Novel feed enzyme delivery method: Binding exogenous feed enzymes to *Bacillus* spores to improve enzyme stability and total tract digestibility

by

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Abstract

The effects of binding feed enzymes to *Bacillus subtilis* (strain RK28) spores on enzyme stability and digestion in cattle were studied in comparison to unbound enzymes. Optimal conditions for adsorbing six different enzymes (α-amylase, β-glucanase, feruloyl esterase, phytase, purified xylanase, and a commercial crude xylanase) to spores were first determined. Spore-bound enzymes were assessed using *in vitro* ruminal batch cultures for their impact on the degradability of feed substrates, including alfalfa: grass hay and a mixture of barley silage and concentrate (75:25 dry matter basis). Each enzyme was examined individually and in combination in either an unbound or spore-bound form. The batch culture studies were used to identify the most effective spore-bound enzyme for use in an *in vivo* cattle study. A commercial xylanase was selected to test the administration of spore-bound enzyme on digestibility of feed in the rumen, post-rumen, and total digestive tract of cattle fed an alfalfa: grass hay diet at 1.15% of body weight. The ability of spores to protect bound enzymes in temperatures representing feed processing, and in simulated gastric and intestinal environments, was also tested *in vitro*. The optimal spore-binding conditions varied according to enzyme being tested, as did the amount of enzyme that could be bound to spores and the retained activity of enzymes once bound to spores. Generally, the addition of enzymes to batch cultures improved degradability, and combinations of enzymes were marginally better than individual enzyme treatments. Spore-bound enzymes did not improve *in vitro* degradation or fermentation parameters compared with unbound enzyme. An exception to this was the spore-bound commercial xylanase, which reduced methane production and had greater xylanase activity during batch cultures, compared with unbound enzyme. When fed to cattle, there were no differences in *in situ* degradability variables for unbound compared with spore-bound commercial xylanase ($P \geq 0.315$). Ruminal digestibility of

organic matter decreased and intestinal digestibility increased, for spore-bound enzyme compared with unbound enzyme ($P \leq 0.028$). In addition, there was a tendency for total tract digestibility of neutral and acid detergent fiber to be 4.9 and 4.0% greater for spore-bound enzyme compared with unbound enzyme ($P \leq 0.082$). Incubating unbound commercial xylanase in intestinal fluid resulted in rapid degradation (>85%) within 3 h, whereas spore-bound enzyme activity decreased to a lesser extent (15%) and there was evidence of enzyme release by spores in the intestines (approximately 125% of 0 h activity after 6 h incubation). Throughout feed processing conditions, spore-bound enzymes also had increased stability compared with unbound enzymes. Thus, adsorption of feed enzymes to spores has potential to increase enzyme stability throughout feed processing and the gastrointestinal tract of ruminants resulting in increased total tract digestibility of neutral and acid detergent fiber.

Preface

This thesis is an original work by C. Rosser. The research chapters in this thesis are not currently published; however, manuscripts have been prepared for publication and are awaiting approval from funding agencies, subsequent to intellectual property evaluation.

C. Rosser was involved in developing all experimental designs, conducting initial lab experiments, *in vitro* experiments, *in vivo* experiments, sample analysis, data analysis, and drafted the manuscripts. T. Alexander was involved in direct supervision of C. Rosser at the Lethbridge Research and Development Centre, Agricutlure and Agri-Food Canada, and provided support throughout the experiments described in this thesis. M. Oba was C. Rosser's supervisor at the University of Alberta. In addition, T. Alexander, M. Oba, and K. Beauchemin were involved in critically evaluating the experimental designs, and editing this thesis and research manuscripts.

All procedures that involved animals followed the guidelines of the Canadian Council of Animal Care (Ottawa, Ontario, Canada). The experimental protocol described in Chapter 6 was reviewed and approved by the Lethbridge Research and Development Centre Animal Care Committee (Protocol TA1611).

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List of Abbreviations

ACX	combination of α-amylase and commercial xylanase
ADF	acid detergent fiber
ADG	average daily gain
ALL	all other treatments
AMYL	α-amylase
BcoaE	*Bacillus coagulans* spore-bound enzyme
BSA	bovine serum albumin
BsubE	*Bacillus subtilis* spore-bound enzyme
CBM	carbohydrate binding module
CON	control treatment
CP	crude protein
CX	commercial xylanase enzyme
D	potentially degradable fraction
DM	dry matter
DMI	dry matter intake
DNA	deoxyribonucleic acid
ED	Effective degradability
EDADF	Effective degradability of acid detergent fiber
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDDM	effective degradability of dry matter

EDNDF effective degradability of neutral detergent fiber

EDOM effective degradability of organic matter

EDTA ethylenediamine tetraacetic acid

ENZY enzyme

FE feruloyl esterase enzyme

FreeE Unbound enzyme treatment

GFACX combination of β-glucanase, feruloyl esterase, α-amylase, and commercial xylanase

GFAX combination of β-glucanase, feruloyl esterase, α-amylase, and pure xylanase

GIT gastrointestinal tract

GLUC β-glucanase

GM genetic modification

I-SBE individually spore-bound enzymes

I-SB-GFACX individually spore-bound β-glucanase, feruloyl esterase, α-amylase, and commercial xylanase which are individually bound to spores then combined

I-SB-GFAX individually spore-bound β-glucanase, feruloyl esterase, α-amylase, and pure xylanase which are individually bound to spores then combined

K_d rate of degradation

K_p rate of passage

NDF neutral detergent fiber

NH_3 ammonia

NHS *N*-hyrdoxysulfosuccinimide

OM organic matter

PHYT commercial phytase

SB-ACX spore-bound combination of α-amylase and commercial xylanase

SB-AMYL spore-bound α-amylase

SB-CX spore-bound commercial xylanase

SBE spore-bound enzyme

SB-FE spore-bound feruloyl esterase

SB-GFACX spore-bound combination of β-glucanase, feruloyl esterase, α-amylase, and commercial xylanase

SB-GFAX spore-bound combination of β-glucanase, feruloyl esterase, α-amylase, and pure xylanase

SB-GLUC spore-bound β-glucanase

SB-PHYT spore-bound phytase

SB-XYL spore-bound xylanase

SEM standard error of the mean

SGF simulated gastric fluid

SIF simulated intestinal fluid

SNT supernatant

Sol soluble fraction

SPR spore

T0 lag time

TMR total mixed ration

TOT total sample (spore-bound enzyme and supernatant combined)

Undeg undegradable fraction

VFA volatile fatty acids

V_{max} maximum reaction rate

WASH wash

XYL pure xylanase enzyme

1.0 Introduction

By 2050, global crop demand is predicted to rise to 100-110% of levels achieved in 2005 (Tilman 2011). Increased demand is expected to result from population growth and increasing affluence in emerging economies leading to increased consumption of meat and milk protein (Keyzer et al., 2005; Alexandratos and Bruinsma, 2012). Greater demands for animal protein present environmental challenges for agriculture, including increased production of greenhouse gases, livestock waste, reduction in available agricultural land, and antimicrobial use associated with intensive livestock production practices (Van Boeckel et al., 2015; Ribeiro et al., 2016). Thus developing technologies to improve the efficiency by which livestock utilize feed would help attenuate the environmental pressures of greater animal meat production. In addition, because feed expenses account for approximately 60 to 70% of the total cost of livestock production, better feed efficiency would also have a significant impact on the profit margins of producers (Barletta, 2010).

Enzymes catalyze chemical and biological reactions, decreasing reaction time, energy input and cost of production. Increased efficiency from the application of enzymes has resulted in their wide spread use throughout the pulp, biofuel, chemical, food, and feed industries (Singh et al., 2016). In 2014 it was estimated that the global market for enzymes accounted for US$5.1 billion, with it forecasted to reach US$11.3 billion by 2020 (Kumar et al., 2014). It has been estimated that feed enzymes account for approximately 20% of this market share (Li et al., 2012). The majority of the feed enzyme market is currently in North America; however, large growth in the Asian market has been predicted due to increasing meat demand.

While administration of exogenous enzymes to improve nutrient digestibility has been successful in monogastrics (Bedford, 2000), responses in ruminants have been variable (Beauchemin et al., 2003). This variability is partially due to the lack of stability of these enzymes, both during feed processing and throughout the gastrointestinal tract. Feed enzymes may be exposed to elevated temperatures during pelleting of dietary ingredients and delivery to animals. In ruminants, consumed feed enzymes are subject to denaturation and degradation in the highly proteolytic environment of the rumen, with some of the activities lasting only several hours (Morgavi et al., 2000b). Thus increasing the stability of feed enzymes would help improve their effectiveness for cattle.

Bacillus spores have been used to immobilize enzymes and increase their stability after adsorption to the spore coat (Huang et al., 2010). Spore-bound proteins have been shown to retain structure after incubation at elevated temperatures (Lee et al., 2010) and passage to the murine small intestine (Song et al., 2012). Studies have also shown that the digestive enzymes phytase (Potot et al., 2010) and ß-galactosidase (Sirec et al., 2012) are functional when anchored to *Bacillus* spore coats. The application of *Bacillus* spores for delivering feed enzymes to cattle is a novel and attractive approach to enhancing digestion for several reasons. *Bacillus* probiotics have a history of safe use in humans and animals, and are commercially available as low-cost direct fed microbials. In addition, binding of proteins to *Bacillus* spores offers a non-transgenic approach to delivering feed enzymes, and avoids regulatory issues resulting from recombinant bacteria as delivery methods. *Bacillus* spores are also heat-resistant and bound proteins have been shown to retain structure after exposure to 45°C for more than 12 months (Lee et al., 2010), making them suitable for long-term storage and incorporation into processed feeds. The overall

objective of this thesis was to develop and test the effectiveness of *Bacillus* spores as a feed enzyme delivery system.

2.0 Literature Review

2.1 Livestock feed

Livestock feeds are inherently variable and include forages, grains, plant by-products, and animal by-products, and require complex digestion processes to break down the chemical constituents. The main components of a plant cell wall are cellulose, hemicellulose, and lignin (Van Soest, 1994). Cellulose is made up of linear glucose chains which are connected by β-1,4-linkages, and which interact with other cellulose chains forming crystalline structures (Ribeiro et al., 2016). Hemicellulose is more complex, containing a xylan core, and various sugar residues that form branches (Van Soest, 1994). Lignin is a complex polymer of aromatic alcohols that can interact with polysaccharides, and due to these interactions, lignin is considered one of the main limiting factors to cell wall digestion (Jung and Allen, 1995). The major carbohydrate present in cereal grains is starch (Beauchemin and Holtshausen, 2010), which is surrounded by the outer layers of the grain, including the aleurone. The main source of phosphorus in grain is in the form of phytate, which is minimally digested in monogastric animals (Walz and Pallauf, 2002).

Monogastric and ruminant animals have differences in their digestive tracts, which impacts what they are fed and the extent of digestion. On average, poultry and swine digest 75 to 85% of their feed dry matter (**DM**; Barletta, 2010), but are typically fed diets with lower complex fibre content. The rumen is highly efficient in degrading lignocellulosic biomass (Flint et al., 2008) and has an incredibly diverse microbiota with overlapping metabolic functions to digest feeds (Bensoussan et al., 2017). Despite this diversity, digestion is incomplete in ruminants. For example, dairy cattle digest 62 to 83% of organic matter (**OM**) and 41 to 83% of the neutral detergent fiber (**NDF**) (Huhtanen et al., 2009). Thus for both monogastric and ruminant animals,

there is a large potential to increase the digestibility of feed and technologies to increase feed utilization are needed.

2.2 Enzymes in livestock feed

The addition of enzymes in livestock feeds can increase both nutrient digestibility and animal performance, reducing the negative environmental impacts associated with animal waste (Barletta, 2010). Due to the complexity of livestock feeds, a multitude of enzymes are required for digestion of the feed. Major enzymes that are involved in cellulose hydrolysis include endoglucanases, exoglucanases, and β-glucosidases (Bhat and Hazlewood, 2001), whereas major enzymes needed for hydrolysis of the xylan core of hemicellulose are β-1,4-xylanase, and β-1,4-xylosidase (Bhat and Hazlewood, 2001). Enzymes that degrade hemicellulose side chains include mannosidases, arabinofuranosidases, glucuronidases, galactosidases, acetyl-xylan esterases, and feruloyl esterases (Beauchemin and Holtshausen, 2010). Starches are degraded by amylases, and phytate can be degraded by phytases (Walz and Pallauf, 2002).

Commercial enzymes are produced by microbial fermentation, where the final enzyme product is separated from fermentation residues, and the source organism. This means that there are no live cells or potentially genetically modified (**GM**) deoxyribonucleic acid (**DNA**) present in the final product (Beauchemin et al., 2003). The types of enzymes, and enzyme activity produced are dependent on the microbe (fungal or bacterial), microbial strain, and fermentation parameters used during production. The main enzyme products used in livestock production are sourced from fungi (*Trichoderma reesei*, *Aspergillus niger*, *Aspergillus oryzae*, and *Saccharomyces cerevisiae*) and bacteria (*Bacillus subtilis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, and *Streptococcus faecium*), (Muirhead, 1996; Pendleton, 1996).

In typical feeding situations exogenous enzymes supply approximately 5 to 15% of the total enzyme activity present in the rumen (Wallace et al., 2001). Even though this is a small amount, exogenous enzymes and rumen microbial enzymes can be synergistic, resulting in greater improvements in degradation within the rumen (Morgavi et al., 2000a). The ability of enzymes to improve digestibility and performance in ruminants has been variable; however, generally animals that have high energy requirements (i.e. dairy cows in early lactation, or finishing beef cattle) benefit from the addition of enzymes to feed (Beauchemin and Holtshausen, 2010).

Some enzymes are endogenously produced by the animal, or its inherent microorganisms (i.e. amylases, proteases), yet addition of exogenous forms of these same enzymes may increase animal performance, but not always. Addition of these exogenous enzymes to diets may not directly improve nutrient digestibility, but can reduce the amount of endogenous enzymes needed to be produced. This results in a potential decrease in maintenance energy and protein requirements of the animals and microbes, allowing more energy and amino acids to be utilised for growth and performance (Cowieson et al., 2003).

Exogenous enzymes generally have optimal activity at high temperatures and low pH, yet these conditions are not present within the gastrointestinal tract of livestock. Therefore, it may improve enzyme performance if feed enzymes could be designed for functionality in the gastrointestinal environment of livestock. There are three main types of enzymes that are utilised in livestock production: carbohydrases, proteases, and phytases (Barletta, 2010).

2.2.1 Carbohydrases

Carbohydrases are enzymes that catalyze the breakdown of carbohydrates into simpler sugars, and these enzymes can target either starch or non-starch polysaccharides. Starch is considered a

simple carbohydrate, containing glucose molecules linked together through alpha-linkages that can be broken down by mammalian enzymes. Fiber is a complex carbohydrate that is found in plant cell walls, and contains hemicellulose, cellulose, and lignin. The monomers are generally linked with beta-linkages and require microbial enzymes for catalysis. Depending on diet, less than 65% of dietary NDF is digested by ruminants (Van Soest, 1994).

Amylase is a starch-degrading enzyme, which is naturally released from the pancreas, and is present in the saliva of some mammalian species. The production of amylase is decreased in young animals compared to mature animals, therefore supplemental amylase has the potential to improve starch digestibility and animal performance in immature animals (Selle, 1996). In addition, supplementation of high-grain diets with amylase can improve total DM digestibility in some livestock species (Tricarico et al., 2007).

Fibrolytic enzymes include a range of classes, including xylanase/xylosidase (involved in hemicellulose hydrolysis), and glucanase/glucosidase (involved in cellulose hydrolysis; Bhat and Hazlewood, 2001). Other fibrolytic enzymes can degrade hemicellulose side chains, as previously discussed. In ruminants, multiple variants of these enzymes are encoded by bacteria, protozoa, and fungi, which function to degrade fibre in conjunction with host attributes including, mastication, buffering, and removal of waste products. More than 80% of fibrolytic ruminal activity is associated with microbial biofilms on feed particles in the rumen (McAllister et al., 2000). The main site of activity for both β-glucanases and xylanases in poultry is the small intestine. The intestinal pH does not degrade the enzymes and the retention time in the small intestine of broiler chickens is relatively long, approximately 1.5 to 2 h (Svihus, 2011). The addition of exogenous enzyme to broiler chicken diets can increase not only starch digestion, but

also apparent metabolizable energy due to a reduction in non-starch polysaccharides (Gracia et al., 2003).

2.2.2 Proteases

Proteases are enzymes that breakdown proteins. Use of exogenous proteases is more important in monogastric animals than in ruminants. Exogenous proteases are generally not used as feed additives for ruminants because the rumen is naturally proteolytic. There are two main proteins present in some feed ingredients such as soybeans which inhibit digestibility: trypsin inhibitors and lectins (Barletta, 2010). Trypsin is an enzyme produced by the pancreas to activate proenzymes, and once these proenzymes are activated they can digest proteins in the intestinal digesta. Trypsin inhibitors reduce trypsin activity; therefore, proenzymes cannot be activated, resulting in reduced protein digestibility (Anderson and Wolf, 1995). Lectins are proteins which bind to sugars on the intestinal epithelial cells, reducing digestibility and absorption of nutrients in the intestinal tract and increasing endogenous protein losses (Vasconcelos and Oliveira, 2004). Because trypsin inhibitors and lectins are both proteins, it is possible to denature them through heat processing (Anderson and Wolf, 1995). However, heat processing can result in the formation of Maillard reaction products, as well as denaturation of other proteins, and bioactive molecules (Inborr and Bedford, 1994). These side effects of heat treatment can reduce animal performance (Silversides and Bedford, 1999). An alternative to heat processing is the addition of proteases to diets, which break down trypsin inhibitors and lectins without having significant negative impacts on other proteins present in the feed (Ghazi et al., 2002). It is interesting to note that protease supplementation in beef cattle diet decreased DM digestibility compared to a control (McGinn et al., 2004). The decrease in digestibility in beef cattle was likely due to alterations in microbial populations and inactivation of some endogenous enzymes.

2.2.3 Phytases

Phytate is a form of phosphorus in plants which can be degraded by phytase; however, monogastric animals produce minimal phytase activity resulting in low availability of phytate phosphorus (Walz and Pallauf, 2002). Selle et al. (2010) summarised that, for common grain sources, phytate as a percent of total P ranged from 60 to 80%. This high content of phytate P results in only 1 to 49% of P being bioavailable for swine (Cromwell, 1992). In addition to reducing P bioavailability, phytate also has the ability to form complexes with other minerals, proteins and starches making these nutrients unavailable for absorption (Selle et al., 2010). Approximately 2/3 of industrial poultry and swine feeds in North America and Europe contain phytase (Barletta, 2010) to improve nutrient digestibility and animal performance, while reducing phosphorus excretion into the environment. The addition of phytase to monogastric diets can decrease phosphate excretion by up to 50% (Greiner and Konietzny, 2010). Excess phosphorus excretion causes eutrophication, which has a significant negative impact on the environment (Daniel et al., 1998). In some countries and states, there is legislation in place to reduce the excretion of phosphorus to the environment, and without the use of phytases, a large proportion of the phosphorus excreted is in the form of indigestible phytate (Barletta, 2010).

Phytase is naturally produced by microbes that are present in the rumen, and is generally produced in adequate amounts. There is a lower concentration of phytate present in forage compared to grains (Ravindran et al., 1994); therefore, with increasing concentrations of grain there is an increasing potential for utilization of phytases in ruminant diets. This is particularly true for barley based diets, which contains phytate in the aleurone layer compared to corn grain which contains phytate in the germ (Beauchemin and Holtshausen, 2010).

2.2.4 Other enzymes

Other enzymes can be utilised in livestock production to improve digestibility and animal performance. For example, feruloyl esterase (ferulic acid esterase) can degrade the ferulic acid bridges that are present between lignin and the cell wall polysaccharides (Jung and Allen, 1995). Applying an exogenous enzyme with ferulic acid esterase activity to poor quality forages increased *in vitro* NDF degradability (Krueger et al., 2008). In addition, many commercial enzymes have a variety of enzyme activities present. This is due to co-extraction of enzymes produced by bacteria or fungi during the production of target enzymes.

Cellulosomes are very organized complexes containing multiple enzymes, and are present on the cell surface of some cellulolytic bacteria (Artzi et al., 2017). Along with enzymes these complexes also can include carbohydrate binding modules (**CBM**) and scaffoldins. The CBM is a module that binds to the substrate, allowing the enzymes on the scaffoldins to degrade the substrate. Scaffoldins are structures that interact with enzymes and CBM, allowing close interaction between substrates and multiple enzymes (Artzi et al., 2017). The presence of scaffoldins result in efficient degradation of substrate by promoting close proximity of synergistic enzymes, without inhibition due to steric hindrance (Fierobe et al., 2002). Although feed enzymes based on cellulosomes have not been used in livestock studies, the concept of adding enzyme mixtures to promote feed degradation has been tested (Wealleans et al., 2017). In addition, research on mimicking cellulosomes by genetically engineering enzyme complexes has been conducted for application in industrial processes (Blanchette et al., 2012; Kim et al., 2012).

2.2.5. Enzyme effectiveness in ruminant diets

The main enzymes used in ruminant diets are cellulases and xylanases; however, responses have been variable in terms of increasing digestibility and performance (Beauchemin et al., 2003). Colombatto et al. (2002) determined that the protein content of enzyme products, and subsequent enzyme activity, can explain approximately 60% of variability in effectiveness, where increasing protein content improves effectiveness. This increase in effectiveness was likely due to greater concentration of enzyme activity within the product, therefore addition of 1 ml of enzyme product containing high protein content would be more effective than 1 ml of product with low protein content. Feed substrate also plays an important factor in variability of enzyme effectiveness, where β-glucanase activity of an enzyme has a large impact on effectiveness when applied to alfalfa hay, whereas β-glucosidase, xylanase, and amylase activity are more important when applied to corn silage (Colombatto et al., 2002). Colombatto et al. (2003) only found one commercial enzyme product, out of 26 products, that improved *in vitro* digestibility in both alfalfa hay and maize silage.

In addition to the biochemical characteristics of an enzyme, dose rate affects the ability of enzymes to increase digestibility and performance in ruminants. Beauchemin et al. (1995) found a nonlinear dose response in digestible dry matter intake (**DMI**) and acid detergent fiber (**ADF**) digestibility when applying a xylanase and cellulase enzyme to alfalfa hay, timothy hay or barley silage. Nsereko et al. (2002) suggested that the decreased response to higher enzyme application rate may be due to the exogenous enzymes attaching to feeds at such an extent that they reduce binding sites for rumen microbes to attach and initiate microbial degradation.

Another major factor affecting detected effectiveness of exogenous enzymes is animal production levels. In general, the addition of enzymes to diets of cattle with high energy requirements (i.e. early lactation dairy cows or growing cattle), has the greatest potential to

improve digestibility and performance (Beauchemin et al., 2003). In order to obtain high levels of productivity these animals require high feed and energy intakes, which can be limited by rumen fill caused by fiber (Dado and Allen, 1995). Increasing ruminal fiber digestibility may allow for increased feed intake, increasing energy intake and animal performance. For example, application of a combination of cellulase and xylanase to a 70% barley/ryegrass silage: 30% rolled barley (DM basis) ration significantly increased average daily gain (**ADG**) to 1.25 kg/d, compared to 1.13 kg/d when no enzyme was applied (McAllister et al., 1999). These results were supported by Beauchemin et al. (1999b), who applied an enzyme containing xylanase and cellulase activities to a 92.2% concentrate diet (DM basis), and increased ADG to 1.53 kg/d compared to 1.40 kg/d for the control. In addition, application of an enzyme product containing predominantly xylanase activity to a dairy ration (50% forage: 50% concentrate; DM basis) increased 3.5% fat corrected milk yield to 41.8 kg/d, compared to 40.7 kg/d for the control (Romero et al., 2016). Together, these studies highlight that digestion of diets, and subsequent animal performance can be improved by utilization of feed enzymes.

2.2.6 Application of enzymes

For enzyme application to feed to be effective, the enzymes must be stable during storage, remain stable during feed processing, be active in the animal, and not inhibit absorption of other nutrients present in the feed (Barletta, 2010). Enzymes can be applied to any ingredient of the diet, or can be included in pellets, and enzyme application generally depends on the species receiving the enzymes as well as convenience of application.

The method of enzyme provision to ruminants has been shown to play a large role in their effectiveness at increasing digestibility and performance. The rumen is a proteolytic

environment, which can result in degradation of vulnerable and unprotected enzymes. Infusing enzymes directly into the rumen has been shown to be ineffective at improving digestibility and animal performance likely due to proteolysis of the enzymes (Beauchemin and Holtshausen, 2010). Interestingly, enzymes applied to a portion of the feed prior to feeding are more effective, suggesting that feed provides partial protection against proteolysis (Fontes et al., 1995), and pre-ingestive digestion may also be initiated (Nsereko et al., 2000b). Hristov et al. (1996) found that application of exogenous enzymes to feed prior to feeding actually resulted in some pre-ingestive degradation of feed. It has been suggested that applying enzyme to drier portions of the diet (such as concentrate or hay), improves enzyme efficacy compared to wetter portions of the diet (such as silage; Feng et al., 1996; Lewis et al., 1999; Rode et al., 1999), though the exact reasons for this are unknown. It is likely that application of enzyme to the drier portion of the feed improves enzyme stability due to the partial protection the feed provides (Fontes et al., 1995).

There are some compounds present in ensiled feeds that inhibit enzyme efficacy (Nsereko et al., 2000a). Adding the enzymes to forage at harvest prior to fermentation can increase rate of aerobic deterioration and decrease nutritive value of the final silage product (Wang et al., 2002). Bowman et al. (2002) determined that applying enzymes to the concentrate portion of a total mixed ration (**TMR**) increased NDF digestibility to a greater extent than when the same amount of enzyme was applied to either the supplement or premix portion of the diet. They suggested this was likely due to the concentrate representing a greater proportion of the diet (45% of TMR; DM basis), compared to the supplement (4% of TMR) or premix (0.2% of TMR).

Providing enzymes through a pellet is a convenient method of supplementation because it ensures a uniform dose. However, the pelleting process results in high temperatures that can denature enzymes. Some methods to avoid enzyme inactivation during feed processing include

spraying enzyme onto pellets after pelleting, vacuum coating pellets with enzyme, or enzyme modification to improve stability (Ding et al., 2008; Lamichhane et al., 2015). Both spraying enzyme and vacuum coating require additional equipment in the feed mill, which are both expensive and can take up a large footprint in the mill, where space is typically limited. In addition to expensive equipment required, Edens et al. (2002) also stated that moisture content is required to be low in the final product to stabilize exogenous enzymes, and it can also be difficult to evenly apply the enzyme throughout the entire batch.

2.2.7 Enzyme survival during feed processing

Feed manufacturing can affect enzyme stability because of the feed ingredients utilised, conditioning temperature, moisture content of steam, pressure during conditioning, and temperature of the mash/pellet. The ability of enzymes to survive during heat treatment in feed processing correlates to the extent of covalent bonds, disulfide bonds and calcium ions present in the enzyme which enhances the rigidity, and subsequent stability of the enzyme (Svihus, 2010). Enzyme denaturation is enzyme-specific. Both Inborr and Bedford (1994) and Silversides and Bedford (1999) determined that pelleting temperatures of 80°C and above decreased non-starch polysaccharidase activity. Contrarily, for a phytase enzyme, activity was decreased by >50% when pelleting temperatures were below 70°C (Slominski et al., 2007). This decrease in enzyme activity during feed processing can have feed companies adding up to twice as much of the desired enzyme activity prior to processing to obtain the desired enzyme activity in the final product (personal communication).

2.2.8 Enzyme survival in the gastrointestinal tract

In addition to decreased enzyme activity during feed processing it is important to note that the physiology of the gastrointestinal tract (**GIT**) differs among livestock species. Therefore, survival of enzymes in the GIT will depend on the livestock species. Retention time of feed from intake to excretion has been estimated to be 12 h for poultry (Tuckey et al., 1958), 32 to 85 h for swine (Svihus, 2010), and 22 to 80 h for ruminants (Schaefer et al., 1978; Hartnell and Satter, 1979). For feed enzymes to be effective they must remain at least partially stable in the GIT, which includes the rumen of ruminants, stomach/abomasum (approximately pH 2.0 to 3.0), and the intestines.

The rumen is a very proteolytic environment, which has the potential to result in degradation of exogenous enzymes. Hristov et al. (1998b) incubated two different enzymes in rumen fluid for up to 6 h, and reported that carboxy-methyl cellulase and xylanase activity generally remained stable, whereas amylase activity decreased after 4 h of incubation. This was partially supported by Morgavi et al. (2001), who found that after incubating two different commercial enzymes in rumen fluid for 6 h, carboxymethyl cellulase, xylanase and β-glucanase activities remained stable, whereas amylase activity was significantly reduced for both enzyme products. Morgavi et al. (2000b) evaluated enzyme stability of four enzymes from different sources after incubation in rumen fluid, and found that enzymes from *A. niger* were generally more stable than the enzymes from other sources. After 6 h of incubation in rumen fluid, xylanase activity was only reduced to 75% of 0 h activity, with the exception of an enzyme sourced from *Irpex lacteus,* which only retained approximately 20% of 0 h activity (Morgavi et al., 2000b). The application of enzymes to feed prior to *in vitro* incubation had a stabilizing effect on the enzyme, increasing resistance to proteolysis and extending ruminal residence time allowing more time to interact with feed sources in the rumen (Hristov et al., 1996).

The stomach and abomasum are acidic environments where the combination of low pH and excretion of pepsin for the purpose of initiating protein degradation, making it an inherently harsh environment for exogenous enzymes. Baas and Thacker (1996) determined that within 30 min of *in vitro* incubation between pH 2.5 and 3.5, the enzyme activity of 10 commercial feed enzymes was significantly reduced compared to pH 4.5 and 5.5. They further examined the ability of these enzymes to retain their activity after 60 min incubation at pH 2.5 followed by rapid pH increase to 5.5 (imitating the passage of digesta from stomach to proximal duodenum). This resulted in enzyme activity ranging from 21 to 62% of original enzyme activity, suggesting that some enzymes are more resistant to low pH present in gastric fluid compared to other enzymes. Morgavi et al. (2001) incubated an enzyme product in simulated gastric fluid (0.1 M glycine/HCl buffer [pH 3.0]) containing 0 to 2.0 mg/ml pepsin, and found that regardless of pepsin concentration cellulase, β-glucosidase, and β-xylosidase activity was decreased. Interestingly, Morgavi et al. (2001) found that xylanase activity remained stable in the gastric fluid, regardless of pepsin concentration.

The intestines are a site of high endogenous enzyme activity, where both carbohydrate and fat digestion can occur. Incubating an exogenous enzyme in simulated intestinal fluid (0.1 M citric phosphate buffer [pH 7.0]) containing pancreatin (0 to 2.5 mg/ml) increased cellulase activity, where increasing concentrations of pancreatin concurrently increased activity until 20 min of incubation (Morgavi et al., 2001). It was suggested that this increase in cellulase activity was because the partial hydrolyzation of the enzyme resulted in a more active form of the enzyme. In the same study, Morgavi et al. (2001) found that xylanase and cellulase 1,4-β-cellobiosidase activity remained stable until at least 40 min of incubation in the simulated intestinal fluid. These

results suggest that if enzymes are able to reach the intestines they may remain active for at least 40 min, with the possibility of an increase in enzyme activity within the intestines.

2.3 Methods to improve enzyme stability

Given their instability, research has been performed to enhance the survival of feed enzymes throughout feed processing and within the GIT. Enzymes can become more heat resistant by coating the enzymes in starch, fiber, protein or fats (Gibbs et al., 1999). However, Svihus (2010) cautioned that coating enzymes has the potential to reduce initial enzyme activity and efficacy in the GIT, particularly in animals with short retention times. Thus methods that allow enzymes to be fully active throughout the GIT are favorable, and include the discovery of enzymes that are naturally resistant to denaturation, engineering enzymes to improve their stability, genetic modification of microbes, and immobilization of enzymes.

2.3.1 Discovery of novel enzymes

With the advent of next generation sequencing methods, and a significant reduction in their costs over the last 10 years, nucleic acid-based technologies are providing significant advances in enzyme discovery. Application of metagenomics and transcriptomics is particularly useful when evaluating microbiomes that have high levels of enzyme activity (Ribeiro et al., 2016). Metagenomics is the study of microbial populations based on DNA sequencing and provides information on the diversity and genetic potential of a microbial community. This type of analysis highlights the potential genes of enzymes but does not provide information on the abundance of enzymes required to digest a substrate. In contrast, transcriptomics is the study of all expressed genes in a community and can be used to evaluate how enzyme production changes when a feed substrate is altered (e.g. from low to high fibre). Both metagenomics and

transcriptomics have been utilised to discover novel enzymes from domesticated ruminants, and wild ruminants, including bison, elk, deer, moose, and muskoxen (Ribeiro et al., 2016). By discovering novel enzymes that are naturally present in ruminants, it is possible that they have increased resistance to the harsh environment of the GIT and may have improved efficacy as feed enzymes.

2.3.2 Engineering enzymes

The structures of feed enzymes have been compared to those sourced from extremophiles and mesophiles in order to analyze differences that may improve thermal stability (Eijsink et al., 2003). Once heat-resistant traits are identified, enzymes can be engineered to incorporate those traits either by altering computation or by statistically determined force fields (Krieger et al., 2002). Methods to increase the rigidity of enzymes and increase stability include introduction of disulfide bridges and salt bridges, or increasing stability of alpha-helices present in the enzyme due to interactions between amino acids (Eijsink et al., 2003; Ding et al., 2008). The alteration of the structure of the proteins can increase thermal stability; however, it is important to note that for the majority of enzymes it also increased optimum temperature for activity. Increasing optimum temperature for activity will reduce efficacy of enzymes in animal production if the animal body temperature is below that required for feed enzyme activity. Thus it is important to test modified enzymes in temperatures representing the GIT of livestock during design.

2.3.3 Genetic modification of microbes

Bacteria and fungi have been genetically altered to overproduce digestive enzymes (Ribeiro et al., 2016). However, to produce adequate amounts of enzyme in the GIT, these microorganisms would be required to successfully colonize and compete against the host microbiota.

Colonization by exogenous bacteria has proven challenging, particularly in ruminants, likely due to the complex microbiota competition for resources (McAllister et al., 2011). The potential for microorganisms to colonize the GIT is influenced by gastric/intestinal emptying rate, gastric acidity, concentration/hydrolytic activity of bile salts, ability to attach to intestinal mucosa, and competition for resources with host microbiota (Bezkorovainy, 2001).

Transgenic bacteria displaying enzymes on their surface have also been researched as delivery vehicles for feed enzymes. For example, enzymes can be displayed on the surface of *Bacillus* spores by creating a fusion between genes coding for the enzyme protein and genes that code for a surface protein on the spore coat (Isticato et al., 2001). Enzymes that are surface displayed on *Bacillus* spores react with substrate similarly to enzymes that are immobilized on the spores' surface, and both are more stable than unbound enzymes (Kwon et al., 2007). Surface displayed enzymes have been shown to retain approximately 30 to 45 % of initial enzyme activity after incubation at 40°C for three hours, whereas unbound enzyme could only retain approximately 5% of initial activity. However, application of transgenic organisms in food production is highly contentious and consumers have shown resistance (Detmer and Glenting, 2006). Regulatory processes involved in the approval of transgenic feed additives are also extensive and costly, and their use may require labelling exceptions.

2.3.4 Enzyme immobilization

Enzyme immobilization is a technique that utilises molecular interactions to bind enzymes to a solid support. Immobilised enzymes generally have a lower V_{max} (maximum reaction rate) compared to unbound enzymes (Gashtasbi et al., 2014; Falahati-Pour et al., 2015), which likely results from changes in enzyme conformation leading to slightly altered active sites (Bower et

al., 1999). Overall, reduction in enzyme activity can be counteracted by an increase in protein stability during immobilization and a resultant increase in substrate conversion. For example, Cunha et al. (2008) immobilized a lipase onto agarose beads, which improved enzyme stability ten times compared to an unbound lipase. In addition, immobilized enzymes have several advantages including: improved reusability and selectivity of enzymes, and reduced inhibition due the medium or by-products of enzymatic reactions (Mateo et al., 2007; Sirisha et al., 2016). Immobilization can be irreversible (covalent binding, entrapment and cross-linking) or reversible (adsorption; Brena et al., 2013). The three main methods that are used in industry are covalent binding, adsorption, and entrapment (Datta et al., 2013).

Covalent immobilization occurs when crosslinks between carboxyl and amine groups are formed between the support material and the enzyme of interest. Covalent binding generally uses chemical cross linkers such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (**EDC**) and *N*-hyrdoxysulfosuccinimide (**NHS**), as indicated in Figure 2.1. Tee and Kaletunc (2009) were able to covalently bind α-amylase to calcium alginate beads more efficiently by pre-treating the beads with ethylenediamine tetraacetic acid (**EDTA**). Pre-treating with EDTA increased the amount of carboxylate groups present on the support material, increasing the sites that EDC could covalently bind to, and subsequently increasing the sites for α-amylase binding. The covalent binding of organophosphorus hydrolase to *B. subtilis* spores resulted in approximately 55% of enzyme immobilization (Falahati-Pour et al., 2015). Similarly, Gashtasbi et al. (2014) used EDC and NHS to covalently bind α-amylase to *B. subtilis* spores at pH 4, 6, 8 and 10 and observed spores aggregating at all pH except for pH 6 (Gashtasbi et al., 2014). The authors also found that covalently binding the α-amylase to *B. subtilis* spores at pH 6 resulted in

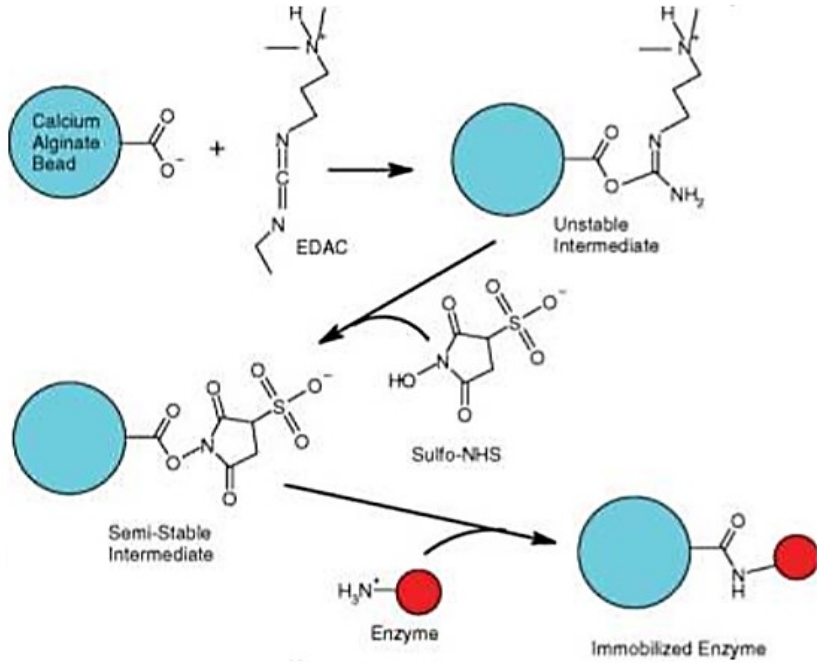

Figure 2.1. Covalent immobilization steps. Using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (NHS) to covalently bind an enzyme to calcium alginate beads. Tee and Kaletunc, 2009.

77% of applied enzyme binding to the spores, and that the reaction was stable, with only 22.3% of bound protein being released after washing in harsh buffers.

Adsorption immobilization is related to hydrophobic and electrostatic interactions, which can be influenced by zeta potential. Zeta potential is the electric potential measured at the shear surface between the stern layer and liquid (Hunter, 1988). Altering the pH of a binding solution will change the zeta potential; an acidic pH will make the zeta potential more positive, whereas a basic pH will make the zeta potential increasingly negative. Schultz et al. (2008) found that for an enzyme with slightly negative zeta potential (-8.2 mV), it had greater adsorption to solid supports with slightly positive potential (+0.6 mV) compared to supports that were slightly negative (-11.4 mV) or very negative (-35.0 mV). The authors suggested that this was due to a decrease in repulsion forces, allowing for the enzyme and support to have closer interaction. These results were supported by Min et al. (2012) who determined that when binding to a support with a very negative zeta potential (-50 mV) an enzyme that had -17 mV zeta potential was able to bind 2.09 mg protein/mg support, compared to 0.626 mg/mg support for an enzyme that had -24 mV zeta potential at binding. While few studies have attempted to feed immobilized enzymes to livestock, these data show that binding conditions are enzyme-specific and should be evaluated and optimized for individual enzymes.

2.3.5 Enzyme immobilization on Bacillus spores

Bacillus spores are highly stable in a range of environments, including extreme temperatures and pH. The surface of *Bacillus* spores has been previously shown to be both hydrophobic and electrostatic (Ahimou et al., 2001), making the spores a favourable material for immobilization. Cho et al. (2011) determined that phytase could be adsorbed to *Bacillus polyfermenticus* through

a combination of electrostatic and hydrophobic interactions, and remained active after adsorption. Gu et al. (2015) also reported that cellobiose 2-epimerase adsorbed to *B. subtilis* spores by hydrophobic properties and electrostatic interaction, where increasing the ionic strength in the solution resulted in dissociation of the enzyme from the spore. Sirec et al. (2012) adsorbed β-galactosidase to wild-type and mutant *B. subtilis* spores, the latter which did not contain proteins involved in formation of the spore crust. The authors found that binding enzymes to spores with a normally formed crust (wild-type) had decreased enzyme activity compared to spores that did not have a normally formed crust (mutants). Thus spore coat composition affects the adsorption potential of spores and likely differs between spore species.

Several studies have investigated the optimal conditions to bind enzymes to *Bacillus* spores, and are summarized in Table 2.1. Gashtasbi et al. (2014) found that adsorption of α-amylase to *B. subtilis* spores was optimized when the binding buffer had a pH of 4. The authors theorized that low pH caused the amylase to have a positive charge and as a result, increased attraction to negatively charged spores. This trend was also found by Cho et al. (2011) who reported optimal binding of a phytase to *B. polyfermenticus* when the buffer pH ranged from 3.0 to 4.0. The concentration of enzyme during adsorption to spores has also been shown to affect binding and retained enzyme activity after binding. In several studies, as enzyme content increased, there was a trend towards decreased spore-bound activity (Gashtasbi et al., 2014, Cho et al., 2011; Gu et al., 2015). This decrease in enzyme activity with increasing protein concentration has been suggested to result from steric hindrance, where continual binding of proteins to spores becomes saturated and results in blockage of active sites (Gashtasbi et al., 2014; Gu et al., 2015). In general, binding enzymes to *Bacillus* spores resulted in decreases in enzyme activity compared to unbound enzyme with the same amount of protein.

Table 2.1. Characterization of the binding of enzymes to the surface of *Bacillus* spores

Spore species	Enzyme (protein applied)	Binding buffer (pH)	Protein binding (%)	Retained activity	Author
B. subtilis (4.0×10^{10})	α-amylase (60 µg/ml)	0.2 M phosphate buffer (pH 4.0)	20.1	---[a]	Gashtasbi et al., 2014
B. polyfermenticus (100 mg)	Phytase (4,150 U/2 mg)	50 mM citric acid buffer (pH 3.0)	66.3	43.6%	Cho et al., 2011
B. subtilis (1.0×10^{11})	Cellobiose 2-epimerase (2.4 mg)	50 mM disodium hydrogen phosphate-citric acid buffer (pH 4.5)	61.3	79.4%[b]	Gu et al., 2015
B. subtilis (1.0×10^{10})	β-galactosidase (0.5 µg)	50 mM sodium citrate buffer (pH 4.0)	--- [c]	49.9%	Sirec et al., 2012

[a] Unbound enzyme activity not published, therefore could not determine retained activity. However, spore bound enzyme activity was 2,843 U/4.0×10^{10} *B. subtilis* spores.

[b]Unbound enzyme activity was not published, therefore retained activity is what was published in the article.

[c] Protein in supernatant was not determined.

Spore-bound enzymes can improve enzyme stability at a range of temperatures and pH. Gashtasbi et al. (2014) measured enzyme activity of unbound amylase, amylase covalently bound to *B. subtilis* spores, and amylase adsorbed to *B. subtilis* spores. They found that the pH range for optimal enzyme activity of unbound amylase was between pH 4.5 to 7.0, whereas for covalently and adsorbed enzymes the optimal pH range was pH 5 to 10 and 6.5 to 10, respectively. Sirec et al. (2012) reported that incubating *B. subtilis* spore-bound β-galactosidase at pH 4.0 for up to 24 h did not reduce enzyme activity; however, enzyme activity of unbound β-galactosidase was decreased to 30% of 0 h activity after 7 h of incubation. Binding phytase to *B. polyfermenticus* spores has been shown to also improve enzyme stability when incubated at 60 to 90°C, compared to unbound phytase (Cho et al., 2011). Similarly, after incubating for 2 h at 70 to 90°C, spore-bound cellobiose 2-epimerase had significantly greater activity (15 to 50%) than unbound cellobiose 2-epimerase (Gu et al., 2011). These studies suggest that spores may confer protection of adsorbed enzymes at temperatures and pH achieved in feed processing and pH found throughout the GIT.

