana et al., 2000; Annan et al., 2008) and passage through the digestive tract (Wang et al., 2016). In addition to improving viability after each challenge, encapsulation can provide controlled release of core materials at a targeted location in the gastrointestinal tract (Anal and Singh, 2007). Encapsulation has broad applications not only in the protection of probiotic bacteria but also in the protection of vitamins, antioxidants, cells and other small molecules (Schrooyen et al., 2001). Identifying effective encapsulation methods and wall materials for application in probiotic delivery could broaden the range of available probiotic species used in the feed industry and potentially enhance the efficacy of existing probiotics.

2.2.1 Encapsulation methods

The two primary methods of encapsulation for probiotic organisms are extrusion and emulsion. These two approaches are relatively gentle, can be adapted to a variety of wall materials and have been broadly researched (Krasaekoopt et al., 2003). Other methods such as spray drying, fluidized bed coating and spray chilling have also been used for probiotic encapsulation (Anal and Singh, 2007) but will be reviewed here in limited detail. The various encapsulation methods can be manipulated to produce capsules of different sizes. Generally, a capsule size between 1 and 1000 μm is considered a microcapsule whereas a size range between 10 and 1000 nm is considered a nanocapsule (Konan et al., 2002; Umer et al., 2011).

2.2.1.1 Extrusion

Extrusion is a relatively easy, gentle and inexpensive way to produce capsules that provide adequate protection of core material (Krasaekoopt et al., 2003). This method has two major steps (**Figure 2.2**). In the first step, a hydrocolloid solution and payload material solution are prepared. In the second step, the solution is extruded through a small outlet nozzle (e.g. syringe needle) to form droplets that are solidified in a cross-linking solution. The size of capsule

generated can be influenced by the protein concentration in the payload-hydrocolloid solution, the cation concentration in the cross-linking solution, the size of the outlet nozzle and the distance between the opening and the surface of the cross-linking solution (Krasaekoopt et al., 2003). Typical capsule size using the extrusion method is in the large range of 2 to 5 mm. Capsules with larger diameters are expected to provide better protection for probiotic payloads compared to smaller sized capsules. However, larger capsules can ruin food texture and may have limited application in feed for smaller animals (Krasaekoopt et al., 2003).

Extrusion has been described as "hard to scale up and slow" (Krasaekoopt et al., 2003). However, recent efforts have improved the potential commercial application of extrusion by increasing the production speed and improving the scalability of the manufacturing process. In theory, the fluid flow rate can be improved through the application of added pressure on the solution at the outlet nozzle. Different approaches for improving extrusion include the jet cutting method and electrostatic droplet generation as described elsewhere (Petrovic et al., 2007; Burgain et al., 2011). Capsules made by extrusion have been successfully used for probiotic encapsulations. For instance, Yeung (2016) observed improved viability (by 2 log cfu/ml) in *L. lactis* encapsulated with 1 % alginate and stored at room temperature after 7 d, when compared to free cells (not dried). Similarly, Chávarri (2010) prepared chitosan-coated alginate capsules containing *L. gasseri* and *B. bifidum.* These capsules protected the coated bacteria placed in gastric acid (pH = 2) or bile salts.

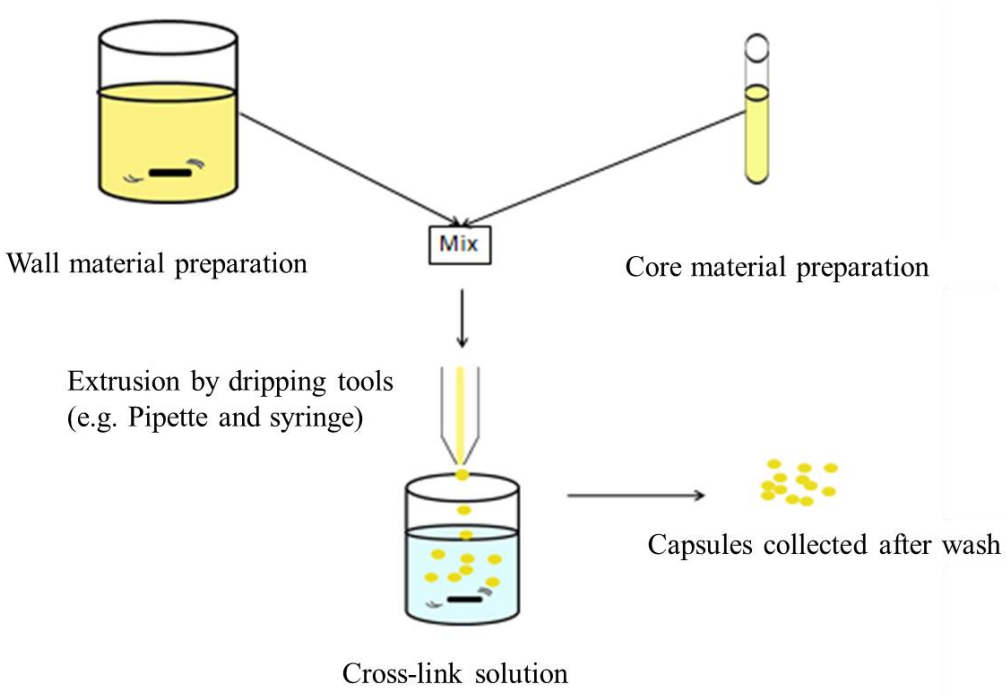


Figure 2.2. Extrusion method for encapsulating (adapted from Krasaekoopt et al., 2003)

2.2.1.2 Emulsion

Emulsion is another common method used to encapsulate probiotics. It is also a two-phase process consisting of a discontinuous phase and a continuous phase. The discontinuous phase consists of the core material and supporting material. Core materials may include probiotics, flavours, proteins and enzymes (Krasaekoopt et al., 2003; Given, 2009; Li et al., 2010), whereas supporting materials may include gum, alginate, chitosan, gelatine and plant proteins (Sultana et al., 2000; Krasaekoopt et al., 2003; Nag et al., 2011; Wang et al., 2014). The continuous phase consists of a vegetable oil (eg. canola oil, sunflower oil, soybean oil). The homogenized discontinuous phase (water) is mixed with the continuous phase (oil) to form a water-in-oil emulsion. A cross-linking solution is then added that hardens the wall material, trapping the core material inside, to form hydrocolloid capsules (Krasaekoopt et al., 2003).

Emulsion has broad applications and produces capsules with a small size (25 µm to 2 mm) compared to extrusion (Krasaekoopt et al., 2003). However, there are some disadvantages to emulsion including the high cost of vegetable oil (Krasaekoopt et al., 2003), difficulties in sterilizing the vegetable oil for aseptic applications (Gbassi and Vandamme, 2012) and the added stress on probiotics caused by rigorous stirring during capsule production. Several studies have noted increased probiotic protection for bacteria encapsulated using the emulsion methodology. For example, increased protection in simulated gastric juice was noted with several lactic acid bacteria including *B. adolescentis* protected with chickpea protein and genipin coating (Wang et al., 2014), *B. bifidum* entrapped with alginate and chitosan (Zhang et al., 2013) and *L. acidophilus* and *Bifidobacterium* spp. coated with alginate and starch (Sultana et al., 2000), compared to non-encapsulated bacteria.

2.2.1.3 Other methods

Spray drying is also commonly applied in probiotic encapsulations. As described above, spray drying is a rapid process whereby a liquid solution containing wall and core material is sprayed into a hot gas resulting in the formation of small (e.g. 10 µm to 500 µm), dry particles (Re, 1998). However, the fast and hot air used during spray drying can cause agglomeration and the stress resulting from exposure to heat and dehydration (during evaporation) can affect sensitive probiotic viability. The application of adapted methods during spray drying can improve probiotic viability. Modified methods include adding protectants and lowering temperature during spray drying or applying vacuum spray drying (Dolly et al., 2011; Jantzen et al., 2013; Lavari et al., 2014). Successfully encapsulated probiotics include *L. reuteri* coated with alginate and chitosan (Malmo et al., 2013), and *Bifidobacterium* BB-12® (Chr. Hansen, Hónsholm, Denmark) with skim milk (Fritzen-Freire et al., 2012).

Fluidized bed coating is an encapsulation method whereby core material is suspended in a temperature and humidity controlled chamber and atomized in the air (Dezarn, 1995). The method uses hot-melt (e.g. fatty acid, waxes) or solvent-based (e.g. starches, gums) wall material for coating, yielding a capsule size of 50 to 500 μm. Core materials may include probiotics, vitamins, organic acid or lactic acid (Gibbs et al., 1999). Several studies applying fluidized bed coating for probiotic encapsulation have demonstrated increased protection of probiotics during *in vitro* simulated gastric juice challenge. For example, *L. reuteri* coated with whey powder and shellac (Schell and Beermann, 2014) and *L. paracasei* coated by trehalose, maltodextrin were successfully encapsulated by fluidized bed coating (Semyonov et al., 2012). The technology has also been successfully commercialized; the company Lallemand Inc. (Saint Simon, Canada) has successfully delivered their *L. acidophilus*, *P. acidilactici*, and *S. cerevisiae* probiotic product using Probiocap® technology which is based on fluidized bed coating. A mixture of fatty acids, vegetable wax and milk powder were used as wall material (Durand and Panes, 2007; Anandharamakrishnan, 2015).

Spray chilling and cooling is another inexpensive method for encapsulation. Unlike in spray drying, where hot air is used to encapsulate core materials, spray chilling uses chilled or cooled air. Spray chilling and cooling is usually used to encapsulate solid materials such as minerals and vitamins (Okuro et al., 2013a) and has recently been applied to probiotic coating (Pedroso et al., 2012). Low melt point wall materials (e.g. lipids) are commonly used in this process. Briefly, melted material containing wall and core material, is injected into a chamber of cold air where the material solidifies to form capsules (Kuang et al., 2010). Spray chilling and cooling has several advantages over spray drying. Compared to spray drying, the cold temperatures used during spray chilling and cooling are better suited for probiotic encapsulation.

Further, the lipid coating material is predicted to delay capsule release until it reaches the intestine, rather than the stomach, based on the site of delivery of pancreatic lipases (Okuro et al., 2013a). The first study to apply spray chilling in probiotic encapsulation was Pedroso (2012). In this study, *B. lactis* and *L. acidophilus* were entrapped in a lipid (palm and palm kernel fat) by spray chilling at 10 °C. Increased protection from encapsulation was observed with exposure to simulated gastric juice, intestinal fluid and storage at low temperatures (-18 °C) for 90 d compared to non-encapsulated bacteria (Pedroso et al., 2012). Cocoa butter was also employed in spray chilling (in place of lipids) and it provided a similar protection for *B. animalis* and *L. acidophilus* (Pedroso et al., 2013). Another study demonstrated increased protection with inulin or polydextrose-coated *L. acidophilus* via spray chilling (Okuro et al., 2013b).

2.2.2 Encapsulation wall material

The wall materials of encapsulation form the structure of the capsules and also provide protection for core bacteria during processing, storage and gastric acid challenge. A variety of chemicals can be used as the wall material for encapsulation including carbohydrates, proteins, and lipids as listed in **Table 2.6**. Each encapsulation method requires wall material suited to the

Table 2.2. Categories and examples of wall materials used in encapsulation (adapted from Zuidam and Nedović, 2010)

Category	Wall materials
Carbohydrate	Starch, κ-Carrageenan gum, pectins, alginate, gellan, chitosan
Proteins	whey, caseins, plant protein isolates (pea, chickpea), gelatin
Lipids	Coconut fat, paraffin wax and beeswax

encapsulation methodology employed such as the ability to form an emulsion or gelling properties (Desai and Park, 2005). More importantly, the unique properties of the wall material

will affect the mechanism of controlled release in the intestine which depends on pH, enzymatic degradation or osmotic pressure (de Vos et al., 2010).

Polysaccharides are complex carbohydrates made up of chains of monosaccharides. Under certain conditions, three-dimensional structured gels are formed by polysaccharides in a process known as gelation (Rinaudo, 1993). Gelation is an important property of polysaccharides and is commonly employed in encapsulation applications to provide core material with added protection. Several polysaccharides have been applied in probiotic encapsulation due to the low cost and non-toxic properties associated with this methodology. Examples are alginate, gellan, chitosan (Chen et al., 2007; Klemmer, 2011a; Caetano et al., 2016).

Protein isolates are also commonly used as an encapsulation wall material, either alone or in combination with polysaccharides. Plant based proteins including pea, lentil and soy protein isolates have been applied in probiotic encapsulation (Klemmer et al., 2012; Khan et al., 2013; Wang et al., 2014). Animal sourced proteins like casein, gelatin and whey protein are also commonly used (Picot and Lacroix, 2004; Annan et al., 2008; Heidebach et al., 2010). Use of plant-based proteins in capsule manufacturing could reduce the possibility of allergy and also broaden the market based on religious, dietary and ethical reasons (Klemmer, 2011a).

More than one wall material can be used to produce capsules if necessary. These combinations usually consist of oppositely charged proteins and polysaccharides. Examples of protein-polysaccharide combinations include alginate and whey protein (Hébrard et al., 2010), chitosan and whey protein (Bastos et al., 2009), and alginate and pea protein (Klemmer, 2011b). Lipids may also be used in combination with alginate or other polysaccharides and are considered a good oxygen and moisture barrier (Weinbreck et al., 2010). This review will mainly focus on alginate and pea protein as example wall materials.

2.2.2.1 Alginate

Alginates are a family of linear unbranched polysaccharides that contain different amounts of 1, 4'-linked β-D-mannuronic acid and α-L-guluronic acid residues (Gombotz and Wee, 1998). Alginate capsule beads can be "hardened" through the addition of divalent cations (including Ca^{2+}, Sr^{2+}, Ba^{2+}, Cu^{2+}, Ni^{2+}) via exchange with sodium ions from guluronic acids. The assembly of these charged guluronic groups form the egg box structures shown in **Figure 2.3** and **Figure 2.4**. Each alginate chain can join together to form a 3-D network. (Gombotz and Wee, 1998).

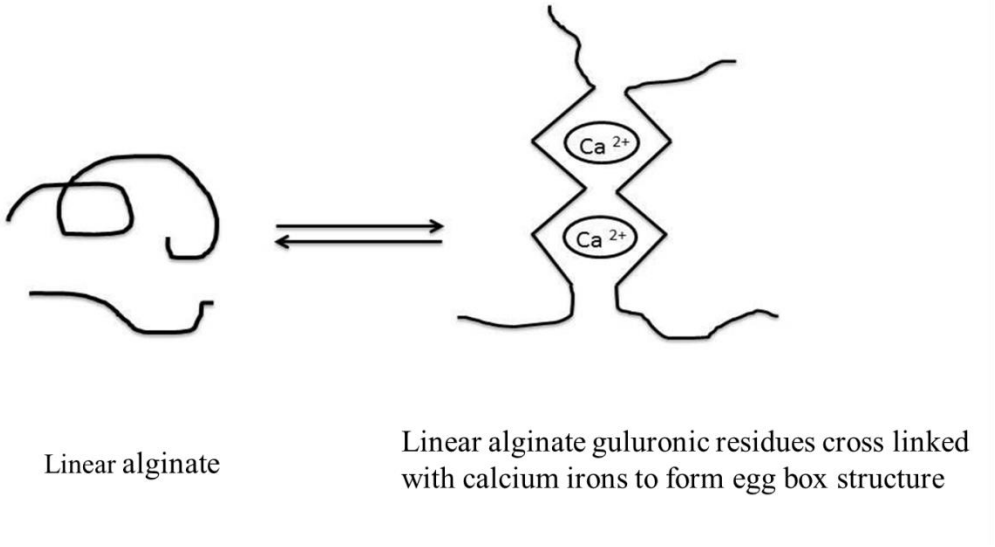


Figure 2.3. Alginate cross-linked by calcium ions to form an egg-box structure (adapted from Gombotz and Wee, 1998).

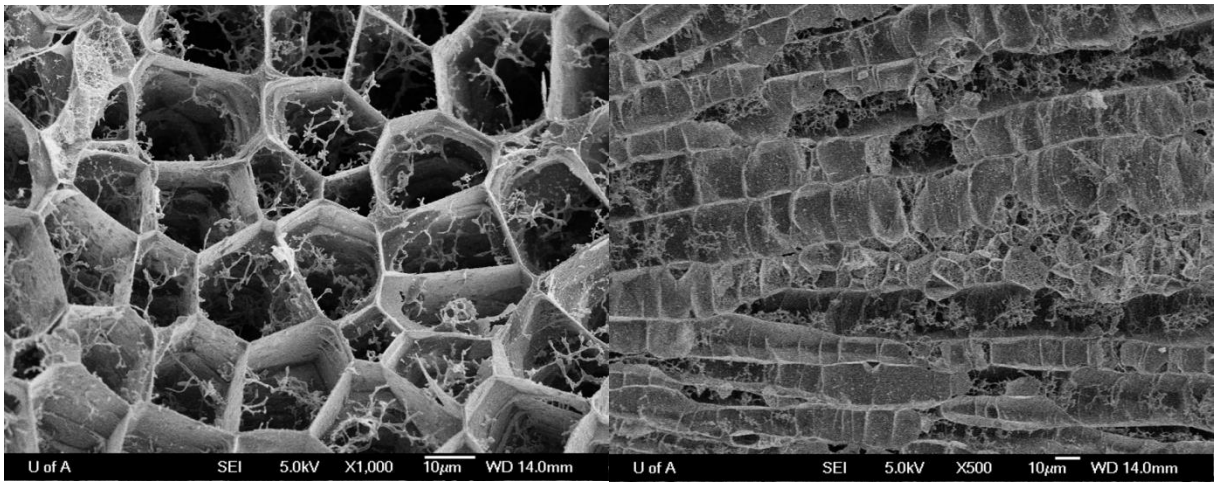


Figure 2.4. Scanning electron microscope picture provided by Dr. Michael T. Nickerson showing the internal structure of pea isolate-alginate capsules.

Various probiotic species have been successfully protected *in vitro* through alginate encapsulation including, *L. acidophilus, L. rhamnosus, L. plantarum, L. casei, B. adolescentis, B. animalis, B. bifidum, B. infantis , B. lactis* and *B. longum* (Rokka and Rantamaki, 2010). Bacteria *B. adolenscentis* 15703T trapped in the alginate-gelatin matrix were challenged with simulated gastric juice (pH = 2.0) and simulated intestinal juice (pH = 7.4). The results of the study showed significant improvement of the viability of *B. adolenscentis* 15703T encapsulated with alginate-gelatin and exposed to gastric conditions. The *in vitro* study also successfully demonstrated controlled-release of the encapsulated bacteria under intestinal conditions (Annan et al., 2008). The addition of a protein source, such as pea or whey protein, has also been found to provide added protection in combination with alginate (Klemmer et al., 2011a).

The percentage of alginate used in encapsulation can affect the capsule size and protection significantly. The percentage of alginate used for encapsulation ranges from 0.5 - 4 % (Lee and Heo, 2000; Gul and Dervisoglu, 2017). Generally, increased alginate concentration results in greater capsule size and improved survival of core bacteria. Mandal (2006)

encapsulated *L. casei* in different alginate concentrations (2, 3, and 4 %) by emulsion in soybean oil. The survival of *L. casei* increased according to alginate concentration during challenge in gastric acid (pH 1.5) for 3 h. Encapsulation reduced the loss of viability of *L. casei* compared to free cells by 1-2 log cfu/mL increasing in a dose-dependent manner with the concentration of alginate used. After heat challenge at 55, 60 and 65 °C for 20 min, a similar trend was found with high alginate concentrations associated with improved survival of *L. casei* at 60 and 65 °C. Furthermore, the increased alginate concentration did not affect the release in simulated colonic conditions (Mandal et al., 2006).

Similarly, Lee and Heo (2000) trapped *B. longum* with different alginate concentrations (2, 3, and 4 %) by extrusion. The survival of *B. longum* was improved in gastric juice challenge (pH = 1.55) with the increased alginate concentration. Loss of viability in simulated gastric juice when capsules were prepared with 4 % alginate was only 0.5 log cfu/bead, whereas there was no protection against loss of viability for free bacteria or bacteria encapsulated with 2 % alginate. Interestingly, the capsule size more than doubled from 1.03 mm to 2.62 mm as alginate concentration increased from 2 to 4 %. The authors indicated that very large capsules would cause coarseness of texture whereas small capsules did not provide sufficient protection suggesting that control of capsule size would be important particularly in human applications (Lee and Heo, 2000). Osman Gul (2017) tested the effect of different alginate concentrations on capsule size, survival of core bacteria during heat challenge, simulated gastric juice and intestinal juice on encapsulated *L. casei*. The best alginate concentration was 2 % and 2.5 % for extrusion and emulsification (Gul and Dervisoglu, 2017).

2.2.2.2 Pea protein isolates

Pea (*Pisum Sativum*) proteins are important components in the human food and animal feed industries due to their physicochemical properties in food preparation as well as their nutritional value (Spielmann et al., 2008; Jezierny et al., 2010). Peas have a high protein concentration (246 g/kg DM) and are also rich in starch (478 – 534 g/kg DM) with crude fat ranging from (15 – 20 g/kg DM) (Jezierny et al., 2010). Pea protein isolate is the protein fraction concentration, which demonstrates good solubility in water, stability in heat, emulsification and foaming capacity (Donsì et al., 2010). Pea protein is an abundant material in Canada and especially in Saskatchewan, which represented 64 % of Canadian pea production in 2014 (Anonymous, 2017b). Development of higher value products using pea proteins is likely to benefit product demand and provide opportunity for further processing. There are already published non-food/feed applications including film formation (Han and Zhang, 2008; Kowalczyk and Baraniak, 2011), stabilization of oil-in-water emulsions (Bogracheva et al., 1994; Franco et al., 2000; Gharsallaoui et al., 2010), and use in producing microspheres. Furthermore, pea proteins have been used for encapsulation of β-carotene (De Graaf et al., 2001) ascorbic acid and α-tocopherol (Pierucci et al., 2006; Pierucci et al., 2007; Pereira et al., 2009).

