

Development of a multi-strain *Bacillus* probiotic for poultry production

by

Uraisha Ramlucken

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**UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA**

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SUPERVISORY COMMITTEE

Supervisor:

Prof MS Thantsha

Department of Biochemistry, Genetics and Microbiology

Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria, 0002

Co-supervisor

Dr R Lalloo

Department of Bioprocess Development

Future, Production: Chemicals

The Council for Scientific and Industrial Research (CSIR)

Pretoria, 0002

Co-supervisor

Dr C Jansen van Ransburg

Department of Animal and Wildlife Sciences

Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria, 0002

DECLARATION

FACULTY OF NATURAL AND AGRICULTURAL SCIENCE

I, Uraisha Ramlucken declare that the thesis/dissertation, which I hereby submit for the degree Doctor of Philosophy (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:.....

DATE:.....

DEDICATION

“If you’ve got your mother’s prayers, you’ve got everything and more”

I dedicate this thesis to my mother, who has showered me with love, encouragement and support through this entire journey. She has given me strength and empowerment in the face of life’s challenges and has been both a mother and father to me these past years. I am forever grateful to her.

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REASERCH ARTICLES

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LIST OF ABBREVIATIONS

AGP- Antibiotic growth promoter

AME- Apparent metabolizable energy

AMPs- Antimicrobial peptide/s

ANF- Anti-nutritional factors

ANOVA- Analysis of variance

BW- Body weight

CaCl₂- Calcium chloride

CE- Competitive exclusion

CFU- Colony forming units

CMC- Carboxymethylcellulose

cm- centimetres

CP-Crude protein

DMEM- Dulbecco modified eagle medium

EDTA- Ethylenediaminetetraacetic acid

EFSA- European food and safety authority

ELISA- Enzyme-linked immunosorbent assay

FCR- Feed conversion ratio

FDA- Food and drug administration

FI- Feed intake

g- Gram

GALTs- Gut-associated lymphoid tissue/s

GIT- Gastrointestinal tract

GRAS- Generally regarded as safe

HCDC- High cell density cultivation

HCL-Hydrochloric acid

H₂O- Water

H₂SO₄- Sulphuric acid

Kg- Kilograms

kPa- Kilopascals

L- Litres

mBar- Millibar

ME-Metabolizable energy

mg- Milligrams

MgSO₄- Magnesium sulphate

mL- Millilitres

mm- Millimetres

MnSO₄- Manganese sulphate

µm- Micrometer

µL- Microliter

NA- Nutrient agar

NaOH- Sodium hydroxide

NE- Necrotic enteritis

NH₄OH- Ammonium hydroxide

NSP- Non-starch polysaccharides

OD- Optical density

PBS- Phosphate buffered saline

PCA- Plate count agar

PCR- Polymerase chain reaction

QPS- Qualified presumption of safety

RPM- Revolutions per minute

RT-PCR- Reverse transcriptase Polymerase chain reaction

SAS- Statistical analysis system

SBM- Soybean meal

SE- Sporulation efficiency

SGF- Simulated gastric fluid

SIF –Simulated intestinal fluid

SLPM- Standard litre per minute

SNE-Subclinical necrotic enteritis

TSA- Tryptone soy agar

TSB- Tryptone soy broth

v- Volume

VCR- Villi height to crypt depth ratio

w- Weight

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SUMMARY

The global demand for poultry products is continuously growing, therefore the poultry industry requires fast growth and high stocking densities to enhance production efficiency. These conditions impact negatively on chicken health driving the indiscriminate use of antibiotic growth promoters (AGPs), which has resulted in an increase in the outbreaks of zoonotic diseases due to antibiotic resistance of poultry pathogens. This has led to restrictions on AGP use in poultry production in many countries. This coupled with consumer preference for more organic food, has created a need for the industry to explore alternative technologies for poultry production. Probiotics can offer a solution to these challenges, as they have been proven to reduce poultry pathogen infections, improve immune responses, aid in digestion of feed and improve general health of chickens. However, due to the vast amounts of probiotics required for poultry production, certain technological parameters need to be taken into consideration regarding stability during feed processing and storage in industrial conditions. For this reason, there has been increased interest in *Bacillus* based products because their endospore-forming ability improves stability during processing, enables longer shelf life and better survival in the chicken gut. This genus also elicits all of the probiotics effects of interest to the poultry industry, making them the ideal target for research and development. This study therefore focused on the development of a multi-strain poultry probiotic product, the associated production processes and demonstration of the efficacy. This study is structured into five chapters which are summarised as follows:

Chapter 1: Literature review

This chapter covers an overview of the poultry industry and the associated challenges. The suitability of probiotics to address the poultry industry challenges especially related to broiler production are justified. A brief overview of the advantages and disadvantages of conventional probiotic species currently popular in the poultry industry are included. The chapter then solely focusses on the advantages of the use of *Bacillus* spp. as an alternative to conventional poultry probiotics. It covers the mechanisms of action of this species within the chicken, giving examples of their benefits that have been proven both *in vitro* and *in vivo*. Auxiliary benefits that address environmental concerns such as ammonia emissions are also addressed including the biosafety and regulatory considerations. The process requirements for efficient production such as high cell density cultivation (HCDC) and high viable spore recovery are reviewed.

Existing information on product shelf life stability and retention of viability through the poultry feed manufacturing process are presented. These technological aspects, are discussed in context of efficiency and cost. The chapter concludes with the future perspectives regarding *Bacillus* based poultry feed probiotics.

Chapter 2 (Experimental 1): Isolation, selection and evaluation of *Bacillus* spp. as potential multi-mode probiotics for poultry

This chapters outlines the isolation and selection of *Bacillus* spp. as putative probiotics. Samples were collected from strategically selected sites associated with poultry production and different types of samples were taken from each site (broiler swabs, faeces, bedding material, feathers and where possible, the gut). A total of 48 pure isolates were obtained. An additional 18 isolates related to animals from an existing database, were added. The best performing strains were selected based on mathematical matrix evaluation using *in vitro* screens mimicking industry relevant criteria. These criteria included the survival and growth in various key stages of the chicken gastrointestinal tract (bile salts, pH 3, 5 and 7), the production of key digestive enzymes (amylase, protease, cellulase and xylanase), the ability to physically break down commercial chicken feed, the antagonistic properties against key poultry pathogens (*Salmonella enteritidis*, *Escherichia coli*, *Clostridium perfringens* and *Listeria monocytogenes*) and adherence properties to Caco-2 epithelial cells. The study resulted in the selection of six isolates designated as CPB 011, CPB 020, CPB 035, D014, HP 1.6 and CPB 029, which displayed a final desirability co-efficient of 76, 75, 70, 69, 67 and 62% respectively. Four of these isolates were identified as *B. subtilis* and two as *B. velezensis*.

Chapter 3 (Experimental 2): Production and stability of a multi-strain *Bacillus* based probiotic product for commercial use in poultry

This chapter demonstrated the technological suitability of *Bacillus* spp. for commercial use. A full production process was successfully developed for each of the six probiotic strains. The high cell density cultivation resulted in spore concentrations exceeding 1×10^{10} CFU.mL⁻¹. Thereafter all strains were subsequently harvested by continuous centrifugation with cell recoveries exceeding 88%. The resultant spore concentrates were then converted to individual powder intermediates and blended into a stable powdered product exceeding 1×10^9 CFU.g⁻¹ containing all six strains. The shelf life of the probiotic product exceeded 2 years under industrial conditions. The probiotic product was then tested in an *in vitro* simulated gastrointestinal assay to verify the viability and germination within the host. The powdered

product was then incorporated into the feed at recoveries exceeding 70% across the feed manufacturing processes.

Chapter 4 (Experimental 3): The efficacy of the probiotic product when tested in a broiler field study

This chapter demonstrated the efficacy of the test probiotic in a 35 day challenged broiler study using two different metabolizable energy (ME) diets. The study included a commercial benchmark, which served as a positive control and a negative untreated control. Broilers fed the standard ME diet with the test probiotic showed significantly improved ($p = 0.001$) feed conversion ratios (FCR) than the negative control from day 21 onwards. Broilers fed a reduced ME diet with the test probiotic achieved higher final body weights ($p = 0.037$) and FCRs ($p = 0.014$) than the negative control. Increased duodenal villi height ($p = 0.012$) and villi height to crypt depth ratio in the duodenum ($p < 0.0001$) and jejunum ($p = 0.0004$) confirmed an improvement in gut health. Additionally, the test probiotic resulted in significantly reduced relative liver weights in both ME groups. The test probiotic performed better than the positive control in most measures, however the differences were not significant in this trial.

Chapter 5 (Conclusion): General conclusions, impact and future recommendations

This study resulted in the successful development of a multi-strain, commercially promising chicken feed probiotic. The general conclusions of the entire project are highlighted in this chapter, along with the academic and industrial impacts. Future recommendations are also discussed.

INTRODUCTION

There is a constant increase in demand for poultry products globally due to affordability, accessibility and consumer preference. On average, 100 million metric tons of poultry is consumed per annum and it has been projected that by the year 2024, an additional 26 million tons of poultry per annum will be produced worldwide, accounting for more than half the total meat production (Poultry, 2018). In South Africa, chicken forms an integral part of the diet, therefore in excess of one billion chickens are produced in South Africa annually (Roosendaal, 2016). To meet the growing demand, producers require fast growth rate and rapid turnaround (4-6 weeks), necessitating intensive high density production. These harsh production conditions such as overcrowding and unfavourable ambient conditions impose stress on the chickens (Meluzzi and Sirri, 2009), increasing susceptibility to disease, immune deficiencies, reduced growth performances and increased mortality (Olori, 2009).

To address these problems, poultry producers have resorted to the use of antibiotics as growth promoters (AGPs) at sub-therapeutic levels as a preventative measure (Hughes and Heritage, 2004, Economou and Gousia, 2015). Currently, many countries, including South Africa, still utilize AGPs as a means of enhancing production and preventing disease (Economou and Gousia, 2015). However, the indiscriminate use of AGPs has resulted in the prevalence of a myriad of multiple drug resistant pathogens. This led to the ban of the prophylactic and nutritive use of AGPs in countries such as Europe, USA and Scandinavia (Casewell *et al.*, 2003, Perreten, 2003, Dibner and Richards, 2005, Grave *et al.*, 2006), which also affects other countries that export to these regions. Furthermore, consumer concerns of food safety, animal welfare and affordability, coupled to preferences for more “organic meat” or “natural food” is also preventing the use of conventional antibiotic treatments and unnatural growth stimulants (Yiridoe *et al.*, 2005, Penz Jr and Bruno, 2011). There is thus a dire need for alternatives to antibiotics (Patterson and Burkholder, 2003). The use of plant extracts, enzymes, organic acids, essential oils and prebiotics are some of the methods currently used to replace AGPs (Yang *et al.*, 2009, Fallah *et al.*, 2013). However, these additives are costly which has restricted wide scale adoption (Yang *et al.*, 2009).

In-feed probiotics, present an attractive alternative. Probiotics are defined in a variety of ways, but the most commonly accepted definition refers to them as “live microorganisms which when

administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2002). Over the past 20 years, probiotics have gained significant attention based on their potential applications in various industries ranging from use in the food industry for the promotion of human health, in the medical field as an alternative to antibiotics and in aquaculture (Gill and Guarner, 2004, Chaucheyras-Durand and Durand, 2009). The use of probiotics in the animal nutrition sector has also been widely accepted and documented (Chaucheyras-Durand and Durand, 2009, Gaggia *et al.*, 2010, Mingmongkolchai and Panbangred, 2018). To a large extent, animal probiotics have been an extension of the research into human probiotics, and there is much scope for the development of tailored and potent probiotics exclusively for animals. Probiotics represent one of the fastest growing and also one of the most reputable segments in animal nutritional supplements (Markowiak and Śliżewska, 2018). However, feed probiotics need to meet criteria that elicits rapid and beneficial responses which requires constant delivery through inclusion in the feed (Simon *et al.*, 2005).