2.4 *Bacillus* spores as probiotics

2.4.1 Bacillus spores survival

Bacillus spores are a dormant life form. They are covered in layers of proteins to protect the DNA inside until there are favourable growth conditions resulting in germination (Nicholson et al., 2000). Layers that are present in *Bacillus* spores include the core, inner membrane, germ cell wall, cortex, outer membrane, spore coat, and exosporium (Figure 2.2). These layers of proteins allow survival in extreme temperatures, a wide range of pH, ultraviolet radiation, gamma radiation, and toxic chemicals, (Setlow, 2005). *Bacillus subtilis* spore coats contain more than 50

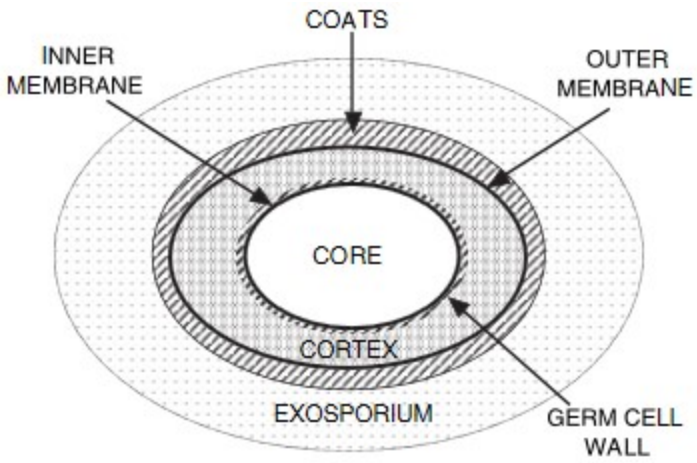

Figure 2.2. *Bacillus* species spore structure. Setlow. 2005.

individual proteins (Driks, 1999) that improve spore survival in the presence of lysis enzymes, but they do not confer resistance to heat, radiation or other chemicals (Nicholson et al., 2000). The inner spore membrane is considered to be the layer that allows the spores to resist degradation by chemical treatment (Cortezzo and Setlow, 2005). The water content in the spore core is low (only 27 to 55%, compared to 80 to 90% for vegetative cells; Henry and Friedman, 1937), and is considered the major cause of resistance to degradation due to wet heat (i.e. steam; Gerhardt and Marquis, 1989).

2.4.2 Probiotic effects

Probiotics are live microorganisms that confer a health benefit to the host (Cutting, 2011). Enhanced health can be achieved by probiotics altering the microbiota, or modulating host immunity (Hong et al., 2005). Since improved animal health typically correlates with animal performance, probiotics are being used increasingly in livestock industries as feed additives.

For ingested spores to have a probiotic effect, it is likely that germination and metabolic activity of vegetative cells would be a precursor. There is evidence that *B. subtilis* spores germinate (potentially acid-induced) then subsequently sporulate in the jejunum and ileum of mice (Casula and Cutting, 2002; Hoa et al., 2001). Tam et al. (2006) found that *B. subtilis* spores were able to survive in the murine GIT, and enter a cycle of germination and sporulation. This was supported by Latorre et al. (2014) who determined that approximately 90% of the spores fed to broiler chickens germinated in the GIT. While Alexopoulos et al. (2004) did not evaluate germination, they determined that supplementation of gestating and lactating diets of sows with a probiotic containing *Bacillus licheniformis* and *B. subtilis* spores improved performance and health status

of the sows and their offspring. These data suggest that *B. subtilis* vegetative cells become metabolically active, and potentially induce health benefits to the host.

Studies that have evaluated the use of *B. subtilis* as probiotics in ruminants have generally focused on calves. It is important to note that in general these additives have either contained vegetative cells alone, or a combination of *B. subtilis* vegetative cells and spores. Sun et al. (2010) supplemented dairy calves with 1.0×10^{10} *B. subtilis* vegetative cells daily until the calves were weaned, and observed increased ADG, serum IgG, and interferon-γ concentration compared to control treatments. The results from the study suggested that supplementation of *B. subtilis* vegetative probiotics enhanced immune response leading to increased animal performance. Sun et al. (2011) also supplemented milk with 1.0×10^{10} *B. subtilis* vegetative cells for dairy calves, and then after weaning incorporated 1.0×10^{10} *B. subtilis* vegetative cells into the calf starter diet. The authors determined that supplementation with *B. subtilis* enhanced rumen development after weaning, and increased the number of rumen papillae throughout the experiment compared to the control. Addition of *B. subtilis* spores (5.0×10^{10}, and 1.0×10^{10} spores/day) to dairy cow diets has been shown to decrease blood non-esterified fatty acid concentrations and rumen acetate: propionate ratios, while increasing fat corrected milk yield and feed efficiency (Peng et al., 2012). Souza et al. (2017) fed late (246 days in milk) and mid-lactation dairy cows (161 days in milk) with 3.0×10^{9} *B. subtilis* spores/day and found no effect on milk production in late-lactation cows, but production of mid-lactation cows increased. *Bacillus subtilis* spores, and vegetative cells may therefore improve cattle health, and as a result, production.

2.5 Knowledge Gap

To improve the efficacy of enzyme use in livestock, enzyme stability needs to be improved during feed processing, and throughout the GIT. Improving enzyme stability will result in increased feed digestibility and animal performance. A viable option to improve enzyme stability is adsorption of enzymes to probiotic species of *Bacillus* spores.

Enzymes have previously been bound to *Bacillus* spores, yet optimal conditions for multiple enzymes have not been established. There are multiple *Bacillus* spore species that are considered to be probiotics, and it has been previously reported that spore species and strain impact protein binding efficiency and enzyme activity (Cho et al., 2011). For example, *B. polyfermenticus* spores could bind 28.2 mg phytase/g spores, compared to 13.3 and 8.1 mg phytase/g of *B. subtilis* spore strains ATCC 6633 and 168, respectively. The stability of spore-bound enzymes when incubated at elevated temperatures has been evaluated (Cho et al., 2011; Gu et al., 2011), but stability of spore-bound enzymes within a ruminant model has not been investigated. In addition, the effect of spore-bound enzymes on total tract digestibility, and site of digestion has not been determined.

2.6 Hypothesis

Binding enzymes to *B. subtilis* spores will improve enzyme stability, both during feed processing and throughout the GIT, resulting in increased digestibility of feed.

2.7 Objectives

The objectives of the studies were:

1) Determine optimal binding conditions for a variety of exogenous enzymes, and survival of spore-bound enzymes during simulated feed processing temperatures.

2) Determine the effect of spore species on *in vitro* degradability and fermentation of forage.
3) Determine the effect of a variety of spore-bound enzymes, both individually and in combination, on *in vitro* digestibility and fermentation of forage.
4) Determine the effect of feeding cattle a commercial enzyme bound to *B. subtilis* spores on ruminal degradability, total tract digestibility, site of digestion, ruminal fermentation, spore survival in the ruminant GIT, and enzyme survival in simulated gastric and intestinal fluid.

3.0 Optimal binding conditions to adsorb enzymes to *Bacillus subtilis* spores for use as livestock feed additives

3.1 Introduction

Adding exogenous enzymes to livestock diets has been shown to improve digestibility and performance (Barletta, 2010). However, efficacy of feed enzymes can be affected by their ability to withstand feed processing prior to consumption, as well as the animal digestive tract after consumption. For example, survivability of feed enzymes throughout the bovine gastrointestinal tract, including resistance to proteolysis in the rumen and acidic conditions in the abomasum has been related to class and source of enzyme (Hristov et al., 1998b). Similarly, the type of enzyme and source organism can significantly impact the ability of enzymes to survive a range of temperatures during the pelleting process of feeds (Spring et al., 1996). Pelleting of feed can be used to deactivate anti-nutritional factors such as trypsin inhibitors (Anderson and Wolf, 1995), while improving the homogeneity of feed ingredients. Addition of enzymes to the mash prior to pelleting also enhances uniform enzyme distribution within the feed, ensuring proper animal dosage. However, pelleting has consistently caused a reduction in enzyme activity for phytase (Jongbloed and Kemme, 1990), glucanase and xylanase (Pickford, 1992). Silversides and Bedford (1999) found that when conditioning temperatures reached 90°C during pelleting, only 10% of the enzyme activity was present in the pellets. To avoid a reduction in enzyme activity during processing, enzymes can be applied post-pelleting in liquid form by spraying onto pellets (Bedford, 2000), or by vacuum coating (Lamichhane et al., 2015). However these methods require additional equipment in feed mills and do not necessarily ensure uniform application throughout batches. Thus a new technology that protects feed enzymes from degradation would be beneficial to the livestock industries.

Solid-support systems such as silica and glass have been used to increase stability of enzymes in industrial applications (Sirisha et al., 2016), and more recently microorganisms have also been researched for displaying enzymes (Potot et al., 2010). For application in livestock, material used to immobilize exogenous enzymes would need to be considered safe for human and animal consumption. Thus, bacteria currently being used as probiotic feed additives to improve animal health may be useful in delivering enzymes. Studies have recently been published showing that enzymes could be adsorbed to *Bacillus* spores and confer protection against proteolysis over a range of temperatures and pH (Cho et al., 2011; Sirec et al., 2012; Gashtasbi et al., 2014; Gu et al., 2015). In addition, antigen proteins that were adsorbed to the surface of *Bacillus subtilis* spores retained immunogenicity after ingestion by mice (Huang et al., 2010). Several species of *Bacillus* are currently used as direct fed microbials in livestock production (Cutting, 2011), offering a safe method of immobilization for feed enzymes. A secondary benefit of using *Bacillus* spores for enzyme delivery may also result from their probiotic properties, as they have been shown to increase milk production (Peng et al., 2012), and immune function (Sun et al., 2010) in dairy cattle as well as improve the health status and performance of sows (Alexopoulos et al., 2004).

Adsorption of proteins to spores occurs through electrostatic interactions (Cho et al., 2011), and therefore optimal conditions for immobilization will vary for different enzymes. Thus, the objectives of the current study were to determine optimal binding conditions of several purified and commercial enzyme mixtures to *B. subtilis* spores and to evaluate survivability of spore-bound enzymes throughout simulated feed processing temperature. Zeta potentials of spores and enzymes were measured to evaluate the stability of spore-bound enzymes.

3.2 Materials and methods

3.2.1 B. subtilis strain and enzymes

Lyophilized *B. subtilis* (RK28) spores were obtained from SporeGen (Egham, Surrey, U.K.). Prior to use, spores were washed sequentially with 1 M KCl, 0.5 M NaCl, and distilled water (three times), by mixing 0.1 g of spore material with 10 ml of appropriate solution, followed by centrifugation (5,000 × *g*, 15 min, 20°C) and decanting of supernatant. After spores were washed they were enumerated by plating heat-treated serial dilutions (65°C, 45 min to kill vegetative cells) onto Luria-Bertani agar, followed by overnight incubation at 39°C. After enumeration the washed spores were diluted with distilled water to a concentration of 1.0×10^{10} *B. subtilis* spores/ml.

Enzymes used in the study included purified solutions of β-glucanase (**GLUC;** 3.2.1.39; *Clostridium thermocellum*: GH81 NZYTech, Lda, Lisboa, Portugal), feruloyl esterase (**FE;** 3.2.1.73; *C. thermocellum*: CE1 NZYTech, Lda, Lisboa, Portugal), and xylanase (**XYL;** E.C.3.2.1.8; *C. thermocellum*: GH11 NZYTech, Lda, Lisboa, Portugal), and commercial crude products of α-amylase (**AMYL;** E.C. 3.2.1.1; *B. subtilis*: #10070 Sigma Aldrich, Darmstadt, Germany), phytase (**PHYT;** E.C. 3.1.3.26; *Escherichia coli* gene expressed in *Schizosaccahomyces pombe*: Phyzyme XP 5000G Danisco Animal Nutrition, Copenhagen, Denmark), and xylanase (**CX;** E.C.3.2.1.8; *Trichoderma longibrachiatum*: Xylanase PLUS Dyadic International, Jupiter, Florida). The enzymes were selected based on their relevance to livestock feeds and ability to potentially enhance degradation of starch, fibre, and phytate.

3.2.2 Enzyme activity assays and protein assay

The activities of α-amylase, β-glucanase, and xylanase were determined by incubating enzymes at 39°C with 1% soluble starch (10 min), 1% laminarin (15 min), and 1% xylan from beechwood

(10 min), respectively, according to Wood and Bhat, (1988). After incubation, 900 µl of a solution containing 3,5-dinitrosalicylic acid (1% w/v) was added to 600 µl of the enzyme/substrate solution in order to stop the reaction. This was followed by boiling the samples for 5 min to allow colour development. Samples were then analysed using spectrophotometry (540 nm) to measure release of reducing sugars and calculate enzyme activity based on standard curves made from glucose for amylase and β-glucanase activity, and xylose for xylanase activity. For both amylase and β-glucanase, one unit of activity was defined as one µmol glucose release/min/ml. For xylanase, one unit of activity was defined as one µmol xylose release/min/ml.

Feruloyl esterase activity was measured according to Mastihuba et al. (2002) using 1 mM 4-nitrophenol trans-ferulate as a substrate, and incubation at 39°C for 20 min. The samples were centrifuged (5,000 × *g*, 4°C, 5 min), and the supernatant was analysed by spectrophotometry (410 nm) to determine enzyme activity, based on a 1 mM 4-nitrophenol standard curve. One unit of feruloyl esterase activity was defined as one µmol nitrophenol release/min/ml.

Phytase activity was determined according to Engelen et al. (1994), utilizing 8 mM phytic acid as a substrate with 30 min incubation at 39°C. A solution (37.6% - 10% ammonium heptamolybdate, 37.6% - 0.235% ammonium vanadate, 24.8% - 65% nitric acid) was added after incubation followed by spectrophotometry (415 nm) to determine enzyme activity, based on a phosphate standard curve. One unit of phytase activity was defined as one µmol phosphate release/min/ml.

Protein content of the enzyme, supernatant, and wash were determined using a commercial protein assay kit from Bio-Rad Laboratories, Inc. (Hercules, CA) and bovine serum albumin as a standard.

3.2.3 Immobilization of enzymes onto B. subtilis spores

Aliquots of *B. subtilis* spores suspended in distilled water (1×10^{10} spores/ml) were centrifuged (5,000 × *g*, 10 min, 4°C), the supernatant removed, and pelleted spores were then re-suspended with 1.0 ml of each enzyme (**ENZY**) diluted with citrate buffer (50 mM) to obtain optimal protein concentration. The spore-ENZY suspensions were vortexed, then mixed in a HulaMixer (InVitrogen, Carlsbad, CA; orbital [30 rpm, 60s], with reciprocal [90°, 10 s], and vibration [5°, 5 sec]) for 1 h. When optimal pH and optimal protein concentration were determined, the enzymes were mixed with spores at room temperature. After mixing, the solution was centrifuged (5,000 × *g*, 10 min, 4°C) and the supernatant (**SNT**) was collected. The pelleted spores were washed with 1.0 ml of distilled water three times, including vortexing and centrifugation steps after each wash, and the wash supernatant was collected and pooled. After the third wash, the spore-bound enzyme (**SBE**) was re-suspended in 1.0 ml of double distilled water. Samples of ENZY, SNT, and WASH were analysed for protein content as described above to determine binding efficiency, and ENZY, SNT, WASH, and SBE were analysed for enzyme activity as described above to determine retained activity after adsorption.

To determine optimal conditions for binding each enzyme to spores, several parameters were tested sequentially. The optimal pH for immobilization was evaluated using 50 mM citrate buffer at pH 2, 3, 4, 5, and 6 for α-amylase, β-glucanase, feruloyl esterase, purified xylanase and commercial xylanase. Buffer pH was altered by varying the proportion of 50 mM citric acid and

50 mM sodium citrate. For the phytase enzyme optimal pH for immobilization was evaluated using 50 mM acetate buffer at pH 2, 3, 4, and 5, to reduce variability when subsequently measuring phytase activity. The pH of the acetate buffer was altered by varying the proportion of 50 mM acetic acid and 50 mM sodium acetate. Using the optimum pH, the effect of enzyme protein concentration on adsorption was evaluated by binding 1.0, 0.50, 0.25, 0.10, and 0.05 mg protein/ml of the enzymes to spores. Optimal time (0, 0.25, 0.5, 1 and 2 h) and temperature (4°C, 25°C, and 40°C) were then analysed using the optimum pH and protein concentrations determined previously. For all criteria tested, optimal conditions were considered conditions which resulted in both high amounts of SBE activity, and high protein binding efficiency.

3.2.4 Zeta potential

The zeta potentials of *B. subtilis* spores, unbound enzymes and the SBE were determined. Aliquots of *B. subtilis* spores (1×10^{10} spores/ml) were centrifuged (5,000 × *g*, 10 min, 4°C) then spores were re-suspended in 1.0 ml of 50 mM citrate buffer at either pH 2, 3, 4, 5, or 6. Unbound enzymes were also diluted in 50 mM citrate buffer for calculating zeta potential. Enzymes were adsorbed to spores using the determined optimized binding conditions for protein concentration, binding time and binding temperature. The SBE were then centrifuged, supernatant removed and the spores were re-suspended in 50 mM citrate buffer (pH 2, 3, 4, 5, or 6). Zeta potentials were measured using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) with folded capillary cells.

3.2.5 Feed processing survival

To determine whether adsorption of enzymes to spores confers protection, both unbound and spore-bound preparations of commercial phytase and xylanase were evaluated using a

temperature to simulate feed processing. For each commercial enzyme, 1.0 mg of protein was adsorbed to 1.0×10^{10} *B. subtilis* spores using the optimized binding buffer pH, incubation time, and temperature. After adsorption, SBE were centrifuged as described above and re-suspended in 1.0 ml of 50 mM binding buffer. The same amount of enzyme bound to spores was also evaluated in unbound form. Both the unbound enzyme and SBE were incubated at 70°C for 0, 5, and 10 min. Afterwards, reactions were immediately placed on ice followed by analysis of respective enzyme activities.

3.2.6 Calculations

Binding efficiency was calculated as follows,

$$\begin{aligned} Binding\ efficiency \\ &= [(Applied\ protein, mg) \\ &- (Supernatant\ protein, mg\ + Wash\ protein, mg)] \\ &\div Applied\ protein, mg\ \times 100 \end{aligned}$$

3.2.7 Statistical analysis

Data from the optimal pH, and protein concentration experiments were analyzed using the Mixed Model procedure of SAS (Version 9.3, Cary, NC), using fixed effect of either pH, or protein content, respectively. Data from the optimal time and temperature experiment was analyzed using the Mixed Model procedure of SAS (Version 9.3, Cary, NC), using fixed effect of time, temperature and the interaction of time and temperature. Significance was declared when $P \leq 0.05$.

3.3 Results and discussion

3.3.1 The effect of buffer pH on zeta potential and enzyme adsorption to spores

As binding buffer pH increased from pH 2 to 6 the zeta potential became increasingly negative for all unbound enzymes, spore-bound enzymes and *B. subtilis* spores (Table 3.1). Zeta potential is the electrical potential measured at the shear plane (Hunter, 1988) and influences the adsorption and stability of particles. An absolute value of the zeta potential greater than 30 mV is considered stable, whereas a zeta potential approaching zero, which is considered the isoelectric point, becomes increasingly unstable, favouring interaction between particles (Schultz et al., 2008). The zeta potential of *B. subtilis* spores was similar at pH 5 and 6 (-24.07 and -26.63 mV, respectively), but increased to -3.53 at pH 3, and 10.42 mV at pH 2. Thus, the zeta potential of the *B. subtilis* spores suggested a greater chance of enzyme adsorption at lower pH, whereas higher repulsion forces at pH 5 and 6 reduced the likelihood of enzyme adsorption (Hunter, 1988). This is supported by previous studies using *B. subtilis* spores, where a lower buffer pH was optimal for adsorbing proteins (Sirec et al., 2012; Gashtasbi et al., 2014; Gu et al., 2015; Huang et al., 2010).

The isoelectric points of unbound enzymes ranged from pH 2 to 3 for amylase, pH 4 to 5 for β-glucanase, and commercial xylanase, and pH 5 to 6 for feruloyl esterase and purified xylanase (Table 3.1). The zeta potential of the phytase product did not appear to reach the isoelectric point with the buffer pH tested, with zeta potential ranging from -0.35 to -1.14 mV. The limited variability in zeta potential for the phytase product is likely due to the fact that it was a commercially obtained crude product, containing a variety of different proteins. The isoelectric point of pure phytase has previously been reported to be pH 4.5 (Cho et al., 2011). Similarly, the zeta potential of the commercial xylanase product was likely affected by multiple proteins present in the crude mixture, as indicated by the non-linear changes in zeta potential from pH 2

Table 3.1. The effect of pH on the zeta potential (mV) of unbound enzymes, *Bacillus subtilis* spore-bound enzymes, and *B. subtilis* spores.

	Zeta Potential, mV (Average ± Standard Error)					
	Purified enzymes			Commercial crude enzymes		
	β-Glucanase	Feruloyl Esterase	Xylanase	α-Amylase	Phytase	Xylanase
Unbound Enzyme						
pH 2	17.00 ± 0.694	4.53 ± 0.014	24.75 ± 0.837	13.90 ± 0.531	-0.35 ± 0.010	1.31 ± 0.727
pH 3	15.30 ± 1.212	7.93 ± 0.049	22.00 ± 1.212	-9.05 ± 0.136	-0.58 ± 0.008	0.51 ± 0.195
pH 4	4.96 ± 0.196	4.95 ± 0.040	5.56 ± 0.078	-7.62 ± 0.117	-0.98 ± 0.001	2.99 ± 0.283
pH 5	-0.59 ± 0.109	0.04 ± 0.001	0.64 ± 0.048	-5.64 ± 0.066	-1.08 ± 0.038	-1.44 ± 0.058
pH 6	-3.16 ± 0.076	-2.01 ± 0.026	-0.29 ± 0.009	-9.43 ± 0.441	-1.14 ± 0.017	-5.00 ± 0.070
Spore-Bound Enzyme						
pH 2	30.80 ± 0.924	28.05 ± 0.144	18.75 ± 0.029	28.37 ± 1.014	8.065 ± 0.251	21.45 ± 0.202
pH 3	19.75 ± 1.357	8.62 ± 0.179	5.46 ± 0.150	-1.05 ± 0.095	-0.99 ± 0.018	4.17 ± 0.179
pH 4	-2.74 ± 0.159	-5.98 ± 0.130	-11.60 ± 0.205	-6.84 ± 0.430	-4.27 ± 0.040	-12.45 ± 0.029
pH 5	-14.63 ± 0.331	-11.63 ± 0.196	-16.45 ± 0.433	-9.93 ± 0.159	-11.55 ± 0.144	-14.55 ± 0.029
pH 6	-15.85 ± 0.318	-11.93 ± 0.054	-16.80 ± 0.231	-13.17 ± 0.119	-9.95 ± 0.085	-14.43 ± 0.098
B. subtilis spores alone						
pH 2	10.42 ± 0.339					
pH 3	-3.53 ± 0.115					
pH 4	-13.75 ± 0.318					

pH 5	-24.07 ±0.166
pH 6	-26.63 ± 0.472

to pH 6, as well as the zeta potential differences between the purified and commercial xylanase enzymes. It is interesting to note that for most enzymes, binding to spores was stable at pH 2, as indicated by highly positive zeta potential values of SBE compared with unbound enzymes. This suggests that the enzymes tested would remain adsorbed to the spores while transiting the low pH environment of the stomach of monogastric stomachs, and the abomasum of ruminants. Retaining SBE activity after transiting the stomach or abomasum would be beneficial, as low gastric pH has previously been shown to be detrimental to feed enzymes such as β-glucanase (Baas and Thacker, 1996). As mentioned previously, antigens bound to spores retained immunogenicity after transiting the murine stomach, which implies the same ability to retain activity may be possible for feed enzymes. It is also interesting to speculate whether the enzymes adsorbed to spores might be released after transit of the stomach, as the pH increases along the small intestine and reaches the isoelectric point of SBE. Adsorption of dyes to nanoparticles has been shown to be reversible when the pH was changed to the isoelectric point of bound particles (Biehl et al., 2018). If this is the case for SBE, the spores may offer a method to deliver unbound enzymes to the lower gastrointestinal tract of livestock.

Generally, spore adsorption of enzymes was greatest when the pH of the buffer was closest to the isoelectric point of enzymes (Figure 3.1). Amylase showed maximum binding efficiency and retained SBE activity at the lowest pH tested (pH 2). Though pH 2 was not analyzed by Gashtasbi et al. (2014), they measured binding of α-amylase to *B. subtilis* spores at pH 4.0, 7.0, and 10.0 and found that an acidic pH was also optimal for spore adsorption. Combined, these data indicate that amylase is favored to bind spores in highly acidic conditions.

Similarly, phytase adsorption was greatest at pH 2, though binding efficiency was high (>90%) across all pH values. High binding efficiency likely resulted from the zeta potential of unbound

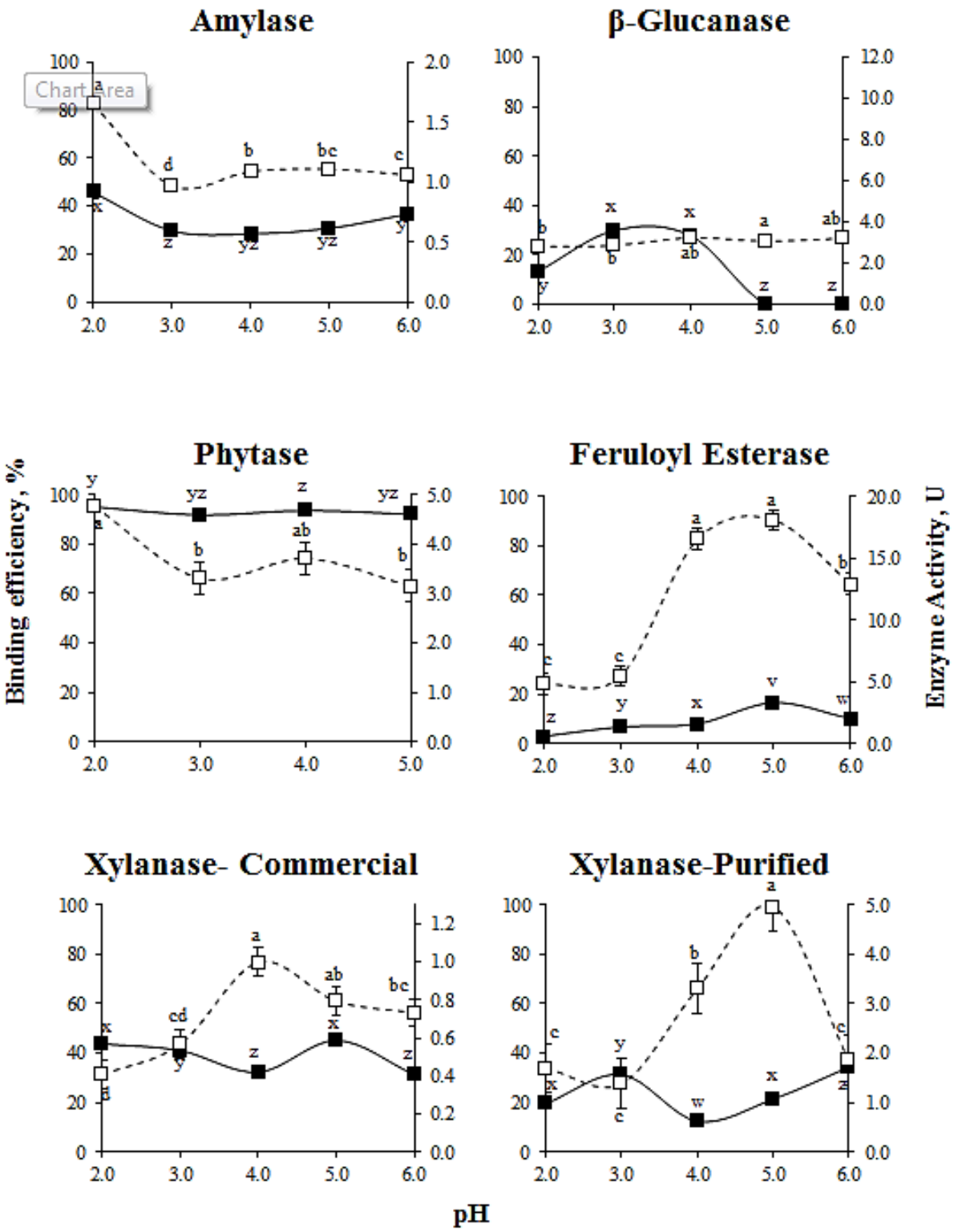
Amylase
β-Glucanase
Phytase
Feruloyl Esterase
Xylanase- Commercial
Xylanase-Purified
Binding efficiency, %
Enzyme Activity, U
pH

Figure 3.1. The effect of binding buffer pH on protein binding efficiency (%; —■—) and retained spore-bound enzyme (SBE) activity (U/ml; - -□- -) for 6 enzymes. α-amylase, β-glucanase, feruloyl esterase, commercial xylanase and purified xylanase were bound to 1.0×10^{10} *Bacillus subtilis* (RK28) spores in 50 mM citrate buffer (pH 2.0, 3.0, 4.0, 5.0 and 6.0) for 1 h at 25°C. The phytase was bound to 1.0×10^{10} *B. subtilis* (RK28) spores in 50 mM acetate buffer (pH 2.0, 3.0, 4.0, and 5.0) for 1 h at 25°C. For both amylase and β-glucanase, one unit of activity was defined as µmol glucose release/min/ml. For xylanase, one unit of activity was defined as µmol xylose release/min/ml. One unit of feruloyl esterase activity was defined a µmol nitrophenol release/min/ml. One unit of phytase activity was defined a µmol phosphate release/min/ml. For each enzyme evaluated spore-bound enzyme activities, and binding efficiencies that were significantly different ($P \leq 0.05$) are indicated by letters (abcd), and (vwxyz), respectively, where points with the same letter are not different ($P > 0.05$).

phytase being close to zero at all pH values. Although SBE activity for phytase also peaked at pH 2, a buffer pH 4 was selected for subsequent characterization due to the tendency of phytase to precipitate after extended incubation at pH 2. In addition, binding buffer pH 4 also resulted in high SBE activity, and interestingly was closer to the isoelectric point that was reported by Cho et al. (2011; pH 4.5). The SBE activity of β-glucanase remained constant across pH values, despite the greatest adsorption occurring at pH 3 to 4. The binding efficiency of purified xylanase had a cubic effect, with a peak in efficiency at pH 6.0, whereas SBE enzyme activity was maximised at pH 5.0. For this reason, pH 5 was selected as the optimal pH in subsequent tests with purified xylanase, in order to have higher SBE activity. Similar to purified xylanase, the pH (pH 5) that resulted in greatest adsorption of commercial xylanase did not correlate to the greatest SBE activity. Thus, pH affected adsorption of each enzyme and potentially also the activity of enzymes once bound to spores. The optimal pH values of binding buffer are shown in Table 3.2.

3.3.2 The effect of enzyme concentration on adsorption to spores

Binding efficiency and SBE activity varied according to protein concentration during adsorption of each enzyme (Figure 3.2). As protein concentration for α-amylase increased there was a decrease in binding efficiency, whereas, SBE activity of amylase peaked at 1.0 mg/ml. Similarly, Gashtasbi et al. (2014) reported that when adsorbing α-amylase to 4×10^{10} *B. subtilis* spores, 60 µg of α-amylase was optimal, and enzyme amounts greater than that decreased SBE activity. The authors attributed the effect to steric hindrance resulting from increasing amounts of amylase adsorbed to the spores. Optimal protein concentrations for β-glucanase and purified xylanase were determined to be 0.5 mg/ml, which was the concentration that resulted in greatest SBE activity (Table 3.2). For both feruloyl esterase and commercial xylanase, SBE activity increased

Table 3.2. Summary of optimal conditions for binding α-amylase, β-glucanase, feruloyl esterase, phytase, purified xylanase and commercial xylanase enzymes to 1.0×10^{10} *Bacillus subtilis* (RK28) spores.

	α-amylase	β-glucanase	Feruloyl Esterase	Phytase	Purified Xylanase	Commercial Xylanase
Optimal pH	2.0	4.0	5.0	4.0	5.0	5.0
Protein Content, mg/ml	1.0	0.5	1.0	1.0	0.5	1.0
Binding Time, h	1	0.5	1	1	1	1
Binding Temperature	4°C	25°C	4°C	40°C	25°C	25°C

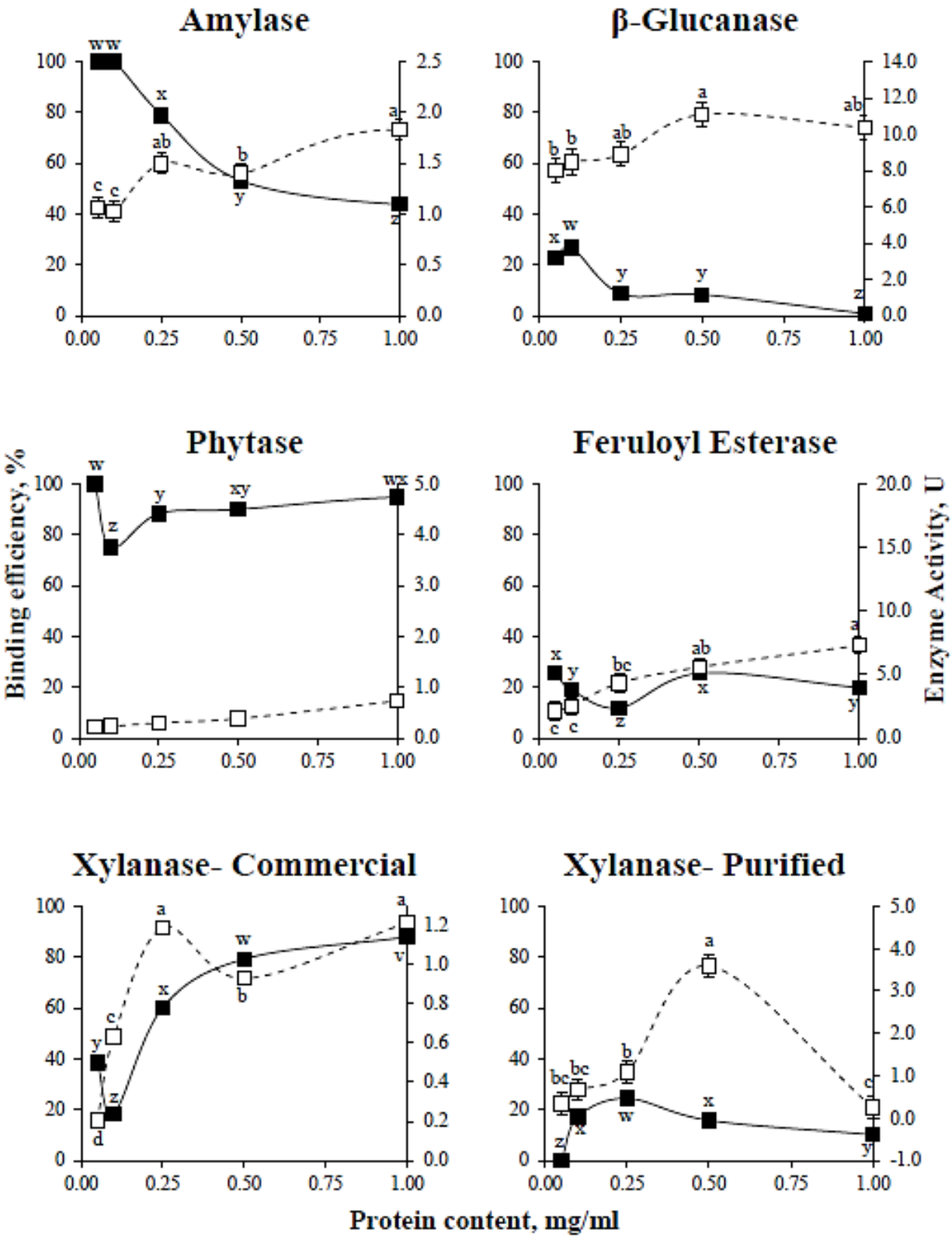
Amylase
β-Glucanase
Phytase
Feruloyl Esterase
Xylanase- Commercial
Xylanase- Purified
Binding efficiency, %
Enzyme Activity, U
Protein content, mg/ml

Figure 3.2. The effect of binding buffer protein concentration on protein binding efficiency (%; —■—) and retained spore-bound enzyme (SBE) activity (U/ml; - -□- -) for 6 enzymes. Enzymes (1.0, 0.50, 0.25, 0.10, and 0.05 mg protein/ml) were bound to 1.0×10^{10} *Bacillus subtilis* (RK28) spores in 50 mM citrate buffer (using optimal pH) for 1 h at 25°C. For both amylase and β-glucanase, one unit of activity was defined as µmol glucose release/min/ml. For xylanase, one unit of activity was defined as µmol xylose release/min/ml. One unit of feruloyl esterase activity was defined a µmol nitrophenol release/min/ml. One unit of phytase activity was defined a µmol phosphate release/min/ml. For each enzyme evaluated spore-bound enzyme activities, and binding efficiencies that were significantly different ($P \leq 0.05$) are indicated by letters (abc), and (vwxyz), respectively, where points with the same letters are not different ($P > 0.05$).

with increasing enzyme concentrations, and were greatest at 1.0 mg/ml. Binding efficiency for phytase was high (>75%) regardless of protein concentration. Cho et al. (2011) showed a similar binding efficiency (66.3%) of phytase from *E. coli*, when 2.0 mg/ml of enzyme was adsorbed to *Bacillus polyfermenticus* spores. Thus, even when using different spores and sources of enzyme, phytase has been shown to efficiently bind *Bacillus* spores.

3.3.3 The effect of binding time and temperature on adsorption to spores

Binding time and temperature resulted in variable binding efficiency and SBE activity for the commercial enzymes amylase and phytase (Figure 3.3). Regardless of time, amylase binding efficiency was less than 20% when bound at 4°C and 25°C; however, when bound at 40°C binding efficiency increased over time. When bound at 4°C there was an initial decrease in SBE activity, followed by an increase in activity at 1 h, whereas when bound at 25°C SBE activity increased from 0 to 0.25 h, followed by slight decreases in activity. Interestingly, the increase in binding efficiency at 40°C resulted in a complimentary decrease in enzyme activity. This decrease in initial enzyme activity is likely due to the enzymes binding in a way that hindered access to the amylase active site (Cho et al., 2011). Gashtasbi et al. (2014) bound amylase to *B. subtilis* spores at room temperature for 1 h, and determined binding efficiency was only 20.07%, which was similar to the binding efficiency measured in the current study at 4°C and 25°C. While increasing temperature to 40°C increased binding efficiency, it resulted in decreased SBE activity. Based on the results obtained optimal binding times for amylase was determined to be at 4°C for 1 h, which maximised SBE activity, and had moderate protein binding efficiency.

As previously mentioned protein binding efficiency for phytase was high regardless of pH (>75%), therefore optimal time and temperature were determined as values that resulted in the

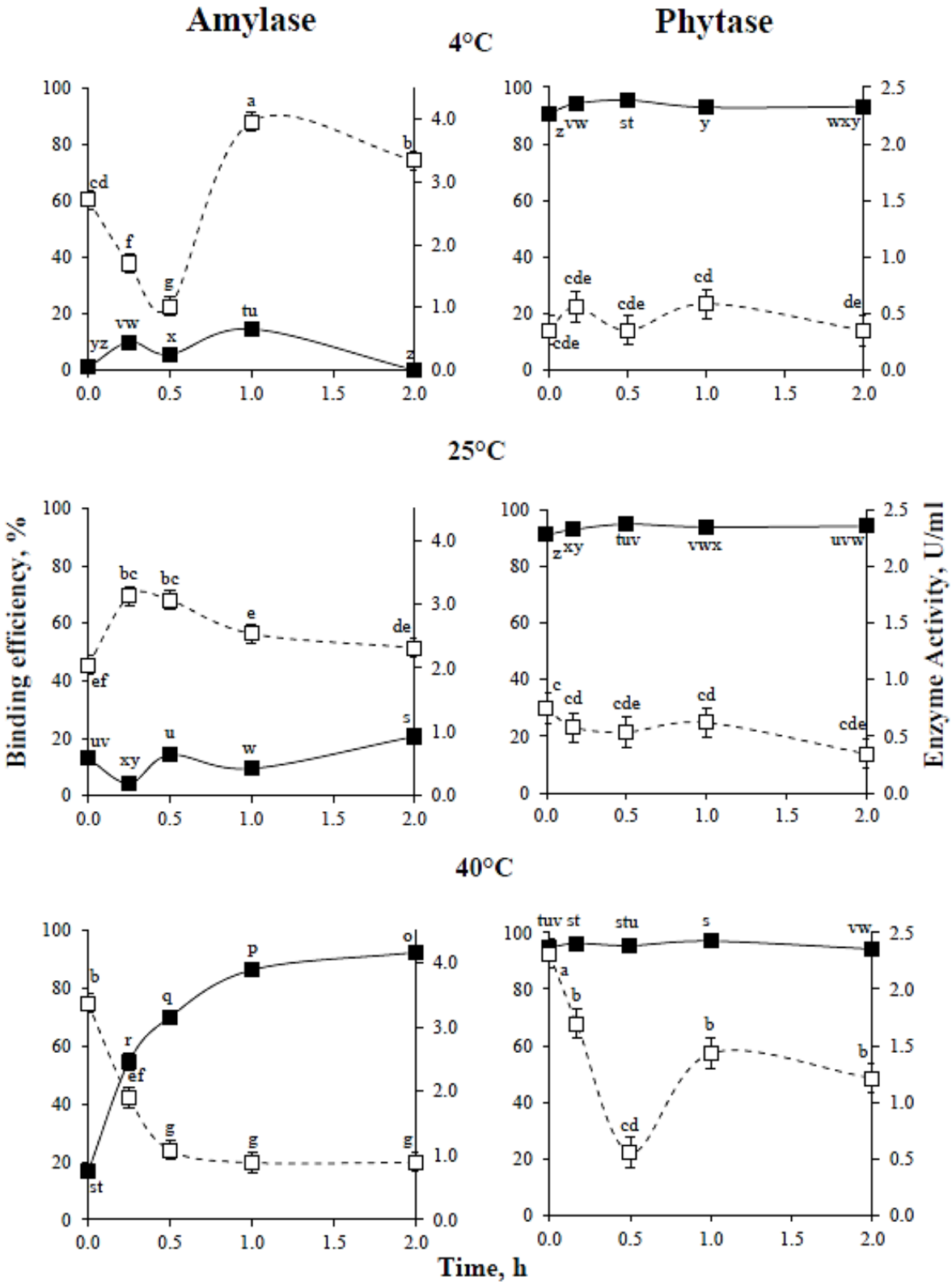
Amylase
Phytase
4°C
25°C
40°C
Binding efficiency, %
Enzyme Activity, U/ml
Time, h

Figure 3.3. The effect of binding time and temperature on protein binding efficiency (%; —■—) and retained spore-bound enzyme (SBE) activity (U/ml; - -□- -) for the commercial enzymes α-amylase and phytase. For α-amylase, optimal protein concentration (1.0 mg/ml) was bound to 1.0 × 10^{10} *Bacillus subtilis* (RK28) spores in 50 mM citrate buffer (pH 2.0) for 0, 0.25, 0.5, 1, and 2 h at 4°C, 25°C, and 40°C. For phytase, optimal protein concentration (2.0 mg/ml) was bound to 1.0 × 10^{10} *B. subtilis* (RK28) spores in 50 mM acetate buffer (pH 4). One unit of amylase activity was defined as one μmol glucose release/min/ml. One unit of phytase activity was defined as one μmol phosphate release/min/ml. For each enzyme evaluated spore-bound enzyme activities, and binding efficiencies that were significantly different ($P \leq 0.05$) are indicated by superscripts (abcdefg), and (opqrstuvwxyz), respectively, where points with the same superscript are not different ($P > 0.05$). Adsorption at both 4°C and 25°C resulted in improved amylase activity compared with 40°C, whereas phytase activity was increased when adsorbed at 40°C compared with 4°C and 25°C ($P \leq 0.05$). Binding efficiency was greater for both α-amylase and phytase at elevated temperature ($P \leq 0.05$).

greatest SBE enzyme activity (Figure 3.3). At all three temperatures tested there was an initial decrease in SBE activity between 0.25 and 0.5 h, then an increase in activity at 1 h. The greatest SBE activity occurred at 40°C. Cho et al. (2011) found that the phytase activity associated with spore-bound enzyme was only 64% of the theoretical activity if all of the protein bound was active. This could be partially due to the phytase not being a pure enzyme, the phytase protein binding to the spores with their active site not being exposed to substrate, or steric hindrance, where some of the active sites were partially blocked by neighbouring phytase proteins (Cho et al., 2011).

Enzyme activity for β-glucanase was relatively stable (ranging from 8 to 10 U/ml) regardless of mixing time or temperature, yet protein binding efficiency varied (Figure 3.4). After 2 h of incubation at 25°C and for the first 0.5 h at 40°C, no enzyme protein appeared to be bound to the spores, yet SBE activity was observed. It is difficult to explain why the glucanase activity was present when absorption was limited. Perhaps, binding β-glucanase to the spores resulted in a release of protein from the spore coat. This resulting protein would be detected in the supernatant, making it appear as though there was no β-glucanase bound to the spores. Overall, a peak in protein binding efficiency occurred at 0.5 h when mixed at 25°C, therefore this was utilised as optimal binding time and temperature for β-glucanase.