Recently, pea protein isolate with alginate capsules (PPC) has shown promising abilities in protecting probiotic bacteria by encapsulation. Kotikalapudi (2009) encapsulated *L. acidophilus* (capsule size 3 mm) in a pea protein isolate (4 %) and alginate (0.5 %) system by extrusion and noted only 1 log cfu/mL loss in simulated gastric juice (pH = 2) when compared to free cells (loss 6 log cfu/mL). Similarly, Wood (2010) entrapped *B. adolescentis* in a 4.0 % pea protein isolate and 1.0 % alginate system (capsule size 2.2 mm) and observed improved survival during gastric juice challenge, whereas alginate alone did not provide any protection. The author suggested that pea protein strengthened the capsule and reduced porosity based on scanning

electron microscopy. Furthermore, Klemmer (2011) encapsulated *B. adolescentis* in pea protein isolate (2 %, 4 %, 6 %) and alginate (0.5 %) with a prebiotic (fructooligosaccharide) and compared various extrusion conditions. Encapsulated *B. adolescentis* survived in a simulated gastric juice (pH = 2) challenge (1 log cfu/mL reduction) compared to a more than 6 log cfu/mL reduction in free cells. Increasing needle (nozzle) size increased the capsule size. However, increase protein and prebiotic concentrations showed similar protection. Finally, capsules have been shown to break down and release probiotic payloads in simulated intestinal juice containing proteases, proposed to hydrolyze capsule protein and high levels of Na^{+} proposed to displace Ca^{++} crosslinks between alginate (Klemmer, 2011a). The efficacy of pea protein and alginate-based capsules have also been examined during *in vivo* passage through the digestive tract as described in detail in the section below.

2.2.3 Application of probiotic encapsulation

2.2.3.1 Effects of encapsulation on probiotic viability following dehydration

Probiotic organisms are commonly dehydrated to minimize environmental conditions required for storage and to extend the shelf-life. Drying without any protection is lethal to many sensitive probiotic strains and protective additives are often used to maintain probiotic viability. Protective additives include skim milk, sugars, glycerol and proteins (Meng et al., 2008). There is limited work investigating the effect encapsulation on microbial viability during dehydration. However, some positive impacts have been demonstrated. Heidebach et al. (2010) encapsulated two strains of probiotic bacteria (*Lactobacillus* F19 and *Bifidobacterium* Bb12) in casein based capsules and examined viability after freeze-drying (at -20 °C). Significant protection against loss of viability was observed in the case of the *Lactobacillus* strain (40 % survive compared to less than 10 %) but not the *Bifidobacterium* strain when compared with non-encapsulated

bacteria (Heidebach et al., 2010). Several studies have investigated on the viability of probiotic organisms after drying. However, comparisons with non-encapsulated controls were not included. Abd-Talib et al. (2013) examined the viability of two strains of *L. plantarum* (B13 and B18) and two species of yeast (*Kluyveromyces lactis* and *Saccharomyces blouradii*) after encapsulation using a combination of gum arabic, gelatin, lecithin and coconut oil prior to spray drying (outlet temperature 70 to 75 °C). Loss of viability differed dramatically between the two bacterial strains; viability of *L. plantarum* B13 was reduced from 1.28 x 10^8 cfu/mL to 2.1 x 10^6 cfu/mL; whereas, *L. plantarum* B18 viability was reduced from 3.25 x10^7 to 2.15 x 10^7 cfu/mL. Loss of viability for the two encapsulated yeast species was similar at approximately 2 log cfu/mL reduction (Abd-Talib et al., 2013). In another study, whey-encapsulated *L. reuteri* was spray dried at 55 and 65 °C, and viability decreased from 1.6 x 10^9 to 1.7 x 10^7 cfu/g (Jantzen et al., 2013) whereas for *Bifidobacterium* PL encapsulated with starch the reduction of viability was only 1 log cfu/mL after spray drying with an outlet temperature at 45 °C (O'riordan et al., 2001).

The effects of encapsulation and cryoprotectant during freeze-drying for probiotic bacteria are similar. A survival rate of 11-15 % after freeze-drying was reported for *P. acidilactici* encapsulated in alginate and alginate-chitosan coated beads. The best survival rate was achieved when the encapsulation formulation included 10 % skim milk (15.79 %), but this was only marginally higher than non-encapsulated cells mixed with 10 % skim milk (11.02 %). Others studies had reported less than 1 % survival of bacterial probiotics (*Pediococcus acidilactici*) following freeze-drying when capsules were made with alginate alone or alginate-chitosan with or without milk addition (Halim et al., 2017).

The effect of encapsulation on probiotics viability during dehydration has largely been carried out using a number of different encapsulation formulations, using different probiotic

strains and with different control treatments. For this reason, it is difficult to draw firm conclusions regarding recommendation for encapsulation formulations to provide consistent protection during dehydration. This trend of variation in wall materials and concentration as well as strain use also applies with respect to studies examining the efficacy of protection against the challenges associated with temperature, storage and *in vivo* animal delivery. A systematic comparison of different formulations across a variety of probiotic strains should be conducted.

2.2.3.2 Effects of encapsulation on probiotic sensitivity to heat challenge

During feed/food processing, probiotic products are subject to several heat challenges as described above in section 2.1.1. Heat challenge is a major stress factor for probiotic organisms with viability markedly decreasing as the temperature and time of exposure increases. However, several studies have shown significant improvement in heat tolerance by encapsulation of probiotic organisms (Teoh et al., 2011; Abbaszadeh et al., 2014; Moumita et al., 2017). Two categories of the heat challenge test that are commonly performed include determination of viability after exposure to temperatures of less than 70 °C for extended periods of time (10 to 60 min.) or after exposure to temperatures above 70 °C for short periods (less than 1 min.). Teoh et al. (2011) tested viability of *L. acidophilus* and *B. pseudocatenulatum* after encapsulation in chitosan coated alginate-starch at 55, 60 and 65 °C over 30 min without dehydration. The results showed that encapsulated cells had significantly improved survival when compared to the free cell at 55 and 60 °C. After challenge at 60 °C for 30 min, the reduction of viability for free cells was 5.00 log cfu/mL, whereas a reduction of 1.99 log cfu/mL for *L. acidophilus* and 0.85 log cfu/mL for *B. pseudocatenulatum* was observed after encapsulation. However, when heat challenge was conducted at 65 °C for 30 min, only encapsulated *B. pseudocatenulatum* was significantly protected by encapsulation (Teoh et al., 2011). In a similar type of study, Sabikhi et

al. (2010) examined the protective effect of encapsulation on probiotic viability after high temperature short duration heat challenge. The authors encapsulated *L. acidophilus* in an alginate - starch matrix and examined viability after the heat challenge for 72, 85 and 90 °C for 30 s without prior dehydration. Encapsulated cells showed significantly improved survival when compared to non-encapsulated cells at all temperatures investigated. Loss of viability increased with increased temperature, however, even with the severest challenge (90 °C for 30 s), the reduction in viability for the encapsulated cells was 4.14 log cfu/mL compared to 9.13 log cfu/mL for non-encapsulated cells (Sabikhi et al., 2010). The effect of encapsulation heat protection is clearly strain dependent. Borges (2012) tested alginate based encapsulation of *L. casei, L. paracasei, L. acidophilus* and *B. animalis* during heat challenge at 55 and 60 °C for 60 min without prior dehydration. Only encapsulated *L. acidophilus* showed an improved protection at 55 °C when compared to free cells.

Limited research had been done to examine the efficacy of dehydrated encapsulation products during heat challenge even though dried products are the preferred product form in the feed industry. Nevertheless, some studies have demonstrated protection against heat challenge of encapsulated and dehydrated products. For instance, spray dried *Bifidobacterium* BB-12 encapsulated with skim milk and prebiotic oligosaccharides was challenged with heat at 55, 65 and 75 °C for 1 and 10 min. There was a significant protection from encapsulation at 55 °C for 10 min although only by about 0.5 log cfu/g. Under challenge at 65 °C for 10 min, there was no detection of viable free cells whereas the viability of encapsulated cells remained high (9 log cfu/g). Encapsulation did not however, protect against challenge at 75 °C (Fritzen-Freire et al., 2013). In another case, freeze-dried alginate-based capsules containing *L. plantarum* were challenged at 75 °C for 30 s and 90 °C for 5 s. The reduction in viability of free cells was about

3.22 log cfu/g whereas a reduction of 1.15 log cfu/g was observed for the encapsulated cells. Similar protection was also noticed at 90 °C for 5 s for encapsulated *L. plantarum* when compared to free cells (Fareez et al., 2017).

Although the efficacy of encapsulation on the heat challenge depends on the encapsulation formula, strain characteristics, challenge temperature and duration, significant protection has been noticed for encapsulation during both low-temperature long duration and high-temperature short duration challenge conditions. However, the heat challenge data on dehydrated products are limited. In the aspect of feed processing, the efficacy of encapsulation during pelleting (temperature can be as high as 90 °C and less than 2 min) is unknown.

2.2.3.3 Effects of encapsulation on probiotic viability during storage

Viability of probiotic organisms during storage varies considerably due to a number of factors including strain differences, water residuals, encapsulation material and methods, storage temperature, control group selection and whether dehydration was employed. Temperature is a primary factor affecting viability during storage. A wide range of temperatures has been investigated reflecting storage conditions for relatively small volumes of product prior incorporation in feed and environmental temperatures commonly experienced during storage of prepared feeds.

For sensitive bacterial strains storage viability represents a significant challenge. Unfortunately, research has shown that encapsulation is ineffective when storage occurs at ambient temperatures (19 to 24 °C) or higher. Weinbreck et al. (2010) tested the viability of *L. rhamnosus* coated with whey protein with or without an extra layer of palm oil during storage at 37 °C for 2 weeks. The results indicated protection was not improved with encapsulation when compared to free cells (Weinbreck et al., 2010). Similarly, O'Riordan et al. (2001) tested

Bifidobacterium PL1 coated with starch by spray drying and stored at ambient temperature (19-24 °C) for 20 d. The viability of the probiotic bacteria decreased markedly from 10 log cfu/g to less than 5 log cfu/g and was not improved by encapsulation (O'riordan et al., 2001). Similar studies have confirmed inefficient protection of encapsulated probiotics during storage at ambient temperature (O'riordan et al., 2001; Martin et al., 2013; Tomás et al., 2015; Xu et al., 2016). However, some exceptional successful cases have been reported. For example, Sánchez et al. (2017) reported viability of a *Lactobacillus* bacteria (detailed information on species and strain was not reported) encapsulated in sodium alginate and calcium carbonate by an emulsification - internal gelation procedure, compared to non-encapsulated bacteria following storage at - 20, 4, 25 °C. Authors reported 100 % of survival of encapsulated bacteria after 150 d of storage independent of storage temperature (Sánchez et al., 2017). Another study of encapsulated *Shewanella putrefaciens* observed a 40 % viability loss after 30 d storage at 22 °C after alginate coating (Rosas-Ledesma et al., 2012).

Several studies have shown a significant protection of encapsulated probiotic bacteria stored under refrigerated conditions. Varankovich et al. (2017) encapsulated *L. rhamnosus* and *L. helveticus* with pea protein-alginate with or without extra chitosan coating. After freeze-drying, capsules were either vacuum packaged or not and stored at 4 and 22 °C for 9 weeks. The overall viability of encapsulated probiotics stored at 4 °C was better than at 22 °C (Varankovich et al., 2017). Rosas-Ledesma (2012) found that alginate coated *S. putrefaciens* maintained its viability over 90 % when stored for one mo at 4 °C. A similar trend was observed *when L. acidophilus* was encapsulated with mucilage and flaxseed protein by spray drying and followed by stored at 4 °C for 45 d (Bustamante et al., 2015). The maximum viability was found when stored at -

20 °C of *L. casei* encapsulated by skim milk, trehalose and maltodextrin compared to other temperature tested (4, 25, 37 °C) (Liao et al., 2017).

In summary, encapsulation can provide protection against loss of viability when storage temperature is below ambient temperature. Few studies have reported any advantage of encapsulation on the viability of probiotic during storage above ambient temperature.

2.2.3.3 Effects of encapsulation on probiotic survival during gastrointestinal delivery in vitro and in vivo

The delivery of viable encapsulated probiotic organisms to the distal digestive tract represents the final challenge in probiotic delivery. Research on the efficacy of encapsulation of probiotic organisms during gastrointestinal tract transit is mostly conducted using *in vitro* testing. Several studies have noted an improved protection during simulated gastric acid challenge (pH as low as 1.2) as well as simulated intestinal juice challenge (Zhang et al., 2013). These studies demonstrated that the addition of wall material and the matrix formed by cross-linking provided additional protection during *in vitro* gastrointestinal challenge. Alginate-based capsules such as alginate-starch (Sultana et al., 2000), alginate-chitosan (Vandenberg et al., 2001), pea protein isolate-alginate (Klemmer et al., 2011b), alginate-skim milk (Shi et al., 2013) and alginate-prebiotics (Iyer and Kailasapathy, 2005) have all been demonstrated to protect viability in simulated upper digestive tract challenges when compared to free cells. Furthermore, in one study, the encapsulated core bacteria were released in the intestinal juice presumably in response to the action of digestive enzymes included in the simulation cocktail (Klemmer et al., 2011b).

The study of encapsulated probiotic organisms during transit in the animal gastrointestinal tract is very limited. These limitations are mainly due to the complex gastrointestinal tract environment and lack of tracking methods for the target encapsulated bacteria. Traditional selective culture methods are typically not strain specific such that

differentiation of the encapsulated probiotic and commensal bacteria resident in the digestive tract is difficult. Whereas molecular methods mainly based on quantitative real-time PCR (Polymerase chain reaction) can improve selectivity, traditional DNA-based PCR techniques do not differentiate live and dead cells. New molecular methods to differentiate live and dead cells, for example, based on cell wall permeability to ethidium monoazide, are being developed, however, their accuracy of these methods are poor (Flekna et al., 2007).

Nevertheless, Rodklongtan et al. (2014) used PCR and RAPD (Random Amplified Polymorphic DNA) to detect the appearance of encapsulated and non-encapsulated *L. reuteri* in excreta of 3 broiler birds. The authors used the appearance in excreta of an indigestible marker incorporated in feed with the probiotic to confirm feed passage. The probiotic was detected in excreta by PCR only when fed in encapsulated form indicating protection of at least probiotic DNA (Rodklongtan et al., 2014). Varankovich et al. (2015) encapsulated *B. adolescentis* in a pea protein isolate-alginate capsules and supplemented the diet to rats. By using qPCR, the authors detected specific *B. adolescentis* DNA in fecal material suggesting probiotic protection during intestinal tract transit. The authors could not establish whether the encapsulated bacterium was viable, and unfortunately, they did not include a non-encapsulated control group making interpretation regarding the efficacy of the encapsulation method impossible. Interestingly in another study, a fluorescence protein-labeling method was applied by Rosas-Ledesma et al. (2012) to test the efficacy of delivery of an alginate coated *Shewanella putrefaciens* on delivery to fish. The feeding trail results showed that encapsulated bacteria could be recovered in excreta of Senegalese sole whereas no recovery was evident in the non-encapsulated probiotic control group.

Two other studies have investigated *in vivo* host responses to encapsulated probiotic are very noteworthy. Pirarat et al. (2015) encapsulated *L. rhamnosus* GG (LGG) with skim milk and alginate by extrusion, followed by freeze-drying. The encapsulated probiotics were fed to Nile tilapia for 30 d with free cells and a non-probiotic group as controls. When encapsulated probiotic was added to feed, fish weight gain improved at 14 and 30 d when compared to both the control group and the non-encapsulated probiotic group. Similarly, encapsulated LGG significantly improved intraepithelial lymphocyte number and protected against challenge with *S. agalactiae*, whereas the non-encapsulated control was not effective (Pirarat et al., 2015). In contrast, Iyer et al. (2013) measured *in vivo* bacteria release pattern using qPCR as well as cytokine expression in mice fed chitosan-coated alginate-starch capsules containing *L. casei*. Encapsulated bacteria were not released in gastric and duodenal contents for 24 h. However, a cytokine expression response was observed in mice fed either the encapsulated or non-encapsulated probiotic compared to the untreated control (Iyer et al., 2013).

In summary, different encapsulation formula provided broad protection for core probiotic strains during *in vitro* gastric challenge when compared to non-encapsulated bacteria. Due to the lack of tracking method for *in vivo* delivery, studies examining efficacy of encapsulation in the animal gastrointestinal tract are limited. However, in the limited studies conducted, encapsulation showed a potential to improve probiotic delivery to the distal intestine. .

2.3 Overall summary

Several probiotic bacterial strains provided significant benefits to animal health and growth performance. However, there are limited strains that can be applied in the commercial feed market for direct application due to the lack of stability during probiotic manufacturing, feed processing and upper gastrointestinal tract challenges. The commercial strains are mainly

spore-forming bacillus, yeast and hardy lactic acid bacteria. In order to broaden the utilization of probiotic strains, additional protections are required. Encapsulation, using one of several available techniques, a variety of wall materials and a variety of formulations, is one solution that has been investigated. Encapsulation has been shown to protect a number of sensitive bacterial strains against dehydration, thermal and chemical stresses experienced during processing, storage and delivery to the intestinal tract. Therefore, this technology provides an approach to broaden the selection of commercial bacterial strains to include those with a health benefit but sensitivity to stressors associated with manufacture, storage and passage through the upper gastrointestinal tract. Alternatively, the technology may simplify the commercial adoption of current *Lactobacillus* probiotic strains that cannot be directly incorporated in to feed.

2.4 Hypothesis and Objectives

Hypothesis:

The encapsulation of probiotic bacteria in a pea protein isolate-alginate matrix will improve the viability of the probiotic during freeze-drying, storage, feed processing and transit through the gastrointestinal tract of animals.

Objectives:

- Develop and scale pea protein-based encapsulation of probiotic bacteria to permit study of encapsulation efficacy on inclusion in feed.
- Determine the efficacy of pea protein-based capsules to protect probiotic bacteria from loss of viability during freeze-drying and storage of dried products.
- Determine the efficacy of pea protein-based capsules to protect probiotic bacteria from loss of viability during challenges associated with feed processing.
- Determine the efficacy of pea protein capsules to enhance viability and colonization of probiotic bacteria in the distal gastrointestinal tract when supplemented in feed.

3 EFFICACY OF PEA PROTEIN ISOLATE-ALGINATE ENCAPSULATION ON VIABILITY OF PROBIOTIC BACTERIUM DURING FREEZE-DRYING AND STORAGE

3.1 Abstract

Several experiments were conducted to determine whether pea protein isolate (PPI) - alginate (AL) capsules (PPC) could improve probiotic viability during drying and storage. Early stationary phase *Bifidobacterium adolescentis* (BA) or *Lactobacillus reuteri* (LR) cultures were suspended in one volume of 10 % skim milk (BA-M and LR-M) or encapsulated in 4.0 % PPI and 0.5 % AL followed by extrusion and crosslinking (BA-PPC and LR-PPC). To examine the additive or synergistic effect of other preservatives, BA capsules were prepared without preservatives (BA-PPC), with 0.25 % skim milk and 0.075 % glucose (BA-PPC-M), 1.5 % glycerol (BA-PPC-G) or 0.02 % cysteine-HCl (BA-PPC-H). Loss of viability during freeze-drying was significantly ($P < 0.0001$) reduced in BA-PPC (1.22 ± 0.02 log cfu/g) compared to non-encapsulated BA without preservatives (5.32 ± 0.13 log cfu/g). However, the addition of milk plus glucose or glycerol to non-encapsulated BA provided similar protection (BA-M: 0.75 ± 0.12 log cfu/g; BA-G: 1.05 ± 0.06 log cfu/g) compared to BA-PPC. Moreover, encapsulation with milk and glucose (BA-PPC-M) further protected ($P < 0.001$) against loss of viability (0.62 ± 0.33 log cfu/g) compared with BA-PPC. The addition of cysteine-HCl did not reduce the loss of viability in non-encapsulated bacteria (5.75 ± 0.09 log cfu/g) or encapsulated bacteria (1.32 ± 0.13 log cfu/g). After storage at -80 °C for 335days, the reduction in viability of BA-M was 5.36 log cfu/g and markedly greater ($P < 0.01$) than observed for BA-PPC (1.99 log cfu/g). The addition of preservatives to BA-PPC or vacuum packaging did not affect the loss of viability. After 14 d storage at room temperature, BA-PPC prepared with any of the added preservatives protected ($P < 0.001$) against loss of viability compared with BA-M. Vacuum packaging

marginally reduced ($P < 0.05$) loss of viability during storage at room temperature for 14 d (2.21 ± 0.15 vs. 2.02 ± 0.23 log cfu/g). *L. reuteri* was more resistant to loss of viability during storage compared to *B. adolescentis*. Loss of viability was less than 0.2 log cfu/g and not different between LR-M and LR-PPC after storage for 84 d at -80, -20 or 4 °C. When stored after 23 d at 37 °C, the viability of both *L. reuteri* decreased rapidly without a difference between LR-M and LR-PPC. Following challenge in simulated gastric juice (SGJ), the loss of viability of *B. adolescentis* was greater ($P < 0.01$) for BA-M compared to BA-PPC. Milk plus glucose or glycerol as preservatives in addition to encapsulation further increased viability during SGJ challenge ($P < 0.05$) when compared to BA-PPC. Encapsulation showed strain and temperature dependency in protecting bacteria during storage. More protection was found when *B. adolescentis* was encapsulated and stored at -80 °C whereas encapsulation did not protect against loss of viability of a more resistant bacteria, *L. reuteri*, during storage at 37 °C. Milk plus glucose or glycerol provided some additional protection against loss of viability of *B. adolescentis* during freeze-drying and SGJ challenge when used in combination with encapsulation.