There are a plethora of information surrounding probiotics and their application in poultry, with the most reported probiotics being *Lactobacillus* spp. (Kalavathy *et al.*, 2003, Tsai *et al.*, 2005, Kabir, 2009, Wang *et al.*, 2015, Jahromi *et al.*, 2016). This genus are natural inhabitants of the GIT of poultry, are generally regarded as safe (GRAS) and generally fulfil most of the criteria required to be successful probiotics. The chicken feed probiotic space is predominately occupied by this species and a myriad of *Lactobacillus* strains have been shown to improve feed conversion ratios (FCR), growth performance, pathogen inhibition and immune responses (Jin *et al.*, 1996, Jin *et al.*, 1997, Jin *et al.*, 1998b, Jin *et al.*, 1998a, Tsai *et al.*, 2005, Higgins *et al.*, 2008). Despite their excellent probiotic characteristics, *Lactobacilli*, along with other conventional probiotics have a critical limitation which is their failure to tolerate harsh environments. Therefore survival through the various production process steps and limited shelf life remains a challenge (Chávez and Ledebøer, 2007).

To address the challenge in stability of *Lactobacillus* spp. and other vegetative probiotics, *Bacillus* spp. have gained attention as feed probiotics. This genera consists of Gram positive, catalase producing, rod shaped bacteria that are aerobic to facultative anaerobes (Cutting, 2011). They are generally found ubiquitously in soil, air and water (Slepecky and Hemphill, 2006) as well as frequently found present within the GIT of chickens (Barbosa *et al.*, 2005, Cartman *et al.*, 2008). The hallmark characteristic of this genus is its ability to form environmentally resistant spores. These metabolically dormant spores are resistant to extreme

temperatures, mechanical stress, chemicals, UV radiation and desiccation. These spores germinate into metabolically active vegetative cells once the conditions becomes favourable (Setlow and Setlow, 1995). This unique characteristic of *Bacillus* spp. is one of the reasons for their success as in-feed probiotics, as the robustness of the spores can be exploited in many harsh manufacturing processes such as cell harvesting, formulation into liquid or powdered products and feed manufacturing (Simon *et al.*, 2005). Furthermore, spores can be viable for several years, thus increasing shelf life under ambient industrial warehouse conditions (Laloo *et al.*, 2010a).

Coupled to the process advantages, this species also elicit several of the probiotic effects of interest to the poultry industry, such as pathogen control, improvement in digestion and absorption, immunomodulation and the reduction of toxic compound in the gut (Lim and Kim, 2009, Lee *et al.*, 2012, Menconi *et al.*, 2013, Murugesan *et al.*, 2014, Lee *et al.*, 2015). These benefits have led to improved broiler performance in terms of body weight, feed intake, feed conversion efficiency, and reduced mortality (Molnár *et al.*, 2011, Abudabos *et al.*, 2013, Jayaraman *et al.*, 2013, Park and Kim, 2014, Lei *et al.*, 2015). Most importantly, *Bacillus* based probiotics serve to address many of the technological challenges faced by conventional probiotics and can provide new alternative options to improve rearing practise, product quality, consumer acceptance and affordability.

Research into this species as poultry probiotics is emerging, specifically in the development of new generation probiotics that improve poultry production and address current challenges faced by the poultry industry. As the gut is a dynamic and complex environment multi-mode, multi strain products are preferred as it gives a myriad of probiotics effects. This is achieved by appropriate isolation, screening and selection procedures which are vital to yield good putative probiotics for use in poultry production (Fontana *et al.*, 2013). Along with research into novel organisms and their potential as probiotic strains, appropriate cost effective upstream processes for biomass production and downstream processes to yield stable products need to be developed. This, including the necessary *in vivo* field testing are areas of research and development that are important to ensure industry adoption and to meet the future demands of the poultry industry.

Problem Statement

The high demand for poultry and poultry products leads to intensive production that relies heavily on AGPs for the prevention of disease, promotion of performance and the reduction of mortality. However increased consumer pressure and regulatory restrictions against the use of artificial growth promoters and antibiotics is driving the search for innovative alternative technology solutions. Probiotics offer such a solution, but challenges remain due to their lack of technological suitability, thus preventing wide scale industry adoption. In contrast to conventional probiotics, *Bacillus* spp. show great promise mainly due to their spore form, which addresses industry needs such as process stability and shelf life, beyond satisfying the typical probiotic mechanism of action. Even though some probiotic products are commercially available, there remain gaps in performance and none are locally manufactured to any reasonable level of quality and scientific credibility using indigenous microbes. This creates an opportunity for development of *Bacillus* probiotics that are used predominantly for growth promotion but also combines other benefits such as competitive exclusion of pathogens and improvement of health. Furthermore, there is a need for the development of efficient and cost effective production processes to meet the local manufacturing needs of developing countries, where poultry remains a major preferred and affordable food source.

Research Aims and Objectives

The aim of this project was to develop a multi-strain *Bacillus* based probiotic product to support poultry production.

The aim of the project was met by the following objectives:

- 1.1 To critically evaluate the poultry probiotic landscape and articulate the advantages of *Bacillus* spp.
- 2.1 To sample, isolate and purify *Bacillus* spp. as probiotic candidates.
- 2.2 To critically evaluate the survival and growth of isolates under poultry GIT conditions.
- 2.3 To screen and select putative isolates against industry relevant probiotic criteria, namely; digestive enzyme production, feed breakdown, adherence and antagonism against pathogens.
- 2.4 To identify and confirm that the selected probiotics are *Bacillus* spp. and are safe for use.
- 3.1 To develop an efficient upstream and downstream production process and to show stability of the probiotic strains through the process and in the product.
- 3.2 To confirm viability and growth of the probiotic product under simulated GIT conditions.
- 3.3 To confirm stability of the probiotic product through the broiler feed manufacturing process.
- 4.1 To demonstrate the functionality and efficacy *in vivo* of the multi-strain probiotic product.

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CHAPTER ONE

LITERATURE REVIEW

Uraisha Ramlucken, Rajesh Laloo, Yrielle Roets, Ghaneshree Moonsamy, Christine Jansen van Rensburg and Mapitsi. S. Thantsha (2019). Advantages of Bacillus based probiotics in poultry production. Livestock Science. (Under Revision)

Advantages of *Bacillus* based probiotics in poultry production

Uraisha Ramlucken^{1,2,3}, Rajesh Laloo¹, Yrielle Roets¹, Ghaneshree Moonsamy¹, Christine Jansen van Rensburg² and Mapitsi. S. Thantsha^{3*}

¹ The Council for Scientific and Industrial Research, Biosciences, Pretoria, South Africa, 0001

² Department of Animal and Wildlife Science, University of Pretoria, Pretoria, South Africa, 0002

³ Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Pretoria, South Africa, 0002

1.1 Abstract

Broiler production is one of the most lucrative food production industries globally, due to the demand for poultry products. Regulations and changing consumer trends in the use of antibiotic growth promoters in animal rearing are becoming stricter, therefore their use has been banned in some countries. Probiotics provide a suitable alternative to address these challenges. *Bacillus* based probiotics, mostly due to their spore-forming ability, are attractive alternatives to conventional probiotics. It has been proven that these organisms possess a myriad of mechanisms that elicit probiotic effects, such as competitive exclusion of common poultry pathogens (*Salmonella* spp., *Clostridium* spp., *Escherichia coli*, and *Campylobacter* spp.); improvement in digestion and absorption via exogenous enzymes and improvement in intestinal morphology; immunomodulation; and the reduction of toxic compounds such as ammonia and aflatoxins. These effects have been proven to reduce disease and mortality, improve feed efficiency up to 5%, enhance health and assist in the environmental sustainability of poultry production. With regards to probiotic development these organisms are easily isolated from environments and show up to 100% survivability in the harsh conditions of the gastrointestinal tract. Besides these important considerations, the key advantages for the use of *Bacilli* as feed probiotics, is their robust nature pertaining to industrial production because spores can be produced at high density up to 1×10^{11} spores.mL⁻¹, retain ~90% viability during the conditions of downstream processing and are stable at a spore concentration of 1×10^9 spores.mL⁻¹ when formulated into probiotic products. Furthermore spores retain more than 90% of their viability when incorporated into feed and the ability of spores to retain metabolic activity and regenerate upon application allows for stable storage and longer product shelf life

up to 5 years. These advantages show great potential for the application of *Bacillus* based probiotics in the poultry industry.

Key words: *Bacillus*; Broiler; Industrial application; Probiotics; Production

1.2 Introduction

The poultry industry is one of the largest meat industries worldwide, producing approximately 23 billion broiler chickens in 2016 (FAOSTAT, 2018). Poultry production is estimated to increase by 24% over the next decade, reaching ~131,255 thousand metric tons by 2025 (Poultry, 2018). This industry results in multi-billion-dollar trade due to the continuous demand for produce, which necessitates high efficiency production and high-stocking densities. This consequently exposes poultry to stressful conditions, resulting in below optimal growth, disease and death.

To prevent losses, antibiotic growth promoters (AGP) are used as a means of enhancing broiler production and reducing the prevalence of infectious zoonotic and other diseases. However, the indiscriminate use of antibiotics for prophylactic and nutritive applications have led to the proliferation of highly resistant pathogens and susceptible organisms also continue to develop antibiotic resistance. For this reason, countries in the EU (Casewell *et al.*, 2003, Perreten, 2003), the US (Mathew *et al.*, 2007) and Scandinavia (Bengtsson and Wierup, 2006) have banned the use of AGPs in livestock production, which will soon become a reality for many other countries. The increase in consumer demand for poultry products that are organic, antibiotic free, and devoid of artificial chemicals, hormones and other harmful substances, further necessitates the requirement for alternative growth promoting-disease suppressing products (Yiridoe *et al.*, 2005).

The response from industry to AGP-free farming has been controversial due to cost, loss of efficiency and deterioration in animal health (Casewell *et al.*, 2003, Maron *et al.*, 2013, Teillant and Laxminarayan, 2015). However, consumer preference for safe foods is driving the development of new technologies that can support industry adoption of alternatives to AGP substances. In order to adapt to new regulations, the broiler industry, including feed manufacturers, had to consider other sustainable options that could replace antibiotics. These include in-feed additives such as organic acids, plant derivatives (phytogenic), enzymes, essential oils and prebiotics. The benefits of these alternatives are covered extensively in

reviews (Huyghebaert *et al.*, 2011, Sethiya, 2016, Gadde *et al.*, 2017a). Despite some successes in broiler health and production, these additives contribute considerably to the cost of poultry production, necessitating the need for more cost effective options (Yang *et al.*, 2009).

Probiotics are an attractive in-feed additive, and this new technology is addressing the challenges of both cost and efficacy. A probiotic is defined as a preparation containing viable or inactivated, known microorganisms in sufficient numbers which exert beneficial effects on the host (Schrezenmeir and de Vrese, 2001). Probiotics have been shown to improve feed utilization, feed conversion ratio (FCR), reduce the prevalence of disease and improve the holistic health and vigor in poultry. Furthermore, being safe and natural, probiotics do not risk the well-being of poultry or consumers with ongoing use (Ghadban, 2002, Patterson and Burkholder, 2003, Kabir, 2009).