Binding of feruloyl esterase to *B. subtilis* spores had similar trends for protein binding efficiency and SBE activity over time, when incubated at 4°C (Figure 3.4). A peak in protein binding efficiency was observed at 1 h of binding, followed by a decrease in the amount of protein bound by 2 h for all temperatures. This decrease in protein binding efficiency suggests that feruloyl esterase storage in the binding buffer would result in a release of protein from the spore surface. The instability observed over time is partially supported by the zeta potential results, which

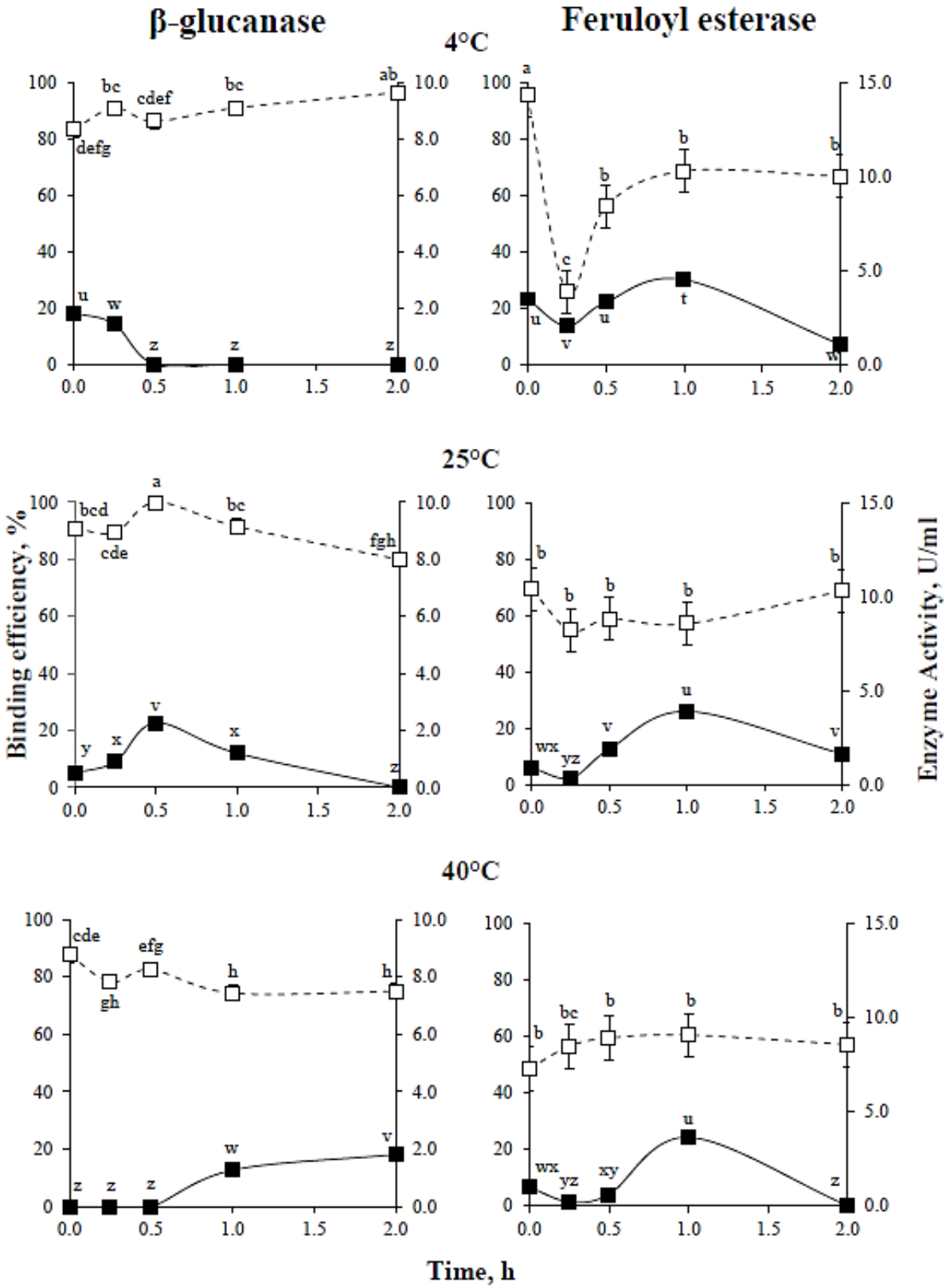
β-glucanase
Feruloyl esterase
4°C
25°C
40°C
Binding efficiency, %
Enzyme Activity, U/ml
Time, h

Figure 3.4. The effect of binding time and temperature on protein binding efficiency (%; —■—) and retained spore-bound enzyme (SBE) activity (U/ml; - -□- -) for the purified enzymes β-glucanase and feruloyl esterase. For β-glucanase, optimal protein concentration (0.5 mg/ml) was bound to 1.0×10^{10} *Bacillus subtilis* (RK28) spores in 50 mM citrate buffer (pH 4.0) for 0, 0.25, 0.5, 1, and 2 h at 4°C, 25°C, and 40°C. For feruloyl esterase, optimal protein concentration (1.0 mg/ml) was bound to 1.0×10^{10} *B. subtilis* (RK28) spores in 50 mM citrate (pH 5.0). For β-glucanase, one unit of activity was defined as one μmol glucose release/min/ml. One unit of feruloyl esterase activity was defined as one μmol nitrophenol release/min/ml. For each enzyme evaluated spore-bound enzyme activities, and binding efficiencies that were significantly different ($P \leq 0.05$) are indicated by letters (abcdefgh), and (tuvwxyz), respectively, where points with the same letter are not different ($P > 0.05$). Temperature significantly influenced enzyme activity of β-glucanase, and binding efficiency for both β-glucanase and feruloyl esterase ($P \leq 0.05$).

indicated that spore-bound feruloyl esterase would be unstable around pH 4 and 5. As a result, it may be better to store spore-bound feruloyl esterase in a lyophilized form, to enhance stability prior to feed application. Because the greatest enzyme activity and protein efficiency occurred after 1 h of mixing for all temperatures, 4°C was chosen as optimal binding temperature since it had both maximum enzyme activity and binding efficiency. To the best of my knowledge there has not been any previous research binding either β-glucanase or feruloyl esterase to *Bacillus* spores.

Protein binding efficiency was generally greater for the commercial xylanase than the purified xylanase (Figure 3.5). For the commercial xylanase there was a general increase in protein binding after 1 h of incubation, with the exception of a slight decrease in binding efficiency after 0.5 h at 4°C, and 0.25 h at 25°C. After 2 h of incubation at both 25°C, and 40°C binding efficiency of the commercial xylanase decreased by at least 25%. In contrast, for the purified xylanase, initial binding efficiency was high, decreasing gradually over time. Greater protein binding for the commercial xylanase did not correspond to increased xylanase enzyme activity, as the spore-bound purified xylanase activity ranged from approximately 4.0 to 8.0 U/ml, whereas the spore-bound commercial xylanase activity ranged from to 0.7 to 1.1 U/ml. As previously mentioned the commercial xylanase was not purified, therefore, it is likely that different proteins adsorbed to the spores, resulting in reduced enzyme activity compared with purified xylanase as a percentage of total adsorbed protein. It is also possible that for the commercial xylanase, these other components present in the crude product may have adsorbed in a way that resulted in steric hindrance and a reduced enzyme activity.

3.3.4 Enzyme survival during feed processing

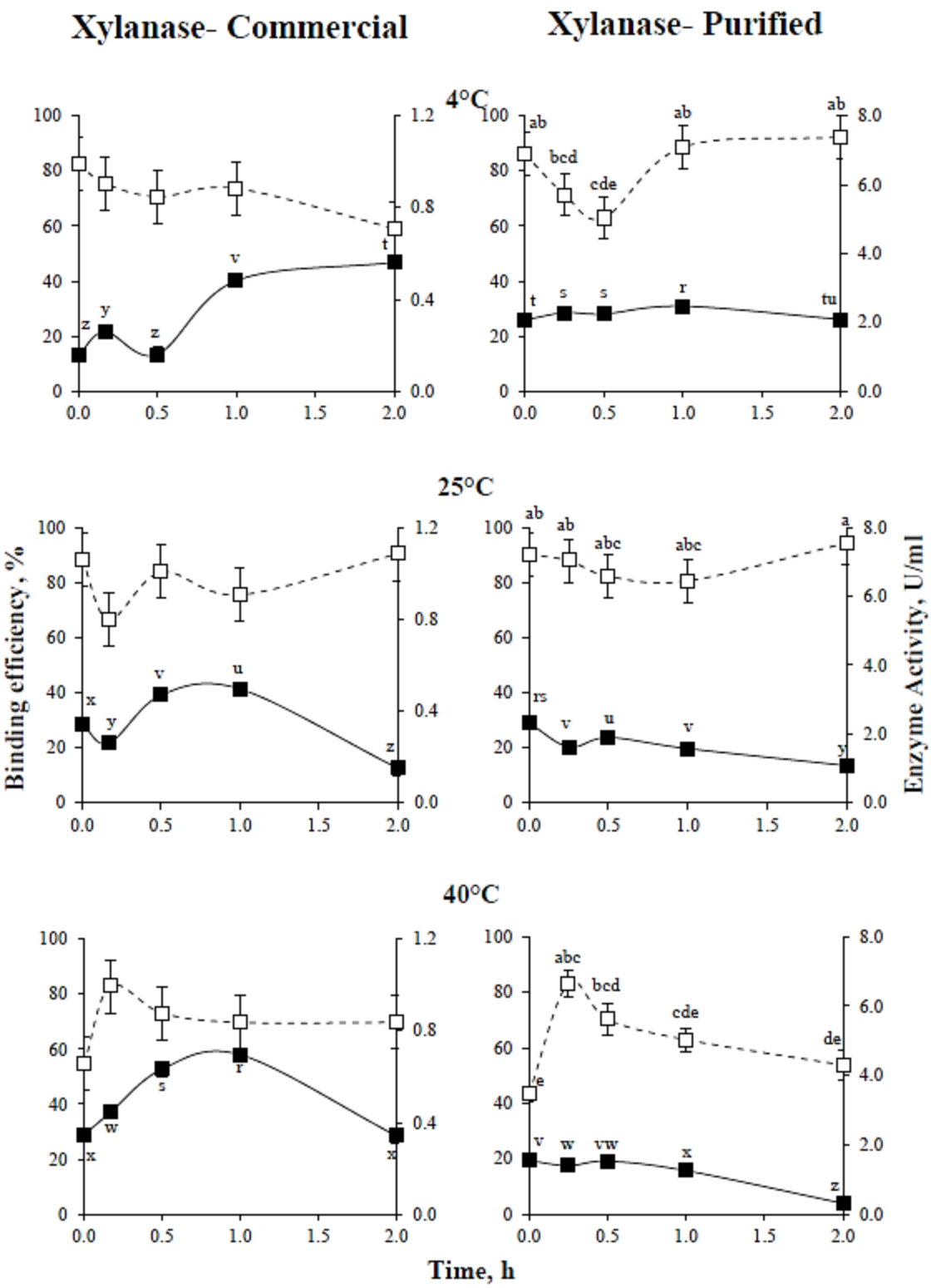
Xylanase- Commercial
Xylanase- Purified
4°C
25°C
40°C
Binding efficiency, %
Enzyme Activity, U/ml
Time, h

Figure 3.5. The effect of binding time and temperature on protein binding efficiency (%; —■—) and retained spore-bound enzyme (SBE) activity (U/ml; - -□- -) for xylanases- commercial and purified. For the commercial xylanase, optimal protein concentration (1.0 mg/ml) was bound to 1.0×10^{10} *Bacillus subtilis* (RK28) spores in 50 mM citrate buffer (pH 5.0) for 0, 0.25, 0.5, 1, and 2 h at 4°C, 25°C, and 40°C. For the purified xylanase, optimal protein concentration (0.5 mg/ml) was bound to 1.0×10^{10} *B. subtilis* (RK28) spores in 50 mM citrate buffer (pH 5.0) for 0, 0.25, 0.5, 1, and 2 h at 4°C, 25°C, and 40°C. One unit of xylanase activity was defined as one µmol xylose release/min/ml. For each enzyme evaluated spore-bound enzyme activities, and binding efficiencies that were significantly different ($P \leq 0.05$) are indicated by letters (abcde), and (rstuvwxyz), respectively, where points with the same letter are not different ($P > 0.05$). Enzyme activity of commercial xylanase was not significantly affected by either time or temperature ($P > 0.05$); however, increasing temperature increased commercial xylanase binding efficiency ($P \leq 0.05$). The purified xylanase had greater enzyme activity and binding efficiency at lower temperatures ($P \leq 0.05$).

When incubated at 70°C, enzyme activity for both phytase and commercial xylanase decreased over time (Table 3.3). After 10 min, enzyme activity was reduced by approximately 85% and 95% for the unbound forms of phytase and xylanase, respectively. In contrast, reduction in activity was less when the enzymes were bound to spores. The spore-bound enzymes experienced only a 75% (phytase) and 85% (commercial xylanase) reduction in activity, which amounted to 1.7 × and 3.2 × better survival compared with the respective unbound enzymes. The greater SBE activity for phytase compared to the xylanase may have resulted from better heat-tolerance of phytase, or differences in the binding conditions. Similar to the current study, Sirec et al. (2012) also found that binding a β-galactosidase to *B. subtilis* spores improved enzyme stability when incubated at 65°C, 75°C and 85°C compared to unbound enzyme. Inborr and Bedford, (1994) reported that mash conditioned at 75°C for either 0.5 or 15 min before pelleting resulted in β-glucanase activity decreasing to 68.3 and 41.3% of 0-h activity, respectively. The greater survivability of β-glucanase in the Inborr and Bedford, (1994) study may have been due to the type of enzyme used or possibly because the mash partially protected the enzyme from heat and steam treatment, resulting in increased retained activity. Regardless, if only 40% of an enzyme applied to feed is active after processing, 2.5 times the concentration would be required to provide livestock an effective dose. These data suggest that utilization of spore-bound enzymes in feed processing can partially overcome enzyme denaturation by improving stability and potentially reducing the amount needed as a feed additive.

3.4 Conclusion

Zeta potentials of enzymes measured at a range of pH influenced protein binding efficiency, which was not always associated with SBE activity. These data allowed us to infer unbound

Table 3.3. The effect of feed processing temperature (70°C) on activity of unbound and spore-bound enzyme (SBE) phytase and commercial xylanase.

	Enzyme Activity, % of 0 h (Average ± Standard Error)	
	Unbound Enzyme	SBE
Phytase		
0 min	100.00 ± 0.972	99.99 ± 1.898
5 min	24.08 ± 0.143	42.79 ± 2.727
10 min	15.21 ± 0.253	25.32 ± 0.572
Commercial xylanase		
0 min	100.00 ± 0.586	100.01 ± 0.686
5 min	7.08 ± 0.104	20.77 ± 0.847
10 min	4.70 ± 0.400	14.97 ± 1.373

enzyme and SBE stability when incubated in solution that ranged from pH 2 to pH 6. Optimal binding conditions to bind a variety of enzymes to *B. subtilis* spores have been determined. The variability in optimal binding parameters indicated that prior to use of this technique as a feed additive in industry, optimal binding conditions will need to be determined for the specific enzyme product. Binding commercial xylanase and phytase to spores increased enzyme stability when incubated at an elevated temperature used for feed processing. This improvement in enzyme stability suggests that less enzyme product would need to be applied to feed prior to processing and feeding to livestock to result in increased animal performance.

4.0 Effect of binding exogenous xylanase to spores of *Bacillus subtilis* and *Bacillus coagulans* on *in vitro* degradability and fermentation in ruminal batch cultures

4.1 Introduction

Enzyme additives have potential to improve fiber digestion and animal performance; however, responses have been variable in ruminants. The effects of exogenous enzymes are confounded by factors such as type of enzyme and feed (Beauchemin et al., 1995) as well as method of application (Nsereko et al., 2000b; Yang et al., 1999). It is generally thought that enzymes applied to feed are slowly released in the rumen (Beauchemin et al., 1999a) and then work in conjunction with ruminal bacteria to enhance digestion (Morgavi et al., 2000a).

Despite recent advancements in enzyme additives, such as novel enzyme discovery (Ribeiro et al., 2016), their efficacy is limited by stability and duration of action in the digestive tract (Morgavi et al., 2000b). Once ingested, feed enzymes are subjected to denaturation in the highly proteolytic environment of the rumen. The ability of enzymes to resist proteolytic denaturation varies greatly by enzyme and source organism, with some of the activities lasting only a couple of hours (Morgavi et al., 2000b). Mixing enzymes with feed substrate prior to ruminal incubation has been shown to reduce their denaturation compared to enzymes directly applied to rumen fluid (Morgavi et al., 2000b). It is possible that premixing enzymes with feed substrate protects the enzymes by limiting their exposure to the proteolytic ruminal environment. Therefore, developing a method that limits feed enzyme proteolysis would increase both the stability and effectiveness of enzymes for ruminants.

Recently, several studies have shown that adsorption of proteins to the coat of *Bacillus* spores can improve protein stability in a range of environments. Adsorption of phytase, β-galactosidase,

α-amylase and cellobiose-2-epimerase to *Bacillus* spores allowed the enzymes to retain their activity over a range of temperatures and pH (Cho et al., 2011; Sirec et al., 2012; Gashtasbi et al., 2014; Gu et al., 2015). The digestive enzymes phytase (Potot et al., 2010), and β-galactosidase (Sirec et al., 2012) have been shown to be fully functional when they are anchored to the surface of *Bacillus* spore coats. Huang et al. (2010) also found that adsorption of protein antigens to the surface of *Bacillus subtilis* spores resulted in an immune response after passage through the gastrointestinal tract. Combined, these data suggest that binding enzymes to *Bacillus* spores may offer protection of exogenous enzymes in the ruminant gastrointestinal tract and enhance enzyme activity.

To my knowledge, binding feed enzymes to spores, as a delivery system to improve enzyme stability and cattle digestibility has not been tested. Therefore, the objective was to determine whether a commercial xylanase feed enzyme bound to the surface of bacterial spores would affect *in vitro* ruminal degradability of alfalfa hay compared with unbound enzyme.

4.2 Materials and methods

4.2.1 Spore preparation

Lyophilized stocks of bacteria (Table 4.1) were cultured in 10 ml of nutrient broth using a shaking incubator (37°C, 200 rpm) for 24 h. The cultures were then streaked onto nutrient agar plates and incubated at 37°C overnight. An individual colony was selected from plates and grown overnight in 5 ml of nutrient broth (37°C, 200 rpm) followed by plating (100 µl) onto AK Agar #2 (BD Difco, Sparks, MD) and incubation at 37°C for 5 d to induce sporulation. Afterwards, spores were removed from the plates by washing with 10 ml phosphate buffered saline (PBS) containing 0.05% Tween 20 (v/v). The mixture was then centrifuged (2,390 × *g*,

Table 4.1. Effect of bacterial spore species on binding efficiency and retained xylanase activity of a commercial xylanase product to 5.0×10^9 spores.

Species	Protein, average ± standard deviation			Enzyme Activity, average ± standard deviation				
	Protein Content, mg[1]		Binding Efficiency, %[2]	Xylanase Activity, U[1]			Total Bound Activity, %[4]	Retained Activity, %[5]
	Supernatant	Wash		SBE[3]	Supernatant	Wash		
Bacillus benzeovorans (DSMZ 5391)	0.391 ± 0.007	0.026 ± 0.004	34.9 ± 1.14	3.84 ± 0.042	27.2 ± 0.985	0.58 ± 0.038	5.1 ± 0.74	24.4 ± 0.27
Bacillus coagulans (B62)	0.294 ± 0.020	0.010 ± 0.002	52.8 ± 2.91	0.35 ± 0.014	20.0 ± 0.682	0.27 ± 0.017	0.3 ± 0.32	1.5 ± 0.06
Bacillus coagulans (L387)	0.488 ± 0.018	0.025 ± 0.010	18.9 ± 1.31	< 0.01	11.0 ± 0.398	0.47 ± 0.021	-	-
Bacillus lichenformis (589)	0.386 ± 0.017	0.014 ± 0.005	37.6 ± 2.18	0.67 ± 0.040	47.4 ± 0.742	0.71 ± 0.204	0.3 ± 0.08	3.9 ± 0.24

Bacillus lichenformis (73)	0.477 ± 0.023	0.013 ± 0.003	24.0 ± 3.84	0.40 ± 0.008	22.2 ± 0.923	0.40 ± 0.015	1.1 ± 0.07	3.6 ± 0.07
Bacillus lichenformis (H1G)	0.417 ± 0.011	0.011 ± 0.005	33.2 ± 1.62	0.53 ± 0.035	25.6 ± 0.490	0.40 ± 0.029	0.2 ± 0.07	3.5 ± 0.23
Bacillus pumilus (3)	0.411 ± 0.018	0.016 ± 0.004	24.8 ± 2.65	1.33 ± 0.074	23.2 ± 1.440	0.44 ± 0.010	2.1 ± 0.57	11.9 ± 0.66
Bacillus pumilus (L106)	0.396 ± 0.054	0.019 ± 0.005	35.6 ± 9.12	0.56 ± 0.018	24.9 ± 1.044	0.47 ± 0.008	0.5 ± 0.49	3.5 ± 0.11
Bacillus pumilus (JS20)	0.448 ± 0.013	0.020 ± 0.009	27.0 ± 1.25	0.61 ± 0.020	27.6 ± 2.615	0.39 ± 0.047	0.4 ± 0.07	5.0 ± 0.17
Bacillus sphaericus (L112)	0.433 ± 0.028	0.024 ± 0.014	28.9 ± 3.77	0.98 ± 0.217	28.2 ± 0.702	0.44 ± 0.010	1.0 ± 0.37	7.5 ± 1.66
Bacillus sphaericus (jbs9)	0.467 ± 0.031	0.040 ± 0.006	21.2 ± 5.48	3.62 ± 0.071	22.9 ± 1.235	0.42 ± 0.015	5.5 ± 0.14	37.9 ± 0.75

Bacillus subtilis (ATCC 6051)	0.384 ± 0.036	0.018 ± 0.003	37.6 ± 5.76	2.13 ± 0.184	42.4 ± 0.355	0.56 ± 0.044	3.9 ± 0.99	12.6 ± 1.08
Bacillus subtilis (RK28)	0.454 ± 0.058	0.017 ± 0.005	26.4 ± 3.87	2.54 ± 0.210	46.2 ± 0.503	0.55 ± 0.042	4.2 ± 0.78	21.3 ± 1.77
Brevibacillus brevis (B405)	0.469 ± 0.043	0.019 ± 0.003	24.2 ± 6.73	0.64 ± 0.059	20.0 ± 1.838	0.43 ± 0.018	0.4 ± 0.04	5.9 ± 0.54
Paenibacillus macerans (ATCC 7069)	0.415 ± 0.017	0.017 ± 0.005	32.9 ± 2.73	3.40 ± 0.240	41.5 ± 3.455	0.52 ± 0.016	5.2 ± 0.45	23.0 ± 1.62
Paenibacillus validus (L186)	0.358 ± 0.030	0.014 ± 0.004	42.2 ± 5.14	< 0.01	28.6 ± 0.351	0.51 ± 0.027	-	-

[1]Enzyme protein applied to the spores was 0.64 mg, and xylanase activity was 45.05 U. One unit of xylanase activity was defined as one μmol xylose release/min/ml.

[2]Binding efficiency is calculated as (Enzyme protein added –[Supernatant protein + Wash protein]) ÷ Enzyme protein added × 100

[3]SBE = Spore-bound enzyme; values indicated as < 0.01 were below the limit of quantification.

[4]Total bound activity was calculated as SBE activity ÷ activity of enzyme protein added × 100.

[5]Retained activity was calculated as SBE activity ÷ theoretical activity of SBE × 100, where theoretical activity = 45.05 U × protein binding efficiency.

4°C, 10 min) and the supernatant discarded. The pellet was washed by resuspending in 20 ml of water and centrifuging (2,390 × *g*, 4°C, 10 min). The pellet was then suspended in another 20 ml of water and incubated at 65°C for 45 min to kill vegetative cells. Afterwards, the suspension was centrifuged and the spore pellet was subsequently washed as described above using individual solutions in the following sequence: 10 ml NaCl (1 M; one time), 10 ml KCl (1 M; one time), 20 ml PBS containing 0.05% Tween 20 (v/v) (two times), and 20 ml water (three times). The final spore pellet was resuspended in water and serial dilutions were incubated at 65° for 45 min, then plated onto Luria-Bertani broth agar (37°C, overnight) for enumeration of spores.

4.2.2 Adsorption of enzyme to spores

A commercial xylanase feed enzyme (E.C. 3.2.1.8; 70.4 U xylanase activity/mg protein) was used for adsorption to the spore species listed in Table 4.1. The feed enzyme was not a pure xylanase and contained crude proteins in the mixture, resulting from its production. The enzyme (**ENZY**) was diluted using 50 mM citrate buffer (pH 5.0) to 0.5 mg protein/ml, then 1.0 ml was added to 5.0×10^9 spores suspended in 0.5 ml citrate buffer (pH 5.0). The spore/enzyme suspensions were mixed using a HulaMixer Sample Mixer (Invitrogen, Carlsbad, CA; orbital [30 rpm, 60s], reciprocal [90°, 10 s], vibration [5°, 5 sec]) for 1 h at room temperature. The samples were then centrifuged (5,000 × *g*, 10 min, 4°) and the supernatant (**SNT**) was removed. The spore pellet was then washed with 1 ml of water, as described above, and vortexed. After vortexing, the spores were then centrifuged again as described above, and the supernatant (**wash**) was collected. This was repeated two more times for a total of three washes. All three washes were pooled and the final spore pellets were resuspended in 1.0 ml of water, which were then

considered the spore-bound enzyme (**SBE**) samples. The ENZY, SBE, SNT, and wash were analysed for xylanase activity and protein content.

4.2.3 Ruminal batch cultures

The treatments used in this experiment were: control (**CON**; water), unbound enzyme (**FreeE**; 0.2 mg protein/ml) and two different SBE treatments (0.2 mg protein/ml of enzyme mixed with 2.0×10^9 spores). The spore species used for the SBE treatments were *Bacillus coagulans* B62 (**BcoaE**) or *B. subtilis* RK28 (**BsubE**). *Bacillus coagulans* B62 was selected because of its high binding efficiency (52.8%; Table 4.1) compared to other spores. *Bacillus subtilis* RK28 was selected because of it having higher retained activity (21.3%; Table 4.1) compared to most spores. The enzyme and spores were mixed using the previously mentioned method.

Three separate *in vitro* batch cultures were conducted on different days. Rumen digesta was collected from different parts of the rumen of three ruminally cannulated dry beef cows 2 h after they were fed an alfalfa hay-based diet (52% alfalfa grass hay, 32% barley silage, 13% barley grain, 3% vitamin/mineral supplement; dry matter (**DM**) basis). The digesta was composited then strained through 4 layers of cheesecloth and the rumen fluid was mixed with Menke's buffer (1:3) under constant CO_2 stream to ensure anaerobic conditions to make inoculant (Menke et al., 1979). Alfalfa hay (55% neutral detergent fiber (**NDF**) and 43% acid detergent fiber (**ADF**); % of DM) ground to pass through a 2-mm screen was used as substrate. The substrate (0.5 g) was weighed into ANKOM F57 bags (25μm pore size), the bags were then sealed and placed into serum vials. Four hours prior to the start of the experiment 0.5 ml of the treatments were applied to the alfalfa substrate in the ANKOM bags. This resulted in a final concentration of 0.1 mg enzyme/0.5 g substrate in the enzyme treatments (FreeE, BcoaE and BsubE), and 1.0×10^9

spores/0.5 g substrate in the SBE treatments (BcoaE, BsubE). At the initiation of the experiment 60 ml of inoculant were added to the vials under CO_2 stream, and the vials were sealed and incubated on a rotary shaker (120 rpm; Unimax 1010; Heidolph Instruments GmbH and CO, Schwabach, Germany) at 39°C for 0, 3, 6, 12, 24 and 48 h. At each time point triplicate vials from each treatment were removed from the incubator and placed onto ice prior to processing. From each vial, 10 ml of gas was collected using a 10 ml syringe with a luer lock tip (Becton Dickinson, Co., Franklin Lakes, NJ) and stored in gas vials at room temperature until methane analysis. Gas production was measured afterwards, using a water displacement apparatus according to Fedorah and Hrudey (1983). The ANKOM bags were then removed from the serum vials and washed in cold tap water, followed by drying (105°C, 48 h) for DM, and subsequent NDF and ADF analysis. Subsamples of the inoculant (1.5 ml) from each vial were added to 0.3 ml of 25% meta-phosphoric acid and stored at -20°C for subsequent analysis of volatile fatty acids (**VFA**). Inoculant sub-samples (1 ml) were also stored for determination of xylanase activity.

4.2.4 Spore persistence

To determine spore persistence, separate batch cultures were conducted in parallel to batch cultures described above using the same inoculant and conditions, but without the addition of ANKOM bags. For this, 1.0×10^9 spores were added to 60 ml of rumen inoculant, and vials were sealed and incubated on a rotary shaker (120 rpm; Unimax 1010; Heidolph Instruments GmbH and CO, Schwabach, Germany) at 39°C for 0, 3, 12, 24 and 48 h. At each time point, vials were immediately placed onto ice and serial dilutions of the rumen inoculant were then incubated at 65°C for 45 min to kill vegetative cells. The dilutions were then plated onto Luria-Bertani agar and incubated at 37°C overnight for enumeration of spores.

4.2.5 Chemical analysis

The NDF and ADF contents were determined sequentially using an ANKOM^{200} Fiber Analyzer (ANKOM Technology, Macedon, NY). The VFA samples were analysed by gas chromatography (model 5890, Hewlett-Packard Lab, Palo Alto, CA) using crotonic acid (trans-2-butenoic acid) as the internal standard. Methane was analysed according to Chaves et al. (2006) using a Varian 3600 chromatograph fitted with a 180-cm Porapak QS (Alltech Associates, Inc., Deerfield, IL) column and a flame ionization detector. The temperature of the column oven was 35°C and nitrogen was used as the carrier gas with a flow rate of 30 mL min^{-1}.

Protein content measured throughout spore absorption tests was analyzed using a Bradford protein assay, with bovine serum albumin (**BSA**) as the standard (Bio-Rad Laboratories, Hercules, CA). Xylanase activity was measured according to Bailey et al. (1992), with some modifications. Briefly, 0.15 ml of 0.1 M citrate phosphate buffer (pH 6.0) was mixed with 0.15 ml of sample, and incubated at 39°C for 10 min. Afterwards, 0.30 ml of 1.0% (w/v) beechwood substrate was added and incubated at 39°C for 10 min, followed by the addition of 0.90 ml of dinitrosalicylic acid solution and incubation at 100°C for 5 min for color development before being placed into an ice bath. The samples were analyzed for absorption values at 540 nm using an Asys UVM 340 microplate reader (BioChrom Ltd. Cambridge, UK) and values were plotted against a xylose standard curve (0 to 0.8 mg xylose/ml). Xylanase activity was then converted to Units (U), where 1 U = 1 μmol xylose min^{-1} ml^{-1} sample.

4.2.6 Calculations and statistical analysis

The enzyme binding efficiencies to spores were calculated as follows:

[Enzyme protein applied – (Supernatant protein + Wash protein)] ÷ Enzyme protein applied × 100

The total bound enzyme activity was calculated as follows:

(SBE activity – intrinsic spore xylanase activity) ÷ ENZY activity × 100

Retained enzyme activity was calculated as follows:

Measured SBE activity ÷ theoretical SBE activity × 100

Where theoretical SBE activity was determined based on protein binding efficiency if 100% of bound enzyme was active.

Corrected xylanase activity was determined by calculating the xylanase activity as a percent of the 0 h activity for each treatment.

Data from the *in vitro* experiments were analyzed by hour using the Mixed Model procedure of SAS (Version 9.3, Cary, NC), using fixed effect of treatment and random effect of batch culture run and vial. Orthogonal contrasts were made to determine significant difference of CON *vs* all treatments (CON *vs* ALL), FreeE *vs* SBE and BcoaE *vs* BsubE for the *in vitro* gas production, degradability, VFA and xylanase activity. Significance was declared when $P \leq 0.05$.

Data from the spore persistence counts were analysed using the Mixed Model procedure of SAS, using fixed effect of time, and significance was declared when $P \leq 0.05$.

4.3 Results

The absorption of xylanase enzyme to 16 spore-forming bacteria was analyzed (Table 4.1). Protein binding efficiency to spores ranged from 18.9 % (*B. coagulans* L387) to 52.8% (*B.*

coagulans B62). However, the total activity of bound xylanase varied from only 0.1 to 5.5% and retained activity from 1.5 to 37.9%, indicating that the majority of enzyme absorbed to the spores was not active after binding. Based on these data, *B. coagulans* B62 was selected as a ruminal batch culture treatment because of its high binding efficiency (52.8%). In addition, *B. subtilis* RK28 was selected for batch cultures because of it having higher retained enzyme activity (21.3%; Table 4.1) compared to most spores and also due to this strain being well-characterized and previously being utilised as a probiotic (Cutting, 2011).

There were differences between the treatments for gas and methane production throughout the ruminal batch cultures ($P \leq 0.011$; Table 4.2). The CON treatment produced the least amount of gas during the incubations ($P < 0.001$), but there were no differences between the FreeE and SBE treatments (FreeE vs SBE: $P \geq 0.106$). There was a tendency for an increased amount of gas produced at 3 and 6 h for BcoaE compared with BsubE (BcoaE vs BsubE: $P = 0.076$). Similarly, methane production was least for CON (CON vs ALL: $P \leq 0.004$). There was no difference in methane produced between FreeE and SBE at 3 h (FreeE vs SBE: $P = 0.544$); however, there was a reduction in methane production for BcoaE and BsubE compared with FreeE at 6 h ($P = 0.032$) and 12 h ($P = 0.047$), and a tendency for reduction by the SBE treatments at 12 and 24 h ($P = 0.085$ and 0.068, respectively).

Dry matter degradability was affected by treatment at 12 and 48 h of incubation. At 12 h, DM degradability was greatest for BcoaE, intermediate for BsubE, and lowest for FreeE and CON (63.5, 61.7, 60.3 and 60.6%, respectively; $P = 0.022$). At 48 h, there was no difference in DM degradability between the enzyme treatments (FreeE vs SBE; $P= 0.639$); however, CON DM degradability was significantly decreased (CON vs ALL; $P = 0.003$). There was a tendency for NDF degradability at 12 h to be greater for BcoaE compared with BsubE (BcoaE vs BsubE: $P =$

Table 4.2. The effect of spore-bound enzyme treatment on *in vitro* gas production, methane production, and degradability.

	Treatments[1]				SEM	*P* value	Contrast *P*-value		
	CON	FreeE	BcoaE	BsubE			CON vs ALL	FreeE vs SBE	BcoaE vs BsubE
Gas production, mL/g DM									
3 h	19.7	23.9	23.5	22.0	8.27	< 0.001	< 0.001	0.106	0.076
6 h	48.3	54.6	54.7	52.6	6.66	< 0.001	< 0.001	0.375	0.076
12 h	101.7	108.8	109.1	107.2	4.18	< 0.001	< 0.001	0.589	0.178
24 h	137.9	142.5	142.4	139.9	2.67	< 0.001	< 0.001	0.451	0.206
48 h	155.9	166.4	165.9	163.3	2.31	< 0.001	< 0.001	0.296	0.204
Methane production, mL/g DM									
3 h	6.6	7.3	7.1	7.3	4.01	< 0.001	< 0.001	0.544	0.185
6 h	11.4	12.2	11.9	11.9	5.62	< 0.001	< 0.001	0.032	0.835
12 h	18.7	20.1	19.7	19.4	7.87	0.001	< 0.001	0.047	0.509
24 h	25.2	26.4	25.9	25.8	9.89	0.009	0.002	0.085	0.524
48 h	29.8	31.3	30.7	30.5	11.76	0.011	0.004	0.068	0.422

Dry matter degradability, %									
0 h	43.4	41.3	41.6	41.1	1.53	0.387	0.093	0.814	0.757
3 h	44.0	43.9	43.5	43.7	1.71	0.484	0.243	0.343	0.704
6 h	49.4	49.4	49.3	49.4	1.04	0.988	0.901	0.904	0.758
12 h	60.6	60.3	63.5	61.7	2.20	0.022	0.169	0.018	0.098
24 h	74.7	73.6	75.4	74.0	1.46	0.361	0.691	0.246	0.194
48 h	78.9	79.9	80.2	79.9	0.49	0.018	0.003	0.639	0.423
NDF degradability, %									
12 h	16.3	16.8	21.8	17.4	1.88	0.091	0.222	0.176	0.068
24 h	43.2	42.6	44.1	42.3	1.94	0.817	0.931	0.735	0.375
48 h	52.2	54.2	54.9	54.1	1.96	0.075	0.014	0.725	0.399
ADF degradability, %									
12 h	18.2	20.0	23.6	20.2	2.72	0.150	0.115	0.361	0.147
24 h	43.2	43.0	44.2	43.3	1.92	0.941	0.854	0.687	0.663
48 h	51.8	54.4	54.0	54.1	1.87	0.184	0.032	0.753	0.922

[1]CON = control; FreeE = commercial xylanase enzyme; BcoaE = commercial xylanase enzyme bound to *Bacillus coagulans* spores; BsubE = commercial xylanase enzyme bound to *Bacillus subtilis* spores

0.068). There was an improvement in NDF degradability at 48 h for BcoaE, BsubE and FreeE (54.9, 54.1 and 54.2%, respectively) compared with CON (52.2%; CON vs ALL: $P = 0.014$). All enzyme treatments had a greater (CON vs ALL: $P = 0.032$) ADF degradability as compared with CON at 48 h of incubation, but not for 12 and 24 h.

There were no differences among treatments for total VFA concentration ($P \geq 0.139$; Table 4.3). At 24 h, the CON had significantly lower acetate proportion (63.5%) compared with the enzyme treatments (65.3%; CON vs ALL: $P = 0.006$) but this effect was not observed at 48 h. However, at 48 h a difference was observed in the proportion of acetate produced between BcoaE and BsubE (64.4 and 63.5%, respectively; BcoaE vs BsubE: $P = 0.047$). All treatments had similar proportions of propionate at 0, 12, and 48 h of incubation ($P \geq 0.493$). However, the proportion of propionate was greater in CON (17.3%) as compared with the other treatments (average 16.7%; CON vs ALL: $P = 0.027$) at 24 h. The proportion of butyrate was not different between 0 and 12 h; however, there was a decrease in proportion of butyrate for all treatments (average 11.8%) compared with CON (12.5%; CON vs ALL: $P = 0.027$) at 24 h. At 48 h there was a difference in butyrate proportions between BcoaE and BsubE (11.7 and 12.1%, respectively; BcoaE vs BsubE: $P = 0.007$).

There was no difference in the spore counts of *B. coagulans* ($P = 0.769$) or *B. subtilis* ($P = 0.263$) after 0, 3, 12, 24 and 48 h incubation (Table 4.4). The average *B. coagulans* spore count was 7.65 log spores/ml, and the average *B. subtilis* (RK28) spore count was 7.75 log spores/ml across all time points.

Overall, xylanase activity decreased throughout the batch culture incubations. At the initiation of the batch culture experiments there was a significant difference in xylanase activity among the

Table 4.3. The effect of spore-bound enzyme treatment on *in vitro* volatile fatty acid (VFA) concentration and molar proportions.

	Treatments[1]				SEM	*P* value	Contrast *P*-values		
	CON	FreeE	BcoaE	BsubE			CON vs All	FreeE vs SBE	BcoaE vs BsubE
Total VFA Concentration, mM									
0 h	33.3	32.0	29.2	29.0	1.636	0.139	0.058	0.162	0.961
3 h	46.0	49.2	47.8	51.6	3.118	0.638	0.331	0.902	0.401
6 h	62.1	62.8	57.5	62.6	3.302	0.633	0.773	0.507	0.281
12 h	74.3	78.1	80.4	73.6	4.409	0.668	0.552	0.831	0.288
24 h	86.3	88.9	90.9	91.8	4.988	0.869	0.467	0.695	0.899
48 h	104.2	96.2	106.5	103.9	6.938	0.738	0.803	0.295	0.790
VFA Molar Proportion, %									
Acetate									
0 h	68.5	68.9	68.0	67.8	1.029	0.884	0.845	0.448	0.907
3 h	68.0	67.8	68.6	68.5	0.697	0.836	0.077	0.412	0.889
6 h	66.8	67.2	67.1	67.2	0.723	0.975	0.675	0.906	0.894

12 h	65.2	66.5	66.3	65.6	0.716	0.547	0.255	0.529	0.526
24 h	63.5	64.8	65.5	65.7	0.546	0.030	0.006	0.246	0.858
48 h	63.8	64.0	64.4	63.5	0.306	0.236	0.675	0.906	0.047
Propionate									
0 h	14.8	15.1	15.4	15.5	0.394	0.493	0.178	0.491	0.812
3 h	16.8	17.0	16.5	16.8	0.311	0.739	0.979	0.394	0.477
6 h	17.3	17.2	17.0	17.2	0.340	0.955	0.713	0.876	0.693
12 h	17.5	17.0	17.1	17.1	0.262	0.648	0.223	0.730	0.997
24 h	17.3	16.8	16.7	16.7	0.212	0.148	0.027	0.599	0.851
48 h	16.8	16.8	16.7	16.9	0.161	0.939	0.828	0.827	0.583
Butyrate									
0 h	11.6	10.9	11.6	11.6	0.599	0.789	0.675	0.360	0.991
3 h	9.9	9.8	9.8	9.6	0.384	0.963	0.753	0.776	0.756
6 h	10.2	10.1	10.2	10.1	0.363	0.990	0.786	0.896	0.884
12 h	11.5	11.0	11.1	11.5	0.367	0.644	0.399	0.503	0.486
24 h	12.5	12.1	11.7	11.6	0.271	0.084	0.027	0.183	0.852
48 h	12.0	11.9	11.7	12.1	0.106	0.045	0.445	0.952	0.007

[1]CON = control; FreeE = commercial xylanase enzyme; BcoaE = commercial xylanase enzyme bound to *Bacillus coagulans* spores; BsubE = commercial xylanase enzyme bound to *Bacillus subtilis* spores

Table 4.4. *In vitro* spore persistence of *Bacillus coagulans* (B62) and *Bacillus subtlis* (RK28) measured at 0, 3, 12, 24 and 48 h of incubation.

	Log spores/mL	
	Bacillus coagulans (B62)	*Bacillus subtilis* (RK28)
0 h	7.74	7.89
3 h	7.61	7.77
12 h	7.67	7.65
24 h	7.58	7.64
48 h	7.67	7.81
SEM	0.087	0.082
P - Value	0.769	0.263

treatments (P = 0.002; Figure 4.1A), where FreeE had the greatest (83.7 U), BcoaE and BsubE treatments were intermediate (73.0 and 68.6 U, respectively) and CON had the least xylanase activity (51.2 U). There was no difference in xylanase activity between any of the treatments at 6 h (P = 0.10), but at 12 h both SBE treatments had greater xylanase activity compared with the CON and FreeE (SBE vs FreeE: P = 0.012). Xylanase activity again became similar for all treatments at 24 h (P = 0.327); however, by 48 h, the three enzyme treatments had greater activity compared with the control (CON vs ALL; P = 0.016). When plotted as relative activity, xylanase activity decreased by approximately 70% for all enzyme treatments by 48 h (Figure 4.1B). Generally, relative xylanase activities were similar between enzyme treatments, except at 12 h when relative activity was greater for both BcoaE and BsubE compared with FreeE (FreeE vs SBE; P = 0.021).

4.4 Discussion

Large variations in both protein binding efficiency (18.9 to 52.8%) and retained enzyme activity (< 0.01 to 5.47%) existed between bacterial spore species and even strains within a species (Table 4.1). Similarly, Cho et al. (2011) reported differences in SBE activities when different spores were used. These differences were likely due to variations in spore size and the content of spore coat proteins influencing the amount of enzyme absorption (Cho et al., 2011), in addition to orientation of the xylanase active site after adsorption. In the current study, there did not appear to be any correlation between the amount of enzyme that bound to the surface of a spore and the amount of enzyme remaining active on the spores. The majority of bound enzyme was inactive, regardless of spore used. The mechanism of enzyme adsorption to spore coats is likely due to a combination of electrostatic attraction and hydrophobicity (Cho et al., 2011). This suggests that the interaction between spore coat proteins and enzymes may result in a portion of

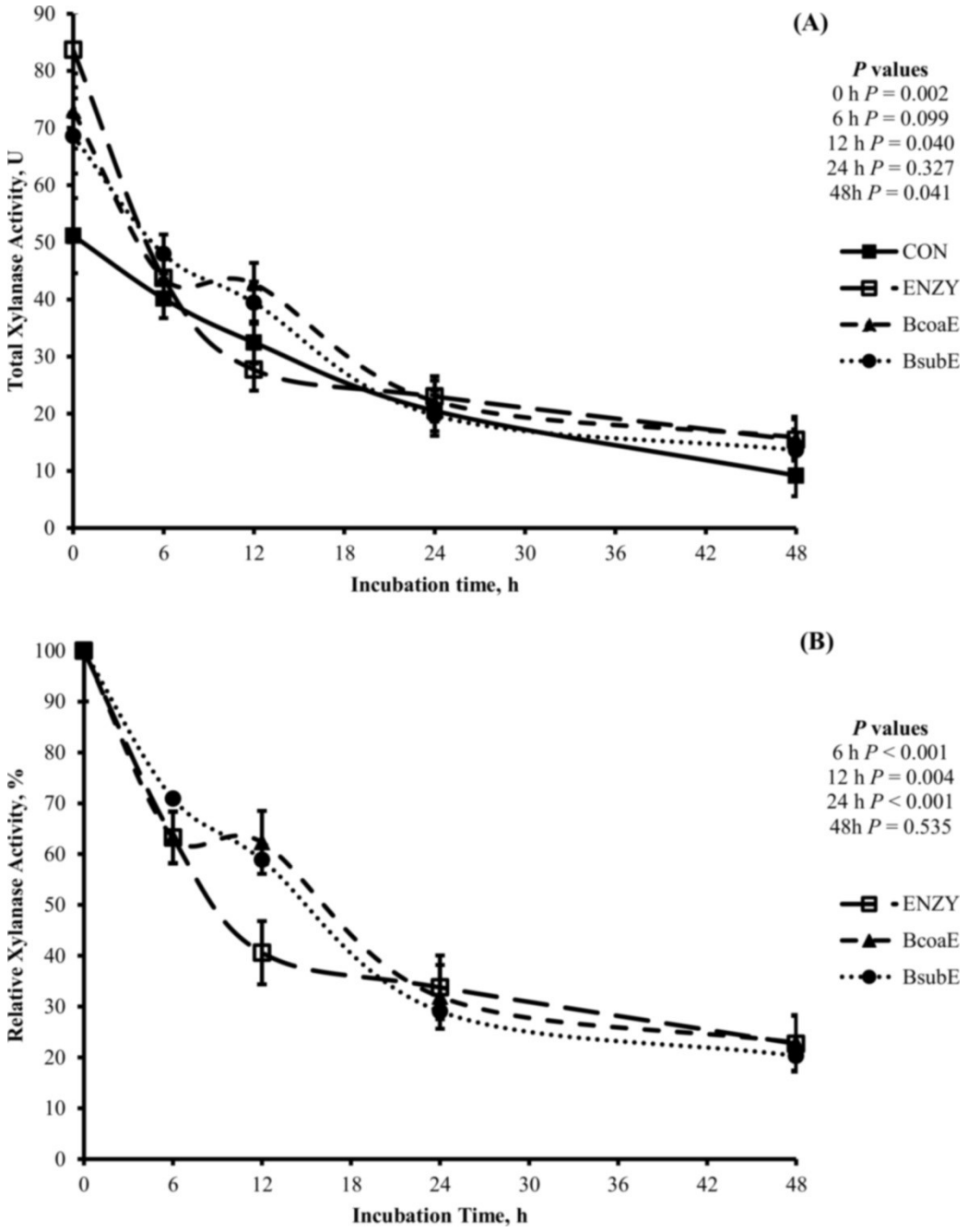

Figure 4.1. The effect of spore binding and bacterial species of spores on total xylanase activity (A) and percentage of original xylanase activity (B) in ruminal batch culture. The treatments were control (CON), commercial xylanase enzyme (ENZY), *Bacillus coagulans* spore-bound enzyme (BcoaE), and *Bacillus subtilis* spore-bound enzyme (BsubE).

enzyme active sites not being accessible to substrate, and reducing their retained activity when bound to spores. As well it has been suggested that the process of adsorption itself may change protein structure, which can reduce enzyme activity (Bower et al., 1999).