3.2 Introduction

The gastrointestinal (GI) tract of mammals harbours a variety of microbes whose numbers can reach up to 10^{12} cfu/mL in the colon (Castillo et al., 2006). The intestinal microbiota has been demonstrated to have significant effects on animal health including the development of immunity, exclusion of pathogens and contribution to numerous metabolic diseases (Duc et al., 2004; Cutting, 2011; Knap et al., 2011). Manipulating the gut microbiota by supplementing probiotics, has shown promising health benefits in humans (Rolfe, 2000; Sanders, 2000). Similarly in the livestock industry, a number of researchers have reported benefits to

probiotic supplementation including increased growth performance (Estrada et al., 2001), improved efficiency of feed utilization (Altaher et al., 2015) and reduced occurrence of diarrhoea (Pieper et al. 2010; Scharek-Tedin et al., 2013) although benefits are not consistently observed (Szabo et al. 2009).

Commercial probiotic products are largely limited to a few "hardy" strains that are naturally resistant to environmental challenges presented during processing and storage. To date, the commercial probiotic supplements used are mainly spore-forming *Bacillus* spp., yeast (*S. cerevisiae*) and *E. faecium* (Anadón et al., 2006). Other products containing *Lactobacillus* or *Bifidobacterium* spp. are primarily stored at 4 °C and, in the case of food animals, applied by mixing with feed, just prior to feeding, due to their short shelf-life (Kalavathy et al., 2003; Mountzouris et al., 2007; Jung et al., 2008). The removal of water (drying), exposure to oxygen, humidity and variations in temperature are all common environmental challenges during processing and storage that can be lethal to many sensitive bacteria limiting their probiotic application (Goderska, 2012). To improve the viability of sensitive bacteria during drying and storage, a number of methods have been investigated including gene mutation, encapsulation or adding preservative compounds with varying degrees of success (Lee et al., 2004; Corbo et al., 2011; Sousa et al., 2012).

Encapsulation entraps probiotics into a coating matrix to provide additional protection against environmental challenges. Encapsulation improved protection of probiotic viability in several studies conducted in liquid-based conditions such as gastric juice (Guérin et al., 2003; Klemmer et al., 2011a), fruit juice (Ding and Shah, 2008) and dairy products (Iyer and Kailasapathy, 2005) typical of human food applications. Encapsulation has also improved viability of bacteria during freeze-drying (Giulio et al., 2005; Tsen et al., 2007; Heidebach et al.,

2010). However, a limited number of studies have investigated the impact of encapsulation on dry-based products and their conclusions are inconsistent. For example, improved protection was observed in protein-carbohydrate-oil matrix coated *B. infantis* when stored at 25 ºC for 5 weeks (Crittenden et al., 2006) as well as a coated *L. acidophilus* stored at 25 and 37 ºC for 20 d (Ann et al., 2007). In contrast, encapsulation did not improve the viability of *L. rhamnosus* when coated with a whey protein, with or without the extra layer of palm oil, and stored at 37 °C for 4 weeks (Weinbreck et al., 2010). Bacteria viability was also not improved for a starch-encapsulated *Bifidobacterium* spp. stored at 19-24 °C for 20 d (O'riordan et al., 2001).

Although encapsulation materials used in the above studies are considered good barriers, a more adequate protection matrix may be needed to ensure consistent protection of probiotics stored as dry products. Alginate is one of the most commonly used probiotic encapsulation materials due to its low cost, non-toxic nature and ability to form a viscous gel after cross-linking (Su et al., 2011; Sathyabama et al., 2014). Numerous studies had shown promising effects of alginate encapsulation in protecting probiotics (Hansen et al., 2002; Annan et al., 2008; Chávarri et al., 2010). However, alginate alone did not show adequate protection (Lee and Heo, 2000). Adding other materials to the encapsulation matrix such as proteins or starch further protected bacteria from environmental challenges (Sultana et al., 2000; Gbassi et al., 2009; Heidebach et al., 2010).

A number of preservatives have been examined to aid in maintaining probiotic viability during processing with and without encapsulation. For example, the addition of cryoprotectants to the encapsulation was shown to enhance protection of bacteria during freeze-drying (Shi et al., 2013). Previous work has demonstrated that isolated pea protein, a non-gene modified plant-based protein, is effective in preserving viability of bacteria on exposure to simulated gastric

juice (Klemmer et al., 2011a; Varankovich et al., 2015) and in improving delivery of viable probiotic bacteria to the distal digestive tract (Wang et al., 2016). The effects of alginate based encapsulation with the addition of pea protein isolate on bacteria viability during storage is largely unknown. Therefore, the objective of the present study was to determine the effect of a pea protein isolate-alginate encapsulation method, with or without added preservatives, on the viability of two model probiotic bacteria (*B. adolescentis* and *L. reuteri*) under different environmental conditions.

3.3 Materials and Methods

3.3.1 Bacterial culture

A single colony of *Bifidobacterium adolescentis* ATCC 15703 (BA) and *Lactobacillus reuteri* ATCC 53608 (LR) [American Type Culture Collection (ATCC), Manassas, VA, USA] were inoculated into 100 mL reinforced clostridial media (RCM, Oxoid Ltd., Basingstroke, England) or de Man, Rogosa and Sharpe (MRS, Oxoid Ltd., Basingstroke, England) media, respectively. The bacteria cultures were incubated for 24 h under anaerobic (80 % N_2, 10 % CO_2 and 10 % H_2) conditions at 37 °C. This 24 h culture was then inoculated into fresh RCM broth media at a 1:1000 ratio and cultured for an additional 20 h (BA) or 14 h (LR) under anaerobic conditions at 37 °C to early stationary phase. *B. adolescentis* and *L. reuteri* were then centrifuged (4200 x g, 10 min, 4 °C) and the bacterial pellet was resuspended in peptone water.

3.3.2 Preparation of pea protein-alginate capsules

Pea protein-alginate capsules (PPC) were prepared using the adapted extrusion method described by Wood (2010). Briefly, pea protein isolate (PPI) (Nutri-Pea Limited, 80% protein, 1.81% lipid, 10.78% cabohydrate, Portage-la-Prairie, MB, Canada) was dissolved into ddH_2O (containing 4 % pea protein; pH adjusted to 8.0 with 1 M NaOH) and mechanically stirred for 30

min in a water bath at 80 ºC. The PPI solution was then cooled to room temperature and adjusted to pH 7.0 with 1 M HCl. Alginate (Sigma-Aldrich, Oakville, ON, CA) was added at 0.5 % (w/w) and the mixture heated to 80 ºC with mechanical stirring for another 45 min until the alginate was completely dissolved. The resulting pea protein and alginate solution (PAS) was cooled to room temperature before BA and LR were added at a ratio of 1 part washed bacteria to 18 parts (w/w) PAS with continuous stirring. The PAS was then extruded (**Figure 3.1)** through a 20 G needle under air pressure and dropped into a cross-linking solution (5 % $CaCl_2$ and 1 % Tween 20). After 30 min hardening time, the capsules were filtered over filter paper (Particle retention> 20µm, Fisherbrand®, EU). Capsules were washed three times with one volume of peptone water (bacteria peptone 1.0 g, NaCl 5 g, dd H_2O adjusted to 1 L) and collected onto aluminium trays prior to freeze-drying.

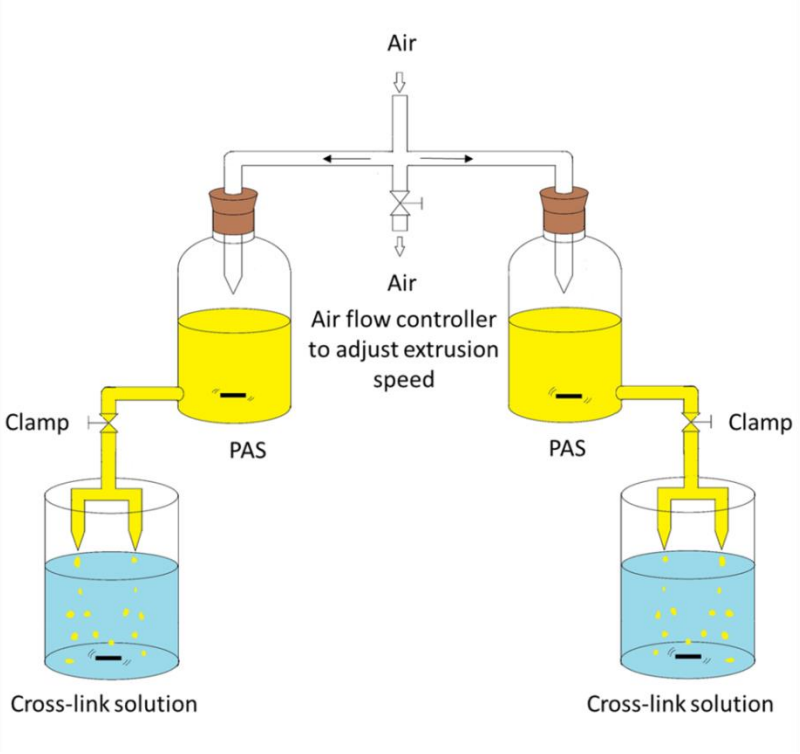


Figure 3.1. Schematic diagram of extrusion system for laboratory-scale production of capsules using a pea protein isolate-alginate solution (PAS) containing core bacteria.

3.3.3 Evaluation of preservatives on viability of *B. adolescentis*

Several preservatives were added to *B. adolescentis* cell preparations prior to freeze-drying to assess their effect on bacteria viability. Non-encapsulated bacteria cells were resuspended in one volume (v/v) of peptone water with of one of four preservatives including, no preservative (BA); 10 % (w/w) skim milk powder and 0.5 volumes of a 10 % (w/w) glucose solution (BA-M); 0.5 % (w/w) cysteine-HCl (BA-H); and 0.3 volumes of glycerol (BA-G). Cell suspensions were freeze dried directly or used to prepare PPC containing no preservative (BA-PPC), containing milk and glucose (BA-PPC-M), containing cysteine-HCl (BA-PPC-H) or containing glycerol (BA-PPC-G).

3.3.4 Freeze-drying

Non-encapsulated bacteria cell suspensions were transferred to Petri dishes to a fluid depth of approximately 5 mm and freeze dried for 48 h (vacuum collector -50 ºC, 0.140 mBar, shelf temperature -20 ºC). Encapsulated bacteria were distributed in Petri dishes to a depth of 8 mm and freeze dried under the same conditions as non-encapsulated cell suspensions. Following freeze-drying, dry matter (DM) and moisture content were determined by oven drying at 55 ºC to a constant weight. Final moisture content was confirmed as below 7 % w/w. The diameter of freeze-dried PPC was (1823.12 ± 570.22 μm) as measured using a laser scattering particle size distribution analyzer (Horiban Instruments Inc., Irvine, CA) in triplicate. The mean diameter of PPC was not affected by the addition of preservatives.

3.3.5 Effect of encapsulation and preservatives on viability of *B. adolescentis* during freeze-drying

To determine the effect of encapsulation and preservatives on bacteria viability during freeze-drying, samples of both non-encapsulated and encapsulated BA were collected before and after freeze-drying and cultured for enumeration. All suspended samples were homogenized three times for 10 s at speed 6 (Omni Macro Homogenizer, Omni International Inc., Marietta, GA) on ice to ensure disruption of capsules. The homogenized samples were further diluted in peptone water, plated on RCM and cultured at 37 °C under anaerobic conditions (BD, Gaspakim. Anaerobe container system, USA) for 48 h. Colonies were enumerated after culture, and viability reported as the difference (log reduction) in log cfu/g dry matter before and after freeze-drying.

3.3.6 Effect of encapsulation, vacuum packaging and preservative addition on viability of *B. adolescentis* during storage

Five formulations containing BA, evaluated for viability after freeze-drying, were also evaluated for viability during storage under different conditions. The five formulations included BA-M, BA-PPC, BA-PPC-M, BA-PPC-H and BA-PPC-G. After freeze-drying formulations were placed in plastic bags and sealed without vacuum or after vacuum removal of air to compare the effect of vacuum packaging versus no vacuum packaging (FoodSaver Vacuum Packing System, V2040, China). Formulations were stored at -80 °C and at room temperature (21 °C). For formulations stored at room temperature, *B. adolescentis* viability was determined in triplicate samples on storage days 1, 3, 5, 7, 9, 14 and 19. For formulations stored at -80 °C, bacteria viability was determined in triplicate samples on storage days 1, 14, 28, 44, 90 and 335.

3.3.7 Effect of encapsulation on viability of *L. reuteri* during storage

A similar comparison was conducted for non-encapsulated *L. reuteri* with milk preservatives (LR-M) and encapsulated but unpreserved *L. reuteri* (LR-PPC). Stored aliquots of

LR-M and LR-PPC were stored at a range of different temperatures. LR-M was diluted with corn starch to 10^9cfu/g. The viability of bacteria stored under different temperatures was determined as follows: on storage days 1, 2, 5, 6, 9, 12, 15 and 23 for formulations stored at 37 °C; on storage days 1, 2, 5, 9, 15, 23, 56 and 84 for formulations stored at 4 °C; and on storage days 6, 56 and 84 for formulations stored at -20 °C and -80 °C.

3.3.8 Effect of encapsulation on viability of *B. adolescentis* in simulated gastric juice

The viability of non-encapsulated freeze-dried *B. adolescentis* with milk as preservative (BA-M) was compared to the viability of *B. adolescentis* in freeze-dried PPC prepared without preservatives (BA-PPC) or with one of the three preservatives described above (BA-PPC-M, BA-PPC-G and BA-PPC-H). The *B. adolescentis* formulations were incubated for 2 h at 37 °C in simulated gastric juice (SGJ: 0.08 M HCl and 0.2 % NaCl (w/v), pH 2.0). The log reduction in viable counts was determined after the 2 h incubation period.

3.3.9 Statistical analysis

A completely randomized design with factorial arrangement was used to assess bacteria viability. The effect of freeze-drying on viability was analyzed by two-way ANOVA using treatment (encapsulation vs. non-encapsulation), preservative (no preservative, milk plus glucose, cysteine-HCl, and glycerol) and their interaction as sources of variation. Bacterial viability during storage was also analyzed as to two-way ANOVA separately for each storage temperature and each experimental day. Treatments (BA-M, BA-PPC, BA-PPC-H, BA-PPC-M and BA-PPC-G) and packaging (vacuum vs. no vacuum) were used as the main effects. A one-way ANOVA was used to analyze the effect of treatment on bacterial viability in SGJ challenge. For all experiments, the log reduction in viability was calculated as the dependent variable with separation of means using Tukey's mean separation procedure. The analysis was conducted using

SAS version 9.4 (Statistical Analysis Software, SAS Institute Inc, 2013, Cary, NC, USA) software with Proc Mixed procedure and probabilities were considered significant at $P < 0.05$.

3.4 Results

3.4.1 Effect of freeze-drying on the viability of non-encapsulated and encapsulated *B. adolescentis*

The log reduction in viability of non-encapsulated and encapsulated *B. adolescentis*, after freeze-drying with different preservatives, is shown in **Table 3.1**. Milk plus glucose and glycerol were equally effective in protecting against loss of viability of non-encapsulated *B. adolescentis* (BA-M and BA-G) limiting the reduction in viability to approximately 1 log cfu/g. In comparison, non-encapsulated *B. adolescentis* viability was reduced by greater than 5 log cfu/g during freeze-drying without added preservatives (BA) or with added cysteine-HCl (BA-H).

Table 3.1. Mean reduction (log10 cfu/g) in the viability of non-encapsulated *B. adolescentis* (BA) and encapsulated *B. adolescentis* (BA-PPC) after freeze-drying without preservative or with milk plus glucose (M), cysteine-HCl (H) or glycerol (G) as preservatives.

	Log reduction (log 10 cfu/g DM)[1]	
	BA[2]	BA-PPC[3]
No preservatives	5.32 ± 0.13*a*	1.22 ± 0.02*bc*
M	0.75 ± 0.12*cd*	0.62 ± 0.33*d*
H	5.75 ± 0.09*a*	1.32 ± 0.13*b*
G	1.05 ± 0.06*bcd*	0.77 ± 0.03*cd*

[1] Values are mean ± SEM (n = 3).
[2] Starting concentration range: 10.02±0.04 to 10.91±0.08 log cfu/g.
[3] Starting concentration range: 8.25±0.04 to 8.57±0.2 log cfu/g.
a-d, An Encapsulation X Preservative interaction was observed; All means with a different letter are different at $P < 0.001$.

Encapsulation alone markedly reduced the loss of viability of *B. adolescentis* (BA-PPC) compared to non-encapsulation without preservatives (BA; $P < 0.001$). Encapsulated *B.*

adolescentis preserved with milk plus glucose (BA-PPC-M) or glycerol (BA-PPC-G) protected bacteria viability to a similar degree as the same treatments applied to non-encapsulated bacteria. However, the addition of milk plus glucose during encapsulation (BA-PPC-M) significantly improved viability compared to encapsulation without any preservatives (BA-PPC; $P < 0.001$). The addition of cysteine-HCl and glycerol to encapsulated bacteria did not significantly alter viability relative to encapsulation without any preservatives. There was also no significant difference in viability between non-encapsulated and encapsulated *B. adolescentis* preserved with milk plus glucose (BA-M and BA-PPC-M, respectively). Glycerol performed similar to milk plus glucose in preserving viability, but the glycerol preservatives formed a viscous gel upon freeze-drying BA, which made sample handling difficult and unsuitable for mechanical processing. Milk plus glucose (BA-M) was therefore selected as the preservative control group in the following storage test.

3.4.2 Shelf-life of *B. adolescentis* storage at -80 °C

There was no significant difference in the viability of *B. adolescentis* stored at -80 °C in vacuum (**Figure 3.2A**) versus non-vacuum packaging (**Figure 3.2B**) throughout the entire storage period. After 10 d of storage at -80 °C, the viability of *B. adolescentis* without encapsulation (BA-M) was reduced significantly ($P < 0.05$) compared to with encapsulation (BA-PPC). This initial rapid decline in viability of non-encapsulated *B. adolescentis* slowed after 10 d, such that the difference in viability between encapsulated and non-encapsulated bacteria remained relatively constant until 91 d. At 335 d of storage, the viability of BA-M was markedly reduced ($P < 0.001$) compared to encapsulated bacteria (BA-PPC, BA-PPC-M, BA-PPC-H and BA-PPC-G). There was no difference in the viability of encapsulated BA when encapsulated with the tested preservatives.

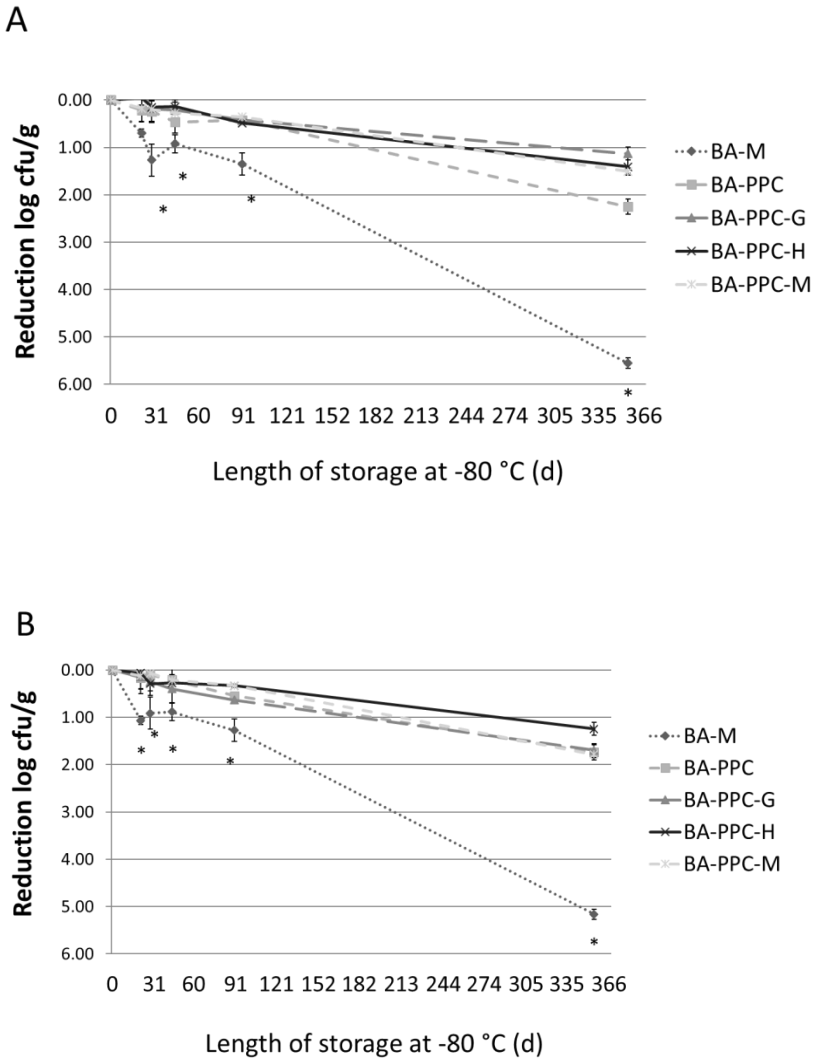


Figure 3.2. Mean (± SEM, n = 3) reduction (log cfu/g) in *B. adolescentis* viability during storage at -80 °C for 335 d. Prior to freeze-drying bacteria were either non-encapsulated with milk plus glucose (BA-M) or encapsulated without preservatives (BA-PPC), with milk plus glucose (BA-PPC-M), with cysteine HCl (BA-PPC-H), or with glycerol (BA-PPC-G) as preservatives. **A**: Vacuum packaged **B**: Non-vacuum packaged. Significant differences are indicated with an asterisk (* $P < 0.001$ between BA-M and BA-PPCs). Starting concentration for BA-M is 12.28±0.65 log cfu/g and for encapsulated PPC group ranged from 7.06±0.22 to 7.30±0.28 log cfu/g.

