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**Efficacy of Pea Protein Isolate-Alginate Encapsulation on Viability of a Probiotic
Bacterium in the Porcine Digestive Tract**

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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.

Keywords: Encapsulation, probiotic, microbiota, swine, *in vivo*

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 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. 2011; 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. 2011; 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. 2011).

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 *Alteromonadaceae shewanella* coated with alginate could be recovered from the ileum of Senegalese sole whereas non-encapsulated *A. shewanella* could not (Rosas-Ledesma et al. 2012). Similarly, chitosan-coated alginate capsules increased *Lactobacillus spp.* abundance in mouse (Iyer et al. 2013) and chicken intestine (Rodklontan 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.

MATERIALS AND METHODS

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

*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 (purchased from ATCC, USA) was inoculated into 10 mL MRS broth and cultured for 24 h at 37°C 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°C.

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% N₂, 10% CO₂, and 10% H₂) for 32 h at 37°C.

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 37°C. 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 days. Colonies formed on MRS (AB+) were enumerated each day to establish retention of antibiotic-resistant LRR.

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 (Propulse™ 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. 2011). Briefly, pea protein isolate (PPI) was dissolved into ddH₂O (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°C. 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°C 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 20 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% CaCl₂ and 1% Tween 20 (Fisher, New Jersey, USA)]. After a 30 min hardening time, the capsules were filtered by filter paper and collected in aluminum trays. All LRR-PPCs and LRR-M were vacuum freeze-dried for 5 days 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 °C until use (**Figure 1**).

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°C for 2 h or simulated intestinal juice [SIJ: 1.25% NaHCO₃ (Fisher, New Jersey, USA), 0.6% (w/v) Difco™ Oxgall (Becton, Dickson and Company, Sparks, MD), 0.09% Pancreatin (Sigma, Oakville, ON)] for 3 h (Klemmer, 2011). 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°C anaerobically for 48 h to enumerate the viable *L. reuteri*.

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°C. 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°C to permit enumeration of LRR.

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-weighted 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-weighted 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 5s in order to physically disrupt capsules.

Statistical analysis

The data analysis was processed by SAS 9.1.3 using the mixed procedure (Statistical Analysis Software, version 9.1.3, SAS Inst. Inc, Cary, NC, USA). 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.

RESULTS

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 days 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.

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 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).

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 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.

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 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 ds 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.

Enumeration of viable LRR in digesta

Figure 2 shows counts of viable antibiotic-resistant lactobacilli in intestinal contents recovered along the gastrointestinal tract. 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.

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; Piatek et al. 2012; Lotfipour 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.

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 (2011) demonstrated the PPC significantly improved acid tolerance of *Bifidobacterium adolescentis* that was otherwise highly susceptible to challenge in SGJ (pH 2.0) (Klemmer et al. 2011). 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 2011). 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.

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 (Latymer et al. 1990; 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 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. 2011). 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.

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.

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Conflict of Interest: Authors declare no conflict of interest.

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TABLES AND FIGURES

Figure 1. Pea protein isolate-alginate capsules (A: Wet capsules after cross-linking and filtering B: Freeze dried capsules).

Table 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	<i>P</i>
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.

Table 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 days^b.

	Treatment		Time (h)		<i>P</i>		
	LRR-PPC	LRR-M	0	24	Treatment	Time	Interaction
Enumeration	5.88 ± 0.2	5.89 ± 0.15	6.29±0.08	5.48 ± 0.06	0.9428	<0.001	0.3414

^aAB+MRS: de Man, Rogosa and Sharpe media containing rifampin (200 µg/mL) and streptomycin (2000 µg/mL).

^bResults are Mean ± SEM for triplicate determinations.

Table 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 resistance lactobacilli at 4.60 log cfu/g. On day 3, two pigs out of eight pigs shed antibiotic resistance 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.

Figure 2. Antibiotic-resistant lactobacilli (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).