The application of SBE did not alter total gas production compared with FreeE; however, it did decrease the amount of methane produced compared with unbound enzyme. Wang et al. (2016) found that addition of a *B. subtilis* additive increased *in vitro* methane concentration although they utilised a live additive whereas in the current study the spores used were washed and had limited background nutrients from spore production. In a previous experiment the effect of *B. subtilis* RK28 and *B. coagulans* B62 spores on ruminal *in vitro* fermentation was evaluated (data not shown), and no difference from a control treatment were observed, indicating the spores by themselves did not affect fermentation. Based on spore counts in the present study (Table 4.4), germination was not occurring in the *in vitro* ruminal inoculant, and therefore active *Bacillus* were not having a direct impact on fermentation. Thus, the difference in methane production between FreeE and SBE was more likely due to the enzyme delivery method rather than the addition of *Bacillus* spores. It is unknown how this may have occurred but could be related to the initial lower xylanase activity of SBE due to spore absorption, compared with FreeE. Methane production is an energy loss during fermentation (Eckard et al., 2010). Therefore, a decrease in methane production suggests that SBE may have the potential to improve feed efficiency, particularly as degradability fermentation parameters (Table 4.2) were not negatively affected by SBE compared with FreeE.

While enzyme addition did not affect total VFA concentrations, acetate proportions were increased. Using a similar xylanase enzyme as the one in the current study, Romero et al. (2015) reported an increase in total VFA when the enzyme was added to ruminal cultures, but no

increase in acetate proportion. These differences may be related to the substrate used; Romero et al. (2015) added bermudagrass haylage to the batch cultures while alfalfa hay was used in the current study. Increased acetate proportion may be indicative of enhanced fiber digestibility (Doane et al., 1997), and this was supported by degradation parameters in the current study. After 48 h of incubation, both ADF and NDF degradability were greater in the FreeE and SBE treatments, compared with the control (Table 4.2).

Despite differences in enzyme binding efficiency and retained activity for BcoaE and BsubE, ruminal xylanase activity between these two treatments was similar. Even though the xylanase activity of all treatments decreased throughout the batch cultures, at 12 h of incubation, xylanase activity for both SBE treatments was greater than FreeE. When represented as relative activity (% of 0 h activity), the SBE treatments had an average of 60.6% remaining xylanase activity, compared with 40.6% for FreeE after 12 h (Figure 4.1B). Because the initial SBE activities were lower and could not account for these differences (Table 4.2), it may be that one or a combination of the following occurred to result in greater xylanase activities in SBE treatments: xylanase was released from the spores, the orientation of spore-bound xylanase shifted throughout rumen fermentation and increased active xylanase on spores, or the SBE treatments stimulated rumen bacterial xylanase production. The latter is less likely because the spores did not germinate and would have had little influence on the microbiota.

It is noteworthy that after 12 h of fermentation, the SBE treatments resulted in greater DM degradability compared with FreeE, which corresponds to when BcoaE and BsubE had relatively greater xylanase levels. Although differences in degradability between the SBE treatments and FreeE did not occur after 24 h and 48 h of fermentation, the greater DM, NDF, and ADF degradabilities of all three enzyme-supplemented treatments compared with CON after 48 h

highlights that the xylanase used in the current study enhanced fiber degradation. Feeding cattle throughout the day with supplemented SBE could potentially result in prolonged elevated enzyme activities compared to the closed system of a batch culture. If spore-bound xylanase elevate xylanase activities in the rumen of cattle, efficacy of enzyme would be expected to increase, which could improve fiber digestion by cattle. This is a concept that needs to be further investigated *in vivo*.

4.5 Conclusion

The binding efficiency and retained activity of xylanase varied according to spore strain and there was no clear relationship between the amount of enzyme binding and SBE activity. When added to ruminal batch cultures, SBE decreased methane production and increased DM degradability, which corresponded to increased xylanase activity after 12 h of fermentation, compared with the unbound enzyme. These results suggest that SBE may have value in prolonging the ruminal activity of feed enzymes fed to cattle.

5.0 The effect of binding a variety of exogenous enzymes to *Bacillus subtilis* spores on gas production, methane production, *in vitro* degradability and volatile fatty acid production

5.1 Introduction

By 2050, global crop demand is predicted to rise to 100-110% of levels achieved in 2005 (Tilman 2011). Increased demand is expected to result from population growth and increasing affluence in emerging economies leading to increased consumption of meat and milk protein (Keyzer et al., 2005; Alexandratos and Bruin, 2012). Greater demands for animal protein present environmental challenges for agriculture, including increased production of greenhouse gases, livestock waste, land clearing, and antimicrobial use associated with intensive livestock production practices (Van Boeckel et al., 2015; Ribeiro et al., 2016). Thus developing technologies to improve the efficiency by which livestock utilize feed would help attenuate the environmental pressures of greater animal meat production.

Despite the vast diversity of microbiota in the rumen of cattle consuming fibre-rich diets (Bensoussan et al., 2017) and the rumen being considered as highly efficient in degrading lignocellulosic biomass (Flint et al., 2008), total tract digestibility of neutral detergent fiber (**NDF**) in dairy cattle averages only 50.4 ± 10.8% (White et al., 2017). To enhance digestion, exogenous enzymes have been investigated as feed additives for ruminants; however, the effect on production efficiency has been variable (Beauchemin et al., 1995; Higginbotham et al., 1996; Lewis et al., 1999). Reasons for this variation or lack of improvement include*:* feed substrate, enzyme dose, application of the enzyme, and physiological state of animal (Beauchemin et al., 2003). In addition, the denaturation of enzymes during feed processing (Inborr and Bedford,

1994), and transit throughout the gastrointestinal tract reduce their efficacy (Baas and Thacker, 1996; Hristov et al., 1998b; Morgavi et al., 2001).

Commercial feed enzymes are produced in large-scale using either unmodified or genetically modified fungi and bacteria. The enzymes are purified to different extents, depending on whether transgenic microorganisms or DNA need to be removed from the product (Ribeiro et al., 2016). There are many different types of enzymes available for use in livestock feeds, which degrade different substrates. Amylases and phytases target starch and phytate, respectively, which are relatively simple substrates. More complex substrates that are fibrous require a cascade of enzymes for degradation, including cellulases, xylanases, pectinases, mannanases, xyloglucanases, glucanases, and glucosidases (Artzi et al., 2017). Esterases are required for degradation of ester bonds that can be formed between resistant lignin and the potentially degradable cell wall polysaccharides (Jung and Allen, 1995). Combinations of exogenous enzymes with different activities have been suggested to be more efficient than single enzyme activities because they can work synergistically to improve accessibility to specific substrates (Hatfield, 1993). A complex array of enzymes is observed in the rumen microbiota as well, with numerous bacteria producing cellulosomes, which are organized multi-enzyme complexes present on the cell surface that improve the efficacy of cellulose degradation. Several studies have tried to mimic cellulosomes by synthesizing complex enzyme structures and adhering them to polystyrene nanospheres (Blanchette et al., 2012), streptavidin (Kim et al., 2012), and DNA scaffold (Mori et al., 2013). Transgenic *Pichia pastoris* (Ou et al., 2014), and *Lactobacillus plantarum* (Morais et al., 2014) have also been developed for the assembly of cellulosomes on the cell surface for future use in bioethanol production. However, these efforts have had limited

success. In addition, transgenic bacteria require strict regulatory approval and are difficult to market.

Non-transgenic approaches to deliver stable combinations of exogenous enzymes are favourable for industry and producers. Adsorption of exogenous enzymes to *Bacillus* spp. spores has previously been reported to improve enzyme stability over a range of temperatures and pH (Cho et al., 2011; Sirec et al., 2012; Gashtasbi et al., 2014; Gu et al., 2015). Huang et al. (2010) adsorbed antigen proteins to the surface of *Bacillus subtilis* spores, and determined that immunogenicity was retained after passage of these spores through the murine gastrointestinal tract. The retained immunogenicity suggests that adsorption of enzymes to *B. subtilis* spores may offer a non-transgenic approach to delivering both individual and combinations of exogenous enzymes to the ruminant gastrointestinal tract, improving enzyme stability and increasing digestibility. The objective of this study was to determine the effect of different spore-bound enzymes (alone and in combination) on *in vitro* gas production, methane production, digestibility, and volatile fatty acid production. It was hypothesized that spore-bound enzymes would improve enzyme stability compared to unbound enzymes, and binding a combination of enzymes would result in greater improvements in degradability than individual spore-bound enzymes.

5.2 Materials and methods

5.2.1 Spores and enzymes

Lyophilized *B. subtilis* (RK28) spores were obtained from SporeGen (Egham, Surrey, U.K.). Prior to use, spores were washed sequentially with 1M KCl, 0.5 M NaCl, and distilled water (three times) by mixing 0.1 g of spore material with 10 ml of each solvent, followed by

centrifugation (5,000 × *g*; 15 min) and decanting of the supernatant. The washed spores were then enumerated by plating heat treated serial dilutions (65°C, 45 min to kill vegetative cells) onto Luria-Bertani agar, followed by incubation overnight at 39°C. After enumeration, wash spores were diluted using water to a concentration of 1.0×10^{10} *B. subtilis* spores/ml. The enzymes used in the study included purified solutions of β-glucanase (**GLUC)**, feruloyl esterase (**FE**), and xylanase (**XYL**), and commercial crude products of α-amylase (**AMYL**), phytase (**PHYT**), and xylanase (**CX**). The commercial crude products were obtained from Sigma-Aldrich Corp. (St. Louis, MO), Danisco Animal Nutrition (Copenhagen, Denmark), and Adisseo France S.A.S (Antony, France), respectively.

Enzymes were adsorbed to 1×10^{10} *B. subtilis* spores as described in Chapter 3, using optimal binding conditions previously determined. For enzyme combinations adsorption was conducted at pH 5.0 for 1 h at 25°C, because these were the most common optimal conditions. The optimal concentrations of enzymes adsorbed to spores were 0.5 mg/ml for GLUC and XYL, and 1.0 mg/ml for AMYL, CX, FE, and PHYT.

5.2.2 Batch cultures

The treatments for the batch cultures were: control (**CON**; distilled water), *B. subtilis* spores (**SPR**; 1.0×10^{10} spores/g substrate), unbound enzyme (**ENZY**; utilizing optimal concentrations described above/g substrate), and spore-bound enzyme (**SBE**; optimal protein concentration of enzyme bound to 1.0×10^{10} spores/g substrate).

For batch cultures, the enzymes were analyzed individually and in the following combinations: AMYL and CX (**ACX**; 1.0 mg/ml AMYL + 1.0 mg/ml CX); GLUC, FE, AMYL, and XYL (**GFAX**; 0.5 mg/ml GLUC + 1.0 mg/ml FE + 1.0 mg/ml AMYL + 0.5 mg/ml XYL); and GLUC,

FE, AMYL, and CX (**GFACX**; 0.5 mg/ml GLUC + 1.0 mg/ml FE + 1.0 mg/ml AMYL + 1.0 mg/ml CX). To test whether proximity of enzymes on the spores would affect digestion, the combined enzymes were either mixed prior to spore adsorption (SBE), or bound individually to spores (using individual optimal adsorption conditions) and combined (I-SBE). A summary of all treatments tested are listed in Table 5.1.

Three separate *in vitro* batch cultures were conducted on different days for all treatments. Rumen digesta was collected and composited from three ruminally cannulated cows 2 h after feeding a diet of 75% barley silage, 22% rolled barley grain, and 3% vitamin/mineral pellet (DM basis). Under anaerobic conditions the composited rumen digesta (strained through four layers of cheesecloth) was mixed with Menke's buffer (1:3) to make the batch culture inoculant (Menke et al., 1979). The substrate in the batch cultures was 75% barley silage and 25% barley grain (DM basis), which was ground to pass through a 2-mm screen. The substrate was weighed (0.5 g) into ANKOM F57 bags (25μm pore size), and four hours prior to the start of the experiment 0.5 ml of each treatment was applied directly onto the substrate, then the bags were heat sealed and placed into serum vials. At the start of each batch culture, 60 ml of inoculant was added to the serum vials (containing the ANKOM bags filled with substrate) under a constant stream of CO_2. The vials were then sealed and incubated on a rotary shaker (39°C) for 0, 3, 6, 12, 24, and 48 h (triplicate vials per time point). At each time point 10 ml of gas was collected from the 48 h vials using a syringe, and stored in pre-evacuated gas vials at room temperature for methane analysis. After the methane samples were collected gas production was measured from all vials remaining in the incubator using a water displacement apparatus described in Fedorah and Hrudey (1983).

The ANKOM bags were removed from the serum vials, washed in cold water (5 times, 1 min/wash), then placed in an oven at 105°C for 48 h to determine DM disappearance. The bags

Table 5.1. Summary of all treatments examined in batch culture experiments

Treatment	Description	Concentration	Conditions
CON	Distilled water		
SPR	*Bacillus subtilis* spores	1.0×10^{10}/g substrate	
AMYL	Unbound α-amylase	1.0 mg α-amylase/g substrate	Buffer pH 2.0
SB-AMYL	Spore-bound α-amylase	1.0 mg α-amylase bound to 1.0×10^{10} *B. subtilis* spores/g substrate	Buffer pH 2.0; bound for1 h at 4°C
CX	Unbound commercial xylanase	1.0 mg commercial xylanase/g substrate	Buffer pH 5.0
SB-CX	Spore-bound commercial xylanase	1.0 mg commercial xylanase bound to 1.0×10^{10} *B. subtilis* spores/g substrate	Buffer pH 5.0; bound for1 h at 25°C
FE	Unbound feruloyl esterase	1.0 mg feruloyl esterase/g substrate	Buffer pH 5.0
SB-FE	Spore-bound feruloyl esterase	1.0 mg feruloyl esterase bound to 1.0×10^{10} *B. subtilis* spores/g substrate	Buffer pH 5.0; bound for1 h at 4°C
GLUC	Unbound glucanase	0.5 mg β-glucanase/g substrate	Buffer pH 4.0
SB-GLUC	Spore-bound glucanase	0.5 mg β-glucanase bound to 1.0×10^{10} *B. subtilis* spores/g substrate	Buffer pH 4.0; bound for 0.5 h at 25°C
PHYT	Unbound phytase	1.0 mg phytase/g substrate	Buffer pH 4.0
SB-PHYT	Spore-bound phytase	1.0 mg phytase bound to 1.0×10^{10} *B. subtilis* spores/g substrate	Buffer pH 4.0; bound for 1 h at 40°C
XYL	Unbound purified xylanase	0.5 mg purified xylanase/g substrate	Buffer pH 5.0
SB-XYL	Spore-bound purified xylanase	0.5 mg purified xylanase bound to 1.0×10^{10} *B. subtilis* spores/g substrate	Buffer pH 5.0; bound for1 h at 25°C

ACX	Unbound combination of α-amylase and commercial xylanase	0.5 mg α-amylase + 0.5 mg/g substrate	Buffer pH 5.0
SB-ACX	Spore-bound α-amylase and commercial xylanase	0.5 mg α-amylase + 0.5 mg bound to 1.0×10^{10} *B. subtilis* spores /g substrate	Buffer pH 5.0; bound for1 h at 25°C
GFACX	Unbound combination of glucanase, feruloyl esterase, α-amylase and commercial xylanase	0.125 mg β-glucanase + 0.25 mg feruloyl esterase + 0.25 mg α-amylase + 0.25 mg commercial xylanase/g substrate	Buffer pH 5.0
SB-GFACX	Spore-bound combination of glucanase, feruloyl esterase, α-amylase and commercial xylanase	0.125 mg β-glucanase + 0.25 mg feruloyl esterase + 0.25 mg α-amylase + 0.25 mg commercial xylanase bound to 1.0×10^{10} *B. subtilis* spores /g substrate	Buffer pH 5.0; bound for1 h at 25°C
I-SB-GFACX	Combination of individually spore-bound glucanase, feruloyl esterase, α-amylase and commercial xylanase	0.125 mg β-glucanase bound to 2.5×10^9 spores + 0.25 mg feruloyl esterase bound to 2.5×10^9 spores + 0.25 mg α-amylase bound to 2.5×10^9 spores + 0.25 mg commercial xylanase bound to 2.5×10^9 spores/g substrate	Buffer pH 5.0; bound for1 h at 25°C
GFAX	Unbound combination of glucanase, feruloyl esterase, α-amylase and purified xylanase	0.125 mg β-glucanase + 0.25 mg feruloyl esterase + 0.25 mg α-amylase + 0.25 mg purified xylanase/g substrate	Buffer pH 5.0
SB-GFAX	Spore-bound combination of glucanase, feruloyl esterase, α-amylase and purified xylanase	0.125 mg β-glucanase + 0.25 mg feruloyl esterase + 0.25 mg α-amylase + 0.25 mg purified xylanase bound to 1.0×10^{10} *B. subtilis* spores /g substrate	Buffer pH 5.0; bound for1 h at 25°C

I-SB-GFAX	Combination of individually spore-bound glucanase, feruloyl esterase, α-amylase and purified xylanase	0.125 mg β-glucanase bound to 2.5×10^9 spores + 0.25 mg feruloyl esterase bound to 2.5×10^9 spores + 0.25 mg α-amylase bound to 2.5×10^9 spores + 0.25 mg purified xylanase bound to 2.5×10^9 spores/g substrate	Buffer pH 5.0; bound for1 h at 25°C

were then utilised for subsequent analysis of neutral detergent fiber (**NDF**) and acid detergent fiber (**ADF**) contents. The inoculant was sampled for analysis of volatile fatty acids (**VFA**; 1.5 ml inoculant + 0.3 ml of 25% meta-phosphoric acid), stored at -20°C until further analysis. The VFA samples were analysed by gas chromatography (model 5890, Hewlett-Packard Lab, Palo Alto, CA) using crotonic acid (trans-2-butenoic acid) as an internal standard.

5.2.3 Statistical analysis

Data were analysed using the Mixed Model procedure of SAS, with fixed effect of treatment and run, and random effect of rep. Contrasts were utilised to determine the difference between SBE vs CON, SBE vs SPR, and SBE vs ENZY. For the enzyme combination batch cultures the following contrast statements were utilised: SBE vs CON, I-SBE vs CON, SBE vs ENZY, I-SBE vs ENZY, and SBE vs I-SBE. Contrasts were declared significantly different when $P \leq 0.050$, and tendencies discussed when $0.05 < P \leq 0.10$.

5.3 Results

Dry matter degradability was not different between SB-GLUC and GLUC, with the exception of at 6 h incubation, where SB-GLUC had decreased DM degradability compared with GLUC ($P = 0.046$; Table 5.2). There was no difference in DM degradability between SB-GLUC and CON or between SB-GLUC and SPR from 3 to 12 h of incubation ($P \geq 0.11$). At 0 h NDF degradability either tended ($P \leq 0.084$) to increase, or increased by approximately 2 to 7% for SB-GLUC compared with the other treatments. However, there was an immediate decrease in NDF degradability at 3 h for SB-GLUC compared with both GLUC and CON ($P \leq 0.064$). At 12 h of incubation NDF degradability was decreased for SB-GLUC compared with both CON and SPR ($P = 0.013$ and 0.009, respectively), then after 24 h incubation SB-GLUC had increased NDF

Table 5.2. The effect of binding β-glucanase (GLUC) to *Bacillus subtilis* (RK28) spores on *in vitro* dry matter (DM), neutral detergent fibre (NDF) and acid detergent fibre (ADF) degradability in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	GLUC	SB-GLUC			SB-GLUC vs CON	SB-GLUC vs SPR	SB-GLUC vs GLUC
DM Degradability, %									
0 h	27.6	28.3	26.2	27.0	0.61	0.014	0.353	0.048	0.160
3 h	28.6	29.2	29.7	29.5	0.85	0.303	0.145	0.628	0.744
6 h	31.8	30.6	32.1	30.6	0.87	0.104	0.114	0.920	0.046
12 h	40.8	41.3	41.2	40.1	1.56	0.710	0.520	0.306	0.325
24 h	48.8	49.7	50.6	52.5	0.97	0.032	0.006	0.023	0.119
48 h	63.6	64.5	63.8	63.3	0.96	0.797	0.816	0.341	0.697
NDF Degradability, %									
0 h	24.6	26.5	29.4	32.0	1.05	< 0.001	< 0.001	< 0.001	0.084
3 h	33.5	30.7	33.5	28.5	3.74	0.120	0.034	0.317	0.062
6 h	34.1	33.9	38.7	38.1	3.86	0.128	0.128	0.106	0.805
12 h	43.4	43.7	38.4	37.6	1.76	0.012	0.013	0.009	0.693
24 h	39.2	39.5	47.3	45.5	1.95	< 0.001	0.003	0.005	0.361
48 h	54.0	55.0	56.2	54.7	1.60	0.660	0.698	0.865	0.402
ADF Degradability, %									
0 h	52.8	53.2	51.8	53.4	1.88	0.917	0.806	0.932	0.518
3 h	53.1	54.5	55.3	51.4	3.35	0.504	0.529	0.258	0.161

6 h	60.2	60.6	60.8	61.4	1.96	0.922	0.503	0.648	0.735
12 h	63.2	64.0	62.3	61.3	2.21	0.383	0.250	0.103	0.551
24 h	61.1	61.1	66.8	65.1	1.33	0.001	0.013	0.020	0.241
48 h	70.3	70.6	71.2	70.6	1.61	0.939	0.876	0.996	0.706

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0 × 10^{10} *B. subtilis* spores/g substrate), β-glucanase (GLUC; 0.5 mg β-glucanase /g substrate) and spore-bound β-glucanase (SB-GLUC; 0.5 mg of β-glucanase bound to 1.0 × 10^{10} *B. subtilis* spores/g substrate).

degradability compared with CON and SPR (P = 0.003 and 0.005, respectively). Degradability of ADF was not different between SB-GLUC and GLUC throughout incubation ($P \geq 0.16$), nor was SB-GLUC different from either CON or SPR at 0 to 12 h of incubation ($P \geq 0.10$).

Addition of SB-FE tended to increase DM degradability compared with either CON or FE at 3 h (P = 0.071 and 0.060, respectively; Table 5.3). The degradability of NDF was not different between SB-FE and FE ($P \geq 0.11$), but SB-FE had greater NDF degradability than FE from 0 to 12 h of incubation ($P \leq 0.005$). There was no difference in ADF degradability between SB-FE and FE, with the exception of 6 h incubation, where SB-FE tended have decreased ADF degradability compared with FE (P = 0.078). At 0, 3, and 12 h of incubation SB-FE had greater ADF degradability compared with SPR ($P \leq 0.034$), and at 3 h SB-FE had greater ADF degradability compared with CON (P = 0.045).

There was no difference in DM or ADF degradability between SB-XYL and XYL ($P \geq 0.10$), but at 0, 6 and 24 h NDF degradability tended to be decreased for SB-XYL compared with XYL ($P \geq 0.057$; Table 5.4). There was also no difference in DM degradability between SB-XYL and SB-XYL with the exception of 6 h, where SB-XYL tended to be greater than SPR (32.3 and 30.6%, respectively; P = 0.081). The degradability of NDF was greater for SB-XYL compared with SPR at 0 and 24 h ($P \leq 0.073$), but there was no difference in ADF degradability between SB-XYL and SPR ($P \geq 0.34$). Degradability of NDF was not different between SB-XYL and CON from 3 to 12, and at 48 h ($P \geq 0.15$).

Dry matter degradability was not different throughout the incubation between SB-AMYL and AMYL ($P \geq 0.12$; Table 5.5). Increases in DM degradability for SB-AMYL compared to CON and SPR were observed throughout incubation ($P \leq 0.045$), with the exception of 24 h ($P \geq$

Table 5.3. The effect of binding feruloyl esterase (FE) to *Bacillus subtilis* (RK28) spores on *in vitro* dry matter (DM), neutral detergent fibre (NDF) and acid detergent fibre (ADF) degradability in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	FE	SB-FE			SB-FE vs CON	SB-FE vs SPR	SB-FE vs FE
DM Degradability, %									
0 h	32.8	27.2	32.7	31.7	0.94	< 0.001	0.173	< 0.001	0.185
3 h	31.0	28.6	31.1	32.9	1.02	< 0.001	0.071	< 0.001	0.060
6 h	35.7	31.1	35.0	35.2	0.90	< 0.001	0.552	< 0.001	0.837
12 h	43.5	40.5	43.0	44.0	1.13	0.005	0.613	< 0.001	0.298
24 h	53.0	49.4	52.4	53.2	1.22	0.007	0.897	0.002	0.466
48 h	63.9	61.1	64.1	64.4	1.06	0.010	0.769	0.008	0.865
NDF Degradability, %									
0 h	33.1	26.8	33.7	34.8	1.62	< 0.001	0.425	< 0.001	0.576
3 h	34.5	29.5	38.4	40.6	2.36	< 0.001	0.001	< 0.001	0.183
6 h	37.8	32.3	37.8	38.8	2.07	< 0.001	0.540	< 0.001	0.541
12 h	42.2	39.3	41.9	44.0	1.92	0.039	0.145	0.005	0.105
24 h	44.1	43.8	46.0	43.5	2.19	0.568	0.734	0.854	0.187
48 h	54.5	53.9	51.5	51.5	1.58	0.156	0.086	0.161	0.993
ADF Degradability, %									
0 h	57.5	53.5	56.6	58.9	2.24	0.054	0.522	0.009	0.260
3 h	55.1	49.5	57.4	58.5	2.36	< 0.001	0.045	< 0.001	0.468

6 h	59.1	56.8	63.7	59.7	2.30	0.015	0.787	0.154	0.078
12 h	59.6	60.3	63.1	64.2	2.30	0.063	0.688	0.034	0.512
24 h	62.4	62.7	62.1	62.0	1.87	0.972	0.785	0.669	0.939
48 h	68.7	69.0	67.3	67.2	1.61	0.421	0.273	0.197	0.944

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0 × 10^{10} *B. subtilis* spores/g substrate), feruloyl esterase (FE; 1.0 mg feruloyl esterase/g substrate) and spore-bound feruloyl esterase (SB-FE; 1.0 mg of feruloyl esterase bound to 1.0 × 10^{10} *B. subtilis* spores/g substrate).

Table 5.4. The effect of binding xylanase (XYL) to *Bacillus subtilis* (RK28) spores on *in vitro* dry matter (DM), neutral detergent fibre (NDF) and acid detergent fibre (ADF) degradability in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	XYL	SB-XYL			SB-XYL vs CON	SB-XYL vs SPR	SB-XYL vs XYL
DM Degradability, %									
0 h	28.4	28.4	27.7	28.2	0.51	0.537	0.735	0.675	0.351
3 h	28.6	29.2	29.8	29.7	0.84	0.467	0.203	0.564	0.882
6 h	31.8	30.6	32.8	32.3	1.03	0.129	0.599	0.081	0.580
12 h	40.8	41.3	42.4	42.6	1.84	0.379	0.146	0.274	0.882
24 h	48.5	49.7	50.8	49.7	0.63	0.080	0.169	0.962	0.150
48 h	63.6	64.5	62.9	64.3	0.80	0.471	0.520	0.865	0.215
NDF Degradability, %									
0 h	24.6	26.5	32.6	29.9	1.19	< 0.001	0.001	0.027	0.085
3 h	33.5	30.7	28.5	33.0	4.05	0.185	0.874	0.415	0.111
6 h	34.1	33.9	39.1	34.6	4.85	0.090	0.836	0.749	0.057
12 h	43.4	43.7	44.0	44.5	1.28	0.907	0.481	0.624	0.759
24 h	39.2	39.5	46.6	43.2	1.68	0.002	0.053	0.073	0.096
48 h	54.0	55.0	53.3	54.3	1.75	0.819	0.868	0.679	0.600
ADF Degradability, %									
0 h	52.8	53.2	54.1	54.8	2.82	0.770	0.340	0.448	0.733
3 h	53.1	54.5	52.4	53.1	4.67	0.615	0.999	0.342	0.688

6 h	60.2	60.6	62.6	60.8	2.11	0.691	0.793	0.933	0.403
12 h	63.2	64.0	64.8	64.6	1.63	0.621	0.300	0.662	0.866
24 h	61.1	61.5	65.5	63.0	1.34	0.027	0.216	0.340	0.101
48 h	70.3	70.6	68.7	69.7	1.62	0.532	0.660	0.537	0.461

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0 × 10^{10} *B. subtilis* spores/g substrate), pure xylanase (XYL; 0.5 mg pure xylanase/g substrate) and spore-bound pure xylanase (SB-XYL; 0.5 mg of pure xylanase bound to 1.0 × 10^{10} *B. subtilis* spores/g substrate).

Table 5.5. The effect of binding α-amylase (AMYL) to *Bacillus subtilis* (RK28) spores on *in vitro* dry matter (DM), neutral detergent fibre (NDF) and acid detergent fibre (ADF) degradability in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	AMYL	SB-AMYL			SB-AMYL vs CON	SB-AMYL vs SPR	SB-AMYL vs AMYL
DM Degradability, %									
0 h	27.0	26.8	31.9	31.2	1.76	< 0.001	< 0.001	< 0.001	0.327
3 h	28.8	28.4	32.2	31.6	1.60	< 0.001	0.004	< 0.001	0.457
6 h	31.8	31.5	36.1	36.3	1.94	< 0.001	< 0.001	< 0.001	0.860
12 h	40.7	39.7	41.9	43.4	2.66	0.013	0.019	0.002	0.177
24 h	48.3	49.5	51.0	49.4	2.80	0.091	0.318	0.876	0.115
48 h	60.7	60.4	63.8	62.9	1.16	0.006	0.045	0.021	0.393
NDF Degradability, %									
0 h	30.0	29.7	27.8	31.3	3.79	0.331	0.504	0.410	0.072
3 h	24.6	25.9	28.9	29.7	3.23	0.001	< 0.001	0.008	0.576
6 h	31.0	31.1	36.7	34.3	1.65	0.037	0.140	0.153	0.275
12 h	40.1	36.2	38.9	39.5	5.64	0.178	0.756	0.082	0.755
24 h	42.0	43.1	43.4	40.1	5.60	0.309	0.310	0.169	0.081
48 h	53.0	49.9	53.3	52.3	1.44	0.342	0.722	0.245	0.635
ADF Degradability, %									
0 h	53.1	53.8	48.8	52.3	3.32	0.041	0.656	0.392	0.060
3 h	47.3	48.0	49.6	50.3	2.98	0.045	0.012	0.053	0.541

6 h	53.3	53.8	58.0	56.1	1.43	0.084	0.171	0.266	0.326
12 h	61.6	58.8	59.9	60.1	4.89	0.524	0.430	0.424	0.911
24 h	61.9	62.1	62.5	59.7	4.78	0.312	0.168	0.190	0.089
48 h	67.8	65.6	67.5	67.0	1.76	0.584	0.633	0.415	0.789

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0 × 10^{10} *B. subtilis* spores/g substrate), amylase (AMYL; 1.0 mg α-amylase/g substrate) and spore-bound amylase (SB-AMYL; 1.0 mg of α-amylase bound to 1.0 × 10^{10} *B. subtilis* spores/g substrate).

0.32). At 24 h, SB-AMYL tended to have a decreased NDF and ADF degradability compared with AMYL ($P \leq 0.089$); however, at all other time points no differences were observed between SB-AMYL and AMYL ($P \geq 0.28$). After 3 h of incubation NDF and ADF degradability were greater for SB-AMYL compared with CON and SPR ($P \leq 0.053$). At 12 h of incubation NDF degradability tended to be greater for SB-AMYL compared with SPR ($P = 0.082$); however, no differences were observed at the other time points ($P \geq 0.15$).

At 0 h DM degradability was greater for SB-PHYT compared with CON and SPR ($P = 0.055$ and 0.031, respectively; Table 5.6), whereas there was no difference between SB-PHYT and PHYT at any time, except for a tendency for a decrease at 6 h for SB-PHYT ($P = 0.056$). There was a tendency for 0 h NDF and ADF degradability to be lower for SB-PHYT compared with CON and PHYT ($P \leq 0.075$); however, at 3 h both NDF and ADF degradability was greater for SB-PHYT compared with CON and PHYT ($P \leq 0.061$). Degradability of NDF at 12 h was greater for SB-PHYT compared with all treatments ($P \leq 0.088$), whereas ADF degradability at 12 h was greater for SB-PHYT compared with SPR and PHYT ($P \leq 0.033$) yet there was no difference between SB-PHYT and CON ($P = 0.42$). At 48 h NDF degradability tended to be decreased for SB-PHYT compared with PHYT (50.1 and 53.6%, respectively; $P = 0.074$).

Both DM and NDF degradability were not different between SB-CX and CX ($P \geq 0.17$; Table 5.7), and ADF degradability was not different between SB-CX and CX with the exception of 3 h, where SB-CX had decreased ADF degradability compared with CX ($P = 0.027$). Degradability of DM and NDF was greater for SB-CX compared with CON and SPR throughout incubation ($P \leq 0.014$), with the exception of NDF degradability at 24 h which was not different ($P \geq 0.11$). At 0, 3, 12 and 24 h of incubation ADF degradability was greater for SB-CX compared with CON

Table 5.6. The effect of binding phytase (PHYT) to Bacillus subtilis (RK28) spores on *in vitro* dry matter (DM), neutral detergent fibre (NDF) and acid detergent fibre (ADF) degradability in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	PHYT	SB-PHYT			SB-PHYT vs CON	SB-PHYT vs SPR	SB-PHYT vs PHYT
DM Degradability, %									
0 h	27.0	26.83	28.01	28.4	1.38	0.086	0.055	0.031	0.588
3 h	28.7	28.40	28.93	28.8	1.68	0.934	0.907	0.629	0.895
6 h	31.8	31.49	34.17	32.6	2.32	0.008	0.305	0.149	0.056
12 h	40.7	40.14	39.92	40.8	2.04	0.629	0.967	0.467	0.282
24 h	48.3	49.52	49.04	49.2	2.25	0.732	0.415	0.793	0.856
48 h	60.7	60.35	62.56	61.7	0.91	0.178	0.351	0.210	0.439
NDF Degradability, %									
0 h	30.0	29.7	26.9	25.9	2.24	0.145	0.056	0.075	0.619
3 h	24.6	25.9	31.1	28.8	2.92	< 0.001	0.008	0.061	0.125
6 h	31.0	31.1	34.1	33.3	3.57	0.648	0.442	0.463	0.792
12 h	40.1	36.2	38.3	43.0	4.73	0.003	0.088	< 0.001	0.009
24 h	42.0	45.6	43.4	44.4	3.36	0.537	0.304	0.630	0.672
48 h	53.0	49.9	53.6	50.1	1.35	0.118	0.130	0.894	0.074
ADF Degradability, %									
0 h	53.1	53.8	49.4	49.0	2.00	0.028	0.039	0.016	0.856

3 h	47.3	48.0	52.9	50.7	2.66	< 0.001	0.012	0.044	0.097
6 h	53.3	53.8	58.4	56.5	2.92	0.267	0.269	0.353	0.521
12 h	62.2	58.8	60.0	63.6	4.18	0.027	0.416	0.006	0.033
24 h	61.9	63.7	62.5	63.6	3.23	0.691	0.323	0.924	0.525
48 h	67.8	65.6	68.3	65.9	1.36	0.179	0.216	0.807	0.120

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0 × 10^{10} *B. subtilis* spores/g substrate), phytase (PHYT; 1.0 mg phytase/g substrate) and spore-bound phytase (SB-PHYT; 1.0 mg of phytase bound to 1.0 × 10^{10} *B. subtilis* spores/g substrate).

Table 5.7. The effect of binding commercial xylanase product (CX) to *Bacillus subtilis* (RK28) spores on *in vitro* dry matter (DM), neutral detergent fibre (NDF) and acid detergent fibre (ADF) degradability in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	CX	SB-CX			SB-CX vs CON	SB-CX vs SPR	SB-CX vs CX
DM Degradability, %									
0 h	27.3	27.3	30.7	30.6	0.96	< 0.001	< 0.001	< 0.001	0.853
3 h	28.6	28.8	32.1	31.4	0.93	< 0.001	0.001	0.002	0.413
6 h	31.8	31.1	34.8	33.8	0.91	< 0.001	0.018	0.001	0.233
12 h	40.8	40.4	43.9	43.6	1.31	< 0.001	0.003	< 0.001	0.815
24 h	49.2	49.6	52.9	52.7	1.23	0.005	0.005	0.013	0.887
48 h	62.2	62.4	64.0	65.0	1.17	0.088	0.019	0.035	0.456
NDF Degradability, %									
0 h	27.0	25.9	42.2	43.6	1.48	< 0.001	< 0.001	< 0.001	0.426
3 h	29.5	28.9	45.6	44.3	2.24	< 0.001	< 0.001	< 0.001	0.459
6 h	31.6	33.0	42.0	40.1	2.27	< 0.001	< 0.001	< 0.001	0.317
12 h	38.6	39.5	44.5	46.3	1.65	< 0.001	< 0.001	< 0.001	0.165
24 h	41.0	41.8	47.6	44.4	2.63	0.015	0.109	0.217	0.176
48 h	52.4	50.5	54.5	56.1	1.64	0.003	0.014	< 0.001	0.295
ADF Degradability, %									
0 h	51.3	50.9	60.0	62.4	1.71	< 0.001	< 0.001	< 0.001	0.192
3 h	49.3	49.8	62.6	60.4	1.70	< 0.001	< 0.001	< 0.001	0.027

6 h	52.5	56.2	61.5	59.2	1.57	< 0.001	0.002	0.116	0.284
12 h	60.1	60.1	62.6	64.1	1.61	0.013	0.003	0.003	0.254
24 h	58.3	56.9	67.7	65.6	2.41	< 0.001	0.004	0.002	0.334
48 h	68.9	66.5	67.5	67.9	1.04	0.186	0.369	0.315	0.792

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0 × 10^{10} *B. subtilis* spores/g substrate), commercial xylanase (CX; 1.0 mg commercial xylanase/g substrate) and spore-bound commercial xylanase (SB-CX; 1.0 mg of commercial xylanase bound to 1.0 × 10^{10} *B. subtilis* spores/g substrate).

and SPR ($P \leq 0.004$). At 6 h of incubation ADF degradability was greater for SB-CX compared with CON (59.2 and 52.5%, respectively; $P = 0.002$).

There was no difference in DM degradability between SB-ACX and ACX between 0 and 24 h ($P \geq 0.11$), but at 48 h SB-ACX had greater DM degradability compared with ACX (66.8 and 64.4%, respectively; Table 5.8). Throughout the 48 h incubation SB-ACX had greater DM degradability compared with SPR ($P < 0.001$), whereas SB-ACX had greater DM degradability compared with CON between 0 and 24 h ($P \leq 0.006$). Degradability of NDF tended to be greater for SB-ACX than ACX at 3 and 48 h ($P \leq 0.083$), and ADF degradability was greater for SB-ACX than ACX at 6 and 48 h of incubation ($P = 0.028$). There was an increase in NDF degradability for SB-ACX compared with CON and SPR at 0 and 3 h incubation ($P \leq 0.032$), and at 6 h SB-ACX was greater than SPR (41.8 and 32.5%, respectively; $P < 0.001$). At 3 h incubation an increase in ADF degradability was observed for SB-ACX (65.8%) compared with SPR (51.3%; $P < 0.001$). No differences in ADF degradability were observed throughout the incubation between SB-ACX and CON ($P \geq 0.18$).

The combination of glucanase, feruloyl esterase, amylase, and commercial xylanase (GFACX) bound to spores either individually (I-SB-GFACX), or in combination (SB-GFACX) resulted in no differences in DM, NDF or ADF degradability ($P \geq 0.12$; Table 5.9). Degradability of DM and NDF were not different between SB-GFACX and GFACX ($P \geq 0.15$), but ADF degradability was greater for SB-GFACX at 24 and 48 h ($P \leq 0.065$). An increase in DM degradability was observed for I-SB-GFACX at 48 h compared with GFACX ($P = 0.048$). A tendency for a decrease in NDF degradability occurred at 12 h for I-SB-GFACX compared with GFACX (42.1 and 44.9%, respectively; $P \leq 0.067$), but by 24 h NDF degradability was greater for I-SB-GFACX compared with GFACX (45.9 and 40.6%, respectively; $P = 0.019$). At the

Table 5.8. The effect of binding a combination of amylase and a commercial xylanase product (ACX) to *Bacillus subtilis* (RK28) spores on *in vitro* dry matter (DM), neutral detergent fibre (NDF) and acid detergent fibre (ADF) degradability in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	ACX	SB-ACX			SB-ACX vs CON	SB-ACX vs SPR	SB-ACX vs ACX
DM Degradability, %									
0 h	32.6	27.3	34.6	36.2	0.92	< 0.001	0.003	< 0.001	0.107
3 h	32.4	28.4	36.7	36.7	1.05	< 0.001	< 0.001	< 0.001	0.973
6 h	35.9	31.1	39.9	39.1	0.90	< 0.001	< 0.001	< 0.001	0.357
12 h	43.6	40.0	47.4	48.0	1.16	< 0.001	< 0.001	< 0.001	0.516
24 h	52.3	49.7	57.1	55.3	1.16	< 0.001	0.006	< 0.001	0.112
48 h	65.5	61.8	64.4	66.8	0.87	< 0.001	0.272	< 0.001	0.047
NDF Degradability, %									
0 h	32.7	28.1	35.2	37.6	1.93	< 0.001	0.032	< 0.001	0.236
3 h	34.8	29.2	39.5	42.4	2.83	< 0.001	< 0.001	< 0.001	0.083
6 h	39.3	32.5	43.2	41.8	3.05	< 0.001	0.145	< 0.001	0.420
12 h	41.9	41.3	44.7	43.2	2.12	0.369	0.518	0.380	0.445
24 h	44.8	42.9	45.7	44.9	2.44	0.587	0.947	0.324	0.662
48 h	54.4	52.4	51.6	55.2	1.77	0.178	0.666	0.129	0.052
ADF Degradability, %									
0 h	59.9	53.5	55.8	56.4	2.56	0.104	0.183	0.182	0.776

3 h	61.8	51.3	59.7	65.8	4.21	< 0.001	0.236	< 0.001	0.901
6 h	61.7	57.2	60.4	62.2	3.39	0.004	0.863	0.121	0.028
12 h	62.8	61.4	63.8	63.3	2.04	0.256	0.776	0.121	0.646
24 h	65.0	60.4	65.2	64.5	2.19	0.052	0.805	0.039	0.744
48 h	69.2	68.1	67.3	70.5	1.68	0.135	0.371	0.093	0.028

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0×10^{10} *B. subtilis* spores/g substrate), combination of amylase and commercial xylanase (ACX; 0.5 mg α-amylase and 0.5 mg commercial xylanase/g substrate) and spore-bound combination of amylase and commercial xylanase (SB-ACX; 0.5 mg of α-amylase and 0.5 mg commercial xylanase bound to 1.0×10^{10} *B. subtilis* spores/g substrate).

Table 5.9. The effect of binding an enzyme combination of glucanase, feruloyl esterase, amylase and commercial xylanase (GFACX) to *Bacillus subtilis* (RK28) spores on *in vitro* dry matter (DM), neutral detergent fibre (NDF) and acid detergent fibre (ADF) degradability in rumen batch cultures.