The most abundantly used probiotics in broiler production are *Lactobacillus* spp. due to their health promoting benefits and as an extension of their use as human probiotics. These probiotics were primarily used to reduce the prevalence of chicken pathogens but also have other positive effects, such as immunomodulation, regulation of the gut microflora, and aiding in digestion and absorption (Kabir, 2009), resulting in improved feed conversion efficiency and growth (Ghadban, 2002, Patterson and Burkholder, 2003, Kabir, 2009). However, the implementation of these organisms in the poultry industry remains challenging because of constraints such as lack of stability in the feed manufacturing process, poor shelf life and limited survival in the gastrointestinal tract (GIT). This leads to a reluctance in adoption of these probiotics by the poultry industry, due to the lack of cost-to-benefit ratio (Mattila-Sandholm *et al.*, 2002).

There is an emerging preference for *Bacillus* based probiotics in the poultry industry, because this genus has characteristics that overcome the challenges associated with conventional probiotics. Their endospore forming ability enables these organisms to be stable during feed manufacture, storage and survival through the gut. For this reason, these organisms have already been successfully applied in other types of animal production, such as aquaculture, ruminants, pigs and domestic animals (Chaucheyras-Durand and Durand, 2009). Although limited, studies are emerging on the use of *Bacillus* spp. as poultry probiotics. This review covers the challenges associated with conventional probiotics and the industry relevant advantages of *Bacillus* spp. as poultry probiotics. The mechanisms of action as probiotics, the

ease of development of technology, the feasibility of commercial production and inclusion in poultry feed are addressed. Further considerations regarding their biosafety and regulatory compliance have been discussed.

1.3 Conventional probiotics used by the poultry industry

There are many species of conventional probiotics currently used in the poultry feed industry, which have enhanced broiler performance, however, their disadvantages have stifled proper industry adoption. Lesser used conventional chicken probiotics include *Saccharomyces* spp., *Aspergillus* spp., *Enterococcus* spp. and *Bifidobacteria*. Although not indigenous to the chicken GIT, *Saccharomyces* spp. offer probiotic advantages such as resistance to mycotoxicosis, coccidiosis and protection against bacterial infections without risking transmission of antibiotic resistance (Reddy *et al.*, 2005, Czerucka *et al.*, 2007, Gao *et al.*, 2008). *Aspergillus* spp. have been reported to improve gut microflora by supporting the growth of beneficial bacteria, reducing serum cholesterol and gas production (Han *et al.*, 1999, Kim *et al.*, 2003, Lee *et al.*, 2006). *Enterococcus* spp. are indigenous to chickens and have been shown to prevent gastrointestinal diseases, colonization of enteric pathogens and increase beneficial bacteria in the GIT (Wendt *et al.*, 1998, Audisio *et al.*, 2000, Samli *et al.*, 2007, Franz *et al.*, 2011). *Bifidobacteria*, also indigenous to chickens, assist in reducing pathogen transmission and produce beneficial compounds (Jung *et al.*, 2008, Baffoni *et al.*, 2012). *Lactobacillus* spp. are most popularly used in broiler production and are considered model probiotics as they are naturally present in the GIT of poultry (Kabir, 2009). *Lactobacillus* spp. have been traditionally used in producing various fermented foodstuffs for years, are considered safe (Soccol *et al.*, 2010) and its probiotic effects in poultry have been shown extensively (Jin *et al.*, 1996, Jin *et al.*, 1998b, Pascual *et al.*, 1999, Kalavathy *et al.*, 2003, Kabir *et al.*, 2004, Tsai *et al.*, 2005, Haghghi *et al.*, 2006, Timmerman *et al.*, 2006, Mookiah *et al.*, 2014, Jahromi *et al.*, 2016).

Contrastingly, several reports indicated that conventional probiotics do not meet some of the key industry criteria regarding performance. A broiler study by Olnood *et al.*, (2015) using four *Lactobacillus* spp. resulted in no significant difference in weight gain and FCR. Similarly, Brzoska *et al.*, (2012) found that *Lactococcus lactis* 847 did not produce a significant difference in body weight, FCR and carcass fat, all crucial parameters required for probiotic acceptance in the poultry industry. A study by Haghghi *et al.*, (2005) showed that treatment with *Lactobacillus acidophilus* and *Bifidobacterium bifidum* did not enhance antibody response in chickens. Possible reasons for the lack of effect when using conventional probiotics are

ascribable to reduced survival against the harsh conditions prevalent within the chicken GIT. Santini *et al.*, (2010) demonstrated the *in vitro* survival of only two of 11 different *Bifidobacterium* and *Lactobacillus* strains tested in a simulated gastric environment. In a study by Shokryazdan *et al.*, (2014) only three out of 42 *Lactobacillus* spp. survived the simulated acid and bile *in vitro* tests, whereas Taheri *et al.*, (2009) showed that none of the *Lactobacilli* they had screened were resistant to a bile concentration of 0.3%, which is usually the minimum lethal dose. Martin *et al.*, (2018), however, demonstrated *Lactobacillus* spp. to be tolerant to bile but are not viable in simulated gastric juice.

Besides issues of viability within the GIT, most conventional probiotics have disadvantages in their production and in subsequent downstream production processes. This is mainly due to the fragile vegetative state which is more susceptible to physical parameters such as pH, temperature, pressure, oxygen and mechanical shear. The larger quantities of feed probiotics needed for animal production than those for human consumption, necessitates efficient production processes (Simon *et al.*, 2005). The two main issues with high intensity cultivation of *Lactobacillus* spp. at industrial scale, are low cell growth rate and a high accumulation of lactate which inhibits production (Elmarzugi *et al.*, 2010), whereas *Bifidobacteria* are sensitive to acidic pH and exposure to oxygen (Ibrahim and Bezkorovainy, 1994). There are ongoing efforts to improve the high cell density cultivation of conventional probiotics, but the fundamental challenges remain (Saarela *et al.*, 2004, Doleyres and Lacroix, 2005, Lacroix and Yildirim, 2007, Chin *et al.*, 2015).

The poultry industry prefers the use of stable powdered products for reasons such ease of handling and blending into the feed, easier administration to the birds and importantly transport and storage considerations. The dry product form dictates that the conventional probiotics require more costly drying processes such as freeze drying, whilst cheaper alternatives such as spray drying and drum drying often require higher temperatures, causing damage to vegetative cells. These methods of drying have been used for *Lactobacillus* spp., *Bifidobacteria* and *Saccharomyces* spp., however, the processing challenges limits the adoption of these products by industry (Wang *et al.*, 2004).

Conventional probiotic products require lower temperatures to preserve viability in the vegetative state, requiring specialized logistics and costly storage. A study by Abd-Talib *et al.*, (2013) showed that *Lactobacillus plantarum* lost 99% of viability after two weeks of non-

refrigerated storage. Conventional probiotics are also susceptible to the process conditions (high temperature, pressure and sheer) involved in feed manufacture. The extrusion and pelletizing processes reach temperatures of 75-85 °C, whereas the tolerant temperature range of some *Lactobacillus* spp. is only 60-65 °C (Teixeira *et al.*, 1997), causing destruction of the majority of viable cells (Kosin and Rakshit, 2006). Other classical probiotics such as *Enterococcus* spp. and *Bifidobacteria* have been shown to withstand temperatures of 50-60 °C, and are therefore destroyed during the higher temperature processes applied during feed manufacture (Lian *et al.*, 2002, Simon *et al.*, 2005).

Due to these limitations, probiotics that are restricted to the vegetative state are not yet considered as ideal as AGP replacements in the poultry industry (Ghosh *et al.*, 2016). This substantiates the exploration of alternate microorganisms to better address the needs of the poultry industry.

1.4 The use of *Bacillus* spp. as poultry probiotics

The genus *Bacillus* are Gram-positive, catalase producing, rod shaped bacteria that are ubiquitous in soil, air and water (Cutting, 2011). Their key advantage over other probiotic species is their inherent ability to form spores that resume viability under favorable conditions. *Bacilli* are renowned work horses of industry with applications in almost every sector (Schallmeyer *et al.*, 2004). Using these organisms as probiotics has gained more recent interest due to their positive attributes.

One of the historical concerns relating to the use of *Bacillus* species as poultry probiotics is their aerobic nature, questioning their growth within the anaerobic regions of the GIT (Cutting, 2011), which may hamper the probiotic effect of this group of organisms (Svihus, 2014). However, it is well known that *Bacillus* spp. can utilize nitrate or nitrite as the terminal electron acceptor, thereby facilitating anaerobic respiration which enables them to survive in anoxic conditions (Cartman *et al.*, 2008). Barbosa *et al.*, (2005) first showed the presence of *Bacilli* within the chicken GIT, thereafter a study by Cartman *et al.*, (2008) proved that *B. subtilis* were able to germinate and grow in the chicken GIT. Furthermore, there have been various other reports of successful isolation of *Bacillus* spp. from the GIT of chickens (Wolfenden *et al.*, 2010, Chaiyawan *et al.*, 2015, Nguyen *et al.*, 2015), mitigating the reservations of the survival of this species within the gut.

Other concerns center around the ability of *Bacillus* spp. to elicit a probiotic effect, as *Lactobacillus* spp. have been considered the gold standard as probiotics, not only in poultry applications, but also in humans. Newer information provides evidence of *Bacillus* spp. showing probiotic characteristics in several *in vitro* and *in vivo* studies (Hong *et al.*, 2005, Cutting, 2011, Grant *et al.*, 2018). The poultry industry is swiftly moving towards the use of *Bacillus* based probiotic products and many companies have successfully commercialized products (Table 1.1). These probiotics have been approved by the EU as safe for use in-feed. *Bacillus subtilis*, in particular, is deemed as one of the most successful probiotic species used in poultry feed (Hong *et al.*, 2005).

Table 1.1: *Bacillus* spp. probiotics used in the poultry industry.

<i>Bacillus</i> Product	Manufacturer	Species	Commercial strain designation	Reference
Calsporin®	Calpis Co. Ltd. Japan	<i>Bacillus subtilis</i>	C-3102	(Maruta <i>et al.</i> , 1996, Fritts <i>et al.</i> , 2000)
GalliPro®	CHR Hansen, Denmark	<i>B. subtilis</i>	DSM 17299,	(Lund <i>et al.</i> , 2005, Abudabos <i>et al.</i> , 2015)
SPORULIN®	Novus International, Inc., USA	Mixture of 3 <i>B. subtilis</i>	unknown	(Kim <i>et al.</i> , 2017, Wang, 2017)
CLOSTAT™	Kemin Industries Inc., USA	<i>B. subtilis</i>	PB6	(Teo and Tan, 2006, Teo and Tan, 2007, Abudabos <i>et al.</i> , 2013)
Enviva® PRO	DuPont Industries, USA	<i>B. amyloliquefaciens</i>	PTA-6507	(Dersjant-Li <i>et al.</i> , 2013, Additives and Feed, 2016b)
B-Act	AgriHealth, Australia	<i>B. licheniformis</i>	DSM 28710	(Additives <i>et al.</i> , 2019)
Alterion NE®	Adisso-Novazyme	<i>B. subtilis</i>	DSM 29784	(Additives <i>et al.</i> , 2017)
BioPlus 2B/ BioGrow	Christian Hansen Hoersholm, Denmark	Mixture of <i>B. licheniformis</i> and <i>B. subtilis</i>	DSM 5749 and DSM 5750	(Additives and Feed, 2016a)
Toyocerin	Asahi Vet S.A., Tokyo, Japan	<i>B. cereus var toyoi</i>	NCIMB- 40112/CNCM- 1012	(Vilà <i>et al.</i> , 2009)

1.5 Modes of action of *Bacillus* spp.

Bacillus species have a wide range of beneficial features which can be categorized as mechanisms that facilitate their corresponding probiotic effect (modes of action). The modes of action of poultry probiotics in general have not been fully elucidated, but some mechanisms have been proposed (Edens, 2003, Ng *et al.*, 2008, Vilà i Miquel *et al.*, 2010). In principle, the mechanisms of action through which *Bacillus* spp. in their vegetative state may function as probiotics, are the same as those for other probiotic organisms. However, *Bacillus* spp. are known to be fastidious and can grow and replicate rapidly within the GIT of chickens (Cartman *et al.*, 2008, Latorre *et al.*, 2014). The intrinsic growth rate of probiotics plays a vital role in the functioning and success of the probiotic, as the growth rate affects all modes of action directly as a consequence of cell number and metabolic activity. With regards to probiotics used in poultry, not much literature is available on direct mechanisms of action, however, there is a significant amount of research showing the improvement of growth and health in animal studies. Mechanisms of action are not mutually exclusive, as a probiotic can function with one or a combination of several mechanisms (Figure 1.1).