3.4.3 Shelf-life of *B. adolescentis* at room temperature

Loss of viability of *B. adolescentis* occurred more rapidly at room temperature compared to -80 °C (**Table 3.2.**). *B. adolescentis* viability was reduced by 1.75 to 2.64 log cfu/g at room temperature after 14 d of storage for all groups. Frequent sampling between 3 to 14 d storage indicated that encapsulation with preservatives improved viability compared with non-encapsulated bacteria. Encapsulation with cysteine-HCl, milk plus glucose or glycerol as a preservative significantly reduced the loss of viability at 14 d storage when compared to BA-M and BA-PPC. Vacuum packaging marginally, but significantly, reduced loss of viability at 7, 9 and 14 d of storage. At the day 3, without vacuum packaging, encapsulation significantly protected bacteria viability during the challenge while the preservatives provided no additional advantages. Under vacuum packaging, PPC and PPC-M significantly protected bacteria when compared to non-encapsulation. For non-encapsulated bacteria, vacuum packaging protected against loss of viability. Encapsulation provided additional protection except in the case of capsules prepared with glycerol and cysteine-HCl.

3.4.4 Shelf-life of *L. reuteri* stored at different temperatures

At storage temperatures of 4, -20 and -80 °C, *L. reuteri* survived well, such that the reduction in viability was less than 0.2 log cfu/g after 84 d of storage (data not shown). The viability of *L. reuteri* was unaffected by encapsulation (data not shown). Loss of viability of *L. reuteri* occurred rapidly when stored at 37 ºC, such that viable counts were below the detection limit for enumeration after 23 d. Encapsulation did not slow the rate of loss of viability for bacteria stored at 37 ºC (**Figure 3.3**).

Table 3.2. Mean (n = 3) reduction (log10 cfu/g) in the viability of encapsulated (PPC) and non-encapsulated *B. adolescentis* (BA) after storage at room temperature for 14 d. Treatments included a non-encapsulated control with milk plus glucose preservative (BA-M), a pea-protein-alginate encapsulation without a preservative (BA-PPC) or encapsulation with milk plus glucose (BA-PPC-M), cysteine-HCl (BA-PPC-H) or glycerol (BA-PPC-G) as preservatives. All treatments were tested with and without vacuum packaging.

	Treatment							Packaging				Treatment
	BA-M	BA-PPC	BA-PPC-H	BA-PPC-M	BA-PPC-G	SEM	*P*	Non Vacuum	Vacuum	SEM	*P*	X Packaging
Day 3	0.98*a*	0.05*c*	0.40*bc*	0.09*c*	0.48*b*	0.09	< **0.0001**	0.30*b*	0.50*a*	0.58	**0.0233**	**0.0117**
Day 5	0.97*ab*	0.92*ab*	1.36*a*	0.63*b*	0.75*b*	0.13	**0.006**	0.94	0.92	0.08	0.8599	0.4288
Day 7	1.34*ab*	1.15*b*	1.75*a*	1.16*b*	1.23*b*	0.11	**0.0046**	1.48*a*	1.18*b*	0.07	**0.0068**	0.5968
Day 9	2.26*a*	1.37*c*	1.87*ab*	1.34*c*	1.45*bc*	0.99	< **0.0001**	1.81*a*	1.51*b*	0.06	**0.0037**	0.6031
Day 14	2.64*a*	2.32*ab*	2.04*bc*	1.75*c*	1.83*c*	0.09	< **0.0001**	2.21*a*	2.02*b*	0.05	**0.0208**	0.4044

a-c: Means with a different letter are significant different at $P < 0.05$.

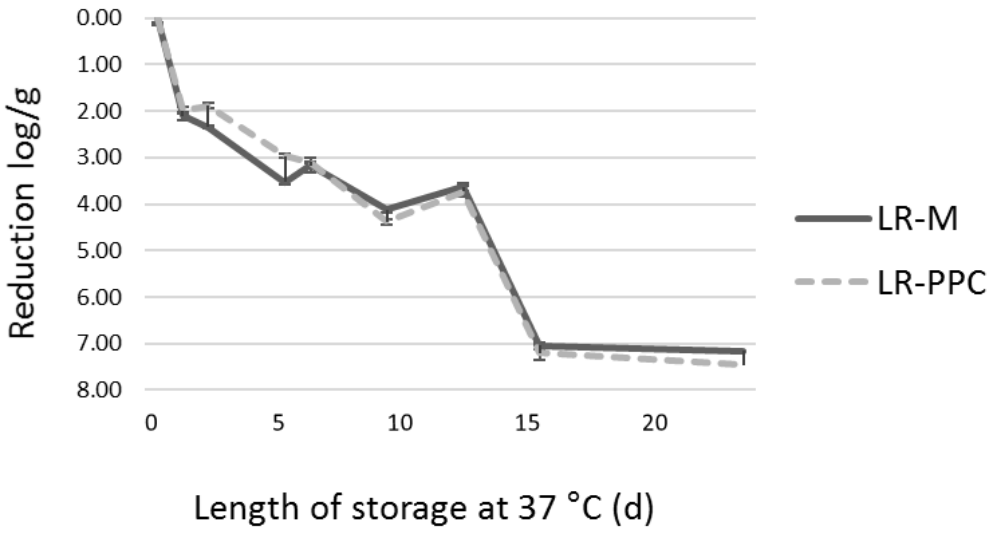


Figure 3.3. Mean (± SEM, n = 3) reduction (log cfu/g) in *L. reuteri* viability stored at 37 °C for 23 d. Prior to freeze-drying bacteria were either non-encapsulated with milk as a preservative (LR-M) or encapsulated without preservative (LR-PPC). Starting concentration for LR-M is 9.82±0.02 log cfu/g and for LR-PPC is 9.46±0.04 log cfu/g.

3.4.5 Effect of encapsulation protection on challenge in simulated gastric juice of *B. adolescentis*

After a challenge in SGJ for 2 h, *B. adolescentis* loss of viability was significantly lower in freeze-dried encapsulated bacteria group (1.43 ± 0.26 log cfu/g) compared to non-encapsulated (BA-M; 4.78 ± 0.15 log cfu/g; **Figure 3.4**). The addition of milk plus glucose (BA-PPC-M; 1.21 ± 0.11 log cfu/g) and glycerol (BA-PPC-G; 1.18 ± 0.13 log cfu/g) further reduced ($P < 0.05$) loss of viability in SGJ compared to PPC without preservatives (BA-PPC; 1.82 ± 0.11 log cfu/g).

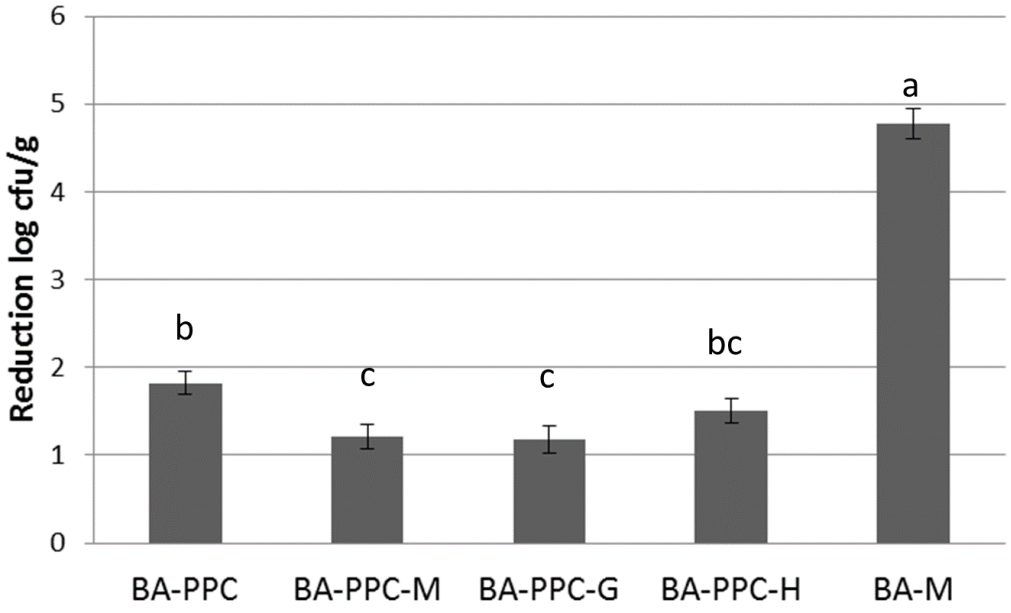


Figure 3.4. Mean (± SEM, n = 3) reduction (log cfu/g) in viable count of *B. adolescentis* after 2 h in simulated gastric juice for non-encapsulated bacteria preserved with milk (BA-M) and bacteria encapsulated without preservatives (BA-PPC) or encapsulated with milk and glucose (BA-PPC-M), cysteine-HCl (BA-PPC-H), or glycerol (BA-PPC-G). Vertical bars represent SEM. Bars with different letters indicate treatments are different at $P < 0.05$. Starting concentration for BA-M is 7.08 ±0.08 log cfu/g and for PPC group is ranged from 6.89±0.08 to 7.18±0.19 log cfu/g.

3.5 Discussion

Probiotic bacteria can provide promising benefits to improve the health of humans (Sanders, 2000) and animals (Musa and Seri, 2016). However, a loss of viability in probiotics sensitive to processing and storage has limited the development and application of probiotic bacteria in the food and feed industries (Anal and Singh, 2007; Rokka and Rantamäki, 2010). This study investigated the potential of a pea-protein and alginate-based encapsulation method, with and without the addition of one of several preservatives, on the viability of model probiotic bacteria during freeze-drying and storage.

3.5.1 Viability after freeze-drying

Although freeze-drying is considered a relatively gentle procedure, water removal and temperature change during drying increases stress on bacterial cells, which can damage the cell membrane (Castro et al., 1997). In the present study, a substantial loss in viability (> 5 log cfu/g DM) was observed in *B. adolescentis* when freeze dried without preservatives. Encapsulation without preservatives protected against loss of viability during freeze-drying. There is limited research on the impact of encapsulation on bacterial viability during freeze-drying. Heidebach et al. (2010) tested the effect of a casein-based microencapsulation on the viability of two bacterial strains (*Bifidobacterium lactis* Bb12 and *Lactobacillus paracasei* subsp. *paracasei* F19) compared to free cells after freeze-drying. Although there is considerable variation in loss of viability of lactic acid bacteria during freeze-drying, the *Bifidobacterium* strain was more resistant to loss of viability during freeze-drying compared to the *Lactobacillus* strain. Heidebach et al. (2010) reported that encapsulation did not improve viability of the hardy *Bifidobacterium* strain, whereas encapsulation markedly improved viability of the more susceptible *Lactobacillus* strain. The studies above suggest encapsulation does offer protection against loss of viability during freeze-drying for sensitive bacteria.

This study also evaluated the effect of different protective preservatives on *B. adolescentis* viability during freeze-drying, with or without encapsulation. The preservatives milk plus glucose and glycerol improved viability during freeze-drying of non-encapsulated bacteria whereas the addition of cysteine-HCL did not improve viability. Skim milk and sugars are commonly used as preservatives during freeze-drying of bacteria. For example, similar to this study, the addition of milk and trehalose improved viability of *L. bulgaricus* after freeze-drying (Castro et al., 1997) and the addition of sucrose improved viability of *E. coli* and *B. thuringiensis*

after freeze-drying (Leslie et al., 1995). Although *B. adolescentis* is an anaerobic bacterium, it was not surprising that cysteine-HCL, an oxygen reducing agent, was not effective in protecting against loss of viability because freeze-drying was carried out under vacuum conditions.

Among the preservatives tested, only the addition of milk plus glucose during encapsulation significantly improved viability of *B. adolescentis* after freeze-drying compared to encapsulation without the preservatives. However, the preservatives milk plus glucose with encapsulation did not further improve viability after freeze-drying compared with milk plus glucose without encapsulation. Thus encapsulation can replace the use of certain preservatives to improve viability of *B. adolescentis* after freeze-drying, but there was limited advantage in combining the preservatives with encapsulation. Our results contrasted Kearney et al. (1990), who showed that Ca-alginate capsules prepared using either glycerol with skim milk or adonitol with skim milk improved viability of *L. plantarum* after freeze-drying compared to the cryoprotectants without encapsulation. Similarly, Martin-Dejardin et al. (2013) observed that sugars (maltose or trehalose) or glycerol improved viability of *B. bifidum* after freeze-drying when incorporated into alginate-pectin capsules. These differences may relate to the use of pea protein and alginate in the capsules prepared in the current study, compared with calcium cross-linked alginate or alginate–pectin used in the other studies. Most of the cryoprotective effects of protein, sugar or glycerol preservatives is likely associated with displacement of water and/or a reduction in the formation of destructive ice crystals (Crowe et al., 1988). Pea proteins incorporated in the capsules in the current study may have provided the cryoprotective effect typically provided by milk proteins such that there was limited added protection with the preservatives milk protein and glucose or glycerol.

3.5.2 Viability during storage

Probiotics with a long shelf-life, which are resistant to fluctuating temperatures, would be ideal for use in the commercial application of probiotic products. However, there is no consensus on the effects of preservatives and encapsulation on the viability of bacteria during storage due to differences in storage temperature, storage solution, dry or liquid products and control groups.

Several studies have observed improved bacteria viability with use of preservatives and/or encapsulation during storage below 0 °C. However, results have varied considerably depending on bacteria strain and storage temperature. Survival of freeze-dried *Streptococcus phocae* was improved when stored with trehalose, glucose, galactose, skim milk and glycerol at a range of temperatures from -20 to 35 °C for up to 6 mo (Kanmani et al., 2011b). However, the author also demonstrated that microcapsules, prepared with alginate and the preservatives trehalose, inulin and Tween 80, were effective in preventing viability loss during storage (Kanmani et al., 2011b). Ying et al. (2012) showed that *L. rhamnosus* GG, encapsulated in whey protein isolate-maltodextrin and stored at 25 °C, was maximized when glucose was added but not inulin (Ying et al., 2012). Recently, improved survival was observed with alginate-encapsulated *Lactobacillus casei* stored at -15 °C compared to free cells (Xu et al., 2016). Sousa et al. (2012) encapsulated several lactic acid bacteria strains in Ca-alginate, with or without cysteine-HCl, and examined the effect of storage (as wet cells) on viability compared with free cells. In their study, encapsulation improved storage viability only at temperatures below 0 °C and cysteine HCl provided an added protective effect. When *L. bulgaricus* was encapsulated in chitosan calcium alginate, viability improved when stored at 22 °C but not at 4 °C, compared to free cells (Lee et al., 2004). Finally, encapsulation improved *B. infantis* viability when stored at 25 ºC when compared to non-encapsulated bacteria (both on dry basis) (Crittenden et al., 2006).

In the present study, encapsulation protected *B. adolescentis* against loss of viability when stored at -80 ºC compared with free cells prepared in milk plus glucose. The strain of *L. reuteri* used in the present study demonstrated no significant loss of viability during storage at 4 ºC and -80 ºC, even when stored as free cells with milk. Storage of *L. reuteri* at 37 ºC resulted in a rapid loss of viability that was not reduced with encapsulation. Unfortunately, viability loss during storage at room temperature was not examined, although this temperature may have resulted in a more gradual loss of viability. However, others have reported that encapsulation is ineffective when bacteria are stored above 0 ºC (O'Riordan et al., 2001; Weinbreck et al., 2010).

Vacuum packaging did not improve the viability of *B. adolescentis* during storage at -80 °C, although some limited protection was observed at room temperature. Vacuum packaging is recommended for storage of dry bacteria because it can control both water activity and oxygen level (Carvalho et al., 2004). Unfortunately, although vacuum packaging was applied in several probiotic encapsulation storage studies (Chen and Mustapha, 2012; Okuro et al., 2013b), none of these studies has compared vacuum versus non-vacuum packaging. However, several studies have observed a prolonged shelf-life with vacuum packaging of probiotic-coated food products (Yingyuad et al., 2006; Jaworska et al., 2011). In the present study, all the samples were stored in sealed bags, with and without vacuum removal of air. Packaging alone may have sufficiently limited oxygen and water activity while bacteria were stored a -80 °C with very low metabolic activity. However, oxygen and water remaining in the sealed bags may have affected viability under the conditions of higher metabolic activity expected at room temperature.

Water percentage is a critical factor for shelf-life. It was reported that less than 4 % water should be present during long-term storage of dry products (Gardiner et al., 2000) whereas higher than 10 % water is not desired (Desmond et al., 2002). In the present study, percent water

for PPC groups averaged from 6.3 to 6.7 %, which was similar to values obtained in another study using whey protein-pullulan capsules (Çabuk and Harsa, 2015). A more extended freeze-drying period did not decrease percent water (data not shown). The moisture content of encapsulated bacteria was typically 1 % higher than free cells, a factor that may have contributed to reduced effectiveness of encapsulation at temperatures above 0 °C.

3.5.3 *In vitro* function

In addition to improved viability during processing and storage, encapsulation of probiotic bacteria may provide protection against the harsh conditions present in the upper gastrointestinal tract of animals and improve probiotic delivery to distal gut regions. In this study, encapsulation of *B. adolescentis* improved the viability of bacteria in SGJ compared to free cells, which has been reported previously for *B. adolescentis* (Klemmer et al., 2011a) and other probiotic strains (Annan et al., 2008; Zhang et al., 2013). This study confirmed that the inclusion of milk plus glucose or glycerol during encapsulation provided added protection against viability loss in *B. adolescentis* in SGJ compared to encapsulation alone. These preservatives may occupy pores present in the pea-protein matrix (Klemmer et al., 2011a), thereby reducing the diffusion of gastric juice into the capsule and preventing it from contacting the bacteria. The inclusion of these preservatives during capsule preparation may improve the *in vivo* efficacy of pea protein capsules delivery of probiotic bacteria to the distal intestine.

3.6 Conclusion

In conclusion, pea protein isolate-alginate encapsulation significantly protected *B. adolescentis* during freeze-drying and gastric-juice challenge. The effects of encapsulation on bacteria storage varied with temperature and bacteria strain. Not surprisingly, the susceptible bacteria (*B. adolensentis*) benefited more from encapsulation relative to the hardy strain (*L.*

reuteri). The addition of protective preservatives, milk plus glucose, in capsules improved viability during freeze-drying and acid challenge. Vacuum packaging of the anaerobe *B. adolescentis*, did not improve viability when stored at -80 °C whereas limited protection was noted at room temperature. Results suggest that encapsulation in a pea protein-alginate matrix can provide protection against loss of bacterial viability during freeze-drying and storage, but the magnitude of the effect is strain dependent. Further work is required to establish techniques to permit long-term viability of sensitive probiotics under ambient temperature conditions commonly found in livestock feeding systems.

4 EFFICACY OF PEA PROTEIN ISOLATE-ALGINATE ENCAPSULATION ON VIABILITY OF PROBIOTIC BACTERIUM DURING FEED PELLETING

4.1 Abstract

Three sets of experiments were designed and conducted to determine the effect of encapsulation using a pea protein isolate (PPI) - alginate (AL) matrix on probiotic bacterial viability in response to thermal and pressure challenges during steam pelleting. Early stationary phase *Bifidobacterium adolescentis* (BA) or *Lactobacillus reuteri* (LR) cultures were suspended in 1 volume of 10 % skim milk (BA-M and LR-M) or encapsulated in a 4.0 % PPI and 1.0 % AL followed by extrusion and crosslinking (BA-PPC and LR-PPC). Loss of viability of bacteria on exposure to 50, 70 and 90 °C for 30, 60 and 180 s was significantly reduced ($P < 0.05$) by encapsulation compared to non-encapsulated BA and LR. Under the most severest challenge (90 °C for 180 s), the reduction in viable counts for BA-PPC and LR-PPC was 1.43 ± 0.03 and 2.43 ± 0.02 log cfu/g, respectively, compared with 3.20 ± 0.25 and 3.47 ± 0.20 log cfu/g, for non-encapsulated bacteria. To examine the combined effect of heat and pressure challenges, freeze-dried bacteria were mixed with complete animal feed and placed in the chamber of a single channel pelleting apparatus with temperature and pressure controls. In separate experiments, 95 MPa pressure was applied for 30 s without additional heat, or at 50, 70 and 90 °C. When the pressure was applied without additional heat, there was no significant difference in loss of viability between LR-M (0.77 ± 0.24 log cfu/g) and LR-PPC (0.43 ± 0.20 log cfu/g). However, encapsulation significantly ($P < 0.05$) improved viability when pressure was applied at 50 °C (0.75 ± 0.17 vs. 1.69 ± 0.14 log reduction cfu/g), 70 °C (1.40 ± 0.08 vs. 2.33 ± 0.08 log reduction cfu/g) and 90 °C (2.09 ± 0.09 vs. 3.43 ± 0.24 log reduction cfu/g). The viability of both encapsulated and non-encapsulated LR was markedly reduced when mixed in completed feed

and pelleted, with or without steam. Encapsulation did not protected bacteria during commercial pelleting.