	Treatments[1]							Contrast *P* - Values				
	CON	SPR	GFACX	SB-GFACX	I-SB-GFACX	SEM	*P*-Value	SB-GFACX vs CON	I-SB-GFACX vs CON	SB-GFACX vs GFACX	I-SB-GFACX vs GFACX	SB-GFACX vs I-SB-GFACX
DM Degradability, %												
0 h	27.3	27.3	32.2	32.0	32.6	0.93	< 0.001	< 0.001	< 0.001	0.770	0.684	0.490
3 h	28.6	28.8	33.8	33.3	34.0	0.97	< 0.001	< 0.001	< 0.001	0.564	0.860	0.451
6 h	31.8	31.1	35.5	36.6	36.1	0.83	< 0.001	< 0.001	< 0.001	0.199	0.529	0.507
12 h	40.8	40.4	43.8	42.6	43.3	1.30	0.012	0.085	0.017	0.308	0.676	0.513
24 h	49.2	49.6	52.1	52.7	53.3	1.30	0.026	0.019	0.008	0.713	0.490	0.747
48 h	62.2	62.4	64.2	64.7	66.7	1.18	0.004	0.041	< 0.001	0.694	0.048	0.123
NDF Degradability, %												
0 h	25.5	25.7	41.7	43.1	43.5	1.02	< 0.001	< 0.001	< 0.001	0.257	0.159	0.775

3 h	29.8	28.9	41.4	41.4	42.0	2.23	< 0.001	< 0.001	< 0.001	0.988	0.677	0.664
6 h	31.9	33.0	43.6	44.1	45.6	2.24	< 0.001	< 0.001	< 0.001	0.746	0.226	0.355
12 h	40.8	40.1	44.9	42.6	42.1	1.89	0.045	0.235	0.390	0.147	0.067	0.719
24 h	39.1	42.0	40.6	43.3	45.9	2.28	0.024	0.059	0.002	0.235	0.019	0.264
48 h	53.1	52.8	58.2	58.3	59.5	1.92	0.001	0.003	0.002	0.952	0.504	0.545
ADF Degradability, %												
0 h	52.7	53.9	60.3	61.8	61.5	1.82	< 0.001	< 0.001	< 0.001	0.387	0.461	0.872
3 h	49.8	52.1	61.2	62.5	60.7	2.36	< 0.001	< 0.001	< 0.001	0.375	0.753	0.228
6 h	55.1	56.8	61.0	62.6	63.7	1.84	< 0.001	< 0.001	< 0.001	0.429	0.164	0.516
12 h	60.3	61.1	63.6	62.3	62.2	1.50	0.050	0.056	0.071	0.268	0.230	0.919
24 h	63.9	64.3	58.0	60.4	61.5	1.93	< 0.001	0.013	0.089	0.065	0.010	0.406
48 h	68.5	68.5	65.4	70.0	70.9	1.34	0.027	0.356	0.129	0.011	0.002	0.601

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0×10^{10} *B. subtilis* spores/g substrate), combination of β-glucanase, feruloyl esterase, α-amylase, and commercial xylanase (GFACX; 0.125 mg β-glucanase, 0.25 mg feruloyl esterase, 0.25 mg α-amylase and 0.25 mg commercial xylanase/g substrate), combination of β-glucanase, feruloyl esterase, α-amylase, and commercial xylanase that was bound to spores (SB-GFACX; 0.125 mg β-glucanase, 0.25 mg feruloyl esterase, 0.25 mg α-amylase and 0.25 mg commercial xylanase bound to 1.0×10^{10} *B. subtilis* spores/g substrate), or a combination of individually spore-bound β-

glucanase, feruloyl esterase, α-amylase, and commercial xylanase (I-SB-GFACX; 0.125 mg β-glucanase bound to 2.5×10^9 *B. subtilis* spores, 0.25 mg feruloyl esterase bound to 2.5×10^{10} *B. subtilis* spores, 0.25 mg α-amylase bound to 2.5×10^9 *B. subtilis* spores and 0.25 mg commercial xylanase bound to 2.5×10^9 *B. subtilis* spores).

beginning of the incubations (0 to 12 h) there was no difference in ADF degradability between either SB-GFACX or I-SB-GFACX and GFACX; however, at 24 h and 48 h spore-bound enzyme treatments tended to increase compared with GFACX ($P \leq 0.065$). The addition of either spore-bound treatments resulted in increased DM degradability for 0 to 48 h ($P \leq 0.085$), and NDF degradability throughout incubation except at 12 h ($P \leq 0.059$) compared with CON. From 0 to 12 h, ADF degradability was greater for the spore-bound treatments compared with CON ($P \leq 0.071$), yet at 24 h, ADF degradability was decreased for the spore-bound treatments compared with CON ($P \leq 0.089$).

No differences between either of the spore-bound treatments and GFAX were observed for DM or NDF degradability ($P \geq 0.11$; Table 5.10). There was no difference between SB-GFAX and I-SB-GFAX in terms of DM, NDF or ADF degradability, with the exception of NDF and ADF degradability at 0 h, which was greater for I-SB-GFAX compared with SB-GFAX ($P \leq 0.012$). At 0 h SB-GFAX had lower ADF degradability compared with GFAX (56.6 and 59.8%, respectively; $P = 0.006$), and at 48 h both SB-GFAX and I-SB-GFAX had lower ADF degradability compared with GFAX ($P \leq 0.013$). Dry matter degradability was increased for both spore-bound treatments compared with CON, with the exception of DM degradability at 24 and 48 h not being different between SB-GFAX and CON ($P \geq 0.18$). Degradability of NDF from 0 to 24 h was greater for the spore-bound enzymes compared with CON ($P \leq 0.026$), but not different at 48 h ($P \geq 0.36$). From 0 to 24 h ADF degradability was increased for both spore-bound treatments compared with CON, with the exception of 6 h ADF degradability not being different between SB-GFAX and CON. Both SB-GFAX and I-SB-GFAX had decreased 48 h ADF degradability compared with CON ($P < 0.001$).

Table 5.10. The effect of binding an enzyme combination of glucanase, feruloyl esterase, amylase and pure xylanase (GFAX) to *Bacillus subtilis* (RK28) spores on *in vitro* dry matter (DM), neutral detergent fibre (NDF) and acid detergent fibre (ADF) degradability in rumen batch cultures.

	Treatments[1]							Contrast *P* - Values				
	CON	SPR	GFAX	SB-GFAX	I-SB-GFAX	SEM	*P*-Value	SB-GFAX vs CON	I-SB-GFAX vs CON	SB-GFAX vs GFAX	I-SB-GFAX vs GFAX	SB-GFAX vs I-SB-GFAX
DM Degradability, %												
0 h	27.3	27.3	32.3	32.8	33.8	0.83	< 0.001	< 0.001	< 0.001	0.630	0.111	0.283
3 h	28.6	28.8	33.6	34.2	33.7	0.74	< 0.001	< 0.001	< 0.001	0.538	0.916	0.638
6 h	31.8	31.1	35.6	36.1	36.3	0.90	< 0.001	< 0.001	< 0.001	0.626	0.449	0.787
12 h	40.8	40.4	45.3	44.4	44.2	1.46	0.001	0.012	0.004	0.526	0.437	0.885
24 h	49.2	49.6	51.3	51.1	53.4	1.20	0.023	0.183	0.002	0.877	0.125	0.115
48 h	62.2	62.4	63.5	63.6	65.5	1.15	0.062	0.236	0.005	0.927	0.116	0.157
NDF Degradability, %												
0 h	25.7	25.6	41.6	39.9	42.9	0.95	< 0.001	< 0.001	< 0.001	0.134	0.282	0.012
3 h	29.2	26.4	38.3	39.1	38.8	1.66	< 0.001	< 0.001	< 0.001	0.639	0.741	0.889

6 h	31.7	32.7	38.1	39.0	38.5	1.98	< 0.001	< 0.001	0.001	0.670	0.823	0.815
12 h	39.2	40.0	43.4	42.5	42.3	1.45	0.050	0.018	0.026	0.492	0.390	0.853
24 h	38.3	41.4	44.7	43.9	44.6	1.64	0.005	0.002	0.002	0.517	0.968	0.630
48 h	51.6	49.5	53.9	52.2	52.8	1.03	0.009	0.621	0.358	0.221	0.441	0.681
ADF Degradability, %												
0 h	50.8	49.5	59.8	56.6	61.2	1.06	< 0.001	< 0.001	< 0.001	0.006	0.186	< 0.001
3 h	49.2	49.5	57.0	56.9	57.4	1.49	< 0.001	< 0.001	< 0.001	0.958	0.784	0.740
6 h	53.2	55.9	57.0	54.7	57.3	2.05	0.176	0.401	0.046	0.226	0.900	0.208
12 h	59.9	60.9	61.8	62.1	62.7	1.34	0.123	0.040	0.012	0.780	0.412	0.576
24 h	58.3	56.5	63.1	62.3	64.7	1.62	0.012	0.057	0.004	0.661	0.371	0.210
48 h	68.5	66.4	67.2	64.2	64.2	1.04	0.001	< 0.001	< 0.001	0.013	0.012	0.936

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0×10^{10} *B. subtilis* spores/g substrate), combination of β-glucanase, feruloyl esterase, α-amylase, and purified xylanase (GFAX; 0.125 mg β-glucanase, 0.25 mg feruloyl esterase, 0.25 mg α-amylase and 0.25 mg purified xylanase/g substrate), combination of β-glucanase, feruloyl esterase, α-amylase, and purified xylanase that was bound to spores (SB-GFAX; 0.125 mg β-glucanase, 0.25 mg feruloyl esterase, 0.25 mg α-amylase and 0.25 mg purified xylanase bound to 1.0×10^{10} *B. subtilis* spores/g substrate), or a combination of individually spore-bound β-glucanase, feruloyl esterase, α-amylase, and purified xylanase (I-SB-GFAX; 0.125 mg β-glucanase bound to 2.5×10^{9} *B. subtilis* spores, 0.25 mg

feruloyl esterase bound to 2.5×10^{10} *B. subtilis* spores, 0.25 mg α-amylase bound to 2.5×10^{9} *B. subtilis* spores and 0.25 mg purified xylanase bound to 2.5×10^{9} *B. subtilis* spores).

Gas production, methane production, methane concentration, total VFA concentration, and VFA molar proportions for all treatments are shown in Appendix 1 (Supplementary Tables 5.1 to 5.18).

5.4 Discussion

The addition of spore-bound enzymes to the incubations generally increased gas production, and consequently, methane production compared with CON and SPR. This result was expected because the SPR should not be metabolically active unless the spores germinate. When *B. subtilis* spores were incubated in ruminal batch cultures for up to 48 h there was no difference in spore counts (Chapter 4), which corroborates that SPR should not enhance fermentation. The addition of enzymes to batch cultures results in increased fermentation, subsequently increasing gas production (Eun et al., 2007). Gas production was not different between SBE and ENZY, which was not surprising since SBE was not centrifuged, therefore both treatments had the same amount of enzyme applied. However, it is interesting to note that even though SBE had high protein binding and low retained activity (eg. phytase had 97% protein binding and 1.43% retained activity; Chapter 3) there was generally no difference in gas production between SBE and ENYZ.

The addition of SB-CX or SB-ACX decreased methane concentrations compared with both CON and SPR. Contrarily, the addition of SB-XYL resulted in increased methane concentration compared to both CON and SPR, suggesting that the decrease in methane concentration observed for crude commercial product may be due to the stabilizers and other components present in the commercial product. Wallace et al. (2001) showed that gas production from two different enzyme products was influenced by the solutions that the enzymes were stored in, where some

enzymes have sugars present which would result in increased gas production. In the current experiment the addition of CX resulted in no difference in gas production compared with CON and SPR, suggesting that the side activities associated with this commercial enzyme were causing gas production to be less than expected. One of the side activities that is associated with this commercial xylanase product is protease, which has been shown to not affect methane production of cattle (McGinn et al., 2004). It is important to note that in the study of McGinn et al. (2004) protease supplementation resulted in a decrease in DM digestibility compared to the control (56.8 and 62.0%, respectively), which was likely due to a change in microbial populations and inactivation of endogenous enzymes. Chung et al. (2012) applied an enzyme containing endoglucanase and xylanase to mixed diet (52% forage: 48% concentrate, DM basis) and found an increase in *Fibrobacter succinogenes* and *Ruminobacter amylophilus*, no effect on total methanogens, but an increase in methane production.

The effect of enzymes on increasing gas production appears to be influenced by both enzyme activity and substrate used in the batch culture. In previous *in vitro* experiments using the same CX, Rosser et al. (Chapter 4) found that addition of enzymes increased gas production compared with CON, whereas in the present experiment gas production was not different between CON and CX. This difference between experiments can likely be explained by the use of different substrates, where in the previous experiment alfalfa grass hay was used as a substrate, whereas in the present experiment 75% barley silage and 25% barley grain was used. Giraldo et al. (2008) conducted batch cultures using three different concentrations of grass hay substrate (30, 50 and 70% of the diet), and found that gas and methane production were effected to a greater extent by the substrate utilised than the addition of enzyme. Applying an enzyme with endoglucanase and xylanase activity to alfalfa hay increased DM, NDF and ADF disappearance *in vitro*; however,

there was no effect when the same enzyme was applied to alfalfa silage or barley silage (Holtshausen et al., 2011).

For phytase there was an initial increase in gas production for SBE; however, after 12 h of incubation there was no difference in gas production between CON and PHYT. The negligible difference after 12 h of incubation suggests that the phytase enzyme was degraded, or that the catalysis of phytate did not result in increased fermentation in the batch cultures. Kincaid et al. (2005) found that addition of phytase to a dairy diet resulted in increased phytate hydrolysis but had no impact on DM digestibility. Based on these results no differences in gas production were expected with the addition of phytase enzyme to *in vitro* batch cultures. The initial increase in gas production for phytase may have been due to either side activities associated with the commercially obtained crude enzyme product, or the phytase activity allowed increased access to nutrients which were previously inaccessible due to phytate.

Enzymes containing AMYL, or CX (alone or in combination) increased degradability, which may be due to the fact they are commercial crude sourced enzymes. Both enzymes are not purified, and have side activities associated with them, which may have resulted in their ability to improve *in vitro* degradability. Ruminant feeds are not homogenous, therefore it should be assumed that exogenous enzymes should either be heterogonous in an attempt to improve digestibility or be an enzyme which represents a limiting step in digestion of the feed. Having a combination of enzymes present generally results in improvements in digestibility when the enzymes complement one another.

The similar response in degradabilities observed for both ENZY and SBE from 0 to 12 h of incubation was expected since the same amount of enzyme protein was applied to each vial.

Interestingly, after 24 h SBE that contain AMYL or CX had an increase in degradability compared with ENZY, suggesting either that SBE were more stable in rumen batch culture or the enzymes were released from the spore surface. Rosser et al. (Chapter 4) incubated SBE in simulated intestinal fluid (pH 7.75), and found that xylanase activity associated with SBE remained stable throughout a 24 h incubation , and concurrently there was an increase in xylanase activity in the supernatant. The increased activity in supernatant suggests that the proteins were bound on top of each other on the spore surface, so as the outer enzyme proteins were released from the spores the inner layers became active, maintaining a stable enzyme activity associated with the SBE while increasing activity in the supernatant. The results from these two studies suggest that binding enzymes to spore surfaces improves enzyme stability in *in vitro* ruminal, gastric and intestinal fluid.

Even though gas production values increased with the addition of SBE suggesting greater fermentation; however, total VFA concentrations did not reflect this increased fermentation. The addition of enzymes (endoglucanase and xylanase) to *in vitro* batch cultures often results in increased total VFA production compared to a control (Tricarico and Dawson, 2005). Contrarily, Chung et al. (2012) found that addition of an enzyme containing endoglucanase and xylanase activities had no effect on total VFA production, VFA molar proportions, or acetate: propionate ratio compared to the control. Even through both Tricarico and Dawson (2005) and Chung et al. (2012) utilised enzymes with endoglucanase and xylanase activities they had variable impacts on total VFA production, which could be due to other side enzyme activities, different substrates utilised.

The addition of both GLUC and FE decreased acetate: propionate ratio compared with CON, whereas addition of AMYL, CX, or ACX did not alter this ratio compared with CON; therefore,

it can be concluded that the decrease in acetate: propionate ratio for SB-GFAX and SB-GFACX compared with CON was influenced by FE and GLUC in the combination. Previous research has shown that addition of xylanase to batch cultures had no effect on acetate: propionate ratio, whereas addition of endoglucanase resulted in decreased acetate: propionate, largely due to an increase in propionate production (Tricarico and Dawson, 2005). Decreasing the acetate: propionate ratio represents a potential improvement in energy efficiency, because it suggests a decrease in methane production and propionate is glucogenic (Wolin, 1960). Methane production for SB-GFAX and SB-GFACX were greater than CON, but methane concentration was generally decreased for the combinations compared with CON.

It was hypothesized that the addition of enzyme combinations would work like cellulosomes to increase *in vitro* degradability compared to individual enzymes; however, this was not observed. Cellulosomes are highly organized systems where enzymes are attached to scaffoldins allowing for efficient degradation of cellulose (Artzi et al., 2017), whereas the unbound enzyme combinations in the current study are not organized, or interconnected by scaffoldins. The provision of synergistic enzymes in close proximity can increase degradation efficiency (Fierobe et al., 2002). In an attempt to obtain close proximity between enzymes were combined, then bound to *B. subtilis* spores (SB-GFAX and SB-GFACX); however, degradation was not increased. Covalent immobilization of cellulase to polystyrene nanoparticles decreased the K_m in the presence of crystalline cellulose compared to the unbound cellulase (Blanchette et al., 2012). The higher affinity to cellulose for the immobilized cellulase was suggested to be due to multiple interactions between the immobilized cellulases and the cellulose reducing diffusion of the enzyme away from the substrate. It is important to note that when both immobilized and unbound cellulase were incubated in CMC there was no difference in either V_{max} or K_m. Unlike

for crystalline cellulose there is no diffusion effect on the unbound cellulase, allowing constant interaction between cellulase and CMC (Blanchette et al., 2012). Kim et al. (2012) determined that binding both an endoglucanase D (CelD), and 3a family carbohydrate-binding module (CBM3a) to either streptavidin, or streptavidin coated cadmium selenide nanoparticles increased the release of reducing sugars from cellulose compared to unbound CelD, CBM3a, or CelD-CBM3a. In addition cellulase activity was greater for the CelD-CBM3a bound to either streptavidin or streptavidin coated cadmium selenide nanoparticles compared with binding either CelD or CBM3a alone. Cellulosome activity is increased when there are both enzymes and carbohydrate-binding modules present (Arai et al., 2003), therefore the increase in activity when both CelD and CBM3a were bound to the same nanoparticles was not surprising. It is important to note that in the current study binding conditions used were not optimal for all enzymes, potentially resulting in only certain enzymes binding to the spores, or orientation of binding resulting in blocked active sites. Additionally, it is likely that the random adsorption of enzymes may have resulted in steric hindrance between the enzymes, resulting in reduced enzyme activities (Fierobe et al., 2002; Cho et al., 2011).

Because SB-GFAX and SB-GFACX binding conditions were not optimal for all enzymes individually spore-bound enzyme treatments (I-SB-GFAX and I-SB-GFACX) were included, where enzymes were individually bound to spores using optimal conditions, then the individually SBE were combined. The use of these I-SBE treatments allowed for optimal binding efficiency, and SBE activity; however, using this technique meant that all enzymes were not present on the same spore, therefore close proximity between the different enzymes could not be ensured. This means both resultant SBE combinations (combined then bound vs individually bound then combined) resulted in either sub-optimal binding, or large proximity between enzymes. The sub-

optimal binding and large proximity between enzymes may explain the observed minimal differences among SBE that contained an individual enzyme, SBE that contained enzyme combinations, and individual spore-bound enzymes which were combined. To improve the efficacy of binding combinations of enzymes to spores future research needs to be conducted where enzymes connected to scaffoldins are then bound to *B. subtilis* spores.

Examining the spore bound enzyme combination data, it suggests that individually binding using optimal binding conditions of each enzyme can result in greater initial retained enzyme activity. As shown in Chapter 3 enzyme activity associated with SBE varied with binding pH, binding temperature and binding time, therefore binding using optimal binding conditions increased retained enzyme activity, whereas using different binding conditions resulted in suboptimal retained enzyme activity. Changing binding conditions may result in a larger amount of protein binding to the spore, but decrease SBE enzyme activity which is what may have occurred for SB-GFAX compared with I-SB-GFAX resulting in the initial increase in NDF and ADF digestibility at 3 h.

5.5 Conclusion

The spore-bound enzymes that had the most positive impact on gas production and *in vitro* degradability were AMYL and CX, either alone or in combination (ACX and GFACX). Binding enzymes to spores has the potential to reduce ruminal methane production compared to the free enzyme treatments, while increasing degradability. When utilizing a combination of enzymes (either GFACX, or GFAX) it was best to individually bind enzymes to spores using optimal binding conditions then combine the spore-bound enzymes compared to binding a combination of enzymes to spores.

6.0 The effect of administering a feed enzyme bound to *Bacillus subtilis* spores on ruminal fermentation and digestibility in cattle

6.1 Introduction

Feed expenses account for approximately 60 to 70% of the total cost of livestock production. Increasing digestibility would reduce total production costs, increase feed utilization, and have beneficial impacts on the environment by increasing land use efficiency and decreasing nutrient excretion and leaching (Barletta, 2010). The rumen is a naturally rich source of microbial fibrolytic enzymes (Ribeiro et al., 2016), yet a meta-analysis showed that the average total tract digestibility of neutral detergent fiber (**NDF**) in dairy cattle was only 50.4% (White et al., 2017). The primary site of NDF digestion is the rumen; however, Bourquin et al. (1994) found that 25 to 38% of NDF digestibility occurred post-ruminally in moderate- to high- forage diets. Therefore, there is significant potential to increase fiber digestibility both ruminally and post-ruminally.

Exogenous feed enzymes, including carbohydrases, proteases and phytases continue to be investigated to increase digestibility in ruminants (Barletta, 2010). The addition of fibrolytic enzymes to ruminant diets can increase total tract digestibility, and animal performance; however, responses have been variable (Beauchemin et al., 2003). Variation in the efficacy of ruminant feed enzymes can be attributed to several factors, including the type of feed and enzyme investigated (Colombatto et al., 2002) and enzyme stability throughout the gastrointestinal tract. For example, survival of feed enzymes in ruminal (Morgavi et al., 2000) and intestinal (Hristov et al., 1998a) fluids can vary according to the source (eg. fungal strain) and class (eg. carboxymethyl cellulase versus xylanase) of enzyme. Thus, increasing the stability

of feed enzymes in both ruminal and post-ruminal environments could increase total tract digestibility and performance of cattle.

Immobilization of enzymes to a support material has been used in industrial processes to increase stability. Common immobilization supports include silica, glass, and synthetic polymers (Sirisha et al., 2016); however, these materials are not suitable for addition to livestock feed. More recently, *Bacillus* spores have been shown to immobilize feed enzymes through a combination of hydrophobic and ionic bond interaction between enzymes and the spore coat (Cho et al., 2011). Enzymes absorbed to *Bacillus* spores retain activity over a range of temperatures and pH (Cho et al., 2011; Sirec et al., 2012; Gashtasbi et al., 2014; Gu et al., 2015). In addition, Huang et al. (2010) found that adsorption of antigens to the surface of *Bacillus subtilis* spores allowed the antigens to illicit an immune response after ingestion by mice, highlighting protection of the antigen from degradation. Thus binding feed enzymes to *B. subtilis* spores may provide a non-transgenic method to enhance enzyme stability throughout the ruminant digestive tract. Additionally, as a feed additive to deliver enzymes, *Bacillus* may have a secondary benefit as a probiotic to enhance animal performance. For example, feeding *B. subtilis* to ruminants has been shown to stimulate rumen development in calves (Sun et al., 2011), increase milk production (Peng et al., 2012), and potentially increase immune function in pre-weaned calves (Sun et al., 2010). The objective of this study was to determine the effect of a spore-bound xylanase on *in situ* ruminal degradability and apparent ruminal and total-tract digestibility in beef heifers. In addition, stability of spore-bound xylanase in the gastrointestinal tract of cattle was investigated using simulated gastric and intestinal fluids. It was hypothesized that spore-bound xylanase would be stable throughout the ruminant gastrointestinal tract, resulting in increased digestibility.

6.2 Materials and methods

6.2.1 Treatments

The treatments administered to cattle in the *in vivo* experiment included: i) *B. subtilis* (RK28) spores provided at 1.0×10^{10} spores ·kg dry matter intake (**DMI**) $^{-1}$ (**SPR**), ii) commercial crude xylanase enzyme provided at 2.5 ml ·kg DMI^{-1} (**ENZY**), iii) spore-bound enzyme provided at 1.0×10^{10} spores and 2.5 ml of commercial xylanase enzyme ·kg DMI^{-1} (**SBE**), and iv) a control treatment without spores or enzyme (**CON**). The xylanase activity of the commercial enzyme was 28,494 U ·ml^{-1}, where one unit of activity was defined as one µmol xylose release/min/ml of product. For the SBE treatment, enzyme was bound to spores by mixing the appropriate amount of spores with commercial enzyme in 40 ml citrate (final volume, pH 4) for 1 h at room temperature using a HulaMixer (InVitrogen, USA). The binding conditions described above have been previously determined for optimal xylanase absorption to *B. subtilis* spores (Chapter 3). Using these binding conditions, 41.38% of the protein content of the commercial xylanase was bound to the spores, and 7.31% of the xylanase activity was retained directly on the spore. In this case, SBE were not centrifuged, therefore all enzyme product mixed to the spores were provided in the treatment, not just the spore-bound enzyme. The same volume of citrate buffer was added to the CON, ENZY and SPR samples, and they were processed the same as the SBE treatment. All treatments were diluted with water for homogenous application to animal feed (15 ml solution ·kg dry matter (**DM**)$^{-1}$). For each animal, the treatments were applied to 1.0 kg of alfalfa grass hay 15 h prior to feeding.

6.2.2 In vivo experimental design

All experimental procedures involving cannulated heifers were pre-approved by the Agriculture and Agri-Food Canada Lethbridge Research and Development Centre Animal Care Committee

(Protocol No. 1611) and followed the guidelines of the Canadian Council of Animal Care (2009). Four duodenally and ruminally cannulated mature heifers (668 ± 25.5 kg) were used in a 4 × 4 Latin square design to determine treatment effects on *in situ* ruminal, apparent ruminal, apparent duodenal and total tract digestibility. The duodenal cannulas were T-shaped, and were placed near to the common bile and pancreatic duct, approximately 10 cm from to the pylorus. Experimental periods included 14 d of adaptation (d 1 to 14) and 7 d of sampling (d 15 to 21), with 7 d of washout (d 22 to 29) between periods where no treatments were applied to feed.

Heifers were weighed the day before the experimental periods started to determine feed offered, and feed refusals were measured throughout. The heifers were fed at 1.15% of body weight a diet consisting of 93% alfalfa-grass hay and 7% vitamin and mineral supplement (DM basis; Table 1). The supplement was provided in a separate feeder at 0900 and after its consumption heifers were provided with 1.0 kg of alfalfa grass hay treated with their respective treatments. This was done to ensure complete consumption of the treatment, after which the remainder of the untreated alfalfa grass hay was provided.

6.2.3 In vivo sampling

Ruminal pH was measured every minute from 0900 on d 10 until 0900 on d 12 using an indwelling pH system (LRCpH, Dascor Inc., USA). Prior to insertion, and following removal from the rumen, the pH systems were standardized in pH buffers 4 and 7 at 39°C (Penner et al., 2006). The minimum, mean and maximum pH was determined from the standardization mV and regression data.

To determine digesta flow out of the rumen $YbCl_3$ dissolved in double distilled water was infused intraruminally using peristaltic pumps at a rate of 0.5 L/d (to provide 2.6 g Yb/ d) on d 13

Table 6.1. Nutrient composition (mean ± standard deviation) of experimental diet.

Nutrient Composition	Hay	Supplement
DM, %	91.51 ± 1.37	94.60 ± 0.058
OM, % of DM	91.21 ± 0.406	70.34 ± 1.83
CP, % of DM	21.76 ± 2.86	18.85 ± 0.34
ADF, % of DM	32.43 ± 1.74	6.32 ± 0.54
NDF, % of DM	48.91 ± 3.98	20.99 ± 1.01

to 21. On d 13 a pulse dose was administered (50% of daily dose) directly into the rumen in an attempt to reduce the amount of time required to achieve a steady state of marker concentration in the rumen.

Ruminal *in situ* digestibility was determined on d 12 to 17 by inserting 30 polyester bags (5 × 10 cm; 53 ± 10 um; BG510; Bar Diamond, Inc. Parma, Idaho) containing 5 g of untreated alfalfa grass hay (ground to pass through a 2-mm screen) into the rumen of heifers. Triplicate bags were removed at 4, 8, 12, 24, 36, 48, 72, 96 and 120 h and were washed with triplicate 0 h bags in cold water (5 times, 1 min/wash with agitation by hand), then immediately frozen (-20°C). The *in situ* bags were later dried in a forced air oven (55°C) for 48 h, weighed, composited by time within cow and then analyzed for DM, organic matter (**OM**), NDF and acid detergent fiber (**ADF**).

Total collections of feces and feed refusals occurred on d 19, 20 and 21 to measure total tract digestibility, with urinary catheters (26 Fr, 75cc balloon; Bard Canada Inc., Oakville, Ontario) inserted on d 18. Total fecal output was weighed daily, thoroughly mixed, and 10% of fecal output was subsampled and stored at -20°C until further analysis.

Samples of ruminal digesta, duodenal digesta and feces were collected at 1000, 1600, and 2200 h on d 19, 0400, 1200, and 1800 h on d 20, 0000, 0600, 1400 and 2000 h on d 21 and 0200 and 0800 h on d 1 of washout. Samples were collected at these time points to represent digesta collected every 2 h over a 24-h time period. At each time point, rumen digesta was collected from the cranial central, central and caudal central region of the rumen (250 ml each) and pooled together. In addition, approximately 250 ml duodenal fluid was collected from the duodenal cannula after ensuring adequate flow (initial flow out of the cannula removed to ensure only fresh duodenal digesta was collected), and 250 g feces (collected rectally) were collected at each

time point, with all samples frozen (-20°C) until subsequent analysis. A separate subsample of rumen digesta was strained through 2 layers of Pecap (355 μm pore size; Tetko Inc., Scarborough, ON, Canada) and 1.5 ml of the fluid fraction was added to 0.3 ml of 25% m-phosphoric acid, or 0.3 ml of 1% sulfuric acid for analysis of volatile fatty acids (**VFA**) and ammonia (**NH_3**) concentrations, respectively. The VFA and NH_3 samples were stored at -20°C until analysis. Also at each time point, 10 ml of duodenal digesta and 10 g of feces were collected and stored at -20°C for subsequent enumeration of spores and to measure xylanase activity.

In situ residues, feed and refusals were dried in a forced air oven at 55°C for 48 h, fecal samples were dried for at 55°C for 72 h, and the pooled duodenal digesta was freeze dried. The samples were then ground using a Retsch ZM200 (Retsch GmbH, Germany) to pass through a 1-mm screen. *In situ* residues, feed, refusals, digesta and feces were analyzed for DM, OM, crude protein (**CP**), NDF and ADF. Dry matter was determined by drying samples at 105°C for 16 h and samples were hot-weighed. The crucibles were placed into a muffle furnace (550°C, 6 h), allowed to cool in a 105°C oven until the residual ash was hot-weighed and OM was determined. Crude protein was analysed on ball ground samples by nitrogen combustion (Carlo Erba Instruments, Italy). Samples (0.5 g) were weighed into F57 ANKOM bags (ANKOM Technology, USA), then analysed for NDF (with addition of α-amylase and sodium sulfite) then sequentially followed by ADF analysis using an ANKOM^{200} Fiber Analyser (ANKOM Technology, USA; Van Soest et al., 1991). The duodenal digesta and digesta marker were analysed for Yb using inductively coupled plasma optical emission spectroscopy according to AOAC (2005; method 968.08). Ruminal VFA was analysed by gas chromatography (model 5890, Hewlett-Packard Lab, USA) using crotonic acid (trans-2-butenoic acid) as an internal

standard. Ammonia was analysed by the method described in Weatherburn (1967), modified to be used with a plate reader.

6.2.5 Spore counts

To enumerate spores, 1.0 g of frozen duodenal digesta or feces was weighed into a stomacher bag with 9.0 ml of phosphate buffered saline. The samples were agitated for 2 min at high speed, then 1.0 ml of sample was incubated at 75°C for 20 min to kill vegetative cells (Reuter et al., 2011). Serial dilutions of the samples were plated onto tryptic soy agar plates and incubated at 37°C for 12 h, followed by enumeration.

6.2.6 Xylanase activity

Xylanase activity was measured in duplicate from duodenal digesta and feces, which was performed as described above, according to the procedure of Wood and Bhat (1988), with some modification. In brief, 150 µl of sample was mixed with 150 µl of 0.1 M citric phosphate buffer (pH 6.0), then incubated at 39°C for 10 min. After incubation 300 µl of substrate (1% xylan from beechwood in distilled water) was added and incubated for another 10 min, followed by addition of a solution that contained 3,5-dinitrosalicylic acid. The samples were immediately boiled for 5 min to allow color development, and then they were placed onto ice. Spectrophotometric readings of the samples at 540 nm were measured to determine the release of xylose, in order to calculate xylanase activity, where one unit of activity was defined as one µmol xylose release/min/ml.

6.2.7 In vitro gastric and intestinal stability

An *in vitro* experiment was conducted to determine stability of xylanase enzyme in unbound form (**ENZY**), or when bound to spores (**SBE**), after incubation in simulated gastric and intestinal fluid (adapted from Gargallo et al., 2006). The simulated gastric fluid was 0.1 M hydrochloric acid (pH 1.9) with pepsin (1 g/L, P-7000, Sigma-Aldrich, USA) and the simulated intestinal fluid was 0.5 M KH_2PO_4 buffer (pH 7.75) containing 6 g/L of pancreatin (P-1750, Sigma-Aldrich, USA). The treatments were control (CON; 0.1 M citric phosphate buffer [pH 6.0]), commercial xylanase enzyme (ENZY; diluted with 50 mM citrate buffer [pH 5.0]) and spore-bound commercial xylanase enzyme (SBE). For the SBE treatment, 1.0 mg protein from commercial xylanase enzyme was bound to 1×10^{10} *B. subtilis* spores according to optimal binding conditions described in Table 3.2. After binding occurred the SBE was centrifuged (5,000 × *g*, 5 min, 4°C) and after removal of supernatant the SBE was resuspended in 50 mM citrate buffer (pH 5.0). Xylanase activity of SBE was measured in a previous experiment, and the ENZY was diluted to contain 1.55 U/ml, which was the same as the xylanase activity associated with SBE. In triplicate, 200 µL of the treatments were either inoculated with 800 µL of simulated gastric fluid then incubated at 39°C for 0, 5, 15, 30 and 60 min, or inoculated with 800 µL of simulated intestinal fluid then incubated at 39°C for 0, 3, 6, 12, 18 and 24 h.

After incubation samples were immediately placed into an ice bath, then subsampled for the xylanase activity assay. For the SBE samples a 500 µL aliquot was collected to measure total xylanase activity (**TOT**) and the remaining sample was centrifuged (5,000 × *g*, 5 min, 4°C). The supernatant (**SNT**) was collected to determine xylanase activity that was no longer associated with the SBE, and the SBE pellet was re-suspended in 500 µL of 0.1 M citrate phosphate buffer (pH 6.0), then analyzed for xylanase activity still associated with the SBE.

6.2.8 Calculations

The residues from the *in situ* incubations were fit to a first order kinetics model according to McDonald (1981), using the nonlinear procedure in SAS (SAS Inst. Inc., Cary, NC). The model used was,

$$\boldsymbol{R(t) = Undeg + D \times e^{-Kd \times (t-T0)}},$$

where R(t) = residue at each time point (%); **Undeg** = undegradable fraction (%); **D** = potentially degradable fraction (%); **Kd** = degradation rate of D (%/h); and **T0** = lag time.

Effective degradability (**ED**) of nutrient components were calculated according to Orskov and McDonald (1979), using the following equation,

$$\boldsymbol{ED = Sol + D \times Kd \div (Kd + Kp)},$$

where **Sol** = soluble fraction (%); D = potentially degradable fraction; Kd = degradation rate of D (%/h) and **Kp** = passage rate, which was set at 5%/h.

Duodenal flow was calculated according to France and Siddons (1986). Total tract, ruminal and intestinal digestibility were calculated as follows,

$$\boldsymbol{Total\ tract\ digestibility}\ (\%) = [\boldsymbol{Intake}\ (\boldsymbol{kg/d}) - \boldsymbol{Fecal\ output}\ (\boldsymbol{kg/d})] \div \boldsymbol{Intake}\ (\boldsymbol{kg/d}) \times \mathbf{100}$$

$$\boldsymbol{Ruminal\ digestibility}\ (\%) = [\boldsymbol{Intake}\ (\boldsymbol{kg/d}) - \boldsymbol{Duodenal\ flow}\ (\boldsymbol{kg/d})] \div \boldsymbol{Intake}\ (\boldsymbol{kg/d}) \times \mathbf{100}$$

where duodenal flow was calculated by dividing Yb infused into the rumen (g/d) by Yb concentration (g/kg DM) measured in duodenal digesta samples.

$$\boldsymbol{Intestinal\ digestibility}\ (\%) = \boldsymbol{Total\ tract\ digestibility} - \boldsymbol{Ruminal\ digestibility}$$

6.2.9 Statistical analysis

The *in situ* and *in vivo* data were analyzed using the Mixed Model procedure of SAS, with fixed effect of treatment and period, and random effect of cow. Period was considered a repeated measure, and simple covariance structure was determined to be the best fit out of simple, first order autoregressive, heterogonous first order autoregressive, Toeplitz, and heterogonous Toeplitz covariance structures. Contrasts were used to determine the difference between SBE vs CON, SBE vs SPR, and SBE vs ENZY.

The *in vitro* data were also analyzed using the Mixed Model procedure of SAS with fixed effect of treatment. Contrasts were used to determine the difference between unbound enzyme and the total activity from the SBE samples (ENZY vs TOT), unbound enzyme and the activity associated with the supernatant (ENZY vs SNT) and the unbound enzyme and the activity associated with the SBE (ENZY vs SBE). To determine if xylanase activity as a percent of the 0 h time point was different than 100% a t-test was conducted.

For all data significance was declared when $P \leq 0.05$ and tendencies were noted when $0.05 \leq P \leq 0.10$.

6.3 Results

6.3.1 In situ ruminal degradation

For all *in situ* parameters measured (Table 6.2) there were no differences between SPR and SBE ($P \geq 0.15$) or ENZY and SBE ($P \geq 0.078$). The ruminal degradation rate (Kd) of DM, NDF and ADF were not affected by treatment ($P \geq 0.12$); however, there was a tendency ($P = 0.086$) for a difference in Kd of OM with CON having the lowest rate (7.36%). In addition, there was a

Table 6.2. The effect of binding commercial xylanase to *Bacillus subtilis* (RK28) spores on ruminal *in situ* degradation rate (K_d), lag time for digestion, soluble fraction (Sol), potentially degradable fraction (D), undegradable fraction (U) and effectively degradable (ED) nutrient components.

	Treatments[1]						Contrast *P*-Values		
	CON	SPR	ENZY	SBE	SEM	*P*-Value	SBE vs CON	SBE vs SPR	SBE vs ENZY
Dry matter									
Kd, %/h	9.22	11.58	13.73	12.48	1.213	0.115	0.074	0.589	0.315
Lag, h	1.8	2.3	2.6	2.1	0.45	0.640	0.633	0.760	0.449
Sol, %	35.2	34.3	34.8	34.8	0.58	0.709	0.621	0.529	0.976
D, %	30.7	34.9	35.4	35.5	1.28	0.023	0.009	0.665	0.973
U, %	34.1	30.9	29.8	29.8	1.17	0.032	0.011	0.398	0.988
ED, %	41.7	48.7	50.5	49.7	1.71	0.048	0.019	0.681	0.632
Organic Matter									
Kd, %/h	7.36	11.95	10.54	9.94	1.314	0.086	0.076	0.247	0.672
Lag, h	1.7	2.2	2.5	2.3	0.39	0.450	0.250	0.786	0.706
Sol, %	34.1	33.1	33.6	33.6	0.57	0.628	0.570	0.477	0.944
D, %	30.7	34.1	33.9	34.1	0.64	0.001	< 0.001	0.892	0.788
U, %	35.2	32.8	32.5	32.3	0.59	0.013	0.004	0.475	0.771
ED, %	38.6	46.9	45.6	44.8	1.64	0.030	0.017	0.426	0.690
Neutral Detergent Fiber									
Kd, %/h	6.05	7.58	7.89	6.79	0.676	0.300	0.437	0.525	0.316

Lag, h	3.6	2.5	3.2	2.5	0.92	0.716	0.370	0.978	0.519
Sol, %	26.3	24.9	25.7	26.1	1.06	0.761	0.883	0.424	0.813
D, %	41.1	44.7	46.9	44.8	1.45	0.006	0.010	0.933	0.078
U, %	32.6	30.4	27.4	29.1	1.58	0.079	0.074	0.450	0.319
ED, %	36.9	39.6	44.2	40.9	2.10	0.173	0.195	0.715	0.339
Acid Detergent Fiber									
Kd, %/h	6.58	8.37	7.39	8.08	0.605	0.250	0.107	0.756	0.411
Lag, h	6.1	3.1	4.4	4.8	0.86	0.162	0.308	0.170	0.685
Sol, %	29.8	26.5	27.6	28.3	0.87	0.110	0.227	0.153	0.553
D, %	41.2	44.2	43.8	44.1	0.62	< 0.001	< 0.001	0.655	0.427
U, %	29.0	29.4	28.6	27.7	1.17	0.561	0.303	0.207	0.480
ED, %	40.3	42.7	42.4	44.2	1.92	0.521	0.173	0.615	0.508

[1]Cattle were fed a diet containing alfalfa grass hay and were administered the following treatments: 1.0×10^{10} *B. subtilis* spores, kg DMI^{-1} (SPR); 2.5 ml of commercial xylanase, kg DMI^{-1} (ENZY); 2.5 ml of commercial xylanase absorbed to 1.0×10^{10} *B. subtilis* spores, kg DMI^{-1} (SBE); or no enzyme and spore (CON).

tendency for SBE to have greater DM Kd compared with CON (12.48 and 9.22%, respectively; $P = 0.074$). There was no effect of treatment on lag time or the rumen soluble fraction of DM, OM, NDF or ADF ($P \geq 0.11$). The degradable DM fraction was increased for SBE compared with CON (35.5 and 30.7%, respectively; $P = 0.009$), however there was no difference between SBE and SPR or ENZY ($P = 0.67$, and 0.97, respectively). The difference between SBE and CON was also observed in the undegradable fraction, (29.8 and 34.1%, respectively; $P = 0.011$). There was a significant difference in the degradable OM fraction between CON (30.7%) and SBE (34.1%; SBE vs CON, $P < 0.001$) but SBE was not different than the other treatments (SBE vs SPR, $P = 0.89$; SBE vs ENZY, $P = 0.79$). The degradable NDF and ADF fractions were also greater for SBE compared with CON (SBE vs CON; $P = 0.010$ and $P < 0.001$, respectively). Effectively degradable DM and OM was greater for SBE compared with CON (SBE vs CON; $P \leq 0.019$); however, there was no difference between SBE and SPR ($P \geq 0.43$) or SBE and ENZY ($P \geq 0.63$). In contrast, ED of NDF and ADF were not different among treatments ($P \geq 0.17$).

6.3.2 In vivo digestibility

For all nutrients, intakes were not different between treatments ($P \geq 0.108$; Table 6.3), which was expected as cows were fed DM as a percent of body weight. Duodenal flows of DM, and OM were not different between SBE and CON ($P \geq 0.112$); however, SBE tended to have increased duodenal flow of CP ($P = 0.086$) compared to CON. While there were no differences in ruminal digestibility of DM and OM between SBE and CON ($P \geq 0.33$), SBE had greater intestinal digestibility of DM and OM compared to CON ($P \leq 0.083$). Although total tract digestibility of DM and OM was not different between SBE and CON ($P \geq 0.14$), there was a tendency for NDF and ADF total tract digestibility to increase by approximately 4% for SBE compared with CON ($P \leq 0.095$). A tendency for a shift in the site digestion of OM towards the

Table 6.3. The effect of binding commercial xylanase to *Bacillus subtilis* (RK28) spores on intakes and digestibility.

	Treatments[1]						Contrast *P*-Values		
	CON	SPR	ENZY	SBE	SEM	*P*-Value	SBE vs CON	SBE vs SPR	SBE vs ENZY
Dry matter									
Intake, kg/d	7.71	7.74	7.85	7.88	0.115	0.271	0.148	0.182	0.780
Duodenal flow, kg/d	5.42	5.42	5.10	5.69	0.122	0.038	0.174	0.133	0.007
Fecal output, kg/d	3.25	3.17	2.95	2.84	0.136	0.041	0.013	0.032	0.434
Rumen digestibility, %	28.9	29.6	32.5	24.8	2.14	0.179	0.330	0.155	0.072
Intestinal digestibility, %	26.2	29.3	27.0	35.9	2.50	0.118	0.083	0.091	0.043
Total tract digestibility, %	56.0	59.0	59.5	60.7	1.33	0.487	0.145	0.372	0.535
Rumen, % of total tract	51.7	50.2	55.1	40.9	3.31	0.121	0.189	0.145	0.040
Intestinal, % of total tract	48.3	49.9	44.9	59.1	3.31	0.121	0.189	0.145	0.040
Organic matter									
Intake, kg/d	6.78	6.91	6.85	6.98	0.156	0.788	0.364	0.761	0.547
Duodenal flow, kg/d	4.41	4.44	4.22	4.69	0.094	0.059	0.112	0.105	0.012
Fecal output, kg/d	2.48	2.94	2.48	2.66	0.121	0.048	0.067	0.574	0.064

Rumen digestibility, %	34.4	35.7	38.4	32.8	1.49	0.130	0.518	0.211	0.028
Intestinal digestibility, %	24.6	20.6	25.5	28.3	1.29	< 0.001	0.001	< 0.001	0.002
Total tract digestibility, %	58.47	57.5	63.3	61.4	1.20	0.019	0.143	0.042	0.249
Rumen, % of total tract	60.3	62.1	60.3	53.1	2.61	0.075	0.045	0.020	0.041
Intestinal, % of total tract	39.7	37.9	39.7	46.9	2.61	0.075	0.045	0.020	0.041
Crude protein									
Intake, kg/d	1.64	1.77	1.71	1.72	0.026	0.108	0.104	0.153	0.740
Duodenal flow, kg/d	0.96	1.14	1.06	1.09	0.034	0.124	0.086	0.328	0.650
Fecal output, kg/d	0.36	0.35	0.33	0.34	0.012	0.230	0.339	0.515	0.228
Rumen digestibility, %	41.4	40.2	38.0	36.9	1.38	0.274	0.116	0.135	0.589
Intestinal digestibility, %	38.0	39.4	42.7	43.1	1.60	0.246	0.122	0.146	0.843
Total tract digestibility, %	79.4	79.6	80.6	80.0	0.70	0.701	0.647	0.707	0.539
Rumen, % of total tract	52.1	50.4	47.0	46.0	1.81	0.239	0.109	0.133	0.724
Intestinal, % of total tract	47.9	49.6	53.0	54.0	1.81	0.239	0.109	0.133	0.724
Neutral detergent fiber									
Intake, kg/d	3.62	3.65	3.56	3.60	0.083	0.729	0.794	0.548	0.645
Fecal output, kg/d	1.79	1.81	1.74	1.70	0.066	0.453	0.261	0.168	0.628

Total tract digestibility, %	50.3	49.9	50.8	54.9	1.22	0.156	0.044	0.048	0.082
Acid detergent fiber									
Intake, kg/d	2.37	2.38	2.31	2.35	0.057	0.641	0.748	0.578	0.527
Fecal output, kg/d	1.32	1.36	1.30	1.28	0.052	0.508	0.496	0.173	0.713
Total tract digestibility, %	44.0	42.5	43.3	48.0	1.37	0.178	0.095	0.050	0.078

[1]Cattle were fed a diet containing alfalfa grass hay and were administered the following treatments: 1.0×10^{10} *B. subtilis* spores, kg DMI^{-1} (SPR); 2.5 ml of commercial xylanase, kg DMI^{-1} (ENZY); 2.5 ml of commercial xylanase absorbed to 1.0×10^{10} *B. subtilis* spores, kg DMI^{-1} (SBE); or no enzyme and spore (CON).

intestines was observed for cattle fed SBE compared to CON ($P = 0.045$).