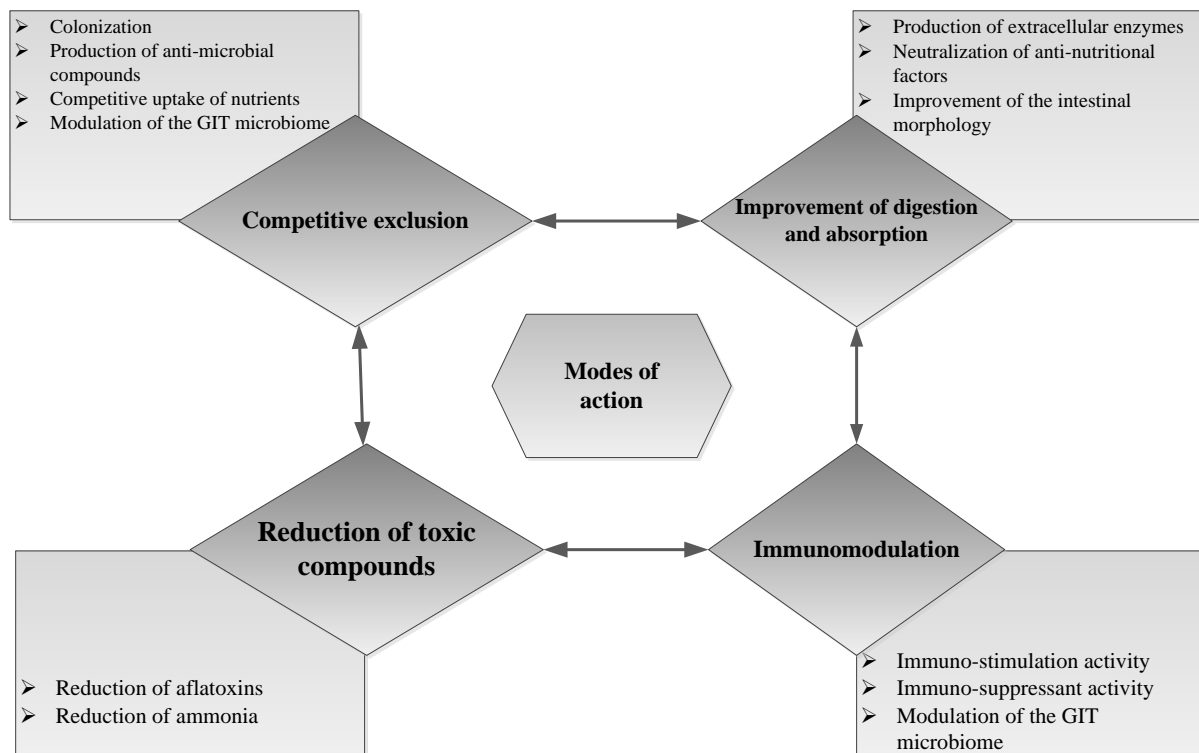


Figure 1.1: Modes of action (diamonds) of *Bacillus* probiotics and associated mechanisms of actions (boxes) relevant to the poultry industry.

1.5.1 Probiotic effect 1: Competitive exclusion (CE)

The main drivers to finding suitable replacements to antibiotics are prevention of antibiotic resistance in chicken pathogens and consumer resistance to foods containing antibiotics (Dhama *et al.*, 2013a). Such substitutes are important to the poultry industry, as zoonotic diseases such as necrotic enteritis caused by *Clostridium perfringens* can eradicate an entire production flock with detrimental economic effects (Hafez, 2011). Other zoonotic diseases such as listeriosis (Dhama *et al.*, 2013b) and salmonellosis (Boyle *et al.*, 2007) have more seriously led to consumer fatalities.

Competitive exclusion (CE) relates to the exclusion of undesirable pathogens by probiotic organisms (Callaway *et al.*, 2008). The mechanisms used by probiotics to reduce the growth of pathogenic species vary, including: competition for physical attachment sites and space, direct and indirect competition for essential nutrients, production of antimicrobial compounds, regulation of the gut microbiome and synergistic interactions of two or more of the above mechanisms (Callaway *et al.*, 2008, Bermudez-Brito *et al.*, 2012). Generally, probiotic organisms will occupy a particular niche within the intestinal tract and dominate that niche at the detriment of undesirable microorganisms (Callaway *et al.*, 2008).

Colonization occurs when probiotic microorganisms adhere more strongly to the epithelial cells of the gut, thereby excluding opportunistic pathogens by spatial domination (Dhama *et al.*, 2011). This strategy has been more frequently used to control endemic and zoonotic agents in poultry, especially in day-old chicks where the gut microbiome is entirely populated by exogenous organisms (Pan and Yu, 2014). Chicks are immunologically immature until about 3-4 weeks of age, and are prone to rapid and persistent colonization by both commensal and pathogenic bacteria (Hughes, 2008). The introduction of probiotics enables colonization of only beneficial bacteria at a young age thereby reducing disease propensity. An emerging field of probiotic research is the *in ovo* stimulation of the embryonic guts with probiotics, to promote early colonization which results in improved productivity and health (Cox and Dalloul, 2015, Rubio, 2019).

Bacillus spp. have been shown to populate the gut of chicks and broilers (Barbosa *et al.*, 2005), however the evidence for adherence to epithelial cells by *Bacillus* spp. have been mostly demonstrated *in vitro*. The consensus is that these genera of bacteria are more transient in nature compared to *Lactobacillus* spp. (Latorre *et al.*, 2014). Jadamus *et al.*, (2001) suggested that *B.*

cereus var *toyoi* persisted in the broiler GIT for 35 days, but did not necessarily colonize it. Probiotics have been shown to function in a transient state and the adhesion capacity of microorganisms is not obligatory to confer a probiotic effect (Vilà i Miquel *et al.*, 2010). The persistence of *Bacillus* spp. in the GIT of poultry could be attributable to the formation of biofilms which aid attachment to the gut epithelia, therefore increasing their persistence in the intestinal mucosa and preventing colonization by enteropathogens (Latorre *et al.*, 2016). Besides enhanced adhesion to the intestinal mucus, biofilms are proposed to have a protective role, shielding the probiotic from antimicrobial substances and gastric juices (Hong *et al.*, 2009). Although *in vivo* data of *Bacillus* based poultry probiotics forming biofilms are scarce, there are several *in vitro* assessments where biofilm formation has been shown (Barbosa *et al.*, 2005, Larsen *et al.*, 2014, Prieto *et al.*, 2014, Latorre *et al.*, 2016). The colonization of the GIT of probiotic organisms is not only attributable to adhesion and biofilm production, but also cell motility, which allows for the extensiveness of colonization through various regions of the gut as demonstrated by Aguiar *et al.*, (2013), who reported on competitive exclusion of *Campylobacter jejuni* due to motility of a *Bacillus* probiotic.

CE by probiotics can also be achieved by the competitive uptake of essential nutrients that are necessary for pathogen growth. The faster uptake of nutrients such as carbon, glucose and iron enable the probiotic to competitively exclude pathogens from growing. Being fastidious, heterotopic *Bacillus* spp. have a high organic carbon utilization rate, which enables them to outcompete pathogens based on specific nutrients (Slepecky and Hemphill, 2006). The synthesis of siderophores by *Bacillus* spp., which are low molecular weight chelating compounds, facilitate competitive uptake of iron. Its role in pathogen exclusion was shown by Patel *et al.*, (2009) and Laloo *et al.*, (2010b). The competition for essential nutrients has mostly been shown *in vitro*, however, the decrease in pathogen load associated with the presence of probiotics in the chicken GIT is an indication of this mechanism *in vivo* (La Ragione and Woodward, 2003).

The production of antimicrobial compounds is one of the main mechanisms of CE and is well reviewed in literature, specifically using *Lactobacillus* spp. (Jin *et al.*, 1997, Ghadban, 2002, Patterson and Burkholder, 2003). *Bacillus* spp. are also capable of producing a large number of antimicrobial peptides (AMP) such as lipopeptides, surfactins, bacteriocins and bacteriocin-like inhibitory substances (Urdaci and Pinchuk, 2004, Baruzzi *et al.*, 2011). These peptides fall under two categories, (i) ribosome-produced AMPs which enable the bacterium to have a

narrow antimicrobial range against closely related organisms and (ii) non-ribosomal AMPs that exert a broader antimicrobial range. The common mechanisms of bacteriocin-mediated killing include the destruction of pathogenic cells by pore formation and/or inhibition of cell wall synthesis and disruption of DNA, RNA and protein metabolism function which occurs within the cell (Bermudez-Brito *et al.*, 2012). The antimicrobial activity of bacteriocins in poultry production specifically with *Bacillus* spp. are difficult to study *in vivo*, however, this has been extensively shown *in vitro* during pathogen inhibition studies (Lim and Kim, 2009, Khochamit *et al.*, 2015).

It is important to note that the use of certain microorganism may elicit an antimicrobial effect due to the production of antibiotics, which is a highly undesirable trait as pathogens develop resistance to this class of AMP. When screening for probiotics it is important to investigate the properties of the bacteriocins produced (Gruenheid and Le Moual, 2012, Cotter *et al.*, 2013). However, in an extensive review, Grant *et al.*, (2018) showed that *Bacillus* spp. can produce a range of AMPs, which are mediated through the disruption of bacterial membranes making the development of pathogen resistance unlikely. Evidence of this was shown by Fernandes *et al.*, (2007), in which two non-ribosomal produced AMPs isolated from *B. subtilis* were effective against 25 multi-drug resistance bacteria. Specifically regarding poultry, Lee *et al.*, (2010a) demonstrated that *Bacillus* spp. were able to produce AMPs that are cytotoxic to *Eimeria* spp., therefore reducing the prevalence of avian coccidiosis and subsequent colonization of *C. perfringens*. Others have shown the narrow spectrum of activity against a variety of chicken pathogens such as *C. difficile* (Rea *et al.*, 2010), *Listeria monocytogenes* (Kamoun *et al.*, 2011) and *Enterococcus faecalis* (Fuchs *et al.*, 2011), using *Bacillus* based AMPs.