Pea protein isolate-alginate-based encapsulation provided significant protection against loss of probiotic viability when compared to non-encapsulated bacteria following a heat and pressure challenge. However, encapsulation was not sufficient to provide adequate protection against loss of viability under commercial pelleting conditions.

4.2 Introduction

Following their introduction to the feed industry in 1974 (Fuller, 1992), probiotics have been used as feed additives and as an alternative to prophylactic antibiotics to improve animal health and growth performance in farm animals and pets (Verschuere et al., 2000; Benato et al., 2014; Prieto et al., 2014). Probiotics are viable microorganisms that when supplied in sufficient numbers, could alter the microflora (by implantation or colonization) and exert beneficial health effects in host (Schrezenmeir and de Vrese, 2001). This definition underlines the requirement for delivery of viable microorganisms in order to be considered a probiotic product. A major challenge in the application of probiotics in the feed and livestock industry has been the harsh conditions that feeds are exposed to during processing, storage and delivery. These conditions limit probiotic viability and have to date restricted probiotic inclusion in feeds to only a few species of microorganisms (Anadón et al., 2006; Amerah et al., 2013).

Feed pelleting is a common processing method that results in the agglomeration of small feed particles into pellets by applying moisture, heat and pressure (Falk. 1985). Pelleted feed is widely employed in the food animal industry to improve palatability, average daily gain and feed conversion rate while reducing foodborne pathogens and decreasing feed wastage (Kjeldsen and Dahl, 1999; Medel et al., 2004; Doyle and Erickson, 2006). The pelleting process typically

includes a conditioning step where heat and optional steam are applied to mash feed (Abdollahi et al., 2013a). Steam conditioning increases feed binding characteristics and improves pellet formation rate as the mash is forced through a metal die (Skoch et al., 1981).

The conditioner temperature, retention time and die size are commonly adjusted to control pellet quality and feed nutritional value (Briggs et al., 1999; Abdollahi et al., 2010; Abdollahi et al., 2013c). Generally, for monogastric feed production, conditioning times are less than 2 min with a temperature range of 70 to 90 °C (Thomas et al., 1997; Doyle and Erickson, 2006). The size of die is also routinely changed, between 0.5 to 10 mm in diameter, to maximize pellet performance in a particular application (Bertipaglia et al., 2010; Gopal et al., 2010; Abdollahi et al., 2013b) with smaller dies requiring a higher pressure and longer time to force feed pass through die. Higher temperatures, steam application and smaller die size produce harsher conditions and are associated with improved control of feed-borne pathogens but may also reduce the activity of sensitive feed additives such as enzymes (Spring et al., 1996; Jones and Richardson, 2004; Doyle and Erickson, 2006). Due to the harsh conditions employed during pelleting, only a few probiotic organisms, including spore-forming bacteria *Bacillus* spp. (Amerah et al., 2013); yeast *S. cerevisiae* (Mathew et al., 1998); and *E. faecium* (Simon et al., 2005), have been demonstrated to retain viability after pelleting.

Encapsulation of probiotic bacteria is an emerging method to protect against loss of viability during harsh environmental conditions including feed processing. Encapsulation immobilizes probiotic bacteria inside coated material and can protect bacteria from storage losses (Corbo et al., 2011; Klemmer et al., 2011b). Encapsulation can also protect against the harsh conditions of the upper gastrointestinal tract (e.g. gastric acid and bile acids) improving distal gut delivery. Alginate encapsulation has been shown to be effective as a thermal protectant reducing

loss of viability for 8 strains of *Lactobacillus* and *Bifidobacterium* compared to non-encapsulated bacteria when challenged at 65 ºC for 1 h (Ding and Shah, 2007). Similarly, a study with alginate coated *Lactobacillus casei* showed heat protection after challenge at 55, 60 and 65 °C for 20 min (Mandal et al., 2006). This suggests encapsulation could provide protection against loss of viability during pelleting.

However, industrial pelleting processes generally exceed these temperatures and include the addition of moisture and pressure that can lead to further loss of viability for probiotics. Pressure over 90 MPa resulted in a linear reduction in *L. acidophilus* viability (Chan and Zhang, 2002). An extensive search of the literature showed that there have been no peer-reviewed reports examining the effect of encapsulation on probiotic viability during feed pelleting. Therefore, the objective of this study was to investigate the efficacy of pea protein encapsulation in protecting the viability of model probiotic bacteria against exposure to conditions observed during commercial pelleting.

4.3 Material and Methods

4.3.1 Preparation of pea protein-alginate encapsulated and non-encapsulated bacteria

Single colonies of *B. adolensentis* (BA, ATCC 15703) and *L. reuteri* (LR, ATCC 53608) were inoculated in 10 mL broth of Reinforced Clostridial Agar (RCM, Oxoid Ltd., Basingstroke, England) or de Man Rogosa and Sharpe (MRS, Oxoid Ltd., Basingstroke, England), respectively. The bacteria cultures were incubated for 24 h under anaerobic (GasPak™ Anaerobic container system, Becton, Dickson and Company, Sparks, MD, USA) conditions at 37 °C. This 24 h culture was then inoculated into fresh RCM or MRS broth at a 1:1000 ratio and cultured for an additional 20 h (BA) or 14 h (LR) under anaerobic conditions at 37 °C until early stationary phase. Cultures were then centrifuged (4000 x g, 10 min at 4°C) and washed with peptone water

[Bacto™ peptones 1.0 g/L (Becton, Dickson and Company, Sparks, MD, USA)]. Non-encapsulated bacteria were suspended in 1 volume of 10 % skim milk (LR-M). Pea protein-alginate capsules (PPC) were prepared for each bacterium (BA-PPC, LR-PPC) by the adapted extrusion method as described before (Wood, 2010). Briefly, a Pea Protein-Alginate Solution (PAS) was prepared using 4 % (w/w) pea protein isolate (Nutri-Pea Limited, 80% protein, 1.81% lipid, 10.78% cabohydrate, Portage-la-Prairie, MB, Canada) and 1 % (w/w) alginate (Acros organics, CAS: 9005383, New Jersey,US) in dd H_2O. BA or LR was added into the PAS with mixing at a ratio of 1:18 (w/w) prior to extrusion and crosslinking in a solution containing 5 % $CaCl_2$ and 1 % Tween 20 for 30 min. Both encapsulated bacteria and non-encapsulated bacteria were vacuum freeze-dried for 7 d with a shelf temperature at -20 °C, a collector temperature at -50 °C and a pressure of 11 Pa (Freezone 6L benchtop freeze dry systems, Labconco®, USA).

4.3.2 Thermal challenge

Early stationary phase BA and LR cultures were centrifuged (Beckman J6-MC, Palo alto, CA USA; 4000 x g, 10 min at 4 °C), washed and resuspended in peptone water, separately. Resuspended BA or LR and freshly prepared PPC containing BA or LR, were diluted 1:10 in peptone water in triplicate and 10 mL was placed in a water bath at 50, 70 and 90 ºC. After 0, 30, 60 and 180 s, tubes were removed from the water bath and cooled to room temperature. The contents of all tubes were homogenized on ice (Fisher Scientific™ PowerGen™ Model 125 Homogenizer, USA) at speed 6 (30,000 rpm) for 3 x 10 s to disrupt the PPC, serially diluted and plated on Reinforced Clostridial Media (RCM, Oxoid, Basingstroke, England) for BA and de Man Rogosa and Sharpe agar (MRS, Becton, Dickson and Company, Sparks, MD, USA) for LR. Colonies were enumerated and log reduction in viable count compared to time zero determined after anaerobic culture for 24 h (LR) and 48 h (BA), at 37 ºC.

4.3.3 Single channel pelleting apparatus challenge

Freeze-dried non-encapsulated and encapsulated LR were added to a complete mashed feed (containing, wheat, barley, soybean meal, corn distiller's grains with solubles, and micro ingredients) to a concentration of 10^7 cfu/g diet. Approximately 1 g of mixed mash feed was placed into a tabletop pelleting apparatus (Instron 3366R4848, MA, USA) permitting production of single pellets under controlled temperature and pressure conditions. Pellets were produced at 50, 70 or 90 ºC for 30 s at a pressure of 95 MPa with die size of 6.35 mm. The resulting feed pellets, generated in triplicate sets, were cooled to room temperature and ground by mortar and pestle. A 1:10 (w/w) dilution was prepared in peptone water, followed by homogenization on ice as described above to disrupt PPC. The homogenized solutions were again serially diluted with peptone water and plated on MRS agar. Colonies were enumerated after anaerobic culture for 24 h at 37 ºC and the log reduction in the viable count was compared to the non-pelleted control.

4.3.4 Steam pelleting

Mashed feed (2.5 kg) was mixed with LR-PPC or LR-M to approximately 10^7 cfu/g diet and pelleted using a laboratory scale feed pelleting machine (CPM P/N 3-3093-15, Crawfordsville, IN, USA) operated at 90 ºC with a die size of 4.7 mm. Pellets were produced in triplicate experiments with addition of steam (1.4 kg/h at 241 kPa) or without steam. Approximately 200 g of pelleted samples were removed from the last 1 kg of feed produced. Conditioner temperature was measured by five probes located along the conditioner during pelleting; die temperature was measured by a digital infrared thermometer and pellet temperature was measured by both digital infrared thermometer and probes placed at exit points on pelleting machine. Pelleted feeds were cooled to room temperature and then 5 g of feed was mixed with 25 mL peptone water and homogenized on ice as described above. The homogenized solutions

were serially diluted with peptone water and plated on MRS agar for culture at 37 ºC for 24 h under anaerobic conditions. Colonies were enumerated and log reduction in viable counts versus pre-pelleted control were reported.

4.3.5 Statistical analysis

The effects of the encapsulation on the viability of model bacteria during thermal challenge, single channel pelleting and commercial pelleting were analyzed by one way ANOVA using Proc Mixed procedure in SAS version 9.4 (Statistical Analysis Software, SAS Institute Inc, 2013, Cary, NC, USA) with Tukey's mean separation procedure. Microbial counts were $\log_{10}$ transformed prior to analysis.

4.4 Results

4.4.1 Thermal challenge

Encapsulation significantly and consistently reduced ($P < 0.05$) the loss of viability after heat treatment for both *L. reuteri* (**Figure 4.1**) and *B. adolescentis* (**Figure 4.2**) compared to non-encapsulated bacteria at all tested temperature and exposure times. As expected, without encapsulation, both bacterial species lost viability during heat challenge demonstrating increased loss of viability with increased temperature and time of exposure. Encapsulation protected both bacteria strains from loss of viability such that, with the exception of the severest challenge (90 ºC for 180 s), loss of viability was less than 0.5 log cfu/g for encapsulated bacteria. At the severest challenge, the reduction in viable counts for encapsulated *B. adolescentis* and *L. reuteri* was 1.43 ± 0.03 and 2.43 ± 0.02 (log cfu/g), respectively, compared with 3.20 ± 0.25 and 3.47 ± 0.20 log cfu/g, respectively, for non-encapsulated bacteria.

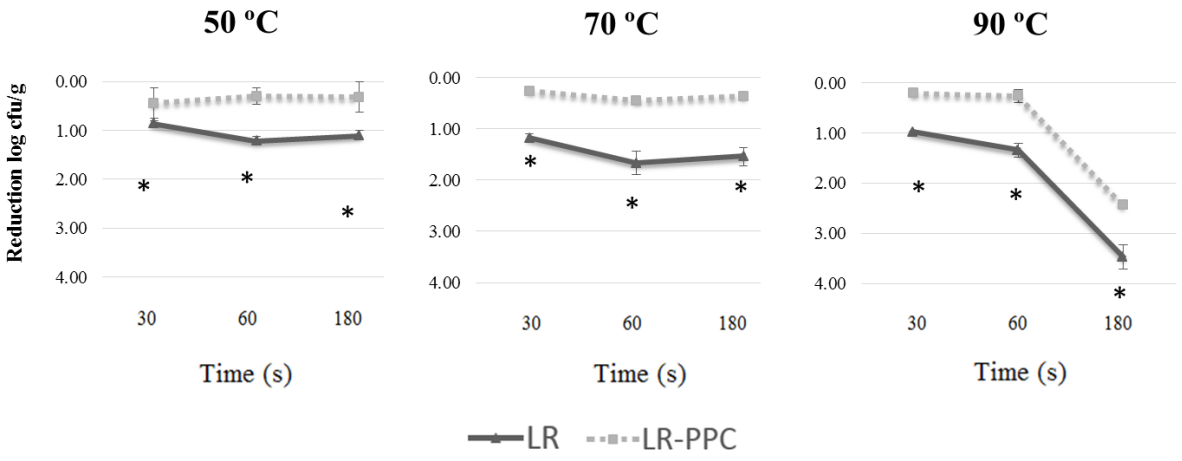


Figure 4.1. Mean (± SEM, n = 3) reduction (log cfu/g) in the viable count after heat treatment of non-encapsulated *L. reuteri* (LR) and pea protein encapsulated *L. reuteri* (LR-PPC) at 50, 70 and 90 ºC for 30, 60 and 180 s. Time points denoted with asterisks are significantly different ($P < 0.05$). Starting concentration for LR is 8.15±0.08 log cfu/g and LR-PPC is 7.37±0.09 log cfu/g.

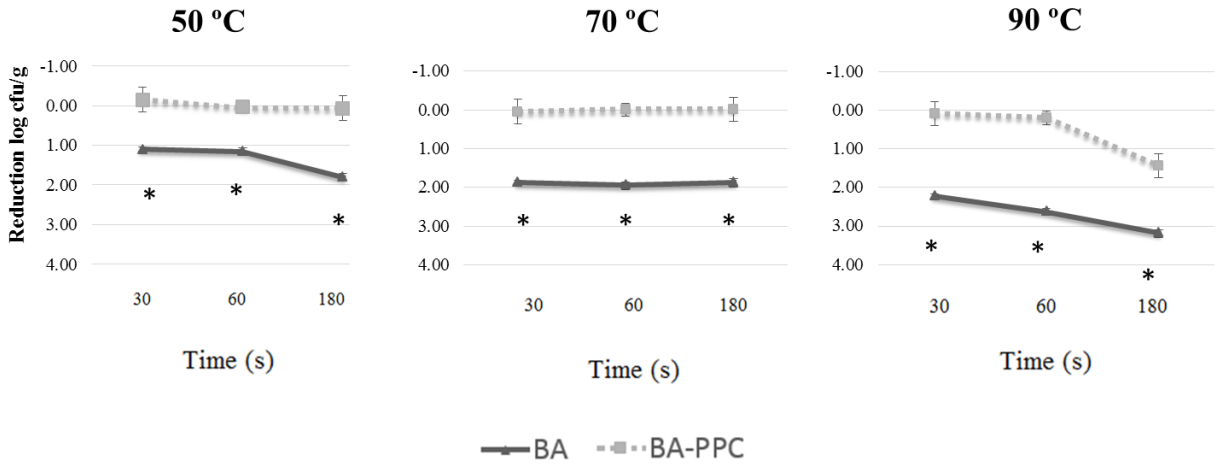


Figure 4.2. Mean (± SEM, n = 3) reduction (log cfu/g) in the viable count after heat treatment of non-encapsulated *B. adolensentis* (BA) and pea protein encapsulated *B. adolensentis* (BA-PPC) at 50, 70 and 90 ºC for 30, 90 and 180 s. Time points denoted with asterisks are significantly different ($P < 0.05$). Starting concentration for BA is 7.84±0.02 log cfu/g and BA-PPC is 6.94±0.03 log cfu/g.

4.4.2 Single channel pelleting apparatus challenge

Encapsulation protected against loss of viability of *L. reuteri* during pelleting in a single channel apparatus at 50, 70 and 90 ºC ($P < 0.05$) using a constant pressure of 95 MPa for 30 s (**Figure 4.3**). The reduction in viable counts at 50, 70 and 90 ºC was 1.69 ± 0.14, 2.33 ± 0.08 and 3.43 ± 0.09 log cfu/g, respectively for non-encapsulated bacteria whereas the reduction in viable counts after encapsulation was 0.75 ± 0.17, 1.40 ± 0.08, 2.09 ± 0.09 cfu/g, respectively. When a pellet was produced at 95 MPa for 30 s without supplemental heat no significant difference in bacterial loss. Viability was observed between encapsulated (0.43 ± 0.20 log cfu/g) and non-encapsulated *L. reuteri* (0.77 ± 0.24 log cfu/g).

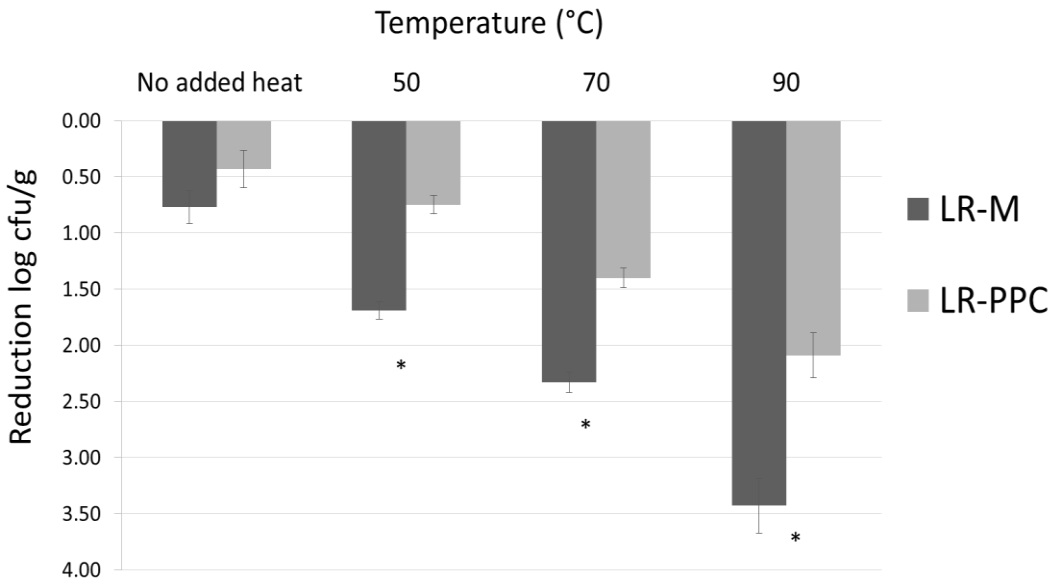


Figure 4.3. Mean (± SEM, n = 3) reduction (log cfu/g) in viable count using a single channel apparatus to pellet a mash feed mixed with non-encapsulated *L. reuteri* (LR-M) and pea protein encapsulated *L. reuteri* (LR-PPC) without supplemental heat or at 50, 70 and 90 ºC for 30 s. Time points denoted with asterisks are significantly different ($P < 0.05$).Vertical bars represent standard error of mean. Starting concentration for LR-M is 7.78±0.08 log cfu/g and LR-PPC 7.02±0.14 log cfu/g.

4.4.3 Steam pelleting

Steam pelleting using a small scale pelleting machine resulted in a marked loss of viability of *L. reuteri* (**Figure 4.4**). Bacterial counts in steam pelleted samples were below the detection limit for 3 of 9 samples containing LR-PPC and 5 of 9 samples containing LR-M. Detection limit 2.08 log cfu/g was applied to these samples. When pelleting was performed without steam addition, approximately half of the feed did not form into pellets such that this material was separated into pelleted feed and non-pelleted mash components for analysis of viable counts of *L. reuteri*. For the mashed feed, the loss of encapsulation of *L. reuteri* was smaller than non-encapsulated form (LR-PPC: 0.52 ±0.46 and LR-M 1.40±0.34 cfu/g, P=0.08) (**Figure 4.4**). There was no significant difference between the viability in pelleted feed for both groups.

The temperature of the die, pellets and conditioner observed during pelleting, with and without the addition of steam, are given in **Table 4.1**. The temperature in each location did not differ between pelleting runs conducted using feed containing encapsulated or non-encapsulated bacteria. There was no significant difference between each run of LR-PPC and LR-M. Without steam, the temperature of the conditioner, die and pellets was approximately 64, 61 and 70 ºC, respectively. With the addition of steam, the temperature in the conditioner increased by approximately 20 ºC and in the die by approximately 5 ºC, resulting in newly-formed pellets approximately 15 °C warmer.

Figure 4.4. Mean (± SEM, n = 3) reduction (log cfu/g) in the viable count after pelleting with or without steam treatment, of feed mixed with non-encapsulated resistant *L. reuteri* (LR-M) and pea protein encapsulated *L. reuteri* (LR-PPC) at a conditioner temperature 90 ºC. Time points denoted with an asterisk are significantly different ($P < 0.05$). Vertical bars represent standard error of mean. Starting concentraion for LR-M is 7.11±0.10 log cfu/g and for LR-PPC is 6.93±0.09 log cfu/g.