Duodenal flow of DM, OM, and CP were not different between SBE and SPR ($P \geq 0.105$).While ruminal digestibility of DM, OM, and CP were similar for SBE compared with SPR ($P \geq 0.135$), intestinal digestibility of OM was increased for SBE compared with SPR (28.3 and 20.6%, respectively; $P < 0.001$). This increase in intestinal digestibility resulted in increased total tract digestibility of OM for SBE compared to SPR (61.4 and 57.5%, respectively; $P = 0.042$). Along with the increase in total tract digestibility of OM, there was also an increase in total tract digestibility of NDF and ADF for SBE compared with SPR ($P \leq 0.048$). There was a shift in the site of OM digestion towards the intestines for SBE compared with SPR ($P = 0.020$).

While there was an increase in the flow of duodenal DM and OM for SBE compared with ENZY ($P = 0.007$, and 0.012), duodenal flow of CP was not different between these treatments ($P = 0.650$). This increased duodenal flow of DM and OM resulted in a reduction in ruminal digestibility of DM and OM ($P = 0.072$ and 0.028), and an increase in DM and OM intestinal digestibility ($P \leq 0.043$). No differences were observed in total tract digestibility of DM, or OM ($P \geq 0.25$); however, total tract digestibility of NDF and ADF tended to be greater for SBE compared with ENZY ($P \leq 0.082$).

Ruminal NH_3 concentrations and maximum ruminal pH were not affected by treatment ($P \leq 0.19$; Table 6.4). Total VFA concentrations were decreased for SBE compared with CON (109.05 mM and 123.89 mM, respectively; $P < 0.001$); however, there was no difference in the proportions of acetate, propionate, isobutyrate, butyrate, or isovalerate ($P \geq 0.29$). There were no differences in acetate: propionate ratio or ruminal pH parameters between SBE and CON, ($P \geq 0.19$).

Table 6.4. The effect of binding commercial xylanase to *Bacillus subtilis* (RK28) on ruminal fermentation parameters.

	Treatments[1]						Contrast *P*-Values		
	CON	SPR	ENZY	SBE	SEM	*P*-Value	SBE vs CON	SBE vs SPR	SBE vs ENZY
Ruminal ammonia concentration, mM									
	13.03	12.00	12.18	12.50	0.485	0.268	0.339	0.371	0.566
Total volatile fatty acid concentration, mM									
	123.89	116.64	114.56	109.05	4.670	< 0.001	< 0.001	0.021	0.091
Volatile fatty acid proportion, mol/100 mol									
Acetate	73.98	73.90	74.10	74.06	0.331	0.733	0.675	0.403	0.839
Propionate	15.41	15.51	15.21	15.24	0.243	0.187	0.292	0.091	0.834
Isobutyrate	1.30	1.34	1.35	1.34	0.034	0.644	0.411	0.999	0.661
Butyrate	6.25	6.28	6.23	6.20	0.131	0.831	0.560	0.362	0.702
Isovalerate	1.65	1.62	1.67	1.65	0.047	0.922	0.942	0.642	0.828
Valerate	1.30	1.28	1.32	1.41	0.036	0.064	0.038	0.012	0.091
Acetate: Propionate ratio									

	4.84	4.79	4.93	4.90	0.095	0.086	0.267	0.065	0.636
Ruminal pH									
Minimum	6.53	6.53	6.63	6.56	0.062	0.024	0.436	0.318	0.064
Mean	6.83	6.85	6.91	6.85	0.045	0.031	0.497	0.975	0.030
Maximum	7.23	7.21	7.21	7.16	0.067	0.575	0.190	0.315	0.320

[1]Cattle were fed a diet containing alfalfa grass hay and were administered the following treatments: 1.0×10^{10} *B. subtilis* spores, kg DMI^{-1} (SPR); 2.5 ml of commercial xylanase, kg DMI^{-1} (ENZY); 2.5 ml of commercial xylanase absorbed to 1.0×10^{10} *B. subtilis* spores, kg DMI^{-1} (SBE); or no enzyme and spore (CON).

Total VFA concentrations were slightly decreased for SBE compared with SPR ($P = 0.021$); however, the only differences in VFA molar proportions were a tendency for a decrease in propionate proportion ($P = 0.091$), and an increase in valerate ($P = 0.012$). This alteration in propionate proportion resulted in a tendency for acetate: propionate ratio to be greater for SBE than SPR (4.90 and 4.79, respectively; $P = 0.065$). No differences were observed in ruminal pH parameters between SBE and SPR ($P \geq 0.32$).

A tendency for a decrease in total VFA concentration was observed for SBE compared with ENZY (109.05 mM and 114.56 mM, respectively; $P = 0.091$), yet there was no difference in acetate, propionate, isobutyrate, butyrate, or isovalerate proportion, and no difference in acetate: propionate ratio ($P \geq 0.64$). Minimum and mean ruminal pH tended to and was decreased for SBE compared with ENZY ($P = 0.064$, and 0.030, respectively).

Spore counts in duodenal digesta and feces were greater for SBE compared with both CON and ENZY ($P < 0.001$; Table 6.5). There was no difference in fecal spore counts between SBE and SPR ($P = 0.45$); however, spore counts were decreased in duodenal digesta for SBE compared with SPR (3.43 and 3.72 log 10/g; $P = 0.038$). This difference in spore counts between SBE and SPR is minimal (0.29 log 10/g), and may be due to the insignificant difference in duodenal flow of DM between SBE and SPR (5.69 and 5.42 kg/d, respectively). Xylanase activity of the duodenal digesta was lower for cows fed SBE compared with cows fed CON (0.492 and 0.507, respectively; $P = 0.023$), but there was no difference in activity between SBE and either SPR or ENZY treatments ($P \geq 0.12$). There was no difference in fecal xylanase activity between SBE and any other treatments ($P \geq 0.42$).

6.3.3 In vitro enzyme stability

Table 6.5. The effect of binding a commercial xylanase product to *Bacillus subtilis* (RK28) spores on spore counts and xylanase activity in duodenal digesta and feces.

	Treatments[1]				SEM	*P* -Value	Contrast *P*-Values		
	CON	SPR	ENZY	SBE			SBE vs CON	SBE vs SPR	SBE vs ENZY
Spore counts, log 10/g									
Duodenal digesta	0.62	3.72	1.29	3.43	0.157	<0.001	<0.001	0.038	< 0.001
Feces	1.99	5.08	2.12	4.96	0.201	< 0.001	< 0.001	0.450	< 0.001
Activity, U/g									
Duodenal digesta	0.507	0.487	0.487	0.492	0.0069	0.005	0.023	0.307	0.123
Feces	0.565	0.575	0.553	0.566	0.0140	0.080	0.871	0.446	0.416

[1]Cattle were fed a diet containing alfalfa grass hay and were administered the following treatments: 1.0×10^{10} *B. subtilis* spores, kg DMI^{-1} (SPR); 2.5 ml of commercial xylanase, kg DMI^{-1} (ENZY); 2.5 ml of commercial xylanase absorbed to 1.0×10^{10} *B. subtilis* spores, kg DMI^{-1} (SBE); or no enzyme and spore (CON).

Xylanase activity of ENZY and SBE prior to incubation with either simulated gastric or intestinal fluid was 1.55 U/ml. Upon addition to simulated gastric fluid, SBE xylanase activity decreased slightly to 1.05 U/ml; however, there was an immediate release of some enzyme from SBE resulting in a 0 h xylanase activity of 2.05 U/ml for the SNT (Figure 6.1A). Between 5 and 30 min incubation, SBE and SNT xylanase activity averaged 89% of 0 h activity; however, after 60 min incubation the xylanase activity of SBE and SNT was not different than 0 h activity (Figure 6.1B; $P \leq 0.05$). Interestingly, throughout the incubation in simulated gastric fluid ENZY activity was stable, with no differences observed between sampling times and 0 h samples.

Incubation in simulated intestinal fluid resulted in an initial decrease in activity for all treatments (Figure 6.2A). At 0 h ENZY and SBE activity was 1.05 and 1.23 U/ml, respectively, and after 3 h of incubation, ENZY activity was reduced to 0.13 U/ml (14.3% of 0 h activity), whereas SBE activity was reduced to 1.07 U/ml (85.5% of 0 h activity), after which both treatments remained relatively stable (Figure 6.2B). Even through SBE xylanase activity remained stable after 3 h incubation there was a peak in activity associated with the SNT at 6 h of incubation (125% of 0 h activity; $P \leq 0.05$). This activity associated with SNE then decreased to 31.3% of 0 h activity by 24 h of incubation.

6.4 Discussion

Previous studies have shown that the effects of exogenous enzymes on *in situ* degradability of feed is highly variable (Feng et al., 1996; Yang et al., 1999; Romero et al., 2013), and hence the present study explored the potential of improving enzyme stability by binding enzymes to *Bacillus* spores. The observed increase in rumen *in situ* potential degradability of DM, OM, NDF and ADF for SBE compared with CON suggests the enzyme was effective when bound to

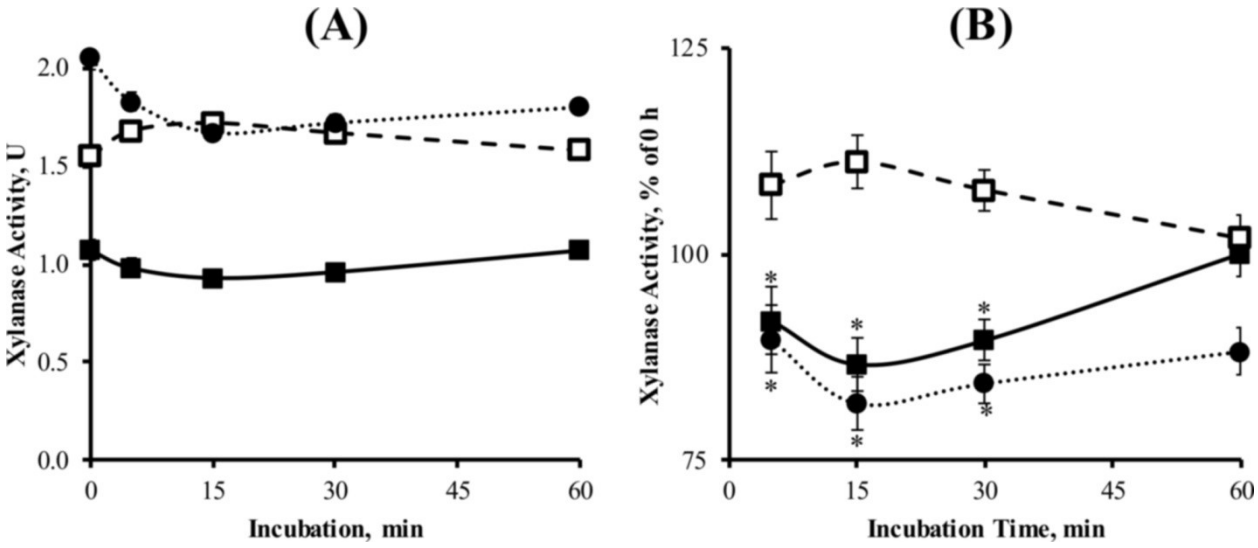

Figure 6.1. Xylanase activity (A) and activity as a % of 0 h (B) of unbound commercial xylanase enzyme (ENZY; open box) and commercial xylanase enzyme bound to 1×10^{10} *Bacillus subtilis* spores (SBE; closed box) after incubation in simulated gastric fluid. After incubation, samples were centrifuged and the supernatant was collected (SNT; closed circle) to allow determination of activity associated with the SBE and xylanase activity that was released from the surface of the spore. Xylanase activity (% of 0 h) that is significantly different from 0 h activity ($P \leq 0.05$) is indicated by *.

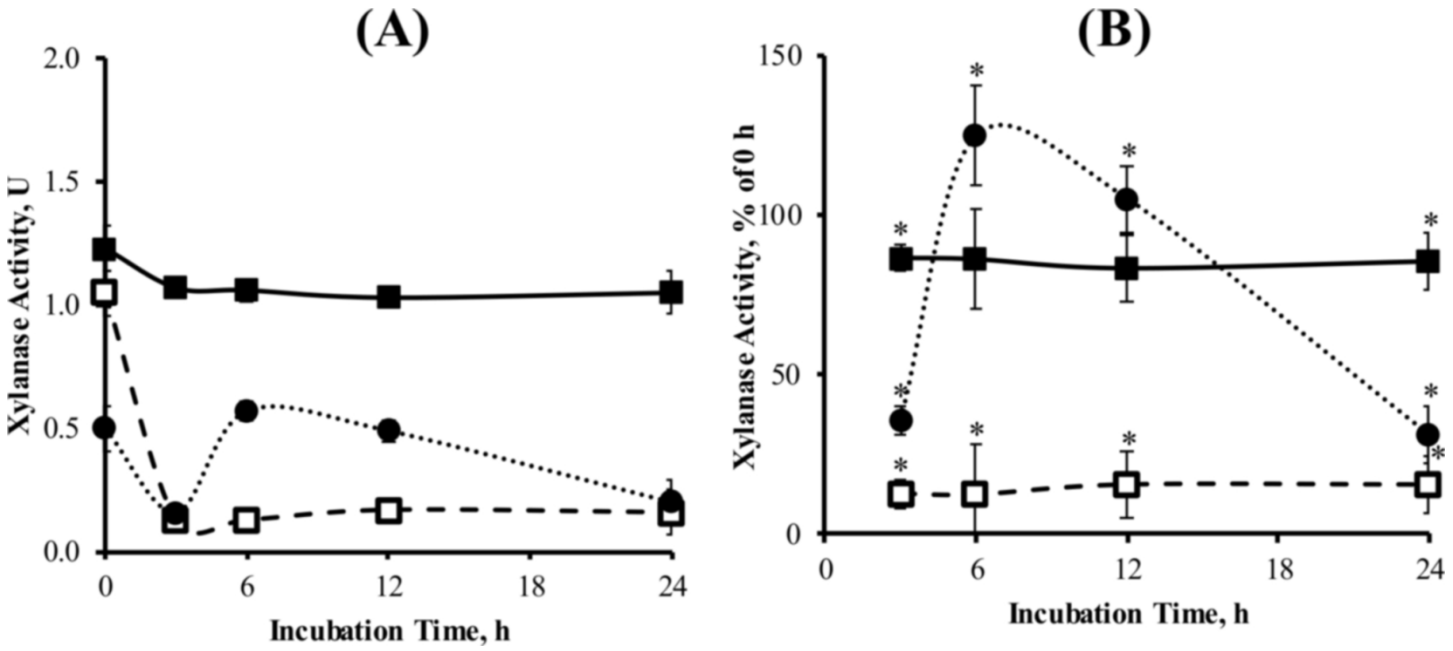

Figure 6.2. Xylanase activity (A) and activity as a % of 0 h (B) of unbound commercial xylanase enzyme (ENZY; open box) and commercial xylanase enzyme bound to 1×10^{10} *Bacillus subtilis* spores (SBE; closed box) after incubation in simulated intestinal fluid. After incubation, SBE samples were centrifuged and the supernatant was collected (SNT; closed circle) to allow determination of activity associated with the SBE and xylanase activity that was released from the surface of the spore. Xylanase activity (% of 0 h) that is significantly different from 0 h activity ($P \leq 0.05$) is indicated by *.

spores, thereby resulting in increased digestibility. Romero et al. (2016) reported no effect on *in situ* degradation when the same commercial xylanase used in the current study was investigated in dairy cattle. However, in the study by Romero and colleagues, cattle were fed a total mixed ration (10% bermudagrass silage, 35% corn silage, 5% alfalfa-orchardgrass hay mixture, and 50% concentrates, DM basis) and the xylanase product was administered at a rate of 1 ml/kg of DM feed, which was less than the application rate applied in the current study (2.5 ml/kg DM). Both the type of feed substrate and enzyme application rate have previously been shown to affect performance. For example, increased weight gain in steers was observed with alfalfa hay treated with low to moderate (900 to 4733 IU per kg DM) levels of a xylanase, whereas a higher enzyme dose (12,000 IU per kg DM) was required for increased gain when steers were fed timothy hay and no effect was observed when any dose was applied to barley silage (Beauchemin et al., 1995). Furthermore, the lack of differences in degradability between SBE and ENZY indicates that binding the enzyme to spores did not further enhance enzymatic activity in the rumen.

Surprisingly, *in situ* degradability parameters were not different between SBE and SPR, yet there were differences between SBE and CON, which suggests that the addition of spores may affect feed digestion in the rumen. A previous study found that administering 20 g of *B. subtilis natto* fermentation product (containing 1×10^{11} *B. subtilis natto*) per day to Holstein cows resulted in no significant differences on 24 h *in situ* DM degradability, but it increased milk production (Sun et al., 2013). The *Bacillus* product used in the study by Sun et al. (2013) contained both *B. subtilis natto* vegetative cells and spores, and the authors suggested that the increased performance was the result of *B. subtilis natto* increasing total, proteolytic and amylolytic rumen bacteria. In a subsequent study, Sun et al. (2016) found that the addition of live *B. subtilis natto* (vegetative and spores) and autoclaved (spore only) *B. subtilis natto* to ruminal batch cultures

increased *Butyrivibrio fibrisolvens* compared with the control. However, increases in *B. fibrisolvens* were also observed when only the fermentation product from growing *B. subtilis* was added to batch cultures, though to a lesser extent than when the bacteria were also present. *Butyrivibrio fibrisolvens* has been associated with fibre digestion (Dehority, 1991), thus an increase in this species may enhance digestion of plant cell walls. In the current study, the spores were washed, and therefore minimal material from the production of the spores would have been administered to the cattle. While *Bacillus* spores have been shown to germinate in the jejunum and ileum of mice (Casula and Cutting, 2002), there is limited information on their fate throughout the ruminant digestive tract. Based on the VFA proportions observed in the current study for SBE compared with CON and SPR there appeared to be little impact of the spores on the rumen microbiota and fermentation pathways. Despite the increase in spore counts in duodenal digesta and feces, addition of *B. subtilis* spores altered *in situ* degradation. Whether this alteration in ruminal degradation resulted from germination and colonization of the rumen requires further investigation.

While SBE and SPR had similar effects on *in situ* ruminal degradability of forage, total tract digestibility of OM tended to be greater for SBE, suggesting that the presence of the enzyme bound to spores increased digestibility. The addition of fibrolytic enzymes to grass hay has previously been shown to increase DM, NDF and ADF total tract digestibility in cattle (Lewis et al., 1996). Using the same enzyme product the present study, Romero et al. (2016) found no treatment effect on total tract digestibility of DM, OM, CP, NDF, hemicellulose, or ADF in dairy cows. However, as mentioned above, the diet, application rates and animal type differed among the two studies and these factors have previously been shown to affect digestion (Beauchemin et

al., 1995; McAllister et al., 1999). These factors may not only affect digestibility, but also passage rate throughout the gastrointestinal tract.

The addition of SBE resulted in an increase in duodenal flow of DM compared with ENZY, which accounts for the tendency for a reduction in the proportion of DM digested in the rumen. Increasing the rate of passage from the rumen reduces the amount of time ruminal bacteria can degrade feed residues, resulting in decreased ruminal digestibility (Mertens, 1987). However, reduced ruminal digestibility for SBE compared with ENZY, due to increased rumen outflow, was not observed. Thus, the observed increase in rumen degradability of forage appeared to have offset the reduction in retention time of feed in the rumen, and consequently ruminal digestibility of feed was not affected. The addition of fibrolytic enzymes to grass hay has been shown to increase particulate passage rate and decrease rumen retention time (Feng et al., 1996). However, this effect can be variable and may depend on the feed substrate. For example, Romero et al. (2013) found a decrease in particulate rate of passage when fibrolytic enzymes were applied to hay harvested at early maturities and an increase in passage rate when applied to hay harvested at later maturity. While difficult to explain, increased duodenal flow was only observed for SBE, not ENZY. This is likely the reason rumen digestibility of OM was lower for SBE compared with ENZY. Interestingly, total tract digestibility of OM was not different between SBE and ENYZ, suggesting that there was compensatory digestion occurring in the lower digestive tract for SBE.

It was hypothesized that the SBE treatment would protect xylanase bound to the spores, resulting in increased feed enzyme being delivered to the lower digestive tract, potentially enhancing total tract digestibility. According to Morgavi et al. (2000), after 6 h of incubation in rumen fluid three enzyme products were able to retain 75% of their 0 h xylanase activity. In addition, Morgavi et

al. (2001) determined that xylanase activity remains stable when incubated in simulated gastric fluid, and when incubated in simulated intestinal fluid remained stable for at least 40 min of incubation. These data suggest that while no differences were observed in xylanase activity of duodenal digesta it is possible that this was due to dilution of the commercial enzyme throughout the gastrointestinal tract, not degradation of the xylanase.

The increase in spore counts for SBE compared with CON and ENZY indicates that administered spores passed to the lower digestive tract as intended. To evaluate whether spore-bound xylanase remained active in the lower digestive tract, *in vitro* simulated gastric and intestinal incubations were performed. Both unbound xylanase and spore-bound xylanase were stable in gastric fluid, indicating denaturation was limited for xylanase reaching and passing through the abomasum. In comparison, unbound xylanase was rapidly degraded in simulated intestinal fluid, suggesting that xylanase which survived passage through the rumen and abomasum would have limited ability to degrade fibre in the lower digestive tract. The rapid decrease in xylanase activity for the unbound xylanase is in contrast to a previous study that showed inactivation of xylanases to primarily occur from the low pH of the abomasum, and not degradation by pepsin (Hristov et al., 1998a). Such differences are likely dependent on the enzyme being studied. When bound to spores, xylanase remained stable throughout incubation in simulated intestinal fluid and there was even an increase in activity associated with the supernatant. This suggests a release of some enzyme which was bound to outer layers of the spore, allowing enzymes bound closer to the spore-coat to interact with the substrate. Thus, binding enzymes to spores may be a method to not only protect bound enzyme against proteolysis in the ruminal digestive tract, but to also deliver enzymes to the lower digestive tract. The improvements in enzyme stability and release of enzyme into the lumen are important,

especially if binding enzymes to spores reduces overall activity. For example, in Chapter 3, 41.38% of enzyme protein applied to the spores was absorbed, but retained xylanase activity on the spores was only 7.31%. The decrease in retained activity indicates that absorption inhibited xylanase activity, possibly due to steric hindrance blocking enzyme active sites (Cho et al., 2011), or altered conformation of enzyme active site due to binding (Bower et al., 1999). Release of enzyme would circumvent loss of activity when bound to spores.

It is possible that the increased intestinal digestibility observed for SBE in the *in vivo* study resulted from both xylanase activity remaining on spores reaching the intestines, and release of enzyme from the SBE into the intestinal lumen. The combination of increased enzyme stability throughout the gastrointestinal tract, and release of enzyme from SBE would support the observation that total tract digestibility was similar among treatments, despite a reduction in rumen digestibility for SBE. A release of active xylanase into the intestinal lumen for SBE may be the reason there was a tendency for increases in total tract digestibility of NDF and ADF compared with unbound enzyme. Shifting digestion towards the lower digestive tract of ruminants may have some benefits, such as a decrease in methane production (Johnson and Johnson, 1995) and increase in protein efficiency.

6.5 Conclusion

Overall, the current study suggests that even though enzymes bound to *Bacillus* spores did not increase total tract digestibility compared with unbound enzymes, they did increase the proportion of total tract digestibility occurring in the intestinal tract. Spore-bound enzymes increased enzyme stability in *in vitro* gastric and intestinal fluid compared with unbound enzymes, and while enzyme activity associated with the spore remained stable there was an

increase in enzyme activity released from the spore in *in vitro* intestinal fluid. Binding enzymes to *Bacillus* spores can increase protein stability, and may be a useful method for delivery of proteins to the lower digestive tract of ruminants.

7.0 General discussion

Enzymes have been used as feed additives to improve both feed digestibility and animal performance, resulting in increased production efficiency. While enzymes have been successfully used in monogastric animal production (Bedford, 2000), their efficacy in ruminant production has been variable (Beauchemin et al., 2013). This variability is partially due to lack of enzyme stability during feed processing and within the gastrointestinal tract of livestock species. One method that can improve enzyme stability is adsorption of enzymes to spores (Cho et al., 2011; Sirec et al., 2012; Gashtasbi et al., 2014; Gu et al., 2015).

The interaction between the enzymes and spores that result in adsorption include a combination of hydrophobic and electrostatic forces (Cho et al., 2011). Therefore, it is likely that individual enzymes require different physical and chemical conditions to optimize adsorption and activity when bound to spores. In Chapter 3, optimal binding conditions were indeed variable depending upon the enzyme of interest, with wide ranges observed in optimal buffer pH, protein concentration, and binding time/temperature to improve adsorption. In general, optimal buffer pH values were acidic. This could partially be explained by the zeta potential of the *Bacillus subtilis* spores becoming more positive at low pH, thus promoting interaction between the spores and enzymes. Similarly, enhanced enzyme adsorption to spores under acidic conditions were also observed by Cho et al. (2011), Sirec et al. (2012), Gashtasbi et al. (2014) and Gu et al. (2015), who found that increased adsorption ranged from pH 3.0 to 4.5 (Table 2.1). We also showed that the concentration of enzyme, time and temperature of adsorption can affect enzyme binding efficiency and retained activity. Due to the large range in optimal binding conditions observed in Chapter 3, and subsequent effects on both protein binding and spore-bound enzyme activity, it is

suggested that any future research evaluating spore-bound proteins should first consider optimal binding conditions for the protein of interest.

The composition of spore coats, and spore sizes are highly variable among species (Dickinson et al., 2004; Carrera et al., 2006), and can affect spore-binding properties. To test the effect of spore species on adsorption of commercial xylanase, we evaluated 16 species/strains of spores using the same binding conditions (1.0 mg/ml commercial xylanase, pH 5.0, 1 h, 25°C; Chapter 4). The results showed a wide range of protein binding efficiency (18.9 to 52.8%) and retained enzyme activity (< 0.01 to 37.9%). Even strains within a species had different binding efficiencies, and retained enzyme activities. It was notable that the adsorption and retained efficiency of *B. subtilis* (RK28) was different if the spore was cultured from large-scale production and small-scale lab production. This highlights that the conditions of spore growth can potentially alter coat content, or nutrients present in the spore solution. Cho et al. (2011) also reported differences in SBE activities when using different species of spores, resulting in the authors selecting *Bacillus lichenformis* for their studies, as it had better adsorption potential compared to *B. subtilis*. The authors suggested that SBE activity was altered not only by the size of the spores, but also by the orientation of the bound enzymes potentially resulting in obstruction of the enzyme active sites.

Optimal binding conditions for protein adsorption did not necessarily correspond to optimal binding conditions which maximized retained SBE activities. This may be due to several reasons. For the commercial enzymes used (α-amylase, phytase and commercial xylanase) it is important to note that they were not purified enzymes, and therefore these products had multiple enzymes and proteins present in their matrix. For example, the commercial xylanase product information sheet lists side activities of cellulase, and β-glucanase; though the content and

activities of these enzymes are not guaranteed. As such, altering the binding conditions to achieve maximized protein adsorption does not necessarily equate to maximized activity of the enzyme of interest (i.e. amylase, phytase and xylanase for the commercial sources of these enzymes). It is possible that when optimal protein binding was achieved, secondary proteins and enzymes with side activities were also binding. Additionally, increasing spore-bound enzyme activity requires not only protein to be bound to the spore surface, but the orientation of binding must be such that the active site of the enzyme can interact with substrates (Sirisha et al., 2016). Similarly, it is important to note that as described by Bower et al. (1999), adsorption of enzymes to a solid support can alter the active site of the enzyme. This alteration in active site could be the reason that multiple studies that immobilized enzymes onto spores have found a decrease in the V_{max} for the bound enzyme (Gashtasbi et al., 2014; Falahati-Pour et al., 2015). A decrease in the rate of reaction for bound enzymes would be detrimental; however, if enzyme stability is increased there is the potential for overall substrate degradability to be greater for bound enzymes compared to unbound enzymes. This would be especially true when exposed to harsh conditions such as those present in feed processing and the ruminant digestive tract.

The high temperatures associated with feed processing can potentially degrade feed enzymes. To achieve an efficacious feed enzyme dose when feed is pelleted, the enzyme must either be applied post-pelleting or a greater dose must be applied pre-pelleting to account for the loss of activity due to thermal denaturation. Slominski et al. (2007) determined that for a specific commercial phytase enzyme, over 50% of activity was lost after pelleting occurred at temperatures below 70°C. While such a large decrease in enzyme activity is not applicable for all commercial enzymes, it is important to note that more enzyme product would need to be applied prior to pelleting to obtain optimal enzyme dose in the final product. Application of enzymes to

pellets post-pelleting has limitations as well due to difficulty ensuring homogenous distribution of enzyme throughout the entire pelleted batch. The use of post-pelleting enzyme application technologies, such as vacuum coating, requires the purchase of expensive equipment (Lamichhane et al., 2015), which is generally not economically viable for most feed mills.

It was predicted that spore-bound enzymes would result in improved stability during feed processing, allowing for pre-pelleting application of the enzyme. As a result, less enzyme would need to be applied pre-processing in order to obtain an effective dose in the final product. As determined in Chapter 3, after 5 min of incubation at 70°C unbound phytase activity was decreased to 24% of the original activity at 0 h, whereas spore-bound phytase was only decreased to 43%. Similarly unbound xylanase activity was decreased to 7% of 0 h, while spore-bound xylanase activity was decreased to 21% of 0 h activity. It is important to note that this preliminary experiment was conducted in liquid form, not in the presence of a feed matrix which could have altered survivability compared to actual processing conditions (Inborr and Bedford, 1994). However, if these trends are similar in commercial feed processing, use of unbound phytase of xylanase would require 4.2 and 14.3 times the volume to be applied pre-pelleting to ensure an optimal dose in the final pelleted product. Contrarily, spore-bound phytase or spore-bound xylanase would only require 2.3 and 4.8 times the volume applied pre-pelleting, suggesting a decrease in enzyme product volume required per feed batch by half. Reducing the volume of enzyme product required for feed products would result in significant savings for both the feed industry, as well as livestock producers. The improved stability during thermal processing suggests that this method of enzyme delivery may improve the efficacy of enzyme use, both in ruminant and monogastric animal production.

Post-consumption denaturation can cause significant reduction in exogenous enzyme activity in ruminant digestive tract, as a result of proteolysis and exposure to the low pH environment of the abomasum (Morgavi et al., 2001). Thus improving feed enzyme stability while transiting the GIT has potential to improve enzyme efficacy at increasing digestibility. In Chapters 4 and 6, spore-bound enzymes were shown to retain greater enzyme activity when incubated in rumen inoculant, and simulated intestinal fluid, compared to unbound enzymes. In addition, enzyme activity was only slightly decreased for spore-bound enzyme activity compared to unbound enzyme when incubated in simulated gastric fluid (approximately 85 vs 105% of 0 h, respectively). Therefore, binding enzymes to spores did increase stability in conditions representing the ruminant GIT. Data presented in Chapter 6 also indicated that release of xylanase from the spore surface occurred when incubated in simulated intestinal fluid. Thus this technique presents a potential delivery method to not only improve bound enzyme stability, but also to deliver active biomolecules to the intestines. As a consequence, the goal of optimal binding conditions should be dependent on where enzyme activity is being targeted; where if delivery to the lower digestive tract is the goal then optimal binding conditions should focus on maximising adsorption rather than retained activity.

Enhanced enzyme stability and potential release of active enzyme after spore adsorption may have contributed to the tendency for NDF digestibility to increase for spore-bound commercial xylanase compared to unbound commercial xylanase both *in vitro* (Chapter 4 and 5) and *in vivo* (Chapter 6). The increase in NDF digestibility was generally correlated to an increase in the proportion of acetate produced. A similar correlation has been previously observed by Doane et al. (1997); however, Romero et al. (2015) found no change in acetate proportion measured *in vitro* when using the same commercial xylanase enzyme applied to bermudagrass haylage. This

difference in acetate proportion between their study and ours was likely due to the substrate used in the batch cultures (bermudagrass haylage, vs alfalfa: grass hay or barley silage: concentrate (75:25 DM basis), respectively). As shown by Giraldo et al. (2008) batch culture substrate has a greater influence on total VFA concentration and acetate: propionate ratio compared to the addition of enzyme.

The *in vivo* study performed in Chapter 6 showed that spore-bound commercial xylanase tended to increase total tract NDF digestibility by 4.6 percentage units compared with the control, and 4.1 percentage units compared with unbound enzymes. Increasing the number of animal replicates in a future study would help evaluate whether the response was actually different, and repeatable. However, if spore-bound xylanase can increase digestibility in commercial livestock production to the same extent as observed in the current research studies, it would have significant impact on animal performance. This is especially true when it is noted that the cows used in Chapter 6 were mature animals (668 ± 25.5 kg) which were not growing, gestating, or lactating, and therefore only had maintenance energy requirements and were less likely to benefit from the addition of exogenous enzymes (Beauchemin et al., 2003). Enhancing *in vivo* total tract NDF digestibility without altering DMI would increase digestible energy intake (Oba and Allen, 1999), and corresponding animal performance. For example, the addition of a combination of xylanase and cellulase applied to alfalfa hay, and fed to steers (289 kg) resulted in an increased ADF digestibility of 5.2 percentage units, and a 0.25 kg/d increase in average daily gain compared to the control (Beauchemin et al., 1995). While there would be a positive economic impact from increased digestibility, the environmental impact would also be reduced, as a result of a decrease in the amount of feed required to be produced and reductions in manure and greenhouse gas production by ruminants (Ribeiro et al., 2016).

The spore-bound enzymes reduced methane production in ruminal *in vitro* batch cultures (Chapter 4 and 5). While the mechanism for this decrease is unclear, it may be related to the initial reduction in xylanase activity for spore-bound enzyme at the initiation of the batch cultures, as observed in Chapter 4. Though xylanase activity of the spore-bound treatments were eventually greater than unbound xylanase the lag would have decreased the initial production of reducing sugars, which may have resulted in delayed formation of methane. The reduction in methane production *in vitro* (Chapter 4 and 5) was supported by the alteration in VFA proportions, specifically propionate and acetate (Wolin, 1960), and suggest that there were changes in the microbiota related to spore-bound enzymes.

While methane emissions were not measured *in vivo*, it is tempting to speculate that the spore-bound enzyme treatment has potential to reduce emissions from cattle based on the increased duodenal flow rate, and alterations in site of digestion observed for spore-bound enzyme treatment compared to unbound enzymes (Chapter 6). Increasing duodenal flow rate can reduce ruminal retention time and ruminal digestibility (Mertens, 1987). This was observed in Chapter 6, where there was an overall reduction in the proportion of digestibility occurring in the rumen, and corresponding increase in proportion of digestibility occurring intestinally for spore-bound enzyme treatment compared to unbound enzymes. Shifting the site of digestion has previously been shown to reduce methane production (Johnson and Johnson, 1995), allowing for increased feed efficiency and animal performance, given that methane emissions represent a loss of energy for animal utilization (Eckard et al., 2010). In addition to potentially reducing methane emissions, an increase in duodenal flow will also increase rumen undegradable protein, and the efficiency of protein utilization. It is important to note that while an increase in duodenal flow was observed for spore-bound enzymes in Chapter 6, how the spores interacted with the ruminal

digesta is not known (i.e. association with liquid or particulate matter in the rumen). If spore-bound enzymes are further evaluated in ruminants it would be of interest to measure both particulate (small and large particles) and fluid phase passage from the rumen, as well as spore counts associated with these digesta phases. This would allow for a more accurate evaluation of the retention time of spore-bound enzymes in the rumen, and subsequent delivery of enzymes to the intestines.

In summary, this thesis showed that the technique of adsorbing feed enzymes to spores was variable, and depended on spore strain, enzyme structure, and physical/chemical conditions during adsorption. Enzymes bound to *B. subtilis* spores had increased stability in environmental conditions representing feed processing and the gastrointestinal tract of ruminants, and also resulted in increased digestibility. Before spore-bound enzyme technology can be utilised commercially, some aspects require further research. For example, stability of spore-bound enzymes during feed processing will need to be evaluated within a feed mill, to compare enzyme stability at different temperatures and within a feed matrix throughout pelleting. The stability of spore-bound enzymes needs to also be evaluated to optimize formulation stability (eg. liquid, dry), storage temperature (eg. -20, 4, 20°C), and delivery method (eg. concentrated, diluted with carrier). To minimize variation in efficacy, any spore-bound enzyme should also be tested on a variety of feedstuffs (eg. silage, high grain diets, poor quality hay, straw, etc.) and livestock species (monogastric and ruminant animals). These studies should measure both animal performance, and digestibility as these two indicators are not always related. Lastly, in Chapter 6, it was observed that spores have the potential to release bound enzymes in the small intestine. This finding warrants further investigations, as the delivery of bioactive molecules to the intestines has application to both feed and pharmaceutical industries.

8.0 Conclusion

In an effort to increase stability of feed enzymes and digestibility in cattle, enzymes were adsorbed to bacterial spores. The optimal binding conditions varied according to the enzymes and spores evaluated. Adsorption was significantly impacted by binding buffer pH, due to changes in the zeta potential of the spores and was favored in acidic conditions. While protein binding efficiency was generally moderate to high, spore-bound enzyme activity did not appear to correlate with the amount of protein bound. This suggested that adsorption resulted in steric hindrance and a resultant reduction in enzyme activity associated with the spore-bound enzymes. The species of spore used for adsorption also had a significant impact on efficiency of enzyme binding and retained enzyme activity, likely due to differences in the composition of spore coats. Based on enzyme binding efficiency and retained activity utilizing 16 spore strains, a *Bacillus subtilis* and *Bacillus coagulans* strain were tested for their effect on *in vitro* degradability after binding a commercial xylanase. From the *in vitro* study *B. subtilis* RK28 was determined to be the optimal strain, and due to its enzyme adsorption properties, effect on ruminal batch cultures, and history of safe use as a probiotic, it was selected for additional studies. Overall, binding feed enzymes to *B. subtilis* spores improved enzyme stability when incubated at high temperatures (similar to those present during feed processing) and when subjected to the proteolytic environment of the ruminant gastrointestinal tract. Improvement in *in vitro* degradation was specific to the spore-bound enzymes being analyzed, and there were no improvements observed for addition of combinations of multiple classes of enzymes prior to adsorption. When a spore-bound commercial xylanase was applied to alfalfa grass hay prior to consumption by cattle, there was an increase in duodenal flow compared to unbound enzymes. This resulted in a proportional shift in the site of digestion, from the rumen to the intestines, and a tendency for an increase in

total tract fiber digestibility. *In vitro* incubation of spore-bound enzymes in simulated intestinal fluid indicated that the release of enzymes may be possible in the small intestines. Thus, spores may offer a method to deliver feed enzymes and bioactive molecules to the lower digestive tract of livestock.

Literature Cited

Ahimou, F., M. Paquot, P. Jacques, P. Thonart, P.G. Rouxhet. 2001. Influence of electrical properties on the evaluation of the surface hydrophobicity of *Bacillus subtilis*. J. Microbiol. Meth. 45:119-126.

Alexandratos, N., and J. Bruinsma. 2012. World agriculture towards 2030/2050. FAO, United Nations, Rome.

Alexopoulos, C., I.E. Georgoulakis, A. Tzivara, S.K. Kritas, A. Siochu, and S.C. Kyriakis. 2004. Field evaluation of the efficacy of a probiotic containing *Bacillus licheniformis* and *Bacillus subtilis* spores, on the health status and performance of sows and their litters. J. Anim. Physiol. Anim. Nutr. 88:381-392.

Anderson, R.L., and W.J. Wolf. 1995. Compositional changes in trypsin inhibitors, phytic acid, saponins, and isoflavones related to soybean processing. J. Nutr. 125:581S-588S.

Arai, T., R. Araki, A. Tanaka, S. Karita, T. Kimura, K. Sakka, and K. Ohmiya. 2003. Characterization of a cellulase containing a family 30 carbohydrate-binding module (CBM) derived from *Clostridium thermocellum* CelJ: Importance of the CBM to cellulose hydrolysis. J. Bacteriol. 185:504-512.

Artzi, L., E.A. Bayer, and S. Morais. 2017. Cellulosomes: bacterial nanomachines for dismantling plant polysaccharides. Nat. Rev. Microbiol. 15:83-95.

Association of Official Analytical Chemists. 2005. Official Methods of Analysis. 18th ed. AOAC Int., Gaithersburg, MD.

Baas, T.C., and P.A. Thacker. 1996. Impact of gastric pH on dietary enzyme activity and survivability in swine fed β-glucanase supplemented diets. Can. J. Anim. Sci. 76:245-252.

Bailey, M.J., P. Biely, and K. Poutanen. 1992. Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. 23:257-270.

Barletta, A. 2010. Introduction: Current market and expected developments. pp 1-11. in: Enzymes and farm animal nutrition. 2nd edition . Bedford, M. and G. Partridge, eds. CABI Publishing, London, UK.

Beauchemin, K.A., L.M. Rode, and V.J.H. Sewalt. 1995. Fibrolytic enzymes increase fiber digestibility and growth rate of steers fed dry forages. Can. J. Anim. Sci. 75:641-644.

Beauchemin, K.A., W.Z. Yang, and L.M. Rode. 1999a. Effects of grain source and enzyme additive on site and extent of nutrient digestion in dairy cows. J. Dairy Sci. 82:378-390.

Beauchemin, K.A., L.M. Rode, and D. Karren. 1999b. Use of feed enzyems in feedlot finishing diets. Can. J. Anim. Sci. 79:243-246.

Beauchemin, K.A., D. Colombatto, D.P. Morgavi, and W.Z. Yang. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. J. Anim. Sci. 81:E37-47.

Beauchemin, K.A. and L. Holtshausen. 2010. Developments in enzyme usage in ruminants. pp 206-230.in: Enzymes and farm animal nutrition. 2nd edition. Bedford, M. and G. Partridge eds. CABI Publishing, London, UK.

Bedford, M.R. 2000. Exogenous enzymes in monogastric nutrition – their current value and future benefits. Anim. Feed Sci. Technol. 86: 1-13.

Bensoussan, L., S. Morais, B. Dassa, N. Friedman, B. Henrissat, V. Lombarrd, E.A. Bayer, and I. Mizrahi. 2017. Broad phylogeny and functionality of cellulosomal components in the bovine rumen microbiome. Environ. Microbiol. 19:185-197.

Bezkorovainy, A. 2001. Probiotics: determinants of survival and growth in the gut. Am. J. Clin. Nutr. 73:399-405S.

Bhat, M.K. and G.P. Hazelwood. 2001. Enzymology and other characteristics of cellulases and xylanases. pp 11-60.in: Enzymes and farm animal nutrition Bedford, M.R. and G.G. Partridge eds. CABI Publishing, Wallingford, UK.

Biehl, P., M. von der Luhe, and F.H. Schacher. 2018. Reversible adsorption of methylene glue as cationic model cargo onto polyzwitterionic magenetic nanoparticles. Macromol. Rapd Commun. 1800017:1-6.

Blanchette, C., C.I. Lacayo, N.O. Fischer, M. Hwang, and M.P. Thelen. 2012. Enhanced cellulose degradation using cellulase-nanosphere complexes. PLoS ONE 7:e42116.

Bourquin, L.D., E.C. Titgemeeyr, N.R. Merchen and G.C. Fahey. 1994. Forage level and particle size effects on orchardgrass digestion by steers: I. Site and extent of organic matter, nitrogen, and cell wall digestion. J. Anim. Sci. 72:746-758.

Bower, C.K., S. Sananikone, M.K. Bothwell, and J. McGuire. 1999. Activity losses among T4 lysozyme charge variants after adsorption to colloidal silica. Biotechnol. Bioeng. 64:373-376.

Bowman, G.R., K.A. Beauchemin, and J.A. Shelford. 2002. The proportion of the diet to which fibrolytic enzymes are added affects nutrient digestion by lactating dairy cows. J. Dairy Sci. 85:3420-3429.

Brena, B., P. Gonzalez-Pombo, and F. Batista-Viera. 2013. Immobilization of enzymes: A literature survey. pp 15-31.in: Immobilization of enzymes and cells. 3rd edition. J.M. Guisan ed. Humana Press, New York, USA.

Canadian Council of Animal Care. 2009. CCAC guidelines on the care and use of farm animals in research, teaching, and testing. [online] Available : http://ccac.ca/Documents/Standards/Guidelines/Farm_Animals.pdf

Carrera, M., R.O. Zandomeni, J. Fitzgibbon, and J.-L. Sagripanti. 2006. Difference between the spore sizes of *Bacillus anthracis* and other *Bacillus* species. J. Appl. Microbiol. 102:303-312.