The gut plays a pivotal role in maintaining good health in poultry as it offers the host protection against biological invasion and is generally regarded as the first line of defense (Kabir *et al.*, 2004, Dhama *et al.*, 2011). The optimum functioning of the GIT is of primary interest to the industry because it directly influences the vigor, growth and disease resistance, thus improving production efficiency. Probiotics play a vital role in the regulation and maintenance of the GIT by many interactive mechanisms that serve to enhance one or more modes of actions. For example, the secretion of mucus by the goblet cells provides a barrier to foreign agents and pathogens. It has been shown that the supplementation with *Bacillus* spp. can aid in the upregulation of the mucin-producing gene, *MUC2*, to counteract the inflammation caused by pathogens (Grant *et al.*, 2018). Another gut associated mechanism is the enhancement of the

epithelial barrier integrity by increasing the regulation of tight junction proteins which bind to one another, creating a continuous barrier that forms protection from pathogens (Chichlowski *et al.*, 2007). Gadde *et al.*, (2017b) reported a distinct increase in tight junction genes when challenged broilers were fed diets supplemented with *B. subtilis*.

Probiotics possess the ability to transiently colonize the GIT and positively enhance the composition of the intestinal microflora of chickens via the stimulation of beneficial populations and the CE of pathogenic bacteria, thereby creating a balance in the gut microbiota. (Ng *et al.*, 2008, Keeney and Finlay, 2011). *Bacillus* spp. have the ability to positively affect the growth of the native microorganisms in poultry GIT through the consumption of oxygen, which creates a more favorable environment to facilitate the growth of commensal anaerobic species (Baruzzi *et al.*, 2011). Some of these microbes produce lactic acid thus facilitating the exclusion of pH- sensitive pathogens (Song *et al.*, 2014). There is reported evidence of an increase in *Lactobacillus* spp. in the gut of broilers fed different *Bacillus* based probiotics with a subsequent decrease in enteropathogens (Wu *et al.*, 2011, Lei *et al.*, 2015). Hosoi *et al.*, (2000) proposed that *B. subtilis* was able to enhance the growth of *Lactobacilli*, through production of catalase and subtilisin. The growth of other beneficial gut microbes such as Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, and Acidobacteria were all increased when broilers were fed diets containing *B. subtilis* and *B. coagulans* (Li *et al.*, 2018). Beneficial species such as *Ruminococcus*, *Lachnoclostridium*, and *Anaerostipes* were also found in higher relative abundance in *Bacillus*-treated birds in the ceca (Jacquier *et al.*, 2019).

Bacillus spp. have proven to elicit CE against many species of poultry pathogens, including *Salmonella* spp. (Gil De Los Santos *et al.*, 2005, Thirabunyanon and Thongwittaya, 2012, Menconi *et al.*, 2013, Park and Kim, 2014), *Clostridium* spp. (Teo and Tan, 2005, Abudabos *et al.*, 2013, Jayaraman *et al.*, 2013), *Escherichia coli*, (La Ragione *et al.*, 2001, Wu *et al.*, 2011), *Campylobacter* spp. (Arsi *et al.*, 2015, Guyard-Nicodeme *et al.*, 2015) and also mixtures of pathogens (La Ragione and Woodward, 2003). The exact mechanism in which competitive exclusion is achieved is not always indicated or clear, however it is generally ascribable to one or more of the mechanisms discussed.

1.5.2 Probiotic effect 2: Improvement in digestion and absorption

The function of the digestive system can be improved and regulated by two main probiotic mechanisms, namely production of metabolic enzymes and the alteration of the intestinal villi morphology to improve uptake of nutrients.

Poultry feed is typically made up of approximately 60% carbohydrates, 20% protein and 5% fats. The cost of feed ingredients has been a major challenge to the industry and necessitates the use of cheaper, non-conventional feed ingredients which are less digestible and have negative impacts on feed conversion and gut health (Choct, 2006).

In the case of carbohydrates, feed ingredient cost optimization has resulted in the increased use non-soluble and soluble non-starch polysaccharides (NSP) (Khattak *et al.*, 2006). These diets usually comprise of corn alternatives such as wheat, oats, barley and rye. These NSP-rich diets have high anti-nutritional factors (ANF) (primarily phytate, enzyme inhibitors and resistant starches), and form a gel like viscous consistency within the intestinal tract (insoluble NSP). This leads to reduced absorption of nutrients and ultimately, reduced growth performance. Poultry do not produce enzymes for the hydrolysis of NSPs and they remain un-hydrolyzed resulting in low feed conversion. Besides the use of NSP ingredients, the use of low grade corn can also contain a high concentration of anti-nutritional components (Cowieson, 2005). Additionally, water soluble β -glucans adversely affect uptake of other nutrients, such as protein and starch, and may also increase gut viscosity (Khattak *et al.*, 2006). These ingredients cause several health issues such as foot lesions, hock burns, and carcass downgrading, as well as wet litter (Ravindran, 2013).

Protein, from either plant or animal origin, is one of the most expensive nutrients in broiler feed. Animal products traditionally included fish meal and animal protein concentrates, which represents a considerable proportion of the production costs. In some countries, it is prohibited to incorporate animal meals into broiler feeds, therefore vegetable protein sources are becoming the norm (Tegua and Beynen, 2005). Soybean meal (SBM) is the preferred protein source used in poultry feed manufacturing, due to its high crude protein content. It is, however, costly and contains high concentrations of ANFs, such as protease inhibitors (trypsin and chymotrypsin) which effect protein utilization, lectins that affect carbohydrate utilization, glycinin with goitrogenic activity, saponins which effect palatability and phytic acid that complexes with certain minerals (calcium, phosphorus, magnesium, copper, iron and zinc) thus

reducing their bioavailability (Yasoithai, 2016). SBM is heat treated to neutralize the activity of the ANFs, however, this increases cost and is not always totally effective (Yasoithai, 2016). SBM is sometimes replaced by cheaper legume grains (black beans, groundnut and cowpea) which are also high in ANFs such as protease inhibitors and lectins (Teguia and Beynen, 2005).

Incorporating free enzymes in lower grade feed, alleviates the issues of ANFs and improves digestion by the breakdown of less digestible feed components, which enhances nutrient absorption (Ravindran, 2013). Some disadvantages of free enzyme use include high cost, lack of stability at high temperatures and uncertainty of the amount and combinations of enzymes to be added (Ravindran, 2013).

Bacillus probiotics that produce desirable enzymes offer an alternative to the use of free enzymes. Furthermore, these probiotics will only produce enzymes in the presence of the substrate, and therefore offers a more intelligent system. *Bacilli* have been proven to produce exogenous enzymes such as α -amylase, β -glucanase, xylanase, protease, phytase, lipase and cellulase, which are all important in the broiler industry in terms of carbohydrate, protein and fat digestibility (Latorre *et al.*, 2015). These include the glycosyl hydrolase enzymes that enables the efficient break down of complex NSP compounds into more easily digestible monosaccharides, thus reducing intestinal digesta viscosity and improving uptake (Latorre *et al.*, 2016). The action of these enzymes also result in increasing the availability of apparent metabolizable energy (AME) in low grade feedstuffs, due to hydrolysis of fibrous material. Similarly, probiotic enzymes also enhance nutrient availability to the microbial flora in the GIT. It was demonstrated that broilers fed with *B. coagulans* NJ0516 showed increased amylase and protease activity, which led to enhanced growth of broilers (Wang and Gu, 2010). Additionally, *B. subtilis* spores (GalliPro[®]) used as a feed additive, reduced the requirements of amino acids and protein supplementation, subsequently reducing feed cost (Zaghari *et al.*, 2015). The benefit of enzyme producing probiotics is most impactful in reduced energy diets (cheaper ingredients) because of the improved cost-to-benefit ratio (Harrington *et al.*, 2016).

The mechanism in which enzymes neutralize ANFs can be direct as with the enzyme phytase which breaks down phytic acid, thus releasing minerals for absorption. Furthermore, phosphatase prevents precipitation of penta-calcium phosphate, thereby improving absorption of calcium and phosphorus (Dida, 2016). Indirect examples of ANF neutralization include protease mediated breakdown of SBM, thus negating the effect of trypsin inhibitors and NSPs

breakdown by xylanase and β -glucanase, which hydrolyses resistant starches. There is extensive research on free enzymes neutralizing ANFs, however, studies on probiotics are limited but are gaining traction. A recent study by Farhat-Khenakhlem *et al.*, (2018) showed the ability of *B. amyloliquefaciens* US573 strain to secrete xylanase, β -glucanase and amylase and achieve *in vitro* wheat digestibility of approximately 48%.

The structure of the intestinal epithelium is an important factor contributing to digestibility and gut health (Lei *et al.*, 2015). It is generally recognized that greater villus height and villus height to crypt depth ratio improves nutrient absorption capacity of the small intestine (Montagne *et al.*, 2003). The height of the villi is directly proportional to the rate of absorption, however, crypt depth and crypt depth to villi height ratio are an indication of epithelial turnover and activation of cell mitosis. Improved morphology leads to improved absorption and gut health (Xu *et al.*, 2003). Samanya and Yamauchi (2002) fed broilers with *Bacillus subtilis* var. *natto* and significantly improved villus height, cell area and cell mitosis. Other studies on *Bacillus* spp. showed increased villi height and improved villi crypt depth to height ratio (Sen *et al.*, 2012, Abudabos *et al.*, 2013, Jayaraman *et al.*, 2013, Al-Fataftah and Abdelqader, 2014, Lei *et al.*, 2015, Li *et al.*, 2018, Ramlucken *et al.*, 2019).

The impact of *Bacillus* based probiotics on digestion due to enzyme secretion and improved gut morphology are mainly realized *in vivo* through improvement in FCR (Gil De Los Santos *et al.*, 2005, Zhang *et al.*, 2013, Jeong and Kim, 2014, Park and Kim, 2014, Lei *et al.*, 2015). Studies using *B. subtilis* (Molnár *et al.*, 2011, Jacquier *et al.*, 2019) and *B. coagulans* (Li *et al.*, 2018) as in-feed probiotics resulted in FCR improvement of approximately 5%. In our latest study using a multimode *Bacillus* probiotic, we showed an improvement in FCR due to a combination of enzyme activity and improvement in GIT histomorphology (Ramlucken *et al.*, 2019).

1.5.3 Probiotic effect 3: Immunomodulation

Immunomodulation refers to the alteration of the host's immune response to foreign agents and pathogens either by antibody stimulation (immune-stimulation) or inflammation suppression (immunosuppressant), to maintain the desired level of host immune-protection. (Klasing, 2007). Accordingly, the intestinal immune system must trigger a protective immune response against pathogenic microbes while maintaining tolerance to antigens from food and commensal bacteria. Gut-associated lymphoid tissues (GALTs) represent the largest compartment of the

immune system. Like all other immune systems, a variety of both innate and adaptive immune responses against pathogenic microbes takes place in the intestine (Kim and Lillehoj, 2019). Innate immunity refers to non-specific defense mechanisms that come into play quickly in response to antigens, whereas adaptive immunity is more complex dealing with memory that facilitates future responses against specific antigens. Monoclonal antibodies, cytokines, glucocorticoids, macrophages, immunoglobulins, plasmapheresis, and related agents mainly produced by the GALT are known to alter cellular or humoral immunity (Brisbin *et al.*, 2008, Wigley *et al.*, 2014). Although avian cytokines are not as well defined as those of humans, there are studies that have isolated a specific range of cytokines found predominantly in avian species. These include pro-inflammatory cytokines, T-helper lymphocytes, which induce cell-mediated immunity and chemokines that play a role in immune regulation (Wigley and Kaiser, 2003).