Table 4.1. Temperature of conditioner, die and pellets during pelleting ± SEM

		Conditioner (°C)	Die (°C)	Pellets (°C)
No Steam	LR-PPC	63.6 ± 2.8	70.0 ± 0.0	60.3 ± 2.1
	LR-M	64.4 ± 2.4	70.0 ± 0.0	61.3 ± 1.2
Steam	LR-PPC	84.7 ± 0.4	77.0 ± 1.6	76.0 ± 1.4
	LR-M	83.1 ± 2.6	73.0 ± 3.7	75.0 ± 1.6

4.5 Discussion

Pelleting is commonly used in the feed industry to improve the efficiency of conversion of feed to animal products and reduce waste (Gramkow et al., 2016). However, pelleting poses a challenge to using probiotics in animal feed processing as the high temperature, steam and

pressure generated during the pelleting process are lethal to most bacteria. As a result, bacterial probiotics suitable for in-feed application in pelleted diets have been largely limited to highly thermostable species such as spore-forming *Bacillus* spp. and yeast (Mathew et al., 1998; Amerah et al., 2013). This study examined the effect of a range of temperature, steam and pressure conditions typically produced during the commercial feed pelleting process on the viability of lactic acid bacteria following encapsulation in a pea protein alginate matrix.

4.5.1 Thermal challenge

In the first set of experiments, two lactic acid bacterial species commonly used as probiotics (Cole et al., 1989; Lan et al., 2003) were exposed to heat under the temperature range and exposure duration that occurs during pelleting. Pea protein-alginate encapsulation was highly effective in protecting both species under heat challenge. Indeed, loss of viability of encapsulated bacteria was maintained at less than 0.5 log cfu/g at all temperatures and times tested with the exception of the most severe heat change (90 °C for 180 s). Several other studies similarly observed improved thermal protection with alginate-based encapsulation. For example, alginate capsules protected bacteria at temperatures below 70 °C and for duration of 10 min to 1 h (Mandal et al., 2006; Azim et al., 2012; Borges et al., 2012), although these conditions do not reflect conditions found during feed pelleting. The effects of encapsulation on probiotic viability at higher temperatures were studied by Sabikhi et al. (2010). The authors reported a protective effect of alginate and cornstarch capsules on *L. acidophilus* viability on exposure to challenge temperatures at 72, 85 and 90 °C for durations of 30 s. Loss of viability for non-encapsulated cells was (8.29, 8.51, 9.13 log cfu/mL) compared with coated cell was only (1.19, 2.18, 4.14 log cfu/mL) (Sabikhi et al., 2010), a much greater protective response to encapsulation than observed in the present study.

Several factors may affect the degree of thermal protection provided to probiotic bacteria by encapsulation. For example, increasing alginate concentration reduces the number and length of capsule pores (Hannoun and Stephanopoulos, 1986) and was reported to increase thermal protection (Mandal et al., 2006). Chen (2007) also proposed that alginate alone provided minimum protection against thermal challenge, possibly associated with heat-induced structural changes causing loss of strength in the alginate-calcium. Increasing the protein concentration in the encapsulation formulation increased bacteria survival after heat challenge (Chen et al., 2007). Adding starch (Sabikhi et al., 2010; Teoh et al., 2011) or other polysaccharides including gellan (Chen et al., 2007), bagasse (Shaharuddin and Muhamad, 2015), carrageenan (Cheow and Hadinoto, 2013), or chitosan (Abbaszadeh et al., 2014) to an encapsulation matrix was also reported to improve heat tolerance of bacteria during challenge. Martin et al. (2013) proposed that starch may improve the protection provided by alginate coating by stabilizing alginate particles; starch is synergistic in gelling with alginate and provides nutrients to coated cells (Martin et al., 2013).

In the current study, the capsules were produced with a relatively low concentration of alginate (1 %) compared to previous reports but with the inclusion of pea protein. The addition of 4 % of pea protein in this study may have enhanced thermal protection in the current formulation. Although the interaction of pea protein with alginate requires further investigation, pea protein was proposed stabilize alginate crosslinks and provide nutrients for bacteria. Wood (2010) showed that the addition of pea protein isolates resulted in a denser alginate capsules structure and less porous and smoother capsule surface. These physical changes may have contributed to an improvement in *B. adolesentis* survival after challenge under acid conditions when pea protein was formulated in the capsules compared to capsules that only contained

alginate (Wood, 2010).

In the present study, non-encapsulated *B. adolesentis* was generally more sensitive to heat challenge (with the exception of 180 s exposure at 90 °C) compared to *L. reuteri*. This observation is consistent with previous reports, establishing species and strain variation in heat tolerance (Borges et al., 2012). The degree of thermal protection provided by encapsulation was also greater for *B. adolescentis*, where complete protection against loss of viability for exposures at 50 and 70 °C was observed. In contrast, encapsulation did not provide complete protection for *L. reuteri* even though this bacterium was less sensitive to thermal stress when not encapsulated. Borges *et al.* (2012) also found strain variation in protection against loss of viability due to thermal stress. A comparison of viability of *L. casei*, *L. paracasei*, *L. acidophilus* and *B. animalis* during exposure to heat (55 or 60 °C) for up to 1 h, demonstrated that encapsulation in a 2 % alginate matrix provided thermal protect only for *L. acidophilus* and only at 55 °C (Borges et al., 2012). Because of its greater resistance to thermal stress, *L. reuteri* was selected for use in the remaining experiments.

4.5.2 Single channel pelleting apparatus challenge

Using a single channel pelleting apparatus, encapsulated and non-encapsulated *L. reuteri* cells were exposed to a pressure of 95 MPa to mimic the die pressure produced in a commercial feed pelleting equipment (Thomas et al., 1997). Pressure alone has been shown to inhibit bacterial enzyme activity, affect cell structure, gene transcription and translation (Patterson, 2000; Murchie et al., 2005; Vogel et al., 2005; Abe, 2007). Indeed, pressure can also reduce bacteria viability as observed by Sheehan (2007), who noted a reduction in bacterial viability ranging from 5.9 to 7.9 log cfu/g for six strains of *Lactobacillus* and *Bifidobacterium*, after a high pressure challenge at 400 MPa for 5 min. In another study, Chan and Zhang, (2002)

observed a linear decrease in cell survival rate of freeze-dried *L. acidophilus* when exposed to compression ranging from 90 to 180 MPa with only a 33 % survival after exposure to 180 MPa.

Increasing temperature, in combination with pressure, increased the loss of bacterial viability consistent with the results of the thermal challenge. Interestingly, the loss of viability of *L. reuteri* was greater in the single pellet apparatus compared to the same temperature challenge applied without pressure. The results suggest the pressure has an additive negative effect on viability when combined with the heat challenge. Similarly, previous studies have shown that the combination of high pressure treatment and heat can dramatically improve microbial inactivation in food products (Patterson and Kilpatrick, 1998; Considine et al., 2008).

4.5.3 Commercial pelleting

Due to limits in lab-scale production of pea-protein encapsulated bacteria, limited quantities of supplemented mash feed could be generated requiring a prioritization of the pelleting parameters that could be examined in the present study. Two temperature settings were examined: operating without the addition of steam (conditioner at 64 °C) and the addition of steam (conditioner at 84 °C). Use of steam added moisture, a variable not examined in the previous small-scale experiments. Unfortunately, consistent pellets were not observed when the pelleting machine was operated without steam resulting in half the material exiting the machine in mash form. Encapsulation protected *L. reuteri* in the mash fraction of feed exiting the die. Interestingly, the loss of viability for both encapsulated and non-encapsulated *L. reuteri* was similar in magnitude to that observed in the dry heat experiments at similar temperatures (70 °C for 180 s) as observed here in the pelleting die. The *L. reuteri* in the pelleted feed fraction exiting the pellet machine showed markedly reduced viability (more than 4 log cfu/g reduction in both treatments) compared to the mashed feed fraction. It is likely that the pressure force exerted on

the pelleted fraction while passing through the die, and the duration of heat exposure, was increased for the pelleted fraction relative to the mash fraction resulting in a reduced level of protection by encapsulation.

The addition of steam to the conditioner increased the conditioner temperature by approximately 20 °C to 84 °C and the temperature of the die to approximately 75 °C. Loss of viability of *L. reuteri* exceeded 4 log cfu/g and the viability of some replicates in both treatments was below the detection limit. Encapsulation did not protect against loss of viability. The magnitude of viability loss, in comparison to previous experiments exposing *L. reuteri* to similar levels of heat, or heat and pressure via single channel pelleting, was unexpected. The addition of steam and moisture at the conditioner may have increased the rate of heating and penetration of heat into the pellet. Moisture contact and rehydration of the bacteria may have reduced heat tolerance as reported elsewhere (Zayed and Roos, 2004).

There are limited publications examining the survival of probiotic bacteria during feed pelleting. Resistance to loss of viability during pelleting has only been observed with specific spore-forming or heat-tolerant strains. For example, more than 90 % of *Bacillus subtilis* spores survived after pelleting at a temperature of 90 °C (Amerah et al., 2013), Similarly, *Bacillus cereus* toyoi spores have a survival rate of 95 % at a pelleting temperature of 87 °C. In contrast, *E. faecium* is a non-spore forming bacterium with a reported recovery rate of 35 % at a pelleting temperature of 80 °C (Simon et al., 2005). Finally, probiotic yeast strains demonstrate heat tolerance such that *S. cerevisiae* can be pelleted with only 0.3 log cfu/g loss with the conditioner at 60 °C (Mathew et al., 1998). There are limited reports examining tolerance of lactic acid bacteria during pelleting. The findings of this study confirm the susceptibility of lactic acid bacteria to the conditions found during pelleting. Encapsulation in a pea protein isolate–alginate

matrix was not able to impart significant survival of lactic acid bacteria during pelleting.

4.6 Conclusion

In conclusion, pea protein isolate-alginate encapsulation protected *B. adolescentis* and *L. reuteri* during the thermal challenge. Pea protein isolate-alginate encapsulation also significantly protected bacteria viability during a combined thermal and pressure challenge, designed to mimic conditions found during commercial pelleting. However, lab scale feed pelleting, with and without steam addition, resulted in significant loss of bacterial viability. Encapsulation did not protect bacteria during commercial pelleting. Even low temperature commercial feed pelleting imposes harsh environmental conditions such that marked improvement in encapsulation methodology will be required to permit inclusion of heat sensitive probiotics in pelleted feeds.

5 EFFICACY OF PEA PROTEIN ISOLATE-ALGINATE ENCAPSULATION ON VIABILITY OF A PROBIOTIC BACTERIUM IN THE PORCINE DIGESTIVE TRACT[1]

5.1 Abstract

The aim of this study was to investigate the efficacy of pea protein isolate-alginate capsules (PPCs) on probiotic viability during transit of the porcine gastrointestinal tract. A *Lactobacillus reuteri* ATCC 53608 isolate selected for rifampin plus streptomycin resistance (LRR) was encapsulated in a pea protein isolate and alginate (LRR-PPC) using an extrusion and cross-linking method prior to freeze-drying. An *in vitro* study in simulated gastric juice showed that encapsulation increased ($P < 0.001$) survival in strong acid. After incorporation into the diet of weaned pigs, LRR were recovered from feces and digesta by selective culture. Fecal shedding of LRR from pigs fed LRR-PPC was higher ($P < 0.001$) than from pigs fed non-encapsulated LRR. Viable LRR counts were not different in homogenized stomach contents, however, higher ($P < 0.001$) counts were observed in distal intestinal contents for pigs fed LRR-PPC. Probiotic encapsulation using pea protein-alginate matrix can protect bacteria during upper intestinal transit improving viability in the distal gut and permitting a broader range of sensitive bacterial species candidates for probiotic application.

5.2 Introduction

Probiotic bacteria supplementation holds promise to improve animal and human health as an alternative strategy to in-feed/food prophylactic antibiotic use. A prerequisite for such application of probiotics is to maintain the viability of the probiotic organism at a sufficiently

[1] A version of this chapter has been published: Wang, J., Nickerson, M. T., Low, N. H., & Van Kessel, A. G. (2016). Efficacy of pea protein isolate–alginate encapsulation on viability of a probiotic bacterium in the porcine digestive tract. Canadian Journal of Animal Science, 97(2), 214-222.

high level to be able to colonize the distal gut adequately to affect the host beneficially (Rambaud et al., 1993). Bacteria with probiotic properties may be sensitive to the digestive enzymes, gastric acid, bile salts and other antimicrobial compounds present in the upper gastrointestinal tract (Klemmer et al., 2011b; Zhang et al., 2013; Tee et al., 2014) limiting their viability and thus application as oral supplements. Providing additional mechanisms to protect sensitive bacteria against environmental challenges in the gastrointestinal tract could improve the efficacy of existing probiotics and/or permit application of novel probiotic species.

Encapsulation, which is an approach to immobilize and trap probiotic bacteria in a coating matrix, has been shown to improve the viability of probiotic strains during *in vitro* challenge (Lee and Heo, 2000; Klemmer et al., 2011b; Sathyabama et al., 2014). Several coating polymer materials have been used for encapsulation such as alginate, chitosan, carrageenan (Annan et al., 2008; Tee et al., 2014). Alginate is an inexpensive, non-toxic, gel-forming material broadly used in encapsulation in the presence of calcium ion (Su et al., 2011; Sathyabama et al., 2014). However, alginate alone did not show sufficient protection of probiotic viability *in vitro* (Lee and Heo, 2000). Milk proteins have been widely used in combination with alginate to improve probiotic protection (Picot and Lacroix, 2004; Heidebach et al., 2010). Pea protein isolates are an abundant and low-cost alternative plant protein source which have shown similar protective properties as milk proteins in combination with alginate (Klemmer et al., 2011b).

Although, numerous studies have shown improved bacterial *in vitro* viability following encapsulation, there has been limited investigations on the effect of encapsulation on probiotic viability *in vivo*. Rosas-Ledesma (2012) showed that red fluorescence protein-labelled *Shewanella putrefaciens* coated with alginate could be recovered from the ileum of Senegalese sole whereas non-encapsulated *S. putrefaciens* could not. Similarly, chitosan-coated alginate

capsules increased *Lactobacillus* spp. abundance in mouse (Iyer et al., 2013) and chicken intestine (Rodklongtan et al., 2014). We hypothesized that pea-protein alginate encapsulation of a probiotic would increase the viability of the organism in the digestive tract. Therefore, in the present study, we selected an antibiotic-resistant strain of *L. reuteri* as a model probiotic species, to investigate the efficacy of a pea protein isolate-alginate encapsulation method on probiotic viability in the porcine digestive tract.

5.3 Materials and Methods

All experimental protocols involving animals were approved by the Animal Care Committee of the University of Saskatchewan (Animal protocol #20110065) and the experiments were performed in accordance with recommendations of the Canadian Council on Animal Care (1993).

5.3.1 Selection and stability of antibiotic-resistant *L. reuteri*

To confirm the concentration and class of antibiotic necessary to inhibit growth of resident lactobacilli in pigs, fresh porcine fecal samples (1 g) from weaned piglets (n = 10) were diluted with 9 g of peptone water [Bacto™ peptones 1.0 g/L (Becton, Dickson and Company, Sparks, MD, USA), NaCl 8.5 mM (Fisher Scientific, Ottawa, ON, CA)]. The diluted feces were plated on de Man Rogosa and Sharpe agar (MRS, Becton, Dickson and Company, Sparks, MD, USA) containing rifampin (Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 20, 100 or 200 µg/mL or containing a combination of rifampin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA) where streptomycin was added at ten times the concentration of rifampin (Pedersen and Tannock, 1989; Simpson et al., 2000). Plates were cultured for 48 h at 37 °C under anaerobic conditions (GasPak™ Anaerobic container system, Becton, Dickson and Company, Sparks, MD, USA) for enumeration of live bacteria.

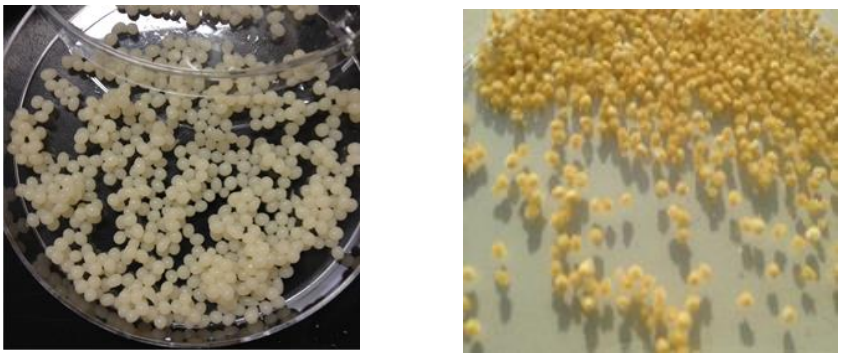
To establish a resistant bacterium, a single colony of *L. reuteri* ATCC 53608 (Manassas, VA, USA) was inoculated into 10 mL MRS broth and cultured for 24 h at 37 ºC anaerobically. An aliquot of 10 µL of starter culture was amplified into 10 mL fresh MRS broth containing rifampin plus streptomycin at 1 µg/mL broth and 10 µg/mL broth, respectively. This subculture procedure was repeated daily with increased concentration of both antibiotics until a concentration of 400 µg/mL rifampin and 4000 µg/mL streptomycin was achieved. An aliquot was then plated on MRS agar containing both antibiotics (400 µg/mL rifampin and 4000 µg/mL streptomycin) to isolate a single resistant colony for overnight culture and storage in 30 % glycerol (w/w) at -80 ºC.

Single colonies of wild-type *L. reuteri* (LRW) and resistance *L. reuteri* (LRR) were inoculated in 10 mL MRS broth with antibiotic (200 µg/mL rifampin + 2000 µg/mL streptomycin, AB+) or without antibiotic (AB-) to establish growth kinetics. Optical density (600 nm) and viable counts were measured every 2 and 4 h, respectively in an anaerobic chamber (80 % N_2, 10 % CO_2, and 10 % H_2) for 32 h at 37 ºC.

The stability of antibiotic resistance was measured by subculturing of LRR in the absence of antibiotic. The frozen stock was cultured on MRS agar (AB-) and a single colony subsequently inoculated into 10 mL MRS (AB-) broth followed by anaerobic culture for 24 h at 37ºC. After 24 h, a 100 µL aliquot was plated on MRS (AB+) containing 200 µg/mL rifampin + 2000 µg/mL streptomycin to enumerate viable antibiotic-resistant LRR. A 10 µL aliquot of the 24 h culture was inoculated into fresh 10 mL MRS (AB-) for another 24 h. This process was repeated every 24 h for 14 d. Colonies formed on MRS (AB+) were enumerated each day to establish retention of antibiotic-resistant LRR.

5.3.2 Preparation of encapsulated and non-encapsulated bacteria

Early stationary phase (12 to 14 h) LRR cultures were centrifuged, washed with peptone water and either resuspended in 1 volume of 10 % skim milk (LRR-M) or encapsulated in a pea protein isolate (PropulseTM Pea protein isolate, 80 % protein, Nutri-Pea Limited, Portage-la-Prairie, MB, Canada)-alginate (Sigma-Aldrich, Oakville, ON, CA) matrix (LRR-PPC). Pea protein-alginate capsules were prepared by an adapted extrusion method as described by Klemmer (Klemmer et al., 2011b). Briefly, pea protein isolate (PPI) was dissolved into ddH_2O (5 % w/w containing 4 % pea protein) at pH 8.0 (adjusted with 1 M NaOH) with mechanical stirring for 30 min in a water bath at 80 ºC. The PPI solution was then cooled to room temperature and adjusted to pH 7.0 with 1 M HCl. Alginate was added at 0.6 % (w/w) and the mixture heated to 80 ºC with mechanical stirring for another 45 min until the alginate was completely dissolved. The pea protein and alginate solution (PAS) was then cooled to room temperature before washed LRR were added at a ratio of 1 part washed bacteria to 18 parts (w/w) PAS with continuous stirring. The PAS was then extruded through a 20 G needle under air pressure and dropped into a cross-link solution [5 % $CaCl_2$ and 1 % Tween 20 (Fisher, New Jersey, USA)]. After a 30 min hardening time, the capsules were filtered by filter paper (Particle retention> 20µm Fisherbrand®, EU) and collected in aluminium trays. All LRR-PPCs and LRR-M were vacuum freeze-dried for 5 d at -20 °C shelf temperature and -50 °C collector temperature with a pressure of 0.11 mBar (Freezone 6 L benchtop freeze dry systems, Labconco®, USA) and stored at -80 ºC until use (**Figure 5.1**).



A B

Figure 5.1. Pea protein isolate-alginate capsules. A) Wet capsules after cross-linking and filtering; B) Freeze-dried capsules.

5.3.3 *In vitro* challenge in simulated gastric juice and simulated intestinal juice

Early stationary phase (12 to 14 h) LRW and LRR cultures were harvested and triplicate samples of freshly prepared LRW, LRR and LRR-PPC were suspended to a concentration of approximately 8 log cfu/mL in simulated gastric juice [SGJ: 0.08 M HCl and 0.2 % NaCl (w/v), pH 1.5 or pH 2.0] at 37 ºC for 2 h or simulated intestinal juice [SIJ: 1.25 % $NaHCO_3$ (Fisher, New Jersey, USA), 0.6 % (w/v) Difco^{TM} Oxgall (Becton, Dickson and Company, Sparks, MD), 0.09 % Pancreatin (Sigma, Oakville, ON)] for 3 h (Klemmer, 2011a). After incubation, SGJ treated samples were neutralized to pH 7.0 using 0.1 M NaOH. In order to physically disrupt the capsules and accurately enumerate viable bacteria, samples were homogenized on ice using a homogenizer (Fisher Scientific™ PowerGen™ Model 125 Homogenizer, USA) at speed 6 (30,000 rpm) for 3 x 5s. The samples were then plated on MRS agar and incubated at 37 ºC anaerobically for 48 h to enumerate the viable *L. reuteri*.