Casula, G. and S.M. Cutting. 2002. *Bacillus* probiotics: Spore germination in the gastrointestinal tract. Appl. Environ. Microbiol. 68:2344-2352.

Chaves, A.V., L.C. Thompson, A.D. Iwaasa, S.L. Scott, M.E. Olson, C. Benchaar, D.M. Veira, and T.A. McAllister. 2006. Effect of pasture type (alfalfa vs. grass) on methane and carbon dioxide production by yearling beef heifers. Can. J. Anim. Sci. 86:409-418.

Cho, E., E. Kim, and J. Pan. 2011. Adsorption immobilization of *Escherichia coli* phytase on probiotic *Bacillus polyfermenticus* spores. Enzyme Microb. Technol. 49:66-71.

Chung, Y.-H., M. Zhou, L. Holtshausen, T.W. Alexander, T.A. McAllister, L.L. Guan, and M. Oba. 2012. A fibrolytic enzyme additive for lactating Holstein cow diets: Ruminal fermentation, rumen microbial populations, and enteric methane emissions. J. Dairy Sci. 95:1419-1427.

Colombatto, D., D.P. Morgavi, A.F. Furtado, and K.A. Beauchemin. 2002. Screening of fibrolytic enzymes as additives for ruminant diets: relationship between enzyme activities and

the *in vitro* degradation of enzyme-treated forages. p. 210 in Proc. Br. Soc. Anim. Annu. Mtg. Penicuik, U.K.

Colombatto, D., G. Hervas, W.Z. Yang, and K.A. Beauchemin. 2003. Effects of enzyme supplementation of a total mixed ration on microbial fermentation in continuous culture, maintained at high and low pH. J. Anim. Sci. 81:2617-2627.

Cortezzo, D.E. and P. Setlow. 2005. Analysis of factors influencing the sensitivity of spores of *Bacillus subtilis* to DNA damaging chemicals. J. Appl. Microbiol. 97:838-852.

Cowieson, A.J., T. Acamovic, and M.R. Bedford. 2003. Supplementation of diets containing pea meal with exogenous enzymes: effects on weight gain, feed conversion, nutrient digestibility and gross morphology of the gastrointestinal tract of growing broiler chicks. Br. Poult. Sci. 44:427-437/

Cromwell, G.L. 1992. The biological availability of phosphorus in feedstuffs for pigs. Pig News and Information. 13:75N-78N.

Cunha, A.G., G. Fernandez-Lorente, J.V. Bevilaqua, J. Destain, L.M. Paiva, D.M. Freire, R. Fernandez-Lafuent, and J.M. Guisan. 2008. Immobilization fo *Yarrowia lipolytica* lipase- a comparison of stability of physical adsorption and covalent attachment techniques. Appl. Biochem. Biotechnol. 146:49-56.

Cutting, S.M. 2011. Review: *Bacillus* probiotics. Food Microbiol. 28:214-220.

Dado, R.G., and M.S. Allen. 1995. Limitations, feeding behaviour, and rumen function of cows challenged with rumen fill from dietary fiber or inert bulk. J. Dairy Sci. 78:118-133.

Daniel, T.C., A.N. Sharpley, and J.L. Lemunyon. 1998. Agricultural phosphorus and eutrophication : A symposium overview. J. Environ. Qual. 27:251-257.

Datta, S., L.R. Christena, Y.R.S. Rajaram. 2013. Enzyme immobilization: an overview on techniques and support materials. Biotech. 3:1-9.

Dehority, B.A. 1991. Symposium on 'Fibre digestion in farm livestock'. Proc. Nutr. Soc. 50:149-159.

Detmer, A. and J. Glenting. 2006. Live bacterial vaccines – a review and identification of potential hazards. Microb. Cell Fact. 5:23-35.

Dickinson, D., M.T. La Duc, W.E. Haskins, I. Gornushkin, J.D. Winefordner, D.H. Powell, and K. Venkateswaran. 2004. Species of differentiation of a diverse suite of *Bacillus* spores by spectrometry-based protein profiling. Appl. Environ. Microbiol. 70:475-482.

Ding, M., Y. Teng, Q. Yin, J. Zhao, and F. Zhao. 2008. The N-terminal cellulose-binding domain of EGXA increases thermal stability of xylanase and changes its specific activities on different substrates. Acta. Biochim. Biophys. Sin. 40:949-954.

Doane, P.H., P. Schofield, and A.N. Pell. 1997. Neutral detergent fiber disappearance and gas and volatile fatty acid production during the in vitro fermentation of six forages. J. Anim. Sci. 75:3342-3352.

Driks, A. 1999. The *Bacillus subtilis* spore coat. Microbiol. Mol. Biol. Rev. 63:1-20.

Eckard, R.J., C. Grainger, and C.A.M. De Klein. 2010. Options for the abatement of methane and nitrous oxide from ruminant production. A review. Livestock Sci. 130:47-56.

Edens, F.W., C.R. Parkhurst, P.R. Ferket, G.B. Havenstein, and A.E. Sefton. 2002. A demonstration of postpellet application of dry phytase to broiler diets. J. Appl. Poult. Res. 11:34-45.

Eijsink, V.G.H., A. Bjork, S. Gaseidnes, R.Sirevag, B. Synstad, B. van den Burg, and G. Vriend. 2003. Rational engineering of enzyme stability. J. Biotechnol. 113:105-120.

Engelen, A.J., F.C. van der Heeft, P.H. Randsdorp, and E.L. Smith. 1994. Simple and rapid determination of phytase activity. J. AOAC. Int. 77:760-764.

Eun, J.-S., K.A. Beauchemin, and H. Schulze. 2007. Use of an *in vitro* fermentation bioassay to evaluate improvements in degradation of alfalfa hay due to exogenous feed enzymes. Anim. Feed Sci. Technol. 135:315-328.

Falahati-Pour, S.K., A.S. Lotfi, G. Ahmadian, and A. Baghizadeh. 2015. Covalent immobilization of recombinant organophosphorus hydrolase on spores of *Bacillus subtilis*. J. Appl. Microbiol. 118:976-988.

Fedorah, P.M., and S.E. Hrudey. 1983. A simple apparatus for measuring gas production by methanogenic cultures in serum bottles. Environ. Technol. Letters. 4:425-432.

Feng, P., C.W. Hunt, G.T. Pritchard, and W.E. Julien. 1996. Effect of enzyme preparations on in situ and in vitro degradation and in vivo digestive characteristics of mature cool-season grass forage in beef steers. J. Anim. Sci. 74:1349-1357.

Fierobe, H.-P., E.A. Bayer, C. Tardiff, M. Czjzek, A. Mechaly, A. Belaich, R. Lamed, Y. Shoham, and J.-P. Belaich. 2002. Degradation of cellulose substrate by cellulosome chimeras: Substrate targeting versus proximity of enzyme components. J. Biol. Chem. 277:49621-49630.

Flint, H.J., E.A. Bayer, M.T. Rincon, R. Lamed, and B.A. White. 2008. Polysaccharide utilization by gut bacteria: Potential for new insights from genomic analysis. Nat. Rev. Microbiol. 6:121-131.

Fontes, C.M.G.A., J. Hall, B.H. Hirst, G.P. Hazelwood, and H.J. Gilbert. 1995. The resistance of cellulases and xylanases to proteolytic inactivation. Appl. Microbiol. Biotechnol. 43:52-57.

France, J., and R.C. Siddons. 1986. Determination of digesta flow by continuous marker infusion. J. Theor. Biol. 121:105-120.

Gargallo, S., S. Calsamiglia, and A. Ferret. 2006. Technical note: A modified three-step in vitro procedure to determine intestinal digestion of proteins. J. Anim. Sci. 84:2163-2167.

Gashtasbi, F., G. Ahmadian, and K.A. Noghabi. 2014. New insights into the effectiveness of alpha-amylase enzyme presentation on the *Bacillus subtilis* spore surface by adsorption and covalent immobilization. Enzyme Microb. Technol. 64-65:17-23.

Gerhardt, P. and R.E. Marquis. 1989. Spore thermoresistance mechanisms. pp.43-63. In *Regulation of Prokaryotic Developlment*. Ed. Smith, I., R.A. Slepecky, and P. Setlow. Washington, DC: American Society for Microbiology.

Ghazi, S., J.A. Rooke, H. Galbraith, and M.R. Bedford. 2002. The potential for the improvements of the nutritive value of soya-bean meal by different proteases in broiler chicks and broiler cockerels. Brit. Poult. Sci. 43:70-77.

Gibbs, B.F., S. Kermasha, I. Alli, and C.N. Mulligan. 1999. Encapsulation in the food industry: a review. Int. J. Food Sci. Nutr. 50:213-224.

Giraldo, L.A., M.L. Tejido, M.J. Ranilla, and M.D. Carro. 2008. Effects of exogenous fibrolytic enzymes on *in vitro* ruminal fermentation of substrates with different forage:concentrate ratios. Anim. Feed. Sci. Technol. 141:306-325.

Gracia, M.I., M.J. Aranibar, R. Lazaro, P. Medel, and G.G. Mateos. 2003. A-amylase supplementation of broiler diets based on corn. Poult. Sci. 82:436-442.

Greiner, R., and U. Konietzny. 2010. Phtyases: Biochemistry, enzymology and characteristics relevant to animal feed use. pp 96-128. in: Enzymes and farm animal nutrition. 2nd edition. Bedford, M. and G. Partridge eds. CABI Publishing, London, UK.

Gu, J., R. Yang, Z. Hua, W. Zhang, and W. Zhao. 2015. Adsorption-based immobilization of *Caldicellulosiruptor saccharolyticus* cellobiose 2-epimerase on *Bacillus subtilis* spores. Biotechnol. Appl. Biochem. 62:237-244.

Hartnell, G.F., and L.D. Satter. 1979. Determination of rumen fill, retention time and ruminal turnover rates of ingesta at different stages of lactation in dairy cows. J. Anim. Sci. 48:381-392.

Hatfield, R.D. 1993. Cell wall polysaccharide interactions and degradability. pp. 285-314. in Forage cell wall structure and digestibility. H.G. Jung, D.R. Bruxton, R.D. Hatfield, and J. Ralph. eds. ASA, CSSA, SSSA, Madison, WI.

Henry, B.S., and C.A. Friedman. 1937. The water content of bacterial spores. J. Bacteriol. 33:323-329.

Higginbotham, G.E., E.J. dePetters, S.L. Berry, and A. Ahmadi. 1996. Effect of adding a cell wall degrading enzyme to a total mixed ration for lactating dairy cows. Prof. Anim. Sci. 12:81-85.

Hoa, T.T., L.H. Duc, R. Isticato, L. Baccigalupi, E. Ricca, P.H. Van, and S.M. Cutting. 2001. Fate and dissemination of *Bacillus subtilis* spores in a murine model. Appl. Environ. Microbiol. 67:3819-3823.

Holtshausen, L., Y.-H. Chung, H. Gerardo-Cuervo, M. Oba, and K.A. Beauchemin. 2011. Improved milk production efficiency in early lactation dairy cattle with dietary addition of a developmental fibrolytic enzyme additive. J. Dairy Sci. 94:899-907.

Hong, H.A., L.H. Duc, and S.M. Cutting. 2005. The use of bacterial spore formers as probiotics. FEMS Microbiol. Rev. 29:813-835.

Hristov, A.N., L.M. Rode, K.A. Beauchemin, and R.L. Wuerfel. 1996. Effect of a commercial enzyme preparation on barley silage *in vitro* and *in sacco* dry matter degradability. Proc. Western Section, Am. Soc. Anim. Sci. pp. 282-284.

Hristov, A.N., T.A. McAllister, and K.J. Cheng. 1998a. Effect of dietary or abomasal supplementation of exogenous polysaccharide-degrading enzymes on rumen fermentation and nutrient digestibility. J. Anim. Sci. 76:3146-3156.

Hristov, A.N., T.A. McAllister, and K.J. Cheng. 1998b. Stability of exogenous polysaccharide-degrading enzymes in the rumen. Anim. Feed Sci. Technol. 76:161-168.

Huang, J., H. Hong, H. Van Tong, T. Hoang, A. Brisson, and S.M. Cutting. 2010. Mucosal delivery of antigens using adsorption to bacterial spores. Vacc. 28:1021-1030.

Huhtanen, P., M. Rinne, and J. Nousiainen. 2009. A meta-analysis of feed digestion in dairy cows. 2. The effects of feeding level and diet composition on digestibility. J. Dairy Sci. 92:5031-5042.

Hunter, R.J. 1988. Zeta potential in colloid science: Principles and applications. Academic Press. UK.

Inborr, J. and M.R. Bedford. 1994. Stability of feed enzymes to steam pelleting during feed processing. Anim. Feed Sci Technol. 46:179-196.

Isticato, R., G. Cangiano, H.T. Tran, A. Ciabattini, D. Medaglini, M.R. Oggioni, M. De Felice, G. Pozzi, and E. Ricca. 2001. Surface display of recombinant proteins on *Bacillus subtilis* sproes. J. Bacteriol. 183:6294-6301.

Johnson, K.A. and D.E. Johnson. 1995. Methane emissions from cattle. J. Anim. Sci. 73:2483-2492.

Jongbloed, A.W. and P.A. Kemme. 1990. Effect of pelleting mixed feeds on phytase activity and the apparent absorbability of phosphorus and calcium in pigs. Anim. Feed Sci. Technol. 28:233-242.

Jung, H.G., and M.S. Allen. 1995. Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. J. Anim. Sci. 73:2774-2790.

Keyzer, M.A., M.D. Merbis, I.F.P.W. Pavel, and C.F.A. van Wesenbeeck. 2005. Diet shift towards meat and the effects on cereal use: Can we feed the animals in 2030? Ecol. Econ. 55:187-202.

Kim, D.-M., H. Nakazawa, M. Umetsu, T. Matsuyama, N. Ishida, A. Ikeuchi, H. Takahashi, R. Asano, and I. Kumagai. 2012. A nanocluster design for the construction of artificial cellulosomes. Catal. Sci. Technol. 2:499-503.

Kincaid, R.L., D.K. Garikipati, T.D. Nennich, and J.H. Harrison. 2005. Effect of grain source and exogenous phytase on phosphorus digestibility in dairy cows. J. Dairy Sci. 88:2893-2902.

Krieger, E., G. Koraimann, and G. Vriend. 2002. Increasing the precision of comparative models for YASARA NOVA- a self parameterizing force field. Proteins. 47:393-402.

Krueger, N.A, A.T. Adesogan, C.R. Staples, W.K. Krueger, D.B. Dean, and R.C. Littell. 2008. The potential to increase digestibility of tropical grasses with a fungal, ferulic acid esterase enzyme preparation. Anim. Feed Sci. Technol. 145:95-108.

Kumar, V., D. Singh, P. Sangwan, and P.K. Gill. 2014. Global market scenario of industrial enzymes, in: Beniwal, V., and Sharma, A.K. (Eds.), Industrial enzymes: Trends, scope and relevance, Nova Science Publishers Inc. New York. pp. 173-196.

Kwon, S.J., H.C. Jung, and J.G. Pan. 2007. Transgalactosylation in a water-solvent biphasic reaction system with β-galactosidase displayed on the surfaces of *Bacillus subtilis* spores. Appl. Environ. Microbiol. 73:2251-2256.

Lamichhane, S., K. Sahtout, J. Smillie, and T.A. Scott. 2015. Vacuum coating of pelleted feed for broilers: Opportunities and challenges. Anim. Feed Sci. Technol. 200:1-7

Latorre, J.D., X. Hernandez-Velaso, G. Kallapura, A. Menconi, N.R. Pumford, M.J. Morgan, S.L. Layton, L.R. Bielke, B.M. Hargis, and G. Tellez. 2014. Evaluation of germination, distribution, and persistence of *Bacillus subtilis* spores through the gastrointestinal tract of chickens. Poult. Sci. 93:1793-1800.

Lee, S., B.R. Belitsky, D.W. Brown, J.P. Brinker, K.O. Kerstein, J.E. Herrmann, G.T. Keusch, A.L. Sonenshein, and S. Tzipori. 2010. Efficacy and heat stability and safety of intranasally

administered *Bacillus subtilis* spore or vegetative cell vaccines expressing tetanus toxin fragment C. Vacc. 28:6658-6665.

Lewis, G.E., C.W. Hunt, W.K. Sanchez, R. Treacher, G.T. Pritchard, and P. Feng. 1996. Effect of direct-fed fibrolytic enzymes on the digestive characteristics of a forage-based diet fed to beef steers. J. Anim. Sci. 74:3020-3028.

Lewis, G.E., W.K. Sanchez, C.W. Hunt, M.A. Guy, G.T. Pritchard, B.I. Swanson, and R.J. Treacher. 1999. Effect of direct-fed fibrolytic enzymes on the lactational performance of dairy cows. J. Dairy Sci. 82:611-617.

Li, S., X. Yang, S. Yang, M. Zhu, and X. Wang. 2012. Technology prospecting on enzymes: Application, marketing and engineering. Comput. Struct. Biotechnol. J. 2:1-11.

Mastihuba, V., L. Kremnicky, M. Mastihubova, J.L. Willett, and G.L. Cote. 2002. A spectrophotometric assay for feruloyl esterases. Anal. Biochem. 309:96-101.

Mateo, C., J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, and R. Fernandez-Lafuente. 2007. Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzym. Microb. Technol. 40:1451-1463.

McAllister, T.A., S.J. Oosting, J.D. Popp, Z. Mir, L.J. Yanke, A.N. Hristov, R.J. Treacher, and K.-J. Cheng. 1999. Effect of exogenous enzymes on digestibility of barley silage and growth performance of feedlot cattle. Can. J. Anim. Sci. 79:353-360.

McAllister, T.A., A.N. Hristov, K.A. Beauchemin, L.M. Rode, and K.-J. Cheng. 2000. Enzymes in ruminant diets. pp.273-299. *in* Enzymes in farm animal nutrition. M.R. Bedford, and G.G. Partridge. ed. Wallingford, UK. CABI Publishing.

McAllister, T.A., K.A. Beauchemin, A.Y. Alazzeh, J.Baah, R.M. Teather, and K. Stanford. 2011. Review: The use of direct fed microbials to mitigate pathogens and enhance production in cattle. Can. J .Anim. Sci. 91:193-211.

McDonald, I.M. 1981. A revised model for the estimation of protein degradability in rumen. J. Agric Sci. 92:499-503.

McGinn, S.M., K.A. Beauchemin, T. Coates, and D. Colombatto. 2004. Methane emissions from beef cattle: Effects of monensin, sunflower oil, enzymes, yeast, and fumaric acid. J. Anim. Sci. 82:3346-3356.

Mertens, D.R. 1987. Predicting intake and digestibility using mathematical models of ruminal function. J. Anim. Sci. 64:1548-1558.

Menke, K.H., L. Raab, A. Salewski, H. Steinglass, D. Fritz, and W. Schneider. 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor in vitro. J. Agric. Sci. Camb. 93:217-222.

Min, K., J. Kim, K. Park, and Y.J. Yoo. 2012. Enzyme immobilization in carbon nanomaterials: Loading density investigation and zeta potential analysis. J. Mol. Catal. B Enzym. 83:87-93.

Morais, S., N. Shterzer, R. Lamed, E.A. Bayer, and I. Mizrahi. 2014. A combined cell-consortium approach for lignocellulose degradation by specialized *Lactobacillus plantarum* cells. Biotechnol. Biofuels. 7:112.

Morgavi, D.P., K.A. Beauchemin, V.L. Nserek, L.M. Rode, A.D. Iwaasa, W.Z. Yang, T.A. McAllister, and Y. Wang. 2000a. Synergy between fibrolytic enzymes and enzymes from *Trichoderma longibrachiatum*. J. Dairy Sci. 83:1310-1321.

Morgavi, D.P., C.J. Newbold, D.E. Beever, and R.J. Wallace. 2000b. Stability and stabilization of potential feed additive enzymes in rumen fluid. Enzyme Microb. Biotechnol. 26:171-177.

Morgavi, D.P., K.A. Beauchemin, V.L. Nsereko, L.M. Rode, T.A. McAllister, A.D. Iwaasa, Y. Wang, and W.Z. Yang. 2001. Resistance of feed enzymes to proteolytic inactivation by rumen microorganisms and gastrointestinal proteases. J. Anim. Sci. 79:1621-1630.

Mori, Y., S. Ozasa, M. Kitaoka, S. Noda, T. Tanaka, H. Ichinose, and N. Kamiya. 2013. Aligning an endoglucanase Cel5A from *Thermobifida fiscal* on a DNA scaffold: potent design of an artificial cellulosome. Chem. Commun. (Camb.) 49:6971-6973.

Muirhead, S. 1996. Direct Fed Microbial, pp. 391. In: Enzyme and Forage Additive Compendium. 3rd edn. The Miller Publishing Company, Minetonka, Minnesota,

Nicholson, W.L., N. Munakata, G. Horneck, H.J. Melosh, and P. Setlow. 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. 64:548-572.

Nsereko, V.L., D.P. Morgavi, K.A. Beauchemin, and L.M. Rode. 2000a. Inhibition of ruminant feed enzyme polysaccharidase activities by extracts from silages. Can. J. Anim. Sci. 80:523-526.

Nsereko, V.L., D.P. Morgavi, L.M. Rode, K.A. Beauchemin, and T.A. McAllister. 2000b. Effects of fungal enzyme preparations on hydrolysis and subsequent degradation of alfalfa hay fibre by mixed rumen microorganisms *in vitro*. Anim. Feed Sci. Technol. 88:153-170.

Nsereko, V.L., K.A. Beauchemin, D.P. Morgavi, L.M. Rode, A.F. Furtado, T.A. McAllister, A.D. Iwaasa, W.Z. Yang, and Y. Wang. 2002. Effect of a fibrolytic enzyme preparation from *Trichoderma longibrachiatum* on the rumen microbial population of dairy cows. Can. J. Microbiol. 48:14-20.

Oba, M. and M.S. Allen. 1999. Evaluation of the importance o fthe digestibility of neutral detergent fiber from forage: Effects on dry matter intake and milk yield of dairy cows. J. Dairy Sci. 82:589-596.

Ørskov. E.R., and Y. McDonald. 1979. The estimation of protein degradability in the rumen from determining the digestibility of feeds in the rumen. J. Agric. Sci. 92:499-503.

Ou, J., and Y. Cao. 2014. Incorporation of *Nasutitermes takasagoensis* endoglucanase into cell surface-displayed minicellulosomes in *Pichia pastoris* X33. J. Microbiol. Biotechnol. 24:1178-1188.

Pendleton, B. 1996. The regulatory environment. pp.49. In: Direct-Fed Microbial, Enzyme and Forage Additive Compendium. 3rd edition. Muirhead, S. eds. The Miller Publishing Co., Minetonka, Minnesota.

Peng, H., J.Q. Wang, H.Y. Kang, S.H. Dong, P. Sun, D.P. Bu, and L.Y. Zhou. 2012. Effect of feeding *Bacillus subtilis natto* fermentation product on milk production and composition, blood metabolites and rumen fermentation in early lactation dairy cows. J. Anim. Physiol. Anim. Nutr. 96:506-512.

Penner, G.B., K.A. Beauchemin, and T. Mutsvangwa. 2006. An evaluation of the accuracy and precision of a stand-alone submersible continuous ruminal pH measurement system. J. Dairy Sci. 89:2132-2140.

Pickford, J.R. 1992. Effects of processing on the stability of heat labile nutrients in animal feeds, pp. 177-192. in: Garnsworthy, P.C., W. Haresign, and D.J.A. Cole Eds. Recent advances in animal nutrition. Butterworth-Heinemann, Oxford.

Potot, S., C.R. Serra, A.O. Henriques, and G. Schyns. 2010. Display of recombinant proteins on *Bacillus subtilis* spores, using a coat associated enzyme as the carrier. Appl. Environ. Microbiol. 76:5916-5933.

Ravindran, V., G. Ravindran, and S. Sivalogan. 1994. Total and phytate phosphorus contents of various foods and feedstuffs of plant origin. Food Chem. 50:133-136.

Reuter, T., T.W. Alexander, and T.A. McAllister. 2011. Viability of *Bacillus lichenformis* and *Bacillus theringiensis* spores as a model for predicting the fate of *Bacillus anthracis* spores during composting of dead livestock. Appl. Environ. Microbiol. 77:1588-1592.

Ribeiro, G.O., R.J. Gruninger, A. Badhan, and T.A. McAllister. 2016. Mining the rumen for fibrolytic feed enzymes. Anim. Frontiers. 6:20-26.

Rode, L.M., W.Z. Yang, and K.A. Beauchemin. 1999. Fibrolytic enzyme supplements for dairy cows in early lactation. J. Dairy Sci. 82:2121-2126.

Romero, J.J., Zarate, M.A., Queiroz, O.C.M., Han, J.H., Shin, J.H., Staples, C.R., Brown, W.F., and Adesogan, A.T. 2013. Fibrolytic enzyme and ammonia application effects on the nutritive

value, intake, and digestion kinetics of bermudagrass hay in beef cattle. J. Anim. Sci. 91:4345-4356.

Romero, J.J., Zarate, M.A., and A.T. Adesogan. 2015. Effect of the dose of exogenous fibrolytic enzyme preparations on preingestive fiber hydrolysis, ruminal fermentation, and in vitro digestibility of bermudagrass haylage. J. Dairy Sci. 98:406-417.

Romero, J.J., E.G. Macias, Z.X. Ma. R.M. Martins, C.R. Staples, K.A. Beauchemin, and A.T. Adesogan. 2016. Improving the performance of dairy cattle with a xylanase-rich exogenous enzyme preparation. J. Dairy Sci. 99:3486-3496.

Schaefer, A.L., B.A. Young, and A.M. Chimwano. 1978. Ration digestion and retention times of digesta in domestic cattle (*Bos Taurus*), American bison (*Bison bison*), and Tibetan yak (*Bos grunniens*). Can. J. Zool. 56:2355-2358.

Schultz, N., G. Metreveli, M. Franzreb, F.H. Frimmel, and C. Syldatk. 2008. Zeta potential measurement as a diagnostic tool in enzyme immobilisation. Colloids Surf. B. 66:39-44.

Selle, J.L. 1996. Physiological changes and potential for improvement in gastrointestinal tract function. J. Appl. Poult. Res. 5:96-101.

Selle, P.H., V. Ravindran, A.J. Cowieson, and M.R. Bedford. 2010. Phytate and phytase. pp 160-205.in: Enzymes and farm animal nutrition. 2nd edition. Bedford, M. and G. Partridge, eds. CABI Publishing, London, UK.

Setlow, P. 2005. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J. Appl. Microbiol. 101: 514-525.

Silversides, F.G. and M.R. Bedford. 1999. Effects of pelleting temperature on the recovery and efficacy of a xylanase enzyme in wheat-based diets. Poult. Sci. 78:1184-1190.

Singh, R., M. Kumar, A. Mittal, and P.K. Mehta. 2016 Microbial enzymes: industrial progress in 21st century. Biotech. 3:174.

Sirec, T., A. Strazzuli, R. Isticato, M. DeFelice, M. Moracci, and E. Ricca. 2012. Adsorption of β-galactosidase of *Alicyclobacillus acidocaldarius* on wild type and mutants spores of *Bacillus subtilis*. Microb. Cell Factories. 11:100-111.

Sirisha, V.L., A. Jain, and A. Jain. 2016. Chapter nine- Enzyme immobilization: An overview on methods, support material and application of immobilized enzymes. Adv. Food Nutr. Res. 79:179-211.

Slominski, B.A., T. Davie, M.C. Nyachoti, and O. Jones. 2007. Heat stability of endogenous and microbial phytase during feed pelleting. Livest. Sci. 109:244-246.

Song, M., H.A. Hong, J. Huang, C. Colenutt, D.D. Khang, T.C.A. Nguyen, S. Park, B. Shim, H.H. Song, I.S. Cheon, J.E. Jang, J. Choi, K. Stadler, and S.M. Cutting. 2012. Killed *Bacillus subtilis* spores as a mucosal adjuvant for an H5N1 vaccine. Vacc. 30:3266-3277.

Souza, V.L., N.M. Lopes, O.F. Zacaroni, V.A. Silveira, R.A.N. Pereira, J.A. Freitas, R. Almeida, G.G.S. Salvati, and M.N. Pereira. 2017. Lactation performance and diet digestibility of dairy cows in response to the supplementation of *Bacillus subtilis* spores. Livestock Sci. 200:35-39.

Spring, P., K.E. Newman, C. Wenk, R. Messikommer, and M. Vukic Vranjes. 1996. Effect of pelleting temperature on the activity of different enzymes. Poult. Sci. 75:357-361.

Sun, P., J.Q. Wang, and H.T. Zhang. 2010. Effects of *Bacillus subtilis natto* on performance and immune function of preweaning calves. J. Dairy Sci. 93:5851-5855.

Sun, P., J.Q. Wang, and H.T. Zhang. 2011. Effects of supplementation of *Bacillus subtilis natto* Na and N1 strains on rumen development in dairy calves. Anim. Feed Sci. Technol. 164:154-160.

Sun, P., J.Q. Wang, and L.F. Deng. 2013. Effects of *Bacillus subtilis natto* on milk production, rumen fermentation, and ruminal microbiome of dairy cows. Animal. 7:216-222.

Sun, P., J. Li, D. Bu, X. Nan, and H. Du. 2016. Effects of *Bacillus subtilis natto* and different components in culture on rumen fermentation and rumen functional bacteria in vitro. Curr. Microbiol. 72:589-595.

Svihus, B. 2010. Introduction: Effect of digestive tract conditions, feed processing, and ingredients on response to NSP enzymes. pp 129-159. in: Bedford, M. and G. Partridge eds. Enzymes and farm animal nutrition. 2nd edition. CABI Publishing, London, UK.

Tam, N.K.M., N.Q. Uyen, H.A. Hong, L.H. Duc, T.T. Hoa, C.R. Serra, A.O. Henriques, and S.M. Cutting. 2006. The intestinal life cycle of *Bacillus subtilis* and close relatives. J. Bacteriol. 188:2692-2700.

Tee, B.L. and G. Kaletunc. 2009. Immobilization of a thermostable α-amylase by covalent binding to an alginate matrix increases high temperature usability. Biotechnol Prog. 25:436-445.

Tilman, D., C. Balzer, J. Hill, and B.L. Befort. 2011. Global food demand and the sustainable intensification of agriculture. Proc. Natl. Acad. Sci. 108:20260-20264.

Tricarico, J.M., and K.A. Dawson. 2005. Influence of supplemental endoglucanase or xylanase on volatile fatty acid production from ruminant feed by ruminal in vitro cultures. Archives of Anim. Nutr. 59:325-334.

Tricarico, J.M., M.D. Abney, M.L. Galyean, J.D. Rivera, K.C. Hanson, K.C. McLeod, and D.L. Harmon. 2007. Effects of a dietary *Aspergillus oryzae* extract containing alpha-amylase activity on performance and carcass characteristics of finishing beef cattle. J. Anim. Sci. 85:802-811.

Tuckey, R., B.E. March, and J. Biely. 1958. Diet and rate of food passage in the growing chick. Poult. Sci. 37:786-792.

Van Boeckel, T.P., C. Bower, M. Gilbert, B.T. Grenfell, S.A. Levin, T.P. Robinson, A. Teillant, and R. Laxminarayan. 2015. Global trends in antimicrobial use in food animals. Proc. Natl. Acad. Sci. 112:5649-5654.

Van Soest, P.J., J.B. Robertson, and B.A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3582-3597.

Van Soest, P.J. 1994. Nutritional ecology of the ruminant. 2nd edition. Cornell University Press, Ithaca, NY.

Vasconcelos, I.M. and J.T.A. Oliveira. 2004. Antinutritional properties of plant lectins. Toxicon. 44:385-403.

Wallace, R.J., S.J.A. Wallace, N. McKain, V.L. Nsereko, and G.F. Hartnell. 2001. Influence of supplementary fibrolytic enzymes on the fermentation of corn and grass silages by mixed ruminal microorganisms in vitro. J. Anim. Sci. 79:1905-1916.

Walz, O.P., and J. Pallauf. 2002. Microbial phytase combined with amino acid supplementation reduces P and N excretion of growing and fininshing pigs without loss of performance. Int. J. Food Sci. Technol. 37:835-848.

Wang, Y., T.A. McAllister, L.M. Rode, K.A. Beauchemin, D.P. Morgavi, V.L. Nsereko, A. Iwaasa, and W. Yang. 2002. Effects of exogenous fibrolytic enzymes on epiphytic microbial populations and *in vitro* digestion of silage. J. Sci. Food. Agric. 82:760-768.

Wang, Z., Z. He, K.A. Beauchemin, S. Tang, C. Zhou, X. Han, M. Wang, J. Kang, N. Odongo, and Z. Tan. 2016. Comparison of two live *Bacillus* species as feed additives for improving *in vitro* fermentation of cereal straws. Anim. Sci. J. 87:27-36.

Wealleans, A.L., M.C. Walsh, L.F. Romero, and V. Ravindran. 2017. Comparative effects of two multi-enzyme combinations and a *Bacillus* probiotic on growth performance, digestibility of energy and nutrients, disappearance of non-starch polysaccharides, and gut microflora in broiler chickens. Poult. Sci. 96:4287-4297.

Weatherburn, M.W. 1967. Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. 39:971-974.

White, R.R., Y. Roman-Garcia, J.L. Firkins, M.J. Van de Haar, L.E. Armentano, W.P. Weiss, T. McGill, R. Garnett, and M.D. Hanigan. 2017. Evaluation of the National Research Council (2001) dairy model and derivation of new prediction equations. 1. Digestibility of fiber, fat, protein, and nonfiber carbohydrate. J. Dairy Sci. 100:3591-3610.

Wolin, M.J. 1960. A theoretical rumen fermentation balance. J. Dairy Sci. 43:1452-1459.

Wood, T.M., and M.K. Bhat. 1988. Methods for measuring cellulose activities, pp. 87-112. in: Wood, W.A. and S. Kellogg Eds. Methods in enzymology, Vol 160, Academic Press Inc. London.

Yang, W.Z., K.A. Beauchemin, and L.M. Rode. 1999. Effects of an enzyme feed additive on extent of digestion and milk production of lactating dairy cows. J. Dairy Sci. 82:391-403 .

Appendix: Supplementary Tables

Supplementary Table 5.1. The effect of binding β-glucanase (GLUC) to *Bacillus subtilis* (RK28) spores on *in vitro* gas production, and methane production in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	GLUC	SB-GLUC			SB-GLUC vs CON	SB-GLUC vs SPR	SB-GLUC vs GLUC
Gas Production, ml/g digestible DM									
3 h	91.5	93.2	124.7	124.61	9.423	< 0.001	< 0.001	< 0.001	0.985
6 h	121.0	125.6	166.8	166.83	8.381	< 0.001	< 0.001	< 0.001	0.992
12 h	135.4	134.2	193.4	187.44	8.530	< 0.001	< 0.001	< 0.001	0.193
24 h	170.2	176.4	221.3	221.65	7.074	< 0.001	< 0.001	< 0.001	0.950
48 h	176.6	183.3	213.7	213.83	3.122	< 0.001	< 0.001	< 0.001	0.977
Methane production, ml/g digestible DM									
3 h	4.7	5.1	7.8	7.6	0.91	< 0.001	< 0.001	< 0.001	0.686
6 h	10.9	10.8	17.0	16.8	0.82	< 0.001	< 0.001	< 0.001	0.717
12 h	16.7	16.2	25.6	24.6	1.27	< 0.001	< 0.001	< 0.001	0.089
24 h	24.0	24.5	32.4	31.7	0.94	< 0.001	< 0.001	< 0.001	0.430
48 h	25.3	26.2	32.4	32.2	0.74	< 0.001	< 0.001	< 0.001	0.696
Methane concentration, %									
3 h	5.3	5.2	6.2	6.1	0.39	< 0.001	< 0.001	< 0.001	0.480
6 h	9.0	8.6	10.2	10.2	0.38	< 0.001	< 0.001	< 0.001	0.649

12 h	12.3	12.1	13.3	13.1	0.36	< 0.001	< 0.001	< 0.001	0.183
24 h	14.1	13.9	14.6	14.3	0.20	< 0.001	0.154	0.006	0.041
48 h	14.3	14.3	15.2	15.1	0.21	< 0.001	< 0.001	< 0.001	0.428

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0 × 10^{10} *B. subtilis* spores/g substrate), β-glucanase (GLUC; 0.5 mg β-glucanase /g substrate) and spore-bound β-glucanase (SB-GLUC; 0.5 mg of β-glucanase bound to 1.0 × 10^{10} *B. subtilis* spores/g substrate).

Supplementary Table 5.2. The effect of binding β-glucanase (GLUC) to *Bacillus subtilis* (RK28) spores on *in vitro* volatile fatty acids in rumen batch cultures.

	Treatments[1]						Contrast *P*-Values		
	CON	SPR	GLUC	SB-GLUC	SEM	*P*-Value	SB-GLUC vs CON	SB-GLUC vs SPR	SB-GLUC vs GLUC
Total Volatile Fatty Acid Concentration, mM									
3 h	43.8	47.1	39.0	49.9	2.50	0.090	0.139	0.467	0.022
6 h	51.6	56.1	48.2	51.3	3.27	0.459	0.938	0.333	0.539
12 h	61.4	57.9	67.3	65.1	4.79	0.559	0.601	0.326	0.758
24 h	81.9	84.9	79.5	81.5	5.30	0.907	0.959	0.661	0.803
48 h	96.4	97.5	102.2	101.7	0.58	< 0.001	< 0.001	0.002	0.567
Acetate:Propionate Ratio									
3 h	3.8	3.8	3.7	3.5	0.06	0.083	0.027	0.033	0.175
6 h	3.7	3.7	3.3	3.3	0.03	< 0.001	< 0.001	< 0.001	0.786
12 h	3.5	3.5	3.1	3.2	0.05	0.005	0.008	0.010	0.279
24 h	3.2	3.0	2.9	2.9	0.14	0.391	0.180	0.627	0.840
48 h	3.1	3.0	2.8	2.8	0.04	0.004	0.002	0.006	0.991
Acetate Proportion, % of Total Volatile Fatty Acids									
3 h	66.7	66.7	65.6	65.0	0.41	0.064	0.025	0.029	0.351
6 h	65.6	65.6	62.7	62.8	0.18	< 0.001	< 0.001	< 0.001	0.666
12 h	63.6	63.4	61.1	61.4	0.32	0.003	0.003	0.004	0.572
24 h	61.5	60.3	58.9	59.2	0.97	0.310	0.152	0.474	0.788
48 h	58.9	58.4	57.0	57.0	0.37	0.022	0.011	0.042	0.908

Propionate Proportion, % of Total Volatile Fatty Acids									
3 h	17.7	17.8	18.0	18.5	0.18	0.079	0.024	0.030	0.096
6 h	17.7	17.7	18.9	18.9	0.13	< 0.001	< 0.001	< 0.001	0.916
12 h	18.3	18.4	19.8	19.3	0.21	0.006	0.015	0.016	0.177
24 h	19.2	20.3	20.7	20.5	0.68	0.501	0.243	0.877	0.863
48 h	19.3	19.5	20.6	20.6	0.16	0.002	0.001	0.003	0.871
Butyrate Proportion, % of Total Volatile Fatty Acids									
3 h	10.0	10.0	10.7	10.9	0.19	0.028	0.013	0.016	0.470
6 h	10.4	10.5	12.3	12.2	0.12	< 0.001	< 0.001	< 0.001	0.661
12 h	11.9	12.1	13.1	13.1	0.22	0.014	0.008	0.017	0.936
24 h	12.9	13.1	14.2	14.1	0.36	0.098	0.065	0.125	0.730
48 h	14.2	14.5	15.1	14.8	0.19	0.085	0.087	0.302	0.335

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0 × 10^{10} *B. subtilis* spores/g substrate), β-glucanase (GLUC; 0.5 mg β-glucanase /g substrate) and spore-bound β-glucanase (SB-GLUC; 0.5 mg of β-glucanase bound to 1.0 × 10^{10} *B. subtilis* spores/g substrate).

Supplementary Table 5.3. The effect of binding feruloyl esterase (FE) *Bacillus subtilis* (RK28) spores on *in vitro* gas production, and methane production in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	FE	SB-FE			SB-FE vs CON	SB-FE vs SPR	SB-FE vs FE
Gas Production, ml/g digestible DM									
3 h	91.0	88.0	108.8	115.3	3.60	< 0.001	< 0.001	< 0.001	0.058
6 h	107.0	135.8	144.8	148.0	7.80	< 0.001	< 0.001	0.062	0.633
12 h	132.2	147.6	168.7	160.4	6.73	< 0.001	< 0.001	0.008	0.075
24 h	174.8	179.7	218.3	216.6	3.23	< 0.001	< 0.001	< 0.001	0.692
48 h	188.7	182.1	225.1	231.1	2.64	< 0.001	< 0.001	< 0.001	0.033
Methane production, ml/g digestible DM									
3 h	5.5	5.3	7.5	8.0	0.52	< 0.001	< 0.001	< 0.001	0.112
6 h	8.0	11.5	14.7	14.1	1.03	< 0.001	< 0.001	0.035	0.313
12 h	11.7	19.0	20.0	18.4	1.63	< 0.001	< 0.001	0.603	0.066
24 h	22.2	25.6	29.3	29.0	0.88	< 0.001	< 0.001	< 0.001	0.653
48 h	26.0	28.3	30.1	31.0	1.16	< 0.001	< 0.001	< 0.001	0.113
Methane concentration, %									
3 h	5.3	5.9	6.4	6.5	0.43	< 0.001	< 0.001	0.006	0.422
6 h	7.8	9.6	9.6	9.7	0.46	< 0.001	< 0.001	0.649	0.666
12 h	10.8	13.0	11.9	12.1	0.45	< 0.001	< 0.001	< 0.001	0.359
24 h	13.0	14.4	13.8	13.7	0.27	< 0.001	< 0.001	0.002	0.470

48 h	13.7	15.0	14.2	13.8	0.33	< 0.001	0.738	< 0.001	0.008

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0 × 10^{10} *B. subtilis* spores/g substrate), feruloyl esterase (FE; 1.0 mg feruloyl esterase/g substrate) and spore-bound feruloyl esterase (SB-FE; 1.0 mg of feruloyl esterase bound to 1.0 × 10^{10} *B. subtilis* spores/g substrate).

Supplementary Table 5.4. The effect of binding feruloyl esterase (FE) to *Bacillus subtilis* (RK28) spores on *in vitro* volatile fatty acids in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	FE	SB-FE			SB-FE vs CON	SB-FE vs SPR	SB-FE vs FE
Total Volatile Fatty Acid Concentration, mM									
3 h	41.4	47.1	44.6	45.8	2.16	0.360	0.203	0.666	0.720
6 h	48.2	56.1	51.0	49.0	1.56	0.042	0.720	0.018	0.407
12 h	57.6	57.9	60.5	59.8	1.80	0.615	0.402	0.464	0.816
24 h	94.8	84.9	98.9	101.2	11.01	0.742	0.696	0.336	0.886
48 h	113.3	97.5	118.3	115.7	13.36	0.701	0.903	0.371	0.898
Acetate:Propionate Ratio									
3 h	4.0	3.8	3.6	3.6	0.10	0.142	0.049	0.350	0.959
6 h	3.8	3.7	3.3	3.3	0.06	0.001	0.001	0.002	0.764
12 h	3.4	3.5	3.0	3.0	0.03	< 0.001	< 0.001	< 0.001	0.612
24 h	3.1	3.0	2.7	2.7	0.13	0.205	0.109	0.154	1.000
48 h	2.9	3.0	2.6	2.7	0.05	0.004	0.007	0.003	0.957
Acetate Proportion, % of Total Volatile Fatty Acids									
3 h	67.1	66.7	65.2	65.2	0.61	0.141	0.065	0.132	0.956
6 h	65.7	65.6	63.6	63.8	0.47	0.031	0.026	0.029	0.844
12 h	63.9	63.4	62.1	62.0	0.19	< 0.001	< 0.001	0.002	0.719
24 h	61.5	60.3	60.0	59.8	0.88	0.568	0.228	0.708	0.899
48 h	59.2	58.4	57.7	57.8	0.43	0.144	0.056	0.354	0.910

Propionate Proportion, % of Total Volatile Fatty Acids									
3 h	16.9	17.8	18.1	18.1	0.34	0.135	0.046	0.457	0.961
6 h	17.4	17.7	19.5	19.4	0.16	< 0.001	< 0.001	< 0.001	0.619
12 h	18.7	18.4	20.8	20.9	0.10	< 0.001	< 0.001	< 0.001	0.450
24 h	20.2	20.3	22.2	22.1	0.58	0.091	0.060	0.074	0.933
48 h	20.2	19.5	21.9	21.8	0.27	0.002	0.005	< 0.001	0.959
Butyrate Proportion, % of Total Volatile Fatty Acids									
3 h	10.1	10.0	11.0	11.0	0.35	0.173	0.119	0.086	0.895
6 h	11.0	10.5	11.4	11.4	0.29	0.187	0.372	0.068	0.993
12 h	12.0	12.1	11.9	12.0	0.16	0.828	0.980	0.531	0.839
24 h	12.7	13.1	12.4	12.6	0.32	0.429	0.863	0.247	0.686
48 h	13.6	14.5	13.4	13.4	0.22	0.038	0.702	0.016	0.813

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0 × 10^{10} *B. subtilis* spores/g substrate), feruloyl esterase (FE; 1.0 mg feruloyl esterase/g substrate) and spore-bound feruloyl esterase (SB-FE; 1.0 mg of feruloyl esterase bound to 1.0 × 10^{10} *B. subtilis* spores/g substrate).