In the case of probiotics, there is greater evidence of their immune stimulatory activity, whereas their immunosuppressant activity is less studied. Immuno-stimulation occurs through bacterial-epithelial cell crosstalk, which activates innate and adaptive immune responses to antigens. Although, the exact mechanism of the immunomodulatory activities of probiotics is unclear, it has been reported that probiotics stimulate different subsets of the immune system to produce cytokines (Brisbin *et al.*, 2008). Other effects of probiotics on the immune system include the stimulation of macrophages and natural killer cells, as well as enhancing the phagocytic activity of the gut cells (Yang *et al.*, 2009). Furthermore, certain probiotic microorganisms can enhance the function of the intestinal barrier related immune response, however, the details of this mode of action is unclear (Ng *et al.*, 2008, Markowiak and Śliżewska, 2018). These immune activities can reduce the incidence of diseases and promote chicken health, which correlates to improved growth and performance. However, probiotic mediated regulation of the inflammatory response must be functional without being excessive, otherwise it can result in attenuation of immune response and damage to the gut tissue lining (Gabriel *et al.*, 2006).

The ability of *Bacillus* organisms to stimulate a host immune response in chickens is common, although the exact immunomodulatory mechanism is not always clear. There is evidence that suggests a role of *B. subtilis* in the stimulation of the sIgA response which is necessary for immunity against mucosal pathogens (Mingmongkolchai and Panbangred, 2018). Khaksefidi and Ghoorchi (2006) demonstrated that broilers fed *B. subtilis* had a positive effect on antibody production against Newcastle disease and Lee *et al.*, (2015) showed immune responses to

causative necrotic enteritis agents (*Eimeria* spp. and *C. perfringens*). Several other studies using *Bacillus* spp. also demonstrated immunomodulation in chickens (Lee *et al.*, 2010b, Lee *et al.*, 2011, Xu *et al.*, 2012, Lee *et al.*, 2013, Gadde *et al.*, 2017b, Rajput *et al.*, 2017). The augmentation of macrophage function is one way that *Bacillus* based probiotics enhance immunity (Grant *et al.*, 2018). It has been reported that *Bacillus* spores support the development of the GALT, increasing the number of intraepithelial lymphocytes and immunoglobulin producing cells (Molnár *et al.*, 2011). Furthermore, there is a direct correlation of sporulation with the development of the GALT (Tam *et al.*, 2006).

In a study by Wang *et al.*, (2018) *B. subtilis* was able to suppress heat stress related inflammation by increasing levels of the anti-inflammatory cytokines, IL-10 and IL-4. Rhayat *et al.*, (2019) used different *B. subtilis* strains and showed inflammatory responses via different mechanisms, where one strain upregulated the expression of tight junction's proteins, whilst another strain blunted the function of IL-8 which when released initiates a pro-inflammatory response. Jacquier *et al.*, (2019) demonstrated *Bacillus*-induced growth of *Butyrivibrio* spp., which are known to produce anti-inflammatory compounds such as conjugated linoleic acid, illustrating indirect immunomodulation.

1.5.4 Probiotic effect 4: Reduction of toxic compounds in the gut

Probiotics can contribute to the reduction of toxicity in the gut from compounds such as ammonia and aflatoxins, thereby improving health and vigor.

B. subtilis generates subtilin, which may reduce urease generating microbiota in the gastrointestinal lumen, thereby attenuating ammonia release (Wang *et al.*, 2009). Furthermore, another mechanism for the reduction of ammonia in the gut by *Bacillus* spp. is the consumption of ammonia, which prevents excessive ammonia toxicity arising from hydrolyzed uric acid (Ahmed *et al.*, 2014). *B. subtilis* and *B. cereus* were shown to be involved in nitrification and therefore show potential for abatement of ammonia toxicity under different conditions (Nemutanzhela *et al.*, 2014). Ahmed *et al.*, (2014) conducted a study in which ammonia reduction correlated with the concentration of *B. amyloliquefaciens* dosed. Although not clear on the exact mechanism of ammonia reduction, various studies demonstrated a significant decrease in ammonia emissions from the fecal matter of broilers that were fed a *B. subtilis* preparation (Tanaka and Santoso, 2000, Zhang *et al.*, 2013, Jeong and Kim, 2014).

Aflatoxins are potent mycotoxins produced by *Aspergillus* spp. and are a common problem in poultry feed (Fan *et al.*, 2015). The continuous intake of these compounds leads to detrimental effects on the liver of broilers. Studies on the role of probiotics in aflatoxin reduction are limited but Fan *et al.*, (2015) demonstrated that the supplementation of *B. subtilis* ANSB060 reduced aflatoxin levels in the duodenum of broilers and prevented aflatoxicosis. Another study selected probiotics for aflatoxin removal and showed its *in vivo* efficacy in Japanese quails (Bagherzadeh Kasmani *et al.*, 2012).

1.6 Auxiliary advantages of *Bacillus* probiotics

Beyond extensive probiotic effects, *Bacillus* spp. also have auxiliary advantages regarding waste treatment in the poultry industry. The intensive nature of poultry production has raised environmental concerns and producers are under intense pressure to meet regulations (Rodić *et al.*, 2011). The major wastes emanating from the poultry industry comprise of manure, feathers, carcasses, effluents and ammonia emissions (Glatz *et al.*, 2011). With its high levels of nitrogen and phosphorous (Malomo *et al.*, 2018), the impact of indiscriminate disposal of poultry manure and waste water (Gbotosho and Burt, 2013, Damalas and Koutroubas, 2016) contributes to phosphorus and nitrogen load, which ultimately ends up in natural habitats. Ammonia emissions are one of the most pressing environmental concerns and although, more prevalent in laying hens due to the age and rearing time, it still poses problems in the broiler industry (Ritz *et al.*, 2004). The bedding used in broiler production is often re-used for cost effective rearing, resulting in accumulation of ammonia. Prolonged exposure to ammonia concentrations can lead to a decrease in feed efficiency, increased susceptibility to disease, loss of cilia in the lungs and eye damage. Furthermore, it also poses a health hazard for farm workers. Historically, feathers were used in poultry feed, though stricter regulation and consumer resistance is prompting the need for alternate solutions (Forgács *et al.*, 2011).

Bacillus spp. are well known for removing nitrogen and phosphorus from environmental wastes (Kim *et al.*, 2005, Yang *et al.*, 2011, DebRoy *et al.*, 2013) and have been extensively applied in the bioremediation of waste water (Iriye and Takatsuka, 1999, Yang *et al.*, 2017). When *Bacillus* based probiotics are used, they can further contribute to the treatment of wastes downstream of the poultry production. The industry has already adopted the use of *Bacillus* spp. to reduce the concentration of ammonia in fecal matter and subsequently alleviate ammonia emissions (Park *et al.*, 2016). Furthermore, Santoso *et al.*, (1999) showed a reduction in ammonia gas emissions in laying hens fed *B. subtilis*. A study by Stough, (2013)

demonstrated the *in vitro* degradation of ammonia by *B. subtilis* but could not prove its efficacy in used litter *in vivo*. Another study by Chiang and Hsieh (1995) showed the reduction in ammonia in litter, using a consortium of *Streptococcus*, *Lactobacillus* and *Bacillus* spp. This area has not been adequately researched, and the nitrification and denitrification ability of these heterotrophs can be of great environmental benefit in ammonia degradation (Kim *et al.*, 2005).

Biological treatment of poultry waste mostly entails anaerobic digestion, yet feathers, which consist mainly of keratin, degrades poorly under anaerobic conditions (Salminen and Rintala, 2002). Kim *et al.*, (2001) demonstrated the use of three strains of *Bacillus* spp. (*B. subtilis*, *B. pumilis* and *B. cereus*) to effectively degrade feathers attributable to production of keratinase.

Bacillus based probiotics are elegant in that they provide a multiple effect of directly improving poultry production efficiency, improving the rearing environment and the safety of the resultant wastes. This dynamic, although not yet well explored by industry, could be of significant importance in selecting *Bacillus* based probiotics over other species.

1.7 The development of *Bacillus* based probiotic

The development of chicken feed probiotics requires a methodological and systematic approach (Figure 1.2). This includes the targeted isolation of microorganisms, followed by screening according to a set of predefined criteria that are associated with commercially relevant desirable characteristics. The use of *in vivo* studies to select putative probiotics from large numbers of isolates are expensive, time consuming and not easily achievable. It is therefore critical to perform extensive *in vitro* evaluation and selection processes in order to reduce the number isolates (Ehrmann *et al.*, 2002). The biosafety considerations must be evaluated for all probiotics to be used in animals, while conforming to regulatory requirements of countries in which the probiotics are to be used. The ultimate step in the development of probiotics is the validation of its efficacy *in vivo*.

1.7.1 Isolation of *Bacillus* spp. as probiotics

The environments that probiotic candidates are isolated from is a critical consideration, as it is preferable to isolate microorganisms from the host or environments associated with the host because host specific probiotics could be better evolved to elicit desirable probiotic effects (Fuller, 2001). Isolation from the host is however not mandatory as equally functional probiotics have been isolated from other sources (Fontana *et al.*, 2013). Conventional anaerobic

probiotics need careful consideration of storage and samples need to be processed quickly to avoid losses in viability. Due to their endospore-forming abilities, *Bacilli* tolerate adverse conditions better than non-spore forming bacteria (Cutting, 2011), hence samples can be stored and processed easily. Easy protocols can be deployed for purification of spore-forming organisms whilst excluding other genera, such as heat, nutrient depletion, dehydration and desiccation (Lalloo *et al.*, 2007). A rationale and proven approach to obtaining pure cultures involves obtaining broiler related environmental samples such as guts, feces, bedding, feathers and, if possible, swabs from the chickens (Barbosa *et al.*, 2005, Wolfenden *et al.*, 2010) while isolating and purifying *Bacillus* spp. from these samples. The purification of *Bacillus* spp. requires a strategy to induce sporulation, for example, using special enrichment medium to form mature spores in large quantities (Földes *et al.*, 2000). Other spore induction procedures include temperature elevation and dehydration using ethanol (Nemutanzhela *et al.*, 2014). Simple sub-culturing procedures on nutrient agar are generally used to purify individual *Bacillus* cultures, followed by quick verification techniques such as microscopic morphology, Gram stain, catalase reaction and other metabolic tests (Földes *et al.*, 2000, Nemutanzhela *et al.*, 2014).

1.7.2 Ensuring survival under GIT conditions to eliminate unsuitable candidate probiotics

All poultry probiotics must be able to survive the harsh conditions of the chicken GIT, which include the highly acidic environment found within the proventriculus, toxic bile concentrations present in the small intestine, the fluctuating pH of the GIT and the digestive enzymes (pepsin and trypsin). The ability to survive these conditions are obligatory for any putative probiotic to elicit its effect and must be established in the initial stages of development. The spores of *Bacillus* spp. are mostly resistant to the acidic conditions, mechanical shear, hydrolyzing enzymes and bile that are present in the chicken GIT (Cartman *et al.*, 2008). A study that screened for human *Bacillus* probiotics, revealed that 80% of isolates survived the acidic conditions of the GIT (Nithya and Halami, 2013). Chaiyawan *et al.*, (2015) reported a 100% survivability of *Bacillus* isolates obtained from broilers when subjected to simulated gastric juice, and similarly, Lee *et al.*, (2012) showed that isolates were highly tolerant to acidic conditions and the presence of bile. The elimination of non-surviving isolates in the rationale for development of probiotics is important as it eliminates large numbers of unwanted strains that would not be functional as probiotics.