5.3.4 *In vivo* probiotic delivery in pigs

A total of 24 weaned pigs (8.81 ± 0.13 kg body weight, PIC commercial breed) were divided into three treatments (4 pigs/pen) and balanced by weight and gender. All the treatment groups received a non-medicated mash feed based on wheat, barley, soybean meal, and corn

distillers grains with solubles and meeting the nutrient requirements for the weanling pig (NRC 1998). To control cross contamination, pens assigned to the control treatment group were housed on the opposite end of the room from pens assigned to pigs supplemented with bacteria. Boots were cleared of organic matters and sprayed with ethanol (75 %) after each visit of individual pens. Beginning of 6 d post-weaning (experimental d 0), pigs were supplemented with LRR-PPC or LRR-M at a level of 10^6 cfu/g diet. Pigs in the third treatment group (Control) did not receive probiotic bacterial supplementation. Diets containing probiotic bacteria were prepared fresh each morning for three consecutive days using bacterial aliquots stored at -80 ºC. Water and diets were offered *ad libitum* throughout the experiment and feed intake recorded daily. Body weight was recorded at the beginning and end of the supplementation period. To confirm probiotic viability, subsamples (5 g) of feed were collected daily at 09:00 from both orts and freshly mixed feed, diluted in 10 mL peptone water and homogenized on ice. Samples were plated on MRS agar (200 µg/mL rifampin + 2000 µg/mL streptomycin) and cultured anaerobically for 48 h at 37 ºC to permit enumeration of LRR.

5.3.5 Enumeration of LRR in feces and digesta

Fecal samples were collected on days 0, 1, 2 and 3 from the anus of individual pigs by digital manipulation. Fecal subsamples (0.2 g) were collected into pre-weighted 15 mL conical tubes containing 1 mL peptone water to permit enumeration of viable *L. reuteri* on selective MRS agar (200 µg/mL rifampin + 2000 µg/mL streptomycin).

All piglets were killed by captive bolt stunning and pithing at experimental day 4. Total contents (without mucosal scrapings) was collected from the stomach, duodenum (the proximal 10% of small intestinal length), jejunum (1 m before and after intestinal midpoint), ileum (distal 1 m of the small intestine exclusive of 10 cm proximal to the ileo-cecal junction), cecum,

proximal colon (ileocecal junction to apex of colonic spiral) and distal colon (apex to base of colonic spiral). All contents were collected into separate sterile plastic weight boats, mixed and subsampled. Subsamples (200 mg) from each location except for stomach were collected into pre-weighed 15-mL conical tubes containing 1 mL peptone water to permit enumeration of viable antibiotic-resistant *L. reuteri* using MRS agar containing antibiotic. In the case of stomach contents, a subsample (2 g) was collected into pre-weighed 50-mL conical tubes containing 5 mL peptone water. Stomach samples were either plated directly or after homogenization on ice (Fisher Scientific™ PowerGen™ Model 125 Homogenizer) at speed 6 (30,000 rpm) for 3 x 10 s in order to physically disrupt capsules.

5.3.6 Statistical analysis

The data analysis was processed by SAS version 9.4 (Statistical Analysis Software, SAS Institute Inc, 2013, Cary, NC, USA) using the mixed procedure. One-way analysis of variance (ANOVA) was used to assess treatment differences for *in vitro* experiments. A completely randomized design with factorial arrangement was used to assess lactobacillus counts in feed using treatment (LRR-PPC vs. LRR-M) and Time (0 vs. 24 h) as main effects. A repeated measures analysis was used to compare resistant lactobacilli counts in feces and intestinal contents using treatment as a main effect and day post inoculation or gastrointestinal location as repeated sources of variance, respectively. When a significant interaction with the repeated variable was observed, means within the repeated variable were separated by one-way ANOVA. Where treatment effects with more than two levels were significant ($P < 0.05$), means were separated using Tukey's multiple comparisons. For *in vivo* experiments, pig was considered the experimental unit.

5.4 Results

5.4.1 Characteristics of antibiotic-resistant *L. reuteri*

One strain of *L. reuteri* was isolated that was capable of growth in 400 µg/mL rifampin + 4000 µg/mL streptomycin following culture in the presence of gradually increasing antibiotic concentrations. This level of resistance exceeded the level of resistance observed in swine fecal microbiota. No growth of bacteria was observed in the culture of swine feces on selective MRS agar containing at least 200 µg/mL rifampin + 2000 µg/mL streptomycin (data not shown). The growth of wild-type LRW and LRR in MRS broth with antibiotic (200 µg/mL rifampin + 2000 µg/mL streptomycin, AB+) or without antibiotic (AB-) was followed over 32 h. Stationary phase for all the groups was observed from 10 to 18 h followed by exponential growth to about 32 h. The growth pattern of wild-type *L. reuteri* in AB- was similar to resistant *L. reuteri* in AB+. Wild-type *L. reuteri* did not grow in AB+. After daily subculture of LRR for 14 d in AB- MRS broth, the number of viable LRR recovered on the AB+ MRS agar remained consistent for each day at 9 log cfu/mL broth indicating no loss of resistance.

5.4.2 *In vitro* challenge in simulated gastric juice and simulated intestinal juice

After 2 h incubation in SGJ (pH 2.0) and 3 h in SIJ (pH=7.3), there was no difference in the reduction of viability of LRW, LRR or LRR-PPC (**Table 5.1**). However, after samples were challenged in SGJ (pH 1.5) for 2 h, a significant reduction in viability occurred for non-encapsulated LRW and LRR (3.54 ± 0.06 and 3.51 ± 0.09 log cfu/g, respectively) compared to LRR-PPC group (1.13 ± 0.06 log cfu/g).

Table 5.1. Reduction in viable counts (log cfu/g) of freshly prepared wild-type (LRW), antibiotic-resistant (LRR) and encapsulated resistant *L. reuteri* (LRR-PPC) before or after challenge in simulated gastric juice (SGJ, pH 2.0 and pH 1.5) for 2 h and simulated intestinal juice (SIJ, pH = 7.3) for 3 h[a].

	pH	LRR-PPC	LRR	LRW	*P*
SGJ	2	1.02 ± 0.01	1.04 ± 0.23	1.06 ± 0.08	0.9721
	1.5	1.13 ± 0.06*a*	3.51 ± 0.09*b*	3.54 ± 0.06*c*	<0.001
SIJ	7.3	0.04 ± 0.09	0.06 ± 0.45	0.10 ± 0.23	0.9805

Note: Means with a different letter in the same row were significantly different at $P<0.001$ level.
[a] Results are Mean ± SEM for triplicate determinations.

5.4.3 Probiotic viability in feed

Viable count of *L. reuteri* in freshly prepared feed and in feed recovered from feeding troughs after 24 h on each of the three experimental supplementation days is shown in **Table 5.2**. The viable count was not different for feed supplemented with LRR-M or LRR-PPC. However, there was a significant reduction ($P < 0.001$) of viable bacteria after 24 h for both LRR-PPC and LRR-M group. No antibiotic-resistant bacteria were recovered from the control diet.

Table 5.2. Number (log cfu/g feed) of antibiotic-resistant bacteria enumerated on AB+ MRS[a] agar for freshly prepared feed and feed recovered after 24 h for freeze-dried antibiotic-resistant *L. reuteri* with milk (LRR-M) and antibiotic-resistant *L. reuteri* coated with pea protein isolate-alginate capsules (LRR-PPC) supplemented diets for consecutively 3 d[b].

	Treatment		Time (h)		*P* value		
	LRR-PPC	LRR-M	0	24	TRT	T	INT
Enumeration	5.88 ± 0.2	5.89 ± 0.15	6.29±0.08	5.48 ± 0.06	0.9428	<0.001	0.3414

[a]AB+MRS: de Man, Rogosa and Sharpe media containing rifampin (200 µg/mL) and streptomycin (2000 µg/mL).
[b]Results are mean ± SEM for triplicate determinations.
TRT = treatment, T = time, INT = interaction.

5.4.4 Probiotic shedding in feces

Feed intake per pig was 245 ± 14 g/d, and not different among the three treatment groups for each day of supplementation. The number of antibiotic-resistant lactobacilli recovered in feces on each day of the study is shown in **Table 5.3.** There was no recovery of antibiotic-resistant lactobacilli on day 0 for all groups. Statistical comparison of antibiotic-resistant lactobacilli counts in feces from days 1 to 3 in LRR-M and LRR-PPC groups (excluding control group) using a repeated measures approach, indicated a significant ($P < 0.01$) effect of treatment and experimental day and a trend ($P < 0.1$) towards a treatment by day interaction. Fecal resistant lactobacilli count from the LRR-M group was significantly ($P < 0.001$) lower compared with the count in feces from LRR-PPC pigs during each of the 3 d in the feeding period. The shedding of antibiotic resistant lactobacilli increased ($P < 0.05$) on day 2 and day 3 compared with day 1 independent of how LRR were administered (LRR-M or LRR-PPC). A trend towards a significant treatment by day interaction suggested that the increase in fecal shedding of resistant lactobacilli was greater in LRR-PPC as compared to LRR-M. No fecal shedding of resistant lactobacilli was observed on experimental day 1 in the control group. However, two control pigs shed antibiotic-resistant lactobacilli at 4.60 log cfu/g on day 2 and two pigs shed resistant lactobacilli at 4.62 and 4.60 log cfu/g on day 3. Only one pig shed resistant lactobacilli on both days.

Table 5.3. Antibiotic-resistant lactobacilli (log cfu/g feces) in fecal samples from pigs fed diets supplemented with freeze-dried antibiotic-resistant *L. reuteri* with milk (LRR-M) or antibiotic-resistant *L. reuteri* coated with pea protein isolate-alginate capsules (LRR-PPC)[a].

Day post inoculation	Treatment			
	LRR-M	LRR-PPC	Overall	Control[b]
Day 0	n.d.[c]	n.d.	n.d.	n.d.
Day 1	4.96 ± 0.13	5.42 ± 0.02	5.19±0.35*a*	n.d.
Day 2	5.29 ± 0.22	6.02 ± 0.11	5.65±0.61*b*	1.00 ± 0.61
Day 3	5.13 ± 0.16	6.32 ± 0.19	5.72±0.77*b*	0.53 ± 0.50
Overall	5.13 ± 0.10*a*	5.92 ± 0.11*b*		0.51 ± 0.28

Note: Means with a different letter in the same row were significantly different at $P < 0.001$ level.

[a] Results are Mean ± SEM (n = 8). Data were analyzed using Proc Mixed procedure using treatment as a main effect and day post inoculation as a repeated source of variation. Treatment $P < 0.001$, Day $P = 0.0035$, Treatment X Day $P = 0.0857$.

[b] Data from the control group was not included in the statistical analysis. On day 2, two pigs out of eight both shed antibiotic resistant lactobacilli at 4.60 log cfu/g. On day 3, two pigs out of eight pigs shed antibiotic resistant lactobacilli with a mean of 4.60 and 4.62 log cfu/g, respectively. Only one pig shed resistant lactobacilli on both days.

[c] n.d. Means no detectable value.

5.4.5 Enumeration of viable LRR in digesta

Counts of viable antibiotic-resistant lactobacilli in intestinal contents recovered along the gastrointestinal tract are showed in **Figure 5.2**. As expected, counts in control pigs were below 2 log cfu/g in all locations. Interestingly, in contents from stomach and duodenum, viable antibiotic- resistant lactobacilli counts were higher ($P < 0.001$) in LRR-M compared to LRR-PCC pigs. In contrast, antibiotic-resistant lactobacilli counts in the distal small intestine, cecum and colon were higher ($P < 0.001$) in LRR-PPC compared to LRR-M pigs. Homogenization of the contents from stomach increased lactobacilli counts in stomach contents from LRR-PPC pigs without affecting the counts for LRR-M pigs such that homogenized counts were not different between the two treatment groups.

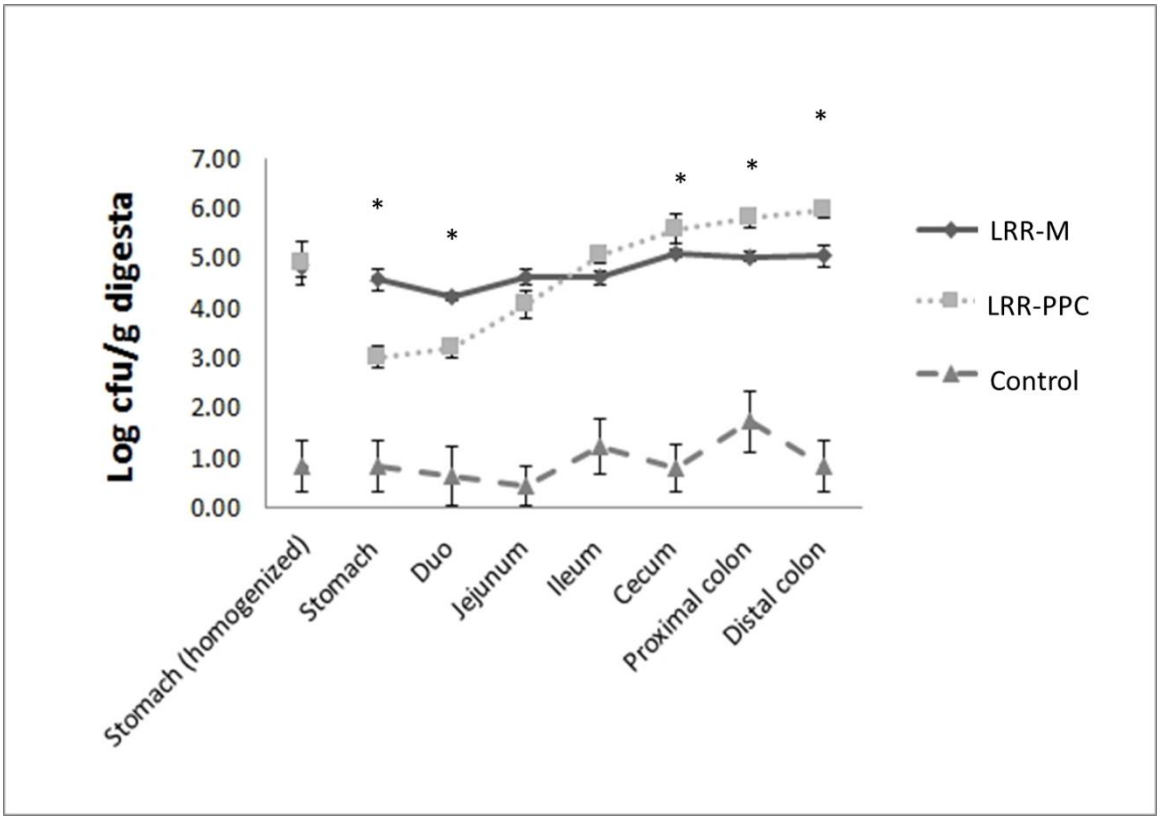


Figure 5.2. Antibiotic-resistant lactobacilli (mean log cfu/g digesta) enumerated in digesta (n = 8) from pigs fed the control diet or diets supplemented with freeze-dried antibiotic-resistant *L. reuteri* with milk (LRR-M) and antibiotic-resistant *L. reuteri* coated with pea protein isolate-alginate capsules (LRR-PPC). Comparison of LRR-M and LRR-PPC treatment groups by repeated measures indicated Treatment by Location Interaction $P < 0.01$. (*within location $P < 0.05$ between LRR-M and LRR-PPC). Vertical bars represent SEM.

5.5 Discussion

Encapsulation has been shown to provide protection to a variety of probiotic bacteria during *in vitro* challenge with simulated gastric juice and bile salts compared to non-encapsulated bacteria (Guérin et al., 2003; Lotfipour et al., 2012; Piątek et al., 2012; Zhang et al., 2013; Cook et al., 2014). Work has also shown improved bacteria viability in acid food products following encapsulation (Martoni et al., 2007; Ortakci and Sert, 2012). However, there are very

few studies establishing the efficacy of encapsulation technologies on the viability of probiotic bacteria in the intestinal tract. One limitation of investigating the viability of encapsulated probiotics is the difficulty in the selective enumeration of the encapsulated probiotic strain. Traditional selective culture methods are generally unable to differentiate probiotic bacterial strains from bacteria commonly found in the digestive tract. Furthermore, while molecular methods such as qPCR demonstrate improved selectivity, differentiation of live and dead bacteria remains difficult with this approach. In the current study, *L. reuteri* was selected as a model probiotic bacterium which has been reported as possessing probiotic properties including secretion of the antimicrobial protein reuterin (Muthukumarasamy et al., 2006) and improving animal growth performance (Agustina et al., 2013). By using natural selection, a strain of *L. reuteri* with stable resistance to antibiotics at concentrations exceeding resistance in lactobacilli colonizing the pig digestive tract was identified. This strain of *L. reuteri* allowed specific enumeration of viable probiotic in porcine feces and gastrointestinal tract after supplementation in feed following encapsulation in a pea protein isolate–alginate matrix or not.

5.5.1 *In vitro* challenge

Generally, lactic acid bacteria show good acid tolerance (van de Guchte et al., 2002). In this study, both wild-type *L. reuteri* ATCC 53608 and the antibiotic-resistant strain, were tolerant to challenge with simulated gastric juice at pH 2.0. However, both strains became susceptible when challenged under SGJ at pH 1.5. The significant protection observed when the pH of SGJ was lowered to 1.5 is consistent with the other studies investigating encapsulation of *Lactobacillus* spp. (Muthukumarasamy et al., 2006; Hassan and Rasco, 2014). Further, PPCs have provided acid protection in other probiotic strains. Klemmer et al. (2011) demonstrated the PPC significantly improved acid tolerance of *Bifidobacterium adolescentis* that was otherwise

highly susceptible to challenge in SGJ (pH 2.0). In the case of simulated intestinal juice (SIJ) challenge, bile salts and pancreatin did not affect the viability of *L. reuteri* used in this study. Instead, SIJ lead to enlarged capsules and may have facilitated the release of encapsulated bacteria (Klemmer et al, 2011a). Given the resistance to SIJ observed for the non-encapsulated *L. reuteri* strain used in this study, it is not surprising that no further enhancement in resistance was observed after encapsulation.

5.5.2 *In vivo* probiotic delivery using pea protein isolate-alginate capsules

In order to confirm that pigs received the same dose of viable probiotic, viability was determined both immediately after mixing with feed and after 24 h in the feed troughs. *L. reuteri* demonstrated significant loss of viability of approximately 1 log cfu /g feed during 24 h in feed at room temperature independent of whether the probiotic was encapsulated or not. Thus, although this observation confirmed that pigs received the same dose of viable probiotic, lack of protection from loss of viability during storage in feed is a significant obstacle to the commercial application of some probiotic strains including lactobacilli, which was not improved by pea protein-alginate encapsulation (Weinbreck et al., 2010). Encapsulation has been previously shown to improve the viable shelf-life of probiotic bacteria when stored frozen but not on storage at room temperature (Heidebach et al., 2010). Given the typical storage times for prepared feeds, loss of 1 log cfu per gram feed per day would not be commercially acceptable, limiting application to mixing with probiotic immediately prior to feeding.

Fecal shedding of antibiotic-resistant *L. reuteri* was observed within 24 h of supplementation consistent with expected. Transit time in the pig digestive tract could be vary by the diet composition and individual pigs and could take from 20 to 102 h to reach to the rectum in pig (Kim et al., 2007). Encapsulation appeared to increase the number of viable *L. reuteri* shed

in feces within 24 h compared to non-encapsulated and supported a further increase in shedding of approximately 0.5 log cfu/g feces over the 3 d feeding period. The observation suggested that encapsulation improved viable probiotic delivery to the distal gastrointestinal tract as previously observed in mice (Iyer et al., 2013). It is unclear why shedding may have increased over time to a greater extent in the encapsulated group. It is unlikely that the efficacy of the capsules improved but perhaps, the delivery of viable bacteria in higher number affected the dynamics of microbial colonization in this complex community permitting *L. reuteri* to occupy a larger niche. Finally, although efforts were made to minimize cross contamination of the probiotic between control and supplemented pigs, low counts of viable antibiotic-resistant lactobacilli were observed in feces of some control pigs 48 h after initiation of the experiment. Similar low levels were found in digesta collected after 3 d of feeding. Those contaminations in the control group may result from transport on air particles within the room or inadvertent contamination carried between pens by staff. Clearly, the design of studies comparing probiotic supplemented groups and control groups should be carefully considered to minimize transfer of the viable organism to control pigs.