Supplementary Table 5.5. The effect of binding xylanase (XYL) to *Bacillus subtilis* (RK28) spores on *in vitro* gas production, and methane production in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	XYL	SB-XYL			SB-XYL vs CON	SB-XYL vs SPR	SB-XYL vs XYL
Gas Production, ml/g digestible DM									
3 h	91.5	93.2	131.8	122.4	9.22	< 0.001	< 0.001	< 0.001	0.049
6 h	121.0	125.6	170.2	167.7	9.76	< 0.001	< 0.001	< 0.001	0.668
12 h	135.4	134.2	221.4	219.3	7.71	< 0.001	< 0.001	< 0.001	0.677
24 h	170.2	176.4	253.0	254.1	6.83	< 0.001	< 0.001	< 0.001	0.817
48 h	176.9	183.3	246.6	243.6	2.65	< 0.001	< 0.001	< 0.001	0.318
Methane production, ml/g digestible DM									
3 h	4.7	5.1	7.5	7.5	0.85	< 0.001	< 0.001	< 0.001	0.795
6 h	10.9	10.8	16.1	17.0	0.83	< 0.001	< 0.001	< 0.001	0.134
12 h	16.7	16.2	32.1	31.8	1.14	< 0.001	< 0.001	< 0.001	0.727
24 h	24.0	24.5	37.8	37.9	0.92	< 0.001	< 0.001	< 0.001	0.867
48 h	25.4	26.2	38.9	38.2	0.68	< 0.001	< 0.001	< 0.001	0.119
Methane concentration, %									
3 h	5.3	5.2	5.6	6.1	0.36	< 0.001	< 0.001	< 0.001	0.002
6 h	9.0	8.6	9.8	10.2	0.35	< 0.001	< 0.001	< 0.001	0.010
12 h	12.3	12.1	14.5	14.5	0.37	< 0.001	< 0.001	< 0.001	0.771
24 h	14.1	13.9	14.9	14.9	0.18	< 0.001	< 0.001	< 0.001	0.801
48 h	14.3	14.3	15.7	15.7	0.21	< 0.001	< 0.001	< 0.001	0.772

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0×10^{10} *B. subtilis* spores/g substrate), pure xylanase (XYL; 0.5 mg pure xylanase/g substrate) and spore-bound pure xylanase (SB-XYL; 0.5 mg of pure xylanase bound to 1.0×10^{10} *B. subtilis* spores/g substrate).

Supplementary Table 5.6. The effect of binding xylanase (XYL) to *Bacillus subtilis* (RK28) spores on *in vitro* volatile fatty acids in rumen batch cultures.

	Treatments[1]						Contrast *P*-Values		
	CON	SPR	XYL	SB-XYL	SEM	*P*-Value	SB-XYL vs CON	SB-XYL vs SPR	SB-XYL vs XYL
Total Volatile Fatty Acid Concentration, mM									
3 h	43.8	47.1	39.4	52.6	1.10	< 0.001	0.001	0.013	< 0.001
6 h	51.6	56.1	57.7	60.0	1.44	0.030	0.006	0.104	0.302
12 h	61.4	57.9	72.5	69.5	3.48	0.074	0.148	0.055	0.562
24 h	81.9	84.9	87.2	86.8	3.44	0.696	0.353	0.719	0.926
48 h	96.4	97.5	106.6	106.6	0.96	< 0.001	< 0.001	< 0.001	0.991
Acetate:Propionate Ratio									
3 h	3.8	3.8	3.5	3.5	0.05	0.013	0.006	0.008	0.367
6 h	3.7	3.7	3.2	3.2	0.04	< 0.001	< 0.001	< 0.001	0.686
12 h	3.5	3.5	2.6	2.6	0.03	< 0.001	< 0.001	< 0.001	0.549
24 h	3.2	3.0	2.5	2.8	0.22	0.217	0.201	0.468	0.415
48 h	3.1	3.0	2.4	2.5	0.03	< 0.001	< 0.001	< 0.001	0.263
Acetate Proportion, % of Total Volatile Fatty Acids									
3 h	66.7	66.7	65.0	64.8	0.32	0.009	0.006	0.007	0.706
6 h	65.6	65.6	62.3	62.2	0.25	< 0.001	< 0.001	< 0.001	0.873
12 h	63.6	63.4	57.6	57.8	0.27	< 0.001	< 0.001	< 0.001	0.581
24 h	61.5	60.3	56.8	58.5	1.49	0.231	0.203	0.423	0.457
48 h	58.9	58.4	55.0	55.8	0.38	< 0.001	< 0.001	< 0.001	0.209

Propionate Proportion, % of Total Volatile Fatty Acids									
3 h	17.7	17.8	18.4	18.7	0.16	0.012	0.005	0.006	0.199
6 h	17.7	17.7	19.6	19.7	0.15	< 0.001	< 0.001	< 0.001	0.620
12 h	18.3	18.4	22.4	22.2	0.09	< 0.001	< 0.001	< 0.001	0.244
24 h	19.2	20.3	22.8	21.4	1.03	0.190	0.182	0.476	0.385
48 h	19.3	19.5	22.9	22.6	0.20	< 0.001	< 0.001	< 0.001	0.427
Butyrate Proportion, % of Total Volatile Fatty Acids									
3 h	10.0	10.0	10.9	10.9	0.13	0.002	0.002	0.002	0.898
6 h	10.4	10.5	12.2	12.2	0.12	< 0.001	< 0.001	< 0.001	0.667
12 h	11.9	12.1	14.2	14.2	0.20	< 0.001	< 0.001	< 0.001	0.890
24 h	12.9	13.1	14.4	13.9	0.53	0.270	0.223	0.339	0.560
48 h	14.2	14.5	14.7	14.6	0.24	0.546	0.334	0.765	0.680

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0×10^{10} *B. subtilis* spores/g substrate), pure xylanase (XYL; 0.5 mg pure xylanase/g substrate) and spore-bound pure xylanase (SB-XYL; 0.5 mg of pure xylanase bound to 1.0×10^{10} *B. subtilis* spores/g substrate).

Supplementary Table 5.7. The effect of binding α-amylase (AMYL) to *Bacillus subtilis* (RK28) spores on *in vitro* gas production, and methane production in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	AMYL	SB-AMYL			SB-AMYL vs CON	SB-AMYL vs SPR	SB-AMYL vs AMYL
Gas Production, ml/g digestible DM									
3 h	104.1	113.9	130.0	127.3	13.18	0.004	0.003	0.077	0.709
6 h	134.4	145.3	168.1	161.8	7.36	< 0.001	< 0.001	0.002	0.201
12 h	165.4	167.4	190.9	183.4	7.01	< 0.001	0.001	0.003	0.138
24 h	192.7	185.9	208.3	201.9	5.53	0.001	0.088	0.005	0.230
48 h	194.8	195.2	206.2	205.2	11.31	0.008	0.013	0.021	0.799
Methane production, ml/g digestible DM									
3 h	8.0	8.3	10.6	10.2	1.53	< 0.001	< 0.001	0.005	0.531
6 h	15.1	16.3	18.9	18.9	1.25	< 0.001	< 0.001	< 0.001	0.974
12 h	24.1	24.3	27.2	26.0	1.63	0.004	0.036	0.055	0.205
24 h	30.0	29.6	32.6	31.5	1.90	0.003	0.071	0.022	0.208
48 h	31.0	32.2	33.6	33.7	2.02	< 0.001	< 0.001	0.018	0.860
Methane concentration, %									
3 h	7.6	7.7	8.2	8.3	0.48	0.013	0.008	0.030	0.894
6 h	11.2	11.2	11.3	11.5	0.56	0.323	0.081	0.134	0.148
12 h	14.6	14.5	14.2	14.2	0.56	0.074	0.035	0.087	0.908
24 h	15.6	15.9	15.6	15.6	0.63	0.525	0.867	0.256	0.977
48 h	16.1	16.3	16.3	16.4	0.57	0.018	0.002	0.348	0.225

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0×10^{10} *B. subtilis* spores/g substrate), amylase (AMYL; 1.0 mg α-amylase/g substrate) and spore-bound amylase (SB-AMYL; 1.0 mg of α-amylase bound to 1.0×10^{10} *B. subtilis* spores/g substrate).

Supplementary Table 5.8. The effect of binding α-amylase (AMYL) to *Bacillus subtilis* (RK28) spores on *in vitro* volatile fatty acids in rumen batch cultures.

	Treatments[1]						Contrast *P*-Values		
	CON	SPR	AMYL	SB-AMYL	SEM	*P*-Value	SB-AMYL vs CON	SB-AMYL vs SPR	SB-AMYL vs AMYL
Total Volatile Fatty Acid Concentration, mM									
3 h	48.2	46.3	47.9	41.8	1.83	0.151	0.049	0.137	0.059
6 h	52.5	53.9	55.3	61.6	2.75	0.202	0.059	0.098	0.161
12 h	65.9	71.3	72.9	81.0	4.52	0.230	0.057	0.180	0.250
24 h	90.8	87.0	92.9	90.2	3.43	0.693	0.900	0.534	0.601
48 h	104.4	104.0	109.3	104.7	1.94	0.275	0.852	0.796	0.151
Acetate:Propionate Ratio									
3 h	3.8	3.8	4.0	4.1	0.06	0.031	0.024	0.009	0.311
6 h	3.7	3.8	3.8	3.8	0.02	0.065	0.058	0.490	0.312
12 h	3.4	3.4	3.5	3.4	0.02	0.152	0.278	0.630	0.193
24 h	3.2	3.1	3.2	3.2	0.04	0.663	0.484	0.252	0.635
48 h	2.9	3.0	3.0	3.0	0.02	0.048	0.010	0.091	0.257
Acetate Proportion, % of Total Volatile Fatty Acids									
3 h	66.8	66.3	67.9	68.5	0.30	0.007	0.006	0.002	0.184
6 h	65.6	65.7	66.6	66.6	0.15	0.005	0.003	0.007	0.975
12 h	63.3	63.3	64.3	64.3	0.12	0.002	0.002	0.001	0.976
24 h	61.3	61.0	62.1	62.2	0.22	0.020	0.033	0.009	0.891
48 h	57.7	58.3	59.3	59.1	0.14	< 0.001	< 0.001	0.004	0.453

Propionate Proportion, % of Total Volatile Fatty Acids									
3 h	17.5	17.6	17.0	16.8	0.19	0.060	0.041	0.021	0.464
6 h	17.8	17.6	17.5	17.7	0.09	0.338	0.554	0.409	0.282
12 h	18.7	18.7	18.7	18.9	0.07	0.205	0.294	0.074	0.078
24 h	19.5	19.6	19.6	19.5	0.20	0.897	0.990	0.703	0.547
48 h	19.7	19.6	19.9	19.6	0.09	0.284	0.553	0.987	0.105
Butyrate Proportion, % of Total Volatile Fatty Acids									
3 h	9.4	9.6	9.1	8.8	0.12	0.010	0.009	0.003	0.140
6 h	10.0	10.0	9.7	9.7	0.10	0.068	0.055	0.029	0.728
12 h	11.6	11.6	11.1	11.0	0.08	0.003	0.002	0.002	0.517
24 h	12.6	12.7	11.9	11.9	0.09	0.001	0.001	0.001	0.957
48 h	14.3	14.0	13.2	13.5	0.08	< 0.001	< 0.001	0.002	0.060

1Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0 × 10^{10} *B. subtilis* spores/g substrate), amylase (AMYL; 1.0 mg α-amylase/g substrate) and spore-bound amylase (SB-AMYL; 1.0 mg of α-amylase bound to 1.0 × 10^{10} *B. subtilis* spores/g substrate).

Supplementary Table 5.9. The effect of binding phytase (PHYT) to *Bacillus subtilis* (RK28) spores on *in vitro* gas production, and methane production in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	PHYT	SB-PHYT			SB-PHYT vs CON	SB-PHYT vs SPR	SB-PHYT vs PHYT
Gas Production, ml/g digestible DM									
3 h	104.1	113.9	122.4	128.8	12.51	0.010	0.002	0.046	0.381
6 h	134.4	145.3	150.5	163.0	10.27	< 0.001	< 0.001	0.005	0.042
12 h	165.4	167.4	178.6	181.7	6.69	0.003	0.002	0.005	0.513
24 h	192.7	185.9	204.2	201.7	5.98	0.013	0.128	0.010	0.667
48 h	195.0	194.5	204.2	193.6	10.30	0.227	0.815	0.899	0.074
Methane production, ml/g digestible DM									
3 h	8.0	8.5	10.1	10.5	1.47	< 0.001	< 0.001	< 0.001	0.444
6 h	15.1	16.3	16.7	18.3	1.39	< 0.001	< 0.001	0.005	0.022
12 h	24.1	24.3	25.8	26.2	1.49	0.015	0.008	0.015	0.584
24 h	30.0	29.6	32.2	31.9	1.81	0.008	0.030	0.009	0.798
48 h	31.0	32.1	31.8	30.3	1.81	0.177	0.464	0.047	0.098
Methane concentration, %									
3 h	7.6	6.8	8.1	8.1	0.66	0.245	0.499	0.076	0.990
6 h	11.2	11.2	11.1	11.3	0.57	0.911	0.781	0.970	0.522
12 h	14.6	14.5	14.4	14.3	0.52	0.312	0.079	0.215	0.619
24 h	15.6	15.9	15.7	15.8	0.58	0.637	0.426	0.771	0.802
48 h	16.1	16.3	15.6	15.7	0.56	< 0.001	< 0.001	< 0.001	0.429

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0 × 10^{10} *B. subtilis* spores/g substrate), phytase (PHYT; 2.0 mg phytase/g substrate) and spore-bound phytase (SB-PHYT; 2.0 mg of phytase bound to 1.0 × 10^{10} *B. subtilis* spores/g substrate).

Supplementary Table 5.10. The effect of binding phytase (PHYT) to *Bacillus subtilis* (RK28) spores on *in vitro* volatile fatty acids in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	PHYT	SB-PHYT			SB-PHYT vs CON	SB-PHYT vs SPR	SB-PHYT vs PHYT
Total Volatile Fatty Acid Concentration, mM									
3 h	48.2	46.3	47.7	42.1	2.72	0.446	0.166	0.323	0.198
6 h	52.5	53.9	53.3	54.3	2.74	0.966	0.656	0.923	0.798
12 h	65.9	71.3	69.4	73.0	2.96	0.436	0.141	0.694	0.422
24 h	90.8	87.0	81.3	89.1	3.30	0.294	0.720	0.672	0.149
48 h	104.9	104.0	106.2	96.3	2.61	0.131	0.075	0.081	0.037
Acetate:Propionate Ratio									
3 h	3.8	3.8	3.8	3.9	0.06	0.642	0.609	0.233	0.560
6 h	3.7	3.8	3.7	3.8	0.04	0.349	0.095	0.353	0.269
12 h	3.4	3.4	3.5	3.4	0.02	0.188	0.648	0.993	0.107
24 h	3.2	3.1	3.2	3.1	0.06	0.845	0.690	0.962	0.459
48 h	2.9	3.0	3.0	3.0	0.01	0.033	0.026	0.815	0.334
Acetate Proportion, % of Total Volatile Fatty Acids									
3 h	66.8	66.3	66.6	66.7	0.26	0.626	0.744	0.370	0.756
6 h	65.6	65.7	66.0	66.3	0.19	0.155	0.043	0.083	0.267
12 h	63.3	63.3	64.1	63.5	0.26	0.235	0.663	0.648	0.167
24 h	61.3	61.0	61.5	61.2	0.39	0.784	0.810	0.739	0.534
48 h	57.7	58.3	58.6	58.4	0.23	0.161	0.098	0.729	0.544

Propionate Proportion, % of Total Volatile Fatty Acids									
3 h	17.5	17.6	17.4	17.2	0.21	0.550	0.350	0.189	0.501
6 h	17.8	17.6	17.7	17.5	0.13	0.463	0.152	0.581	0.323
12 h	18.7	18.7	18.5	18.7	0.09	0.336	0.719	0.703	0.174
24 h	19.5	19.6	19.4	19.6	0.24	0.849	0.631	0.853	0.439
48 h	19.7	19.6	19.6	17.0	0.07	0.856	0.590	0.880	0.843
Butyrate Proportion, % of Total Volatile Fatty Acids									
3 h	9.4	9.6	9.6	9.5	0.05	0.114	0.176	0.314	0.333
6 h	10.0	10.0	10.0	9.9	0.09	0.479	0.320	0.158	0.288
12 h	11.6	11.6	11.4	11.6	0.13	0.583	0.989	0.852	0.260
24 h	12.6	12.7	12.5	12.6	0.20	0.959	0.989	0.865	0.739
48 h	14.3	14.0	14.0	14.0	0.16	0.510	0.268	0.951	0.862

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0 × 10^{10} *B. subtilis* spores/g substrate), phytase (PHYT; 2.0 mg phytase/g substrate) and spore-bound phytase (SB-PHYT; 2.0 mg of phytase bound to 1.0 × 10^{10} *B. subtilis* spores/g substrate).

Supplementary Table 5.11. The effect of binding commercial xylanase (CX) product to *Bacillus subtilis* (RK28) spores on *in vitro* gas production, and methane production in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	CX	SB-CX			SB-CX vs CON	SB-CX vs SPR	SB-CX vs CX
Gas Production, ml/g digestible DM									
3 h	97.8	90.1	112.3	117.1	5.91	0.005	0.007	< 0.001	0.539
6 h	127.7	135.4	125.6	135.8	5.82	0.060	0.112	0.945	0.062
12 h	150.4	150.8	143.8	142.6	8.84	0.173	0.077	0.064	0.790
24 h	181.4	181.1	181.3	180.7	5.96	0.999	0.884	0.926	0.911
48 h	177.6	184.1	205.7	205.3	2.54	< 0.001	< 0.001	< 0.001	0.925
Methane production, ml/g digestible DM									
3 h	6.0	5.4	6.9	7.3	0.66	0.006	0.012	< 0.001	0.420
6 h	12.7	13.6	10.9	12.4	1.12	< 0.001	0.599	0.034	0.017
12 h	20.1	20.5	17.3	17.1	1.80	< 0.001	< 0.001	< 0.001	0.804
24 h	26.7	27.1	24.7	25.3	1.43	0.031	0.107	0.046	0.517
48 h	28.1	29.2	28.8	29.1	1.56	0.296	0.210	0.966	0.690
Methane concentration, %									
3 h	6.5	6.4	5.2	5.4	0.60	< 0.001	< 0.001	< 0.001	0.381
6 h	10.1	9.9	8.4	8.7	0.61	< 0.001	< 0.001	< 0.001	0.330
12 h	13.4	13.3	11.9	11.9	0.58	< 0.001	< 0.001	< 0.001	0.784
24 h	14.6	14.8	13.9	13.9	0.39	0.002	0.012	0.001	0.925
48 h	15.2	15.1	14.5	14.4	0.42	< 0.001	< 0.001	0.003	0.506

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0×10^{10} *B. subtilis* spores/g substrate), commercial xylanase (CX; 1.0 mg commercial xylanase/g substrate) and spore-bound commercial xylanase (SB-CX; 1.0 mg of commercial xylanase bound to 1.0×10^{10} *B. subtilis* spores/g substrate).

Supplementary Table 5.12. The effect of binding commercial xylanase product (CX) to *Bacillus subtilis* (RK28) spores on *in vitro* volatile fatty acids in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	CX	SB-CX			SB-CX vs CON	SB-CX vs SPR	SB-CX vs CX
Total Volatile Fatty Acid Concentration, mM									
3 h	46.0	46.7	51.3	51.3	4.00	0.599	0.289	0.353	0.996
6 h	52.1	55.0	57.2	57.5	4.84	0.745	0.365	0.673	0.960
12 h	63.7	64.6	62.2	63.9	4.71	0.977	0.972	0.899	0.787
24 h	86.3	86.0	80.1	79.7	4.22	0.468	0.215	0.241	0.942
48 h	100.3	100.7	94.4	95.5	2.87	0.251	0.186	0.155	0.768
Acetate:Propionate Ratio									
3 h	3.8	3.8	3.8	3.8	0.13	0.980	0.857	0.937	0.889
6 h	3.7	3.7	3.7	3.7	0.05	0.974	0.829	0.905	1.000
12 h	3.4	3.4	3.5	3.3	0.09	0.300	0.225	0.225	0.071
24 h	3.2	3.1	3.2	3.1	0.14	0.799	0.628	0.824	0.786
48 h	3.0	3.0	3.1	3.1	0.03	0.036	0.074	0.055	0.350
Acetate Proportion, % of Total Volatile Fatty Acids									
3 h	66.8	66.5	66.4	66.5	0.63	0.946	0.723	0.996	0.899
6 h	65.6	65.7	65.7	65.7	0.34	0.991	0.832	0.955	0.956
12 h	63.5	63.4	64.0	62.6	0.59	0.333	0.210	0.269	0.084
24 h	61.4	60.6	61.6	61.2	0.93	0.775	0.849	0.632	0.758
48 h	58.3	58.3	59.5	59.2	0.31	0.026	0.042	0.040	0.412

Propionate Proportion, % of Total Volatile Fatty Acids									
3 h	17.6	17.7	17.7	17.6	0.43	0.994	0.993	0.869	0.888
6 h	17.7	17.6	17.7	17.7	0.18	0.963	0.796	0.904	0.977
12 h	18.5	18.5	18.2	19.1	0.37	0.270	0.203	0.178	0.064
24 h	19.4	19.9	19.6	19.8	0.62	0.750	0.538	0.867	0.831
48 h	19.5	19.6	19.2	19.3	0.15	0.175	0.239	0.182	0.451
Butyrate Proportion, % of Total Volatile Fatty Acids									
3 h	9.7	9.8	10.7	10.6	0.23	0.016	0.012	0.024	0.713
6 h	10.2	10.3	11.0	11.1	0.21	0.012	0.006	0.009	0.756
12 h	11.8	11.9	12.2	12.8	0.31	0.090	0.019	0.028	0.166
24 h	12.8	12.9	13.0	13.1	0.40	0.868	0.444	0.635	0.840
48 h	14.3	14.2	14.1	14.2	0.19	0.816	0.780	0.842	0.563

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0 × 10^{10} *B. subtilis* spores/g substrate), commercial xylanase (CX; 1.0 mg commercial xylanase/g substrate) and spore-bound commercial xylanase (SB-CX; 1.0 mg of commercial xylanase bound to 1.0 × 10^{10} *B. subtilis* spores/g substrate).

Supplementary Table 5.13. The effect of binding a combination of amylase and a commercial xylanase (ACX) to *Bacillus subtilis* (RK28) spores on *in vitro* gas production, and methane production in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	ACX	SB-ACX			SB-ACX vs CON	SB-ACX vs SPR	SB-ACX vs ACX
Gas Production, ml/g digestible DM									
3 h	94.3	91.5	111.8	114.0	5.42	< 0.001	< 0.001	< 0.001	0.642
6 h	107.1	135.4	135.5	137.2	7.87	< 0.001	< 0.001	0.701	0.738
12 h	133.0	150.8	150.0	154.7	9.84	< 0.001	< 0.001	0.459	0.373
24 h	172.6	180.0	192.0	191.8	3.99	< 0.001	< 0.001	0.008	0.960
48 h	193.1	183.3	211.8	212.7	3.41	< 0.001	< 0.001	< 0.001	0.786
Methane production, ml/g digestible DM									
3 h	5.6	5.4	7.0	7.3	0.61	0.001	0.003	0.001	0.640
6 h	7.4	13.1	11.5	11.1	1.27	< 0.001	< 0.001	0.019	0.583
12 h	13.5	19.2	18.4	18.3	1.39	< 0.001	< 0.001	0.330	0.866
24 h	21.8	25.8	24.2	25.0	0.93	< 0.001	< 0.001	0.216	0.208
48 h	25.6	28.5	27.1	28.3	1.24	< 0.001	< 0.001	0.637	0.007
Methane concentration, %									
3 h	5.0	6.4	5.8	5.5	0.62	< 0.001	0.150	0.002	0.324
6 h	7.8	9.7	8.4	8.0	0.50	< 0.001	0.399	< 0.001	0.120
12 h	10.7	13.0	10.7	10.6	0.49	< 0.001	0.461	< 0.001	0.674
24 h	12.7	14.6	12.5	12.8	0.37	< 0.001	0.583	< 0.001	0.065

48 h	13.8	15.0	13.5	13.5	0.33	< 0.001	0.064	< 0.001	0.698

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0 × 10^{10} *B. subtilis* spores/g substrate), combination of amylase and commercial xylanase (ACX; 0.5 mg α-amylase and 0.5 mg commercial xylanase/g substrate) and spore-bound combination of amylase and commercial xylanase (SB-ACX; 0.5 mg of α-amylase and 0.5 mg commercial xylanase bound to 1.0 × 10^{10} *B. subtilis* spores/g substrate).

Supplementary Table 5.14. The effect of binding amylase and a commercial xylanase product (ACX) to *Bacillus subtilis* (RK28) spores on *in vitro* volatile fatty acids in rumen batch cultures.

	Treatments[1]				SEM	*P*-Value	Contrast *P*-Values		
	CON	SPR	ACX	SB-ACX			SB-ACX vs CON	SB-ACX vs SPR	SB-ACX vs ACX
Total Volatile Fatty Acid Concentration, mM									
3 h	41.37	46.26	45.42	46.53	3.90	0.773	0.386	0.964	0.848
6 h	48.20	53.94	51.29	51.76	3.96	0.787	0.549	0.711	0.937
12 h	57.55	71.30	80.13	80.76	10.02	0.397	0.153	0.529	0.966
24 h	94.81	86.98	103.19	100.21	10.32	0.711	0.724	0.400	0.845
48 h	113.34	104.00	112.41	110.42	13.52	0.959	0.884	0.749	0.921
Acetate:Propionate Ratio									
3 h	3.97	3.76	4.06	4.00	0.13	0.453	0.865	0.240	0.752
6 h	3.78	3.75	3.84	3.79	0.12	0.958	0.956	0.807	0.789
12 h	3.43	3.40	3.37	3.43	0.10	0.965	0.986	0.847	0.672
24 h	3.05	3.12	3.01	3.05	0.04	0.348	0.894	0.263	0.509
48 h	2.93	2.97	2.96	2.93	0.05	0.931	0.940	0.600	0.687
Acetate Proportion, % of Total Volatile Fatty Acids									
3 h	67.10	66.31	67.87	67.44	0.72	0.519	0.746	0.308	0.691
6 h	65.68	65.72	66.94	66.40	0.71	0.579	0.500	0.527	0.612
12 h	63.92	63.32	64.44	64.88	0.57	0.331	0.276	0.100	0.603
24 h	61.47	60.99	61.74	62.09	0.36	0.264	0.263	0.071	0.507
48 h	59.21	58.25	60.10	59.92	0.32	0.022	0.163	0.010	0.695

Propionate Proportion, % of Total Volatile Fatty Acids									
3 h	16.92	17.64	16.72	16.88	0.39	0.416	0.951	0.216	0.781
6 h	17.40	17.56	17.45	17.53	0.39	0.989	0.816	0.959	0.882
12 h	18.68	18.65	19.15	18.93	0.39	0.782	0.661	0.628	0.707
24 h	20.19	19.57	20.49	20.34	0.14	0.014	0.470	0.008	0.480
48 h	20.18	19.63	20.32	20.48	0.25	0.190	0.430	0.053	0.676
Butyrate Proportion, % of Total Volatile Fatty Acids									
3 h	10.12	9.59	10.05	10.20	0.33	0.593	0.868	0.239	0.754
6 h	11.01	10.04	10.50	10.75	0.27	0.177	0.522	0.115	0.538
12 h	11.96	11.58	11.49	11.25	0.22	0.249	0.063	0.333	0.469
24 h	12.65	12.67	12.38	11.96	0.24	0.227	0.087	0.079	0.256
48 h	13.56	14.02	12.59	12.68	0.12	< 0.001	0.002	< 0.001	0.607

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0×10^{10} *B. subtilis* spores/g substrate), combination of amylase and commercial xylanase (ACX; 0.5 mg α-amylase and 0.5 mg commercial xylanase/g substrate) and spore-bound combination of amylase and commercial xylanase (SB-ACX; 0.5 mg of α-amylase and 0.5 mg commercial xylanase.

Supplementary Table 5.15. The effect of binding an enzyme combination of glucanase, feruloyl esterase, amylase and commercial xylanase (GFACX) to *Bacillus subtilis* (RK28) spores on *in vitro* gas production, and methane production in rumen batch cultures.

	Treatments[1]							Contrast *P* - Values				
	CON	SPR	GFACX	SB-GFACX	I-SB-GFACX	SEM	*P*-Value	SB-GFACX vs CON	I-SB-GFACX vs CON	SB-GFACX vs GFACX	I-SB-GFACX vs GFACX	SB-GFACX vs I-SB-GFACX
Gas Production, ml/g digestible DM												
3 h	97.8	94.7	166.1	174.6	166.1	7.70	< 0.001	< 0.001	< 0.001	0.073	0.999	0.072
6 h	127.7	135.4	166.1	164.5	164.0	7.20	< 0.001	< 0.001	< 0.001	0.808	0.749	0.939
12 h	150.4	150.8	181.3	188.8	188.0	8.82	< 0.001	< 0.001	< 0.001	0.174	0.226	0.879
24 h	181.4	181.1	213.9	218.5	220.3	6.06	< 0.001	< 0.001	< 0.001	0.420	0.264	0.753
48 h	178.4	184.8	237.7	238.6	235.4	3.50	< 0.001	< 0.001	< 0.001	0.800	0.502	0.383
Methane production, ml/g digestible DM												
3 h	5.1	4.9	10.3	11.9	10.4	0.75	< 0.001	< 0.001	< 0.001	< 0.001	0.861	< 0.001
6 h	12.4	13.5	17.7	16.8	16.5	1.14	< 0.001	< 0.001	< 0.001	0.226	0.101	0.693

12 h	19.9	20.5	24.2	23.9	24.9	1.77	< 0.001	< 0.001	< 0.001	0.768	0.389	0.273
24 h	26.3	26.8	30.5	29.7	30.1	1.45	< 0.001	0.004	< 0.001	0.451	0.725	0.682
48 h	28.2	28.8	35.4	34.9	34.9	1.45	< 0.001	< 0.001	< 0.001	0.532	0.459	0.949
Methane concentration, %												
3 h	6.5	6.4	6.2	6.7	6.2	0.59	0.283	0.323	0.267	0.059	0.982	0.062
6 h	10.1	9.9	10.0	10.2	9.8	0.56	0.415	0.851	0.142	0.541	0.370	0.130
12 h	13.5	13.3	12.8	12.7	12.7	0.56	0.002	0.002	< 0.001	0.900	0.810	0.915
24 h	14.9	14.8	14.1	14.0	14.0	0.45	0.005	0.005	0.004	0.696	0.686	0.999
48 h	15.2	15.0	15.2	15.3	15.0	0.39	0.601	0.596	0.535	0.736	0.463	0.270

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0×10^{10} *B. subtilis* spores/g substrate), combination of β-glucanase, feruloyl esterase, α-amylase, and commercial xylanase (GFACX; 0.125 mg β-glucanase, 0.25 mg feruloyl esterase, 0.25 mg α-amylase and 0.25 mg commercial xylanase/g substrate), combination of β-glucanase, feruloyl esterase, α-amylase, and commercial xylanase that was bound to spores (SB-GFACX; 0.125 mg β-glucanase, 0.25 mg feruloyl esterase, 0.25 mg α-amylase and 0.25 mg commercial xylanase bound to 1.0×10^{10} *B. subtilis* spores/g substrate), or a combination of individually spore-bound β-glucanase, feruloyl esterase, α-amylase, and commercial xylanase (I-SB-GFACX; 0.125 mg β-glucanase bound to 2.5×10^{9} *B. subtilis* spores, 0.25 mg feruloyl esterase bound to 2.5×10^{10} *B. subtilis* spores, 0.25 mg α-amylase bound to 2.5×10^{9} *B. subtilis* spores and 0.25 mg commercial xylanase bound to 2.5×10^{9} *B. subtilis* spores).

Supplementary Table 5.16. The effect of binding an enzyme combination of glucanase, feruloyl esterase, amylase and commercial xylanase (GFACX) to *Bacillus subtilis* (RK28) spores on *in vitro* volatile fatty acids in rumen batch cultures.

	Treatments[1]							Contrast *P* - Values				
	CON	SPR	GFACX	SB-GFACX	I-SB-GFACX	SEM	*P*-Value	SB-GFACX vs CON	I-SB-GFACX vs CON	SB-GFACX vs GFACX	I-SB-GFACX vs GFACX	SB-GFA vs I-SE GFAC
Total Volatile Fatty Acid Concentration, mM												
3 h	46.0	46.7	54.7	54.7	54.2	3.91	0.229	0.086	0.104	0.999	0.920	0.919
6 h	52.1	55.0	59.4	60.1	61.4	4.49	0.442	0.158	0.108	0.902	0.737	0.831
12 h	63.7	64.6	65.6	65.8	66.2	4.48	0.990	0.700	0.644	0.973	0.915	0.942
24 h	86.3	86.0	83.1	83.7	84.4	4.00	0.956	0.590	0.691	0.902	0.798	0.894
48 h	100.3	100.7	99.1	100.4	99.8	2.78	0.990	0.969	0.880	0.719	0.854	0.860
Acetate:Propionate Ratio												
3 h	3.8	3.8	3.5	3.4	3.5	0.13	0.086	0.026	0.046	0.826	0.949	0.776
6 h	3.7	3.7	3.4	3.4	3.4	0.06	< 0.001	< 0.001	0.002	0.993	0.486	0.480
12 h	3.4	3.4	3.2	3.2	3.2	0.06	0.006	0.006	0.005	0.820	0.870	0.948

24 h	3.2	3.1	2.9	2.9	2.8	0.13	0.219	0.089	0.038	0.917	0.581	0.653
48 h	3.0	3.0	2.8	2.8	2.8	0.03	0.001	0.002	0.001	0.588	0.768	0.803
Acetate Proportion, % of Total Volatile Fatty Acids												
3 h	66.8	66.5	64.5	64.1	64.3	0.68	0.022	0.008	0.011	0.701	0.845	0.849
6 h	65.6	65.7	63.2	63.3	63.6	0.35	< 0.001	< 0.001	< 0.001	0.951	0.445	0.480
12 h	63.5	63.4	61.7	61.8	61.8	0.46	0.015	0.011	0.010	0.786	0.827	0.958
24 h	61.4	60.6	60.0	60.1	59.3	0.93	0.456	0.254	0.085	0.941	0.583	0.535
48 h	58.3	58.3	57.6	57.9	57.5	0.32	0.191	0.243	0.055	0.516	0.854	0.409
Propionate Proportion, % of Total Volatile Fatty Acids												
3 h	17.6	17.7	18.7	18.8	18.6	0.43	0.141	0.043	0.086	0.862	0.841	0.709
6 h	17.7	17.6	18.8	18.8	18.6	0.20	< 0.001	< 0.001	0.003	0.962	0.470	0.442
12 h	18.5	18.5	19.6	19.5	19.5	0.22	0.003	0.003	0.003	0.792	0.885	0.905
24 h	19.4	19.9	20.7	20.8	21.1	0.57	0.154	0.053	0.027	0.825	0.573	0.730
48 h	19.5	19.6	20.5	20.5	20.4	0.17	< 0.001	< 0.001	0.001	0.744	0.582	0.820
Butyrate Proportion, % of Total Volatile Fatty Acids												
3 h	9.7	9.8	11.7	11.9	12.0	0.29	< 0.001	< 0.001	< 0.001	0.687	0.553	0.847

6 h	10.2	10.3	12.6	12.5	12.4	0.23	< 0.001	< 0.001	< 0.001	0.692	0.476	0.747
12 h	11.8	11.9	13.3	13.2	13.2	0.32	0.004	0.003	0.003	0.783	0.874	0.907
24 h	12.8	12.9	13.6	13.4	13.8	0.47	0.370	0.266	0.079	0.776	0.682	0.491
48 h	14.3	14.2	14.6	14.6	14.7	0.23	0.452	0.293	0.126	0.960	0.583	0.617

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0×10^{10} *B. subtilis* spores/g substrate), combination of β-glucanase, feruloyl esterase, α-amylase, and commercial xylanase (GFACX; 0.125 mg β-glucanase, 0.25 mg feruloyl esterase, 0.25 mg α-amylase and 0.25 mg commercial xylanase/g substrate), combination of β-glucanase, feruloyl esterase, α-amylase, and commercial xylanase that was bound to spores (SB-GFACX; 0.125 mg β-glucanase, 0.25 mg feruloyl esterase, 0.25 mg α-amylase and 0.25 mg commercial xylanase bound to 1.0×10^{10} *B. subtilis* spores/g substrate), or a combination of individually spore-bound β-glucanase, feruloyl esterase, α-amylase, and commercial xylanase (I-SB-GFACX; 0.125 mg β-glucanase bound to 2.5×10^{9} *B. subtilis* spores, 0.25 mg feruloyl esterase bound to 2.5×10^{10} *B. subtilis* spores, 0.25 mg α-amylase bound to 2.5×10^{9} *B. subtilis* spores and 0.25 mg commercial xylanase bound to 2.5×10^{9} *B. subtilis* spores).

Supplementary Table 5.17. The effect of binding an enzyme combination of glucanase, feruloyl esterase, amylase and pure xylanase (GFAX) to *Bacillus subtilis* (RK28) spores on *in vitro* gas production, and methane production in rumen batch cultures.

	Treatments[1]					SEM	*P*-Value	Contrast *P* - Values				
	CON	SPR	GFAX	SB-GFAX	I-SB-GFAX			SB-GFAX vs CON	I-SB-GFAX vs CON	SB-GFAX vs GFAX	I-SB-GFAX vs GFAX	SB-GFAX vs I-SB-GFAX
Gas Production, ml/g digestible DM												
3 h	97.8	90.7	128.6	148.2	153.0	6.70	< 0.001	< 0.001	< 0.001	0.025	0.004	0.583
6 h	127.7	135.4	170.4	173.9	174.3	7.12	< 0.001	< 0.001	< 0.001	0.582	0.535	0.943
12 h	150.4	150.8	209.3	203.4	196.9	9.11	< 0.001	< 0.001	< 0.001	0.284	0.026	0.235
24 h	181.4	181.1	234.4	227.6	234.4	5.65	< 0.001	< 0.001	< 0.001	0.222	0.999	0.222
48 h	177.9	184.4	245.0	253.4	249.0	3.33	< 0.001	< 0.001	< 0.001	0.045	0.326	0.291
Methane production, ml/g digestible DM												
3 h	5.4	5.0	8.9	9.8	9.5	0.67	< 0.001	< 0.001	< 0.001	0.084	0.211	0.570
6 h	12.1	13.4	19.0	18.9	18.7	0.94	< 0.001	< 0.001	< 0.001	0.931	0.655	0.717
12 h	19.8	18.9	28.5	28.7	27.5	1.48	< 0.001	< 0.001	< 0.001	0.790	0.136	0.073
24 h	26.0	25.8	34.9	32.9	34.0	1.06	< 0.001	< 0.001	< 0.001	0.029	0.289	0.240
48 h	27.4	28.6	37.7	39.9	38.1	1.25	< 0.001	< 0.001	< 0.001	0.010	0.635	0.031
Methane concentration, %												
3 h	6.5	6.2	6.6	6.1	6.2	0.54	0.127	0.146	0.208	0.048	0.072	0.854
6 h	10.0	9.7	10.8	10.5	10.3	0.47	< 0.001	0.019	0.142	0.249	0.040	0.346
12 h	13.2	12.9	13.7	13.6	13.6	0.43	< 0.001	0.010	0.003	0.346	0.660	0.613
24 h	14.4	14.5	14.9	14.8	14.8	0.30	0.184	0.062	0.080	0.685	0.540	0.845

48 h	14.9	14.9	15.9	16.0	15.8	0.32	< 0.001	< 0.001	< 0.001	0.202	0.631	0.088

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0×10^{10} *B. subtilis* spores/g substrate), combination of β-glucanase, feruloyl esterase, α-amylase, and purified xylanase (GFAX; 0.125 mg β-glucanase, 0.25 mg feruloyl esterase, 0.25 mg α-amylase and 0.25 mg purified xylanase/g substrate), combination of β-glucanase, feruloyl esterase, α-amylase, and purified xylanase that was bound to spores (SB-GFAX; 0.125 mg β-glucanase, 0.25 mg feruloyl esterase, 0.25 mg α-amylase and 0.25 mg purified xylanase bound to 1.0×10^{10} *B. subtilis* spores/g substrate), or a combination of individually spore-bound β-glucanase, feruloyl esterase, α-amylase, and purified xylanase (I-SB-GFAX; 0.125 mg β-glucanase bound to 2.5×10^{9} *B. subtilis* spores, 0.25 mg feruloyl esterase bound to 2.5×10^{10} *B. subtilis* spores, 0.25 mg α-amylase bound to 2.5×10^{9} *B. subtilis* spores and 0.25 mg purified xylanase bound to 2.5×10^{9} *B. subtilis* spores).

Supplementary Table 5.18. The effect of binding an enzyme combination of glucanase, feruloyl esterase, amylase and pure xylanase (GFAX) to *Bacillus subtilis* (RK28) spores on *in vitro* volatile fatty acids in rumen batch cultures.

	Treatments[1]					SEM	*P*-Value	Contrast *P* - Values				
	CON	SPR	GFAX	SB-GFAX	I-SB-GFAX			SB-GFAX vs CON	I-SB-GFAX vs CON	SB-GFAX vs GFAX	I-SB-GFAX vs GFAX	SB-GFAX vs I-SB-GFAX
Total Volatile Fatty Acid Concentration, mM												
3 h	46.0	46.7	52.7	53.8	53.5	3.96	0.368	0.125	0.138	0.837	0.882	0.954
6 h	52.1	55.0	60.7	61.1	61.0	4.50	0.385	0.117	0.122	0.942	0.960	0.982
12 h	63.7	64.6	68.8	68.3	67.1	4.78	0.880	0.429	0.559	0.935	0.778	0.841
24 h	86.3	86.0	84.1	85.8	85.8	3.84	0.992	0.911	0.905	0.736	0.741	0.994
48 h	100.3	100.7	103.2	103.3	102.5	3.42	0.926	0.472	0.602	0.980	0.869	0.849
Acetate:Propionate Ratio												
3 h	3.8	3.8	3.5	3.4	3.4	0.13	0.090	0.032	0.036	0.841	0.888	0.953
6 h	3.7	3.7	3.2	3.1	3.1	0.05	< 0.001	< 0.001	< 0.001	0.660	0.821	0.830
12 h	3.4	3.4	2.9	2.9	3.0	0.06	< 0.001	< 0.001	< 0.001	0.870	0.789	0.667
24 h	3.2	3.1	2.7	2.7	2.7	0.12	0.016	0.008	0.005	0.857	0.997	0.861

48 h	3.0	3.0	2.7	2.7	2.7	0.04	< 0.001	< 0.001	< 0.001	0.598	0.984	0.612
Acetate Proportion, % of Total Volatile Fatty Acids												
3 h	66.8	66.5	64.5	64.3	64.2	0.70	0.029	0.013	0.011	0.824	0.737	0.909
6 h	65.6	65.7	62.1	61.9	61.9	0.31	< 0.001	< 0.001	< 0.001	0.602	0.604	0.998
12 h	63.5	63.4	60.3	60.2	60.4	0.44	< 0.001	< 0.001	< 0.001	0.802	0.926	0.731
24 h	61.4	60.6	58.4	58.6	58.5	0.84	0.039	0.017	0.013	0.826	0.936	0.889
48 h	58.3	58.3	56.9	57.2	57.0	0.34	0.013	0.019	0.008	0.512	0.818	0.667
Propionate Proportion, % of Total Volatile Fatty Acids												
3 h	17.6	17.7	18.7	18.8	18.7	0.44	0.134	0.044	0.060	0.821	0.944	0.876
6 h	17.7	17.6	19.7	19.7	19.7	0.15	< 0.001	< 0.001	< 0.001	0.605	0.906	0.688
12 h	18.5	18.5	20.6	20.6	20.5	0.23	< 0.001	< 0.001	< 0.001	0.902	0.637	0.554
24 h	19.4	19.9	21.7	21.6	21.8	0.55	0.010	0.005	0.004	0.868	0.971	0.840
48 h	19.5	19.6	21.1	21.0	21.2	0.19	< 0.001	< 0.001	< 0.001	0.745	0.903	0.656
Butyrate Proportion, % of Total Volatile Fatty Acids												
3 h	9.7	9.8	11.7	11.7	11.9	0.29	< 0.001	< 0.001	< 0.001	0.896	0.627	0.721
6 h	10.2	10.3	12.9	13.0	13.0	0.22	< 0.001	< 0.001	< 0.001	0.709	0.613	0.894

12 h	11.8	11.9	13.8	13.8	13.8	0.33	< 0.001	< 0.001	< 0.001	0.884	0.965	0.850
24 h	12.8	12.9	14.1	14.0	14.1	0.42	0.053	0.027	0.025	0.870	0.895	0.975
48 h	14.3	14.2	14.8	14.7	14.7	0.25	0.374	0.219	0.223	0.717	0.709	0.991

[1]Batch culture treatments were control (CON; 1.0 ml distilled water/g substrate), spore (SPR; 1.0×10^{10} *B. subtilis* spores/g substrate), combination of β-glucanase, feruloyl esterase, α-amylase, and purified xylanase (GFAX; 0.125 mg β-glucanase, 0.25 mg feruloyl esterase, 0.25 mg α-amylase and 0.25 mg purified xylanase/g substrate), combination of β-glucanase, feruloyl esterase, α-amylase, and purified xylanase that was bound to spores (SB-GFAX; 0.125 mg β-glucanase, 0.25 mg feruloyl esterase, 0.25 mg α-amylase and 0.25 mg purified xylanase bound to 1.0×10^{10} *B. subtilis* spores/g substrate), or a combination of individually spore-bound β-glucanase, feruloyl esterase, α-amylase, and purified xylanase (I-SB-GFAX; 0.125 mg β-glucanase bound to 2.5×10^{9} *B. subtilis* spores, 0.25 mg feruloyl esterase bound to 2.5×10^{10} *B. subtilis* spores, 0.25 mg α-amylase bound to 2.5×10^{9} *B. subtilis* spores and 0.25 mg purified xylanase bound to 2.5×10^{9} *B. subtilis* spores).