1.7.3 The selection of putative probiotics against industry relevant criteria

With regards to selecting *Bacillus* isolates for use as poultry probiotics, a specific rationale needs to be implemented, which is not dissimilar to the screening and selection of conventional probiotics. In general, screening is done to test the extent of mechanisms that enable probiotic modes of action that are relevant to the poultry industry (TaHERI *et al.*, 2009). This has already been extensively discussed under modes of action. *Bacillus* spp. do not offer any special advantages in the screening and selection stages, compared to conventional probiotics, as the procedures are generally common. The functional aspects also need to be evaluated using appropriate *in vitro* screening techniques, which are generally well known (Harimurti and Hadisaputro, 2015).

The colonization potential of isolates can be determined by techniques such as auto-aggregation, cell surface hydrophobicity and adherence to epithelial cells, whereas antagonistic effects are elucidated using microbial plate assays co-culturing probiotics and pathogens of interest. Several studies describe various methodologies for testing CE by *Bacillus* spp. (Teo and Tan, 2007, Lee *et al.*, 2012, Menconi *et al.*, 2013, Chaiyawan *et al.*, 2015). Screens for digestion and AME usage, involve testing for key digestive enzymes such as amylase, protease, lipase, cellulase, xylanase and phytase. Enzyme screens on *Bacillus* spp. has been extensively reported (Lee *et al.*, 2012, Latorre *et al.*, 2015, Latorre *et al.*, 2016, Hmani *et al.*, 2017). Common screens to evaluate immunomodulation include cell mediated bioassays, reverse transcriptase PCR (RT-PCR) and enzyme-linked immunosorbent assays (ELISA). Studies on *in vitro* screening are limited due to poor correlation between *in vitro* to *in vivo* tests, thus the majority of studies used to determine immune modulation by *Bacillus* spp. were done in field studies (Lee *et al.*, 2015, Gadde *et al.*, 2017b, Wang, 2017).

During the selection stage, multiple strain probiotics (consortium) are generally preferred, because the potential for holistic effects is better compared to single strains (Chapman *et al.*, 2011). The population dynamics and co-existence of strains in the consortium must therefore also be tested. A rational approach to probiotic selection *in vitro* does not always correlate to the *in vivo* efficacy. That being the case, before a probiotic can be commercialized, efficacy must be proven in field trials, measuring commercially relevant effects such as health and productivity. The validation of selected probiotics *in vivo* following a rationale screening process, has been the approach followed in several studies involving *Bacillus* spp. (Wolfenden *et al.*, 2010, Menconi *et al.*, 2013, Nguyen *et al.*, 2015).

1.8 Biosafety considerations of *Bacillus* spp. and the associated regulations

Once putative probiotics are prioritized, it is imperative to determine the biosafety before final selection. Proper identification of strains provides insight into the safety and techniques such as biochemical API 50 CHB test kits and 16S rRNA sequence analyses are frequently used (Fontana *et al.*, 2013). 16S rRNA sequencing is the preferred method as conserved regions of the genome are compared to known sequences of species within databases compared to other tests, which are based at the phenotypical and biochemical levels (Fontana *et al.*, 2013). Once identified, the taxonomy of the strains can aid in the assessment of its biosafety using information such as scientific literature, history of use and industrial and ecological applications (EFSA, 2007).

There are causes for concern with regards to the use of *Bacillus* spp., specifically as probiotics, because some strains produce enterotoxins and some are pathogenic. *B. anthracis*, *B. thuringiensis* and *B. cereus* are members of the *Bacillus cereus* group of bacteria, commonly isolated when screening for probiotics (Sanders *et al.*, 2003, Hong *et al.*, 2005). *B. anthracis* is never permissible as a probiotic as it causes the fatal disease anthrax and is a potential biological weapon (Helgason *et al.*, 2000). *B. thuringiensis* produces intracellular protein crystals toxic to a wide number of insect larvae and has been implicated in gastroenteritis (Jensen *et al.*, 2002). Although many strains of *B. cereus* are ubiquitous and excellent biological agents, some strains can cause food poisoning (Helgason *et al.*, 2000) and must consequently be shown to be negative for the *B. cereus* enterotoxin and the anthrax genes.

Another concern is that some *Bacillus* strains such as *B. clausii*, *B. cereus*, plasmids of *B. subtilis* and *B. licheniformis* transfer antibiotic resistance genes within the GIT that cause antibiotic resistant pathogenicity (Mingmongkolchai and Panbangred, 2018). Although this has no effect on antibiotic free chicken production, it is useful to check candidate probiotics for susceptibility to commonly used antibiotics such as vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol, to ensure that they do not contain the resistance genes (EFSA, 2015).

At present, guidelines presented for animal probiotics are vague and limited, although in some countries it is customary that aspects such as identification, safety and the health effects are provided for probiotic products (Hamilton-Miller *et al.*, 1999). The European food and safety

authority (EFSA) is the only organization currently that has regulations for the use of feed probiotics which was critically reviewed by Anadon *et al.*, (2006). EFSA have embarked on implementing a system referred to as the qualified presumption of safety (QPS), wherein, biological material is critically assessed for their safety (Ricci *et al.*, 2017). In terms of *Bacillus* spp. over 2000 reports were analyzed and 14 species were recognized as QPS. These species include *B. amyloliquefaciens*, *B. atropheus*, *B. clausii*, *B. coagulans*, *B. flexus*, *B. fusiformis*, *Paenibacillus lentus*, *B. licheniformis*, *B. megaterium*, *B. mojavensis*, *B. pumilus*, *B. smithii*, *B. subtilis* and *B. vallismortis* (EFSA, 2015). The USA allows for probiotics that are GRAS to be commercialized, thus the probiotic species of choice remains *B. subtilis* and *B. coagulans* (Cartman *et al.*, 2008).

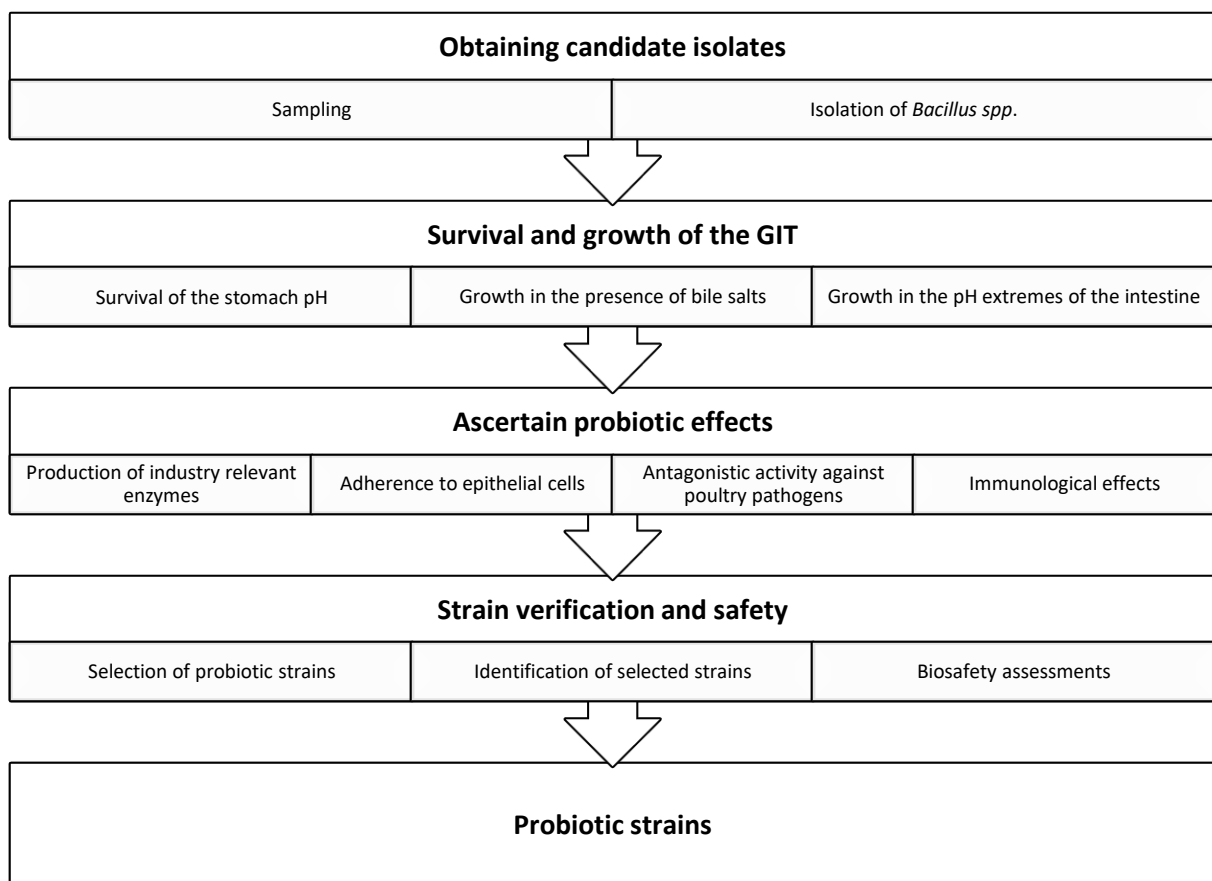


Figure 1.2: The general systematic approach to probiotic development.

1.9 Advantages in the manufacturing of *Bacillus* probiotics

There is immense effort going into probiotic development for use in the poultry industry mainly focused on screening and efficacy. However, a critical aspect of development and which is

often neglected, is the production of selected probiotics at industrial scale. Commercially viable strains must show attractive techno-economic properties in the production process (Lacroix and Yildirim, 2007). Some of the key consideration in ensuring a commercially acceptable production process includes storage of strains in validated master and working cell banks, an inoculum train that delivers a proper quality and quantity of cells for the fermentation process, a high performance fermentation processes, the efficient harvesting of the probiotic cells, and the formulation into a stable product ready for easy incorporation into premixes or feeds. This facilitates the commercial roll-out of probiotic products, which is largely dependent on the efficiency and cost of the production process at industrial scale to deliver shelf stable product in sufficient quantity (Amer and Utkhede, 2000, Patel *et al.*, 2004). A schematic diagram of the steps involved in probiotic production is illustrated in Figure 1.3.

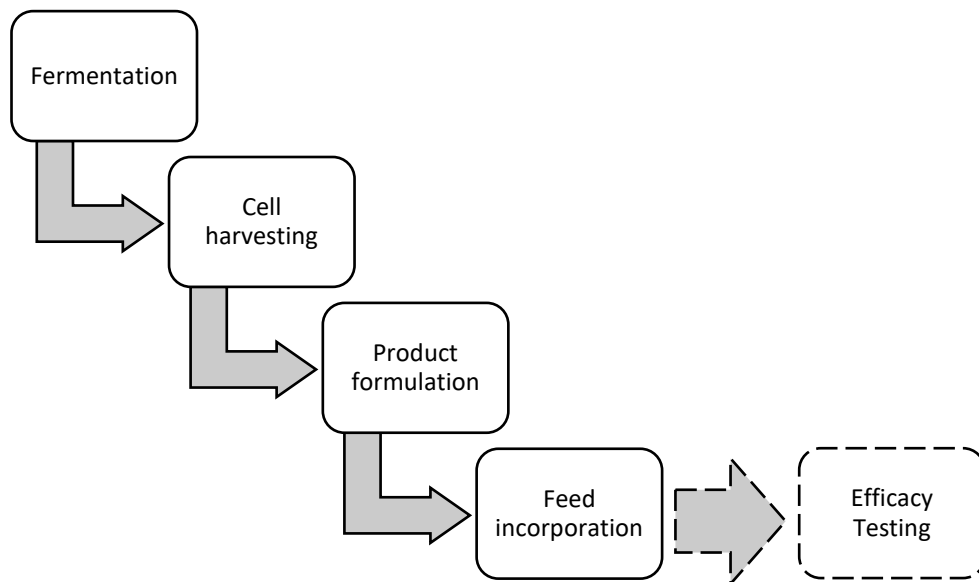


Figure 1.3: Schematic chart showing the steps involved in the production of probiotic augmented feed.

1.9.1 Fermentation and cell separation of *Bacillus* probiotics

1.9.1.1 Cell storage and inoculum train

For the commercial production of probiotics, it is important to have a stable culture that is appropriately preserved. *Bacillus* spp. can be stored in spore form with better stability and viability in contrast to vegetative cells (Monroy *et al.*, 2004, Gao *et al.*, 2007). It is understood

that a well-characterized inoculum train is essential for the bulking of the initial culture into a suitable inoculum for the main production fermentation (Okonkowski *et al.*, 2005, Meyer *et al.*, 2016). Under high cell density cultivation (HCDC), *Bacillus* spp. have been shown to scale well from the starter culture through to flask and pre-fermenter inoculum stages, which is a key requirement to ensure that the main production fermentation is efficient in terms of yield, productivity and cost (Monteiro *et al.*, 2005, Laloo *et al.*, 2009, Monteiro *et al.*, 2014).

1.9.1.2 Fermentation

The poultry industry functions on high-volume-low-margin commodities, therefore the cost of in-feed additives needs to be minimal. Furthermore, losses in viability downstream of the production process, such as product formulation and feed blending, needs to be compensated upstream by higher density fermentation. The production process of probiotics must be designed such that the overall process has increased cell yields, productivities and a lowered cost, which ultimately results in a feasible and economically attractive production process. The cultivation of *Bacillus* spp. at large scale is influenced by various factors such as the composition of the media, physical variables, and cell harvesting, each of which have to be developed to ensure a cost effective production process (Nemutanzhela *et al.*, 2014).

The growth medium that is used to support high productivities in commercial bioprocesses is predominantly formulated with inexpensive nutrient sources and is an essential aspect of process development, because it influences the economic competitiveness of the bioprocess technology (Singh *et al.*, 2017). Conventional probiotics often require more expensive complex nutrients such as tryptones, peptones and yeast extracts, but the cost at commercial scale can be prohibitive (Zhang and Greasham, 1999). *Bacillus* spp. have been shown to grow efficiently on lower cost, locally available waste substrates (Laloo *et al.*, 2009, Singh *et al.*, 2017), but information specifically related to the production of poultry probiotics is limited. HCDC on several agricultural and industrial wastes either as is or as hydrolysates, such as molasses, corn steep liquor, soybean, or wheat, has been proven (Prabakaran *et al.*, 2007, Chang *et al.*, 2008, Laloo *et al.*, 2009, Chen *et al.*, 2010). A study by Khardziani *et al.*, (2017) showed the growth of *B. amyloliquefaciens* B-1895 in various lignocellulosic materials at concentration of 40 g.L⁻¹ yielded a cell concentration of 1 × 10¹⁰ spores.ml⁻¹.

HCDC of *Bacillus* spp. is preferably done in fed-batch fermentation because the concentration of the limiting substrate can be maintained at a low level, thus avoiding substrate inhibition

(Shiloach and Rinas, 2010). For example, Monteiro *et al.*, (2005) observed that limiting glucose concentration to 5 g.L⁻¹ led to an increase in the vegetative cell and spore concentration of *B. subtilis*. In this way there is some control over the organism's growth rate and oxygen demand thus ensuring oxygen sufficiency (Shiloach and Rinas, 2010, Monteiro *et al.*, 2014, Elisashvili *et al.*, 2019). Using a glucose fed-batch procedure coupled with the manipulation of carbon to nitrogen ratio, Panday (2016) was able to produce the probiotic *B. coagulans* at a concentration 3.8×10^{11} cells.mL⁻¹.

The efficient production of *Bacillus* spp. is often reflected in the quantity and quality of spores harvested at the end of the fermentation. Accordingly, cultivations should be optimized per strain to achieve high sporulation efficiencies as shown by Cho *et al.*, (2009), Flores *et al.*, (1997) and Posada-Uribe *et al.*, (2015). The pathway leading from a vegetative cell to a spore is triggered by depletion of certain media components such as carbon, nitrogen, phosphate, vitamins or essential macro and micronutrients (Sonenshein, 2000, Posada-Uribe *et al.*, 2015). Some macro nutrients such as phosphate, ammonium, magnesium and calcium have been shown to enhance sporulation (Laloo *et al.*, 2009, Monteiro *et al.*, 2014).

Physical parameters such as temperature, pH, agitation and aeration have a critical impact on reproducibility and consistency of spore production (Tavares *et al.*, 2013, Xiang *et al.*, 2013). Several studies have evaluated the effect of different physical parameters such as temperature (20-30 °C), pH (5.0–9.0), aeration rates (0.5–2.0 v⁻¹.v⁻¹.m⁻¹) and agitation rates (200–500 rpm), in order to enhance spore production of *Bacillus* spp. concluding that optimum culture conditions are very specific for each strain (Tzeng *et al.*, 2008, Chen *et al.*, 2010, Posada-Uribe *et al.*, 2015). Most microorganisms in the vegetative state are pH and temperature sensitive, though *Bacillus* spp. can generally tolerate wider ranges and are more robust. Laloo *et al.*, (2008) studied the effect of temperature on growth rate, cell concentration and spore germination of *B. cereus* NRRL 100132, concluding that extreme temperatures lowered growth and germination, with an optimum range of 25-30 °C. Their study also showed that there is no significant effect of pH on growth in the range 6-9, which was in accordance with another study by Monteiro *et al.*, (2005) using *B. subtilis*.

Agitation, aeration and pressure primarily influence mixing and mass transfer and the physical effect can be detrimental to more sensitive microorganisms. Nevertheless, most *Bacillus* spp. grow well under high aeration and agitation and the spores are resistant to pressure and

mechanical shear (Feng *et al.*, 2003). Posada-Uribe *et al.*, (2015) concluded that spore concentration was increased by increasing agitation and aeration, wherein 9.33×10^9 spores.mL⁻¹ was achieved at 400 rpm and 12 slpm respectively, whilst sporulation efficiency was not affected. This is in accordance with other reports where an increase in agitation and aeration generated higher biomass in different *Bacillus* spp. (Feng *et al.*, 2003, Yeh *et al.*, 2006), indicating that effective sporulation is dependent on oxygen sufficiency during the growth phases (Flores *et al.*, 1997, Nemutanzhela *et al.*, 2014). Monteiro *et al.* (2005) achieved a high *B. subtilis* spore concentration of 3.5×10^9 spore.ml⁻¹, when dissolved oxygen concentration was maintained above 30% saturation.

The speed of production (cell growth and spore formation) should be maximized to ensure efficient utilization of labor, utility and capital. *Bacillus* spp. have been shown to replicate rapidly, which maximizes productivity, one of the key indicators of process efficiency. Chen *et al.*, (2010) showed a maximum spore concentration of 1.56×10^{10} CFU.mL⁻¹ within 40 hours, whilst Panday (2016) showed an even greater spore concentration of 1.9×10^{11} spores.mL⁻¹ after 32 hours - the latter study showing a higher productivity. The intrinsic substrate utilization efficiency of a specific strain influences the process efficiency because a high cell concentration coupled with lower substrate consumption indicates a better yield of spores for the quantity of raw material used, thus reducing the unit cost of production. In general, the carbon to nitrogen ratio can be manipulated in order to achieve maximum substrate utilization and *Bacillus* spp. have been shown to have excellent yields on substrates in studies conducted by Laloo *et al.*, (2010b), Monteiro *et al.*, (2005) and Panday (2016).

The key challenge in spore production is to maximize sporulation from a high density vegetative cell culture, as a result the sporulation efficiency is critical for *Bacillus* production. Sporulation efficiencies over 90% have been reported for *B. cereus* and *B. subtilis* strains (Laloo *et al.*, 2009, Posada-Uribe *et al.*, 2015). Moreover, *Bacillus* spp. have been proven to yield high spore densities up to 1×10^{10} spores.mL⁻¹ (Monteiro *et al.*, 2005, Laloo *et al.*, 2009, Chen *et al.*, 2010, Khardziani *et al.*, 2017). Panday (2016) reported the highest spore concentration of 1.9×10^{11} spores.mL⁻¹. These studies on high density cultivation of *Bacillus* spores show great promise for commercialization as poultry probiotics. The main technological advantages of this genus as poultry probiotics is illustrated in Figure 1.4.

1.9.2 Cell harvesting

The efficient harvesting and purification of spores from the resultant fermentation broth contributes to the overall commercial attractiveness of the process. *Bacillus* spores are more robust than vegetative cells against damage from harsh process conditions typical in cell harvesting processes, such as pressure and mechanical shear. The most widely used process remains centrifugation because of its simplicity, low cost, consistency and it has been shown to result in recoveries of viable spores exceeding 90% (Villafaña-Rojas *et al.*, 1996, Lalloo *et al.*, 2010a). An added advantage is that the centrifugation process can be continuous, resulting in improved process through-put, while maintaining high cell recoveries (Zamola *et al.*, 1981, Lalloo *et al.*, 2010a). Mature spores that are harvested, need to be stabilized to maintain long term viability and to prevent the cells reverting to the vegetative state, which could result in product intermediate spoilage. It is therefore imperative to develop a stabilization process that results in a spore suspension for later product formulation (Schisler *et al.*, 2004, Brar *et al.*, 2006). *Bacillus* spores, in general, are tolerant of buffers, preservatives and the manipulation of pH during the stabilization process, resulting in high product recoveries in contrast to most conventional probiotics.

1.10 Product formulation considerations for *Bacillus* probiotics

The formulation of the final probiotic product is a key consideration that enables the commercial adoption of the technology (Brar *et al.*, 2006, Prabakaran *et al.*, 2007). The probiotic product should satisfy certain requisites such as deliver adequate number of viable microorganisms to the target host, have a sufficiently long shelf life, and allow for the ease of application and the provision of a product form that commands customer appeal (Moodley *et al.*, 2014). Poultry probiotic products are generally formulated as either a powder or liquid. The liquid form is cheaper and often administered in the potable water fed to chickens but requires large storage areas, higher costs of shipment, and refrigeration (Lacroix and Yildirim, 2007). Powdered products are preferred and commonly utilized by the poultry industry as it is cost effective, alleviates the storage limitations and offers ease of handling and administration. Furthermore, dry forms of probiotics have a longer shelf life and better tolerance to the gastric environment (Markowiak and Ślizewska, 2018). In contrast to human probiotics, the poultry industry cannot absorb the high cost of encapsulation, for this reason, bulk drying techniques to form probiotic powders are preferred (Moënné-Loccoz *et al.*, 2001, Wiwattanapatapee *et al.*, 2004).

Due to the spore-forming nature of *Bacillus* organisms, they do not require specialized techniques to obtain viable spores in either liquid or powdered forms. The spores can be blended with specific carriers and are resistant to high shear powder blending. Transforming liquid product intermediates of spore concentrates into a dried product requires drying at high temperature (~60 °C), but the viability of spores generally remains unaffected. An advantage to powder products is that carriers, protection aids and nutrients that support the germination of the spores can be easily included into the product without negatively affecting shelf life (Moënne-Loccoz *et al.*, 2001, Wiwattanapatapee *et al.*, 2004, Brar *et al.*, 2006). For the poultry industry, common carriers include calcium carbonate and limestone which is incorporated with a sugar additive. A key consideration of powder manufacturing is that the spores must be evenly distributed so that