Enumeration of antibiotic-resistant *L. reuteri* in digesta collected along the length of the pig gastrointestinal tract indicated an interesting pattern. Whereas as viable antibiotic-resistant lactobacilli counts were relatively static in all locations in LRR-M pigs, counts in LRR-PPC pigs were below LRR-M in proximal regions and above LRR-M in distal regions. Although increased probiotic counts in distal locations for pigs fed LRR-PPC was consistent with higher levels of fecal shedding, low counts in proximal regions were unexpected. However, visual observation indicated the presence of intact capsules in the stomach that were present in declining abundance from proximal to distal regions of the small intestine. The few capsules observed in the ileum

appeared swollen and soft relative to the capsules observed in the stomach. Although there is limited research on the *in vivo* delivery of encapsulated probiotic, *in vitro* results have shown the release of *Bifidobacterium adolescentis* from pea protein-alginate capsules, formulated similarly to the current study, slowly in simulated intestinal juice (SIJ) over a 3-h period (Klemmer et al., 2011b). The release mechanism of PPC was proposed to be mediated by the osmotic change, cleavage of amide bonds by pepsin and trypsin, and physical pressure. When stomach chyme from LRR-PPC pigs was homogenized to disrupt the capsules, similar bacteria counts were observed compared to the LRR-M group without pH adjusted to neutral for the stomach fluids. This increase in counts is consistent with our *in vitro* experience regarding enumeration of encapsulated bacteria where homogenization of capsules increased the recovery of viable counts enumerated on agar by 1 to 2 log cfu/g likely via physical disruption permitting bacteria dispersion on the plate. Given the observation of capsules along the length of the small intestine, it is likely that antibiotic resistant *L. reuteri* enumerated in these locations without homogenization underestimated total counts in LRR-PPC pigs.

Although disruption of capsules increased counts in the stomach to the same level of non-encapsulated bacteria, no increase in viability of antibiotic-resistant lactobacilli was evident in either stomach or upper small intestine. Because the pH of stomach contents from this study was above pH 3.0 at the time of euthanasia (data not shown), these observations are consistent with the tolerance of our *L. reuteri* strain to SGJ (pH 2.0). However, the results do not establish a mechanism by which the capsules improved *L. reuteri* colonization in distal gut locations. Interestingly, acid tolerance in lactic acid bacteria is related to H+-ATPase activity (H+ pump) (Matsumoto et al., 2004) while the survival mechanism from bile is unclear but may be related to triggering removal by an efflux pump (Gunn, 2000). Thus, both acid and bile tolerance

mechanisms require metabolic adaptations and ATP consumption. It is possible that while capsules may not improve viability, they could minimize the energy expenditure and metabolic adaptations required to survive in the upper gastrointestinal tract environment, improving their ability to compete for space and nutrients in the distal gut. Partial support for this could be extrapolated from the observation that loss of viable *L. reuteri* occurred when the pH of SGJ was lowered to 1.5.

5.6 Conclusion

An antibiotic-resistant *L. reuteri* was selected to establish the efficacy of pea protein - alginate capsules for *in vivo* delivery of a probiotic to the gastrointestinal tract of pigs. Although *L. reuteri* are resistant to acid conditions and bile found in the stomach and duodenum, respectively, encapsulation improved probiotic counts in the distal gastrointestinal tract and shed in feces. Encapsulation may be a viable approach to expanding the taxonomic repertoire of bacteria suitable for commercial probiotic application to include strains sensitive to environmental conditions in the upper gastrointestinal tract. Loss of probiotic viability in the feed bunk, however, was not improved by encapsulation. Whether encapsulation could also improve probiotic shelf-life under controlled conditions and or improve resistance to feed processing will require further investigation.

6 GENERAL DISCUSSION AND CONCLUSION

A major obstacle for application of probiotic microorganisms in the animal feed industry is the maintenance of microbe viability during several challenges such as storage, processing and gastrointestinal passage. During probiotic product production and delivery to the animal, dehydration, oxidation, enzymatic, acidic, thermal and mechanical pressure are commonly encountered stressors that may all constitute lethal challenges. Exploring the efficacy of a protective matrix to protect against these challenges was the primary objective of this project. Specifically, we investigated the use of an extrusion method and a pea protein isolate–alginate matrix to microencapsulate bacteria and examined the efficacy of microencapsulation to protect the viability of model probiotic bacteria during challenges associated with probiotic production, storage, incorporation into feed and gastrointestinal delivery.

6.1 Scale-up of encapsulation in a lab setup

The current project required a large quantity of capsules (over 500 g) to permit examination of shelf-life over a 1 year period, to account for loss during the impact of commercial pelleting and to allow for inclusion of coated bacteria in the feed of a large animal. Compared to previous work, a relatively small amount of encapsulated probiotic was required to facilitate *in vitro* testing and small animal trials. Thus, the first objective of our project was to develop and scale up the production of a pea protein based encapsulation method (including freeze-drying) as previous work was mostly conducted using wet, freshly prepared capsules.

A device that could produce capsules of consistent size and shape containing viable bacteria in sufficient quantity for an *in vivo* animal trial in a reasonable time frame was desired. Initial experiments involved extrusion using hand pressure with a large volume syringe (50 mL) as well as attempts to extrude using a peristaltic pump to apply extrusion pressure to the PAS

solution. However, these two methods were slow and too laborious to apply as an approach to produce the large quantities of capsules required. After several modifications, an appliance in which pressurized air could be controlled was set up to force the PAS liquid solution through an extrusion needle. Fine control of air pressure was critical in order to manage extrusion rate, capsule size and uniformity. Nitrogen gas was initially used as the pressurizing gas rather than air when encapsulating anaerobic bacteria to reduce the potential stress from oxygen. However, in subsequent work, no difference was found between nitrogen gas and compressed air on the viability of anaerobic bacteria following extrusion (data not shown).

Using the final design with pressurized air (see **Figure 3.1**), the maximum production of one device was approximately 250 g of dried capsules per day. Using two devices production of roughly 500 g of dried capsules per day was possible. A typical production run required about 12 to 17 working days including, culture media preparation, culture of probiotic, glassware and tube sterilization (4 d), extrusion of wet capsules (1 to 3 d depending on the total quantity required) and freeze-drying (7 to 10 d). The average feed intake for a pig (6 to 8 wk olds) ranges from 0.75 kg to 1 kg per day. When capsules are incorporated at 1 % (w/w) of the feed generating a relatively standard supplementation level of 10^5 to 10^6 cfu/g feed, a 3 d extrusion run was sufficient to supplement 50 kg feed. This amount of feed is sufficient to feed 2 weanling pigs for 28 d.

Options to improve production capacity at the laboratory scale include doubling or tripling the number of appliances or to replace the single extrusion needle with multiple extrusion points (e.g. similar to a showerhead). Adaptations to extrusion methods applied in the literature include scaling up production through jet cutting and electrostatic droplet generation (Petrovic et al., 2007; Burgain et al., 2011). Jet cutting techniques use a cutter under the needle

that consists of a motor and cutting wire to continuously cut the fluids coming out from the needle at a high production rate (Pruβe et al., 1998). Electrostatic droplet generation applies electrostatic forces to disrupt the liquid surface at the needle tip producing small and uniform capsules (Poncelet et al., 1999). These methods could be applied in the future to increase the production and uniformity of capsules during extrusion.

A loss of wall material occurred (approximately 5 % of dry matter) during extrusion and cross-linking as assessed by comparing the dry matter content of PAS and the dry matter recovered in freeze-dried capsules. Presumably, some PAS components (pea protein or alginate) leached into the cross-linking solution and could potentially be recovered, although this was not assessed. Similarly, a loss of bacteria (approximately 0.2 %) into the cross-link solution during production was also noticed because bacteria could be cultured from the cross-link solution.

6.2 Efficacy of pea protein isolate-alginate encapsulation on the viability of probiotic bacterium during freeze-drying and storage

There is limited research investigating the efficacy of encapsulation against loss of viability during freeze-drying (Heidebach et al., 2010) and during storage as dried capsules (Weinbreck et al., 2010). The loss of viability during freeze-drying was significantly improved by encapsulation compared to non-encapsulated bacteria in the *B. adolescentis* group. Encapsulation protected *B. adolescentis* during freeze-drying similar to the addition of milk plus glucose and glycerol, a common commercial practice. However, incorporating milk ingredients in the final products may lead to the allergy or religious concerns for human application. Although *B. adolescentis* is anaerobic, the addition of cysteine-HCl as a reducing agent did not improve viability during freeze-drying or storage as observed by others (Pan et al., 2013). Interestingly, glycerol was an effective additive in protecting against loss of viability. However,

the physical characteristics of glycerol after freeze-drying (sticky or gummy) made this additive undesirable given the impact of these characteristics on product "flow ability". We did not examine a range of concentrations in testing additives, but instead the additive concentration applied in this project were based on industry and literature norms. An examination of a range of concentrations of the additives may be warranted in the context of application in combination with encapsulation.

Limited advantages of PPC were observed in protecting against loss of probiotic viability during storage at different temperatures. The most protective effect from PPC was noted when the core bacteria was *B. adolescentis* and stored at -80 °C regardless of the additive used. It is unlikely that the level of protection observed during -80 °C would be of commercial value given that a simpler approach would be to recommend probiotic feed supplementation at a marginally higher rate accounting for storage losses. The rate of loss of bacterial viability increases as storage temperature increases and the protective effect of encapsulation on storage viability also became less as storage temperature increased. Indeed, no protective effect was observed at storage temperatures above 0 °C for neither *B. adolescentis* nor *L. reuteri*.

Storage of probiotic at sub-zero temperatures to maintain probiotic shelf-life is costly and viable in the animal feed industry only for storage of concentrated product prior to addition to feed. Following incorporation into feed, the volume of product precludes sub-zero storage. Thus, the most immediate challenge for the industry is to protect against the loss of probiotic viability during storage at uncontrolled temperatures experienced after incorporation in to feed and which range from sub-zero to over 40 °C. In our study, a significant loss of viability (about 0.8 log cfu/g) was observed during 24 h of storage at room temperature of encapsulated LR after incorporation in feed during the pig feeding period. Such a rate of loss of bacteria viability would

be unacceptable under commercial conditions where feed might be stored at room temperature for months rather than days.

6.3 Efficacy of pea protein isolate-alginate encapsulation on viability of probiotic bacterium during feed pelleting

Feed pelleting involves heat, steam and physical pressure challenges that are detrimental to bacteria viability. Indeed, pelleting is considered a processing method that could be used to control feed contamination by bacterial pathogens (Jones, 2011). There are few studies reporting the viability of probiotic products after pelleting probably because of the harsh condition employed. Indeed, pelleting conditions offer a hurdle to the maintenance of bioactivity of some feed additives, notably feed enzymes included phytases and carbohydrases (Spring et al., 1996; Ribeiro et al., 2003). In the case of probiotic bacteria, published studies are largely limited to spores from *Bacillus* spp. known to be extremely resistant to harsh environmental conditions. Even so, only two studies have critically assessed the effect of pelleting on the viability of *Bacillus* spores (Jadamus et al., 2001; Amerah et al., 2013) and one other study examined the effect of different pelleting conditions on the survival of encapsulated *E. faecium* (Simon et al., 2005).

In the present study, encapsulation of using PPC provided significant protection during heat challenge (50, 70 and 90 °C) for *B. adolescentis* and *L. reuteri* as well as heat plus pressure (50, 70, 90 °C at 95 MPa) challenge for LR. However, there was lack of sufficient protection from encapsulation for core bacteria during the small commercial pelleting process. The significant reduction in viability during pelleting, compared to heat and pressure challenges, may be due to the second heat challenge at the die (70 to 77 °C) and the addition of steam. In this study, although the conditioner temperature was set at 90 °C, the actual temperature of the

conditioner was lower (64 °C without steam and 84 °C with steam) due to machine limitations. Even at a lower temperature of 64 °C, there was an approximately 4 log cfu/g reduction in viability for encapsulated and non-encapsulated bacteria groups. Therefore, PPC did not provide protection against loss of viability during steam pelleting. It will be difficult to develop an encapsulation protocol for vegetative bacteria that is able to protect against harsh pelleting conditions under steam, while also being capable of disintegrating in the gastrointestinal tract to release the organism into distal locations.

For probiotic bacteria, post-pelleting application methods should be investigated as an alternative to inclusion prior to the pelleting step. Examples including reconstituting dehydrated probiotics in water and spraying on feed after pelleting or even just before feeding may be commercially viable (Owunmi et al., 2016). Also, vacuum coating has been applied in the pet food industry to add probiotics after extrusion, an approach that could also be applied post pelleting (Kirejevas, 2007; Pascher et al., 2008). However, in addition to evaluating the impact of post pellet sprays or vacuum coating processes on probiotic viability, attention should be given to the potential for product separation from feed particles and sedimentation. Zhang, et al., (2016) indicated that a particle size larger than 1.5 mm is not recommended for incorporation with the feed due to sedimentation. This may be particularly problematic in the case of capsules of the size employed here, where sedimentation through feed, even in the case of mash is a likely possibility.

6.4 Efficacy of pea protein isolate-alginate encapsulation on viability of a probiotic bacterium in the porcine digestive tract

The final target of delivery of a probiotic product is the animal digestive tract. Encapsulation of PPC significantly protects *B. adolescentis* and *L. reuteri* during *in vitro*

gastrointestinal tract challenge as observed here and by others (Wood, 2010). Moreover, PPC showed significant protection of *L. reuteri* in pig digestive tract. The *in vivo* trial reported here showed that PPC carried and protected *L. reuteri* in the upper gastrointestinal tract and released it in the distal ileum. Compared to the non-encapsulated bacteria group, the PPC group successfully delivered more bacteria into the distal gastrointestinal tract resulting in increased shedding of the antibiotic resistant bacterium in feces. In the current study, the animal trial was a short feeding trial (3 d on the experimental diet) focussed on evaluating the delivery system and the specific recovery of viable probiotic in feces and along the gastrointestinal tract. We did not perform a long-term trial comparing the efficacy of probiotics on performance or health outcomes largely due to the variability of conditions under which probiotic supplementation results in positive responses. We, therefore, focussed on selecting a bacterium we could specifically track through antibiotic resistance. The species of bacterium was selected is commonly used as a probiotic organism. However, there was no consideration whether this strain would provide a health benefit.

The development of an effective and specific recovery method for the animal trial was a challenge in the current study. As noted above, whereas molecular detection methods can be specific they lack the ability to assess viability and whereas culture-based methods permit assessment of viability, they lack specificity. The approach to select a stable, antibiotic-resistant bacterium to permit specific recovery of the encapsulated bacterium followed our experience with a separate study not reported here in which we attempted to assess recovery of encapsulated *B. adolescentis*. Both molecular (specific primer by qPCR) and selective culture (Beerens' agar) based methods were used to recover *B. adolescentis* from pigs that were fed diets containing encapsulated *B. adolescentis* or non-encapsulated *B. adolescentis*, with or without the addition of

FOS. The qPCR method could specifically detect the probiotic at the species level, but it could not differentiate between a probiotic that remained in capsules versus a probiotic that was viable and released. In the case of Beeren's agar, the media was not selective for the probiotic species, which allowed for growth of bacteria species that normally reside in the pig intestine. Accordingly, these analyses did not permit clear interpretation of the efficacy of PPC in delivery and release of viable probiotics.

We investigated the potential to encapsulate other compounds using PPC with the intention of examining the efficacy of PPC to deliver bioactive payloads to the distal intestine (not reported here). We also encapsulated several different molecules including lysozyme, a fluorescein dye and butyrate selected to reflect a range of molecule sizes and chemistry, potential to serve as a capsule tracking system or potential as a beneficial gastrointestinal bioactive. However, the incorporation rate of these molecules (lysozyme 7.9 %, fluorescein isothiocyanate 4 %, butyrate 13-17 %) with capsules was very low and would likely preclude any commercial application.

We were able to produce small amounts of PPC containing food blue dye #1 sufficient to conduct a small *in vivo* trial in poultry with the intent of visually assessing capsule disintegration along the track. As shown in **Figure 6.1A**, intact capsules could be easily identified in the contents collected from the crop. Interestingly, we were able to confirm the effective physical grinding capacity of the avian gizzard as shown in **Figure 6.1B**. The photograph shows the food dye-stained interior lining of the gizzard of chickens fed capsules containing dye and an absence of intact capsules in contents recovered from the gizzard or from distal locations. Although this study did not evaluate of the efficacy of capsules for *in vivo* payload delivery, we did identify poultry as an unsuitable model for this type of capsule.

A B

Figure 6.1. Pea protein isolation-alginate capsules containing food dye in chicken digestive tract. A) Digesta in crop; B) Digesta in gizzard.

6.5 Overall summary and conclusions

An extrusion appliance and methodology were devised to scale-up production of pea protein isolate-alginate based capsules in quantities adequate to conduct efficacy testing in near commercial scale feed processing and in a large animal. This process enabled encapsulation of live probiotic bacteria efficiently. However, encapsulation of bioactive proteins or small molecules occurred with very low incorporation rates. The capsules were effective in protecting bacteria against loss of viability during freeze-drying. However, the efficacy of this capsule to protect probiotic bacteria from loss of viability during storage, especially above 0 °C, was limited. Although encapsulation protected probiotic bacteria during heat and pressure challenge, the capsules were not able to protect the viability of probiotics during pelleting, even at relatively low pelleting temperatures, compared to the industry norm. Lastly, this work was the first to demonstrate that pea-protein-based encapsulation enhanced the viability and colonization of probiotic bacteria delivered to the distal digestive tract when supplemented in the feed of

growing pigs. Therefore, whereas pea-protein based capsule may be useful in delivering sensitive bacteria to the distal gastrointestinal tract of target animals, viability during processing and storage remain a major challenge to commercial application.

6.6 Future studies

An improvement of encapsulation structures, encapsulation methods was recommended by Chen et al., (2017) for future probiotics encapsulation. In this project, although pea protein isolate-alginate encapsulation showed potential to enhance probiotic viability during some of the challenges probiotics experience during animal feed production, this technology alone will not address all challenges without further development and additional innovation. Other encapsulation methods that should also be investigated include emulsion-based methods that produce smaller-sized capsules. However, decreased capsule size could compromise the protection of core bacteria (Burgain et al., 2011). Capsule sizes (diameter) smaller than 100 μm are ideal for the incorporation of encapsulation in food products in order to prevent their effect on food texture (Talwalkar and Kailasapathy, 2004). The balance between effective protection and capsule size requires further investigation.

A systematic evaluation of capsule formulation to maximize efficacy in protection of viability during storage and passage through the digestive tract is required. Due to time and cost, we were unable to evaluate variations in capsule composition including protein content and source, oligosaccharide content and source, and combinations of preservatives. A variety of formulations have been examined in the literature but there has not been a systematic comparison of preservative concentration, source of wall materials and additive combinations. Furthermore, studies have examined different bacterium or groups of bacteria and applied different controls,

preventing conclusions on whether specific formulations should be employed with specific species or strains.

During initial studies, FOS was included in the matrix formulation as a prebiotic so that capsules protected probiotic viability against challenge and supported probiotic colonization in the gastrointestinal tract by serving as a preferred fermentation substrate. Products formulated to contain both prebiotic and probiotic have been termed "synbiotics" reflecting an expected synergy versus providing either prebiotic or probiotic benefits alone (Bielecka et al., 2002). As noted above, we abandoned use of *B. adolescentis* as a model probiotic in favour of developing an antibiotic-resistant *Lactobacillus* to permit specific tracking of live bacteria during gastrointestinal tract passage. Because FOS is a preferred substrate for *Bifdiobacterium* species, its evaluation as a prebiotic in the case of a *Lactobacillus* payload was not warranted. Nevertheless, the efficacy of incorporating prebiotic compounds specific to the capsule payload requires further investigation.

This work identified that a lack of protection during storage is the primary barrier to future probiotic development in animals. Investigation of a second layer of other wall components could be considered as a means of adding additional protection. Examples include an additional coating, such as chitosan or wax. This idea is based on the physical structure of PPC, which has several pores on the surface. An extra coating could block the pores reducing the contact of core bacteria with the outside environment and strengthen the wall matrix. Chitosan (as polycations) could further stabilize alginate-Ca^{++}structure (Krasaekoopt et al., 2006). Chitosan-coated alginate bead reportedly improves bacteria survival during acidic challenges and storage (Chávarri et al., 2010; Varankovich et al., 2017). Wax on the other hand, has been

reported as a good oxygen and moisture barrier (Weinbreck et al., 2010), and warrants further investigation.

The steam pelleting process generates harsh environment conditions posing a substantial challenge to the protection of sensitive bacteria by encapsulation. Thus, research efforts should be directed towards the post-pellet application of probiotics. Options including post pelleting spray application as well as vacuum coating for heat-sensitive feed additives (Cao et al., 2007; Lamichhane, 2015). Post extrusion vacuum coating of probiotics has been reported in extruded pet food but detailed information is patented (Cavadini et al., 1999; verfügbar, 2007). Vacuum coating of encapsulated probiotics may have potential but there is currently no research on vacuum coating for probiotics after pelleting. Post-pellet vacuum coating may reduce the heat stress on probiotic products. However, additional protection is still needed, such as the encapsulation of probiotics during feed storage and delivery in the animal gastrointestinal tract. Furthermore, the encapsulation method applied in the current study may have limited application because the average size of capsules was 2 to 3 mm, which is unlikely to penetrate into a pelleted feed matrix. A smaller sized capsule for probiotics will be needed for the vacuum coating study.

Animal species tested in this thesis was limited to swine and poultry. As noted above, the gizzard in poultry represents a separate challenge for the application of probiotics in this food animal species because the gizzard may not be well suited for the relatively large capsules generated by extrusion. Given that the gizzard lumen is an acidic environment (2.7 to 4.1), physical capsule disruption and release of the payload is likely. An examination of the fate of other types of capsules in the gizzard environment would be interesting given that poultry are a major target for probiotic intervention. Interestingly, given that the current extruded capsule

formulations tended to float in aqueous phase, their use to deliver probiotic bacteria as a pond surface feed for aquatic fish species (e.g. trout) may be of interest.

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APPENDIX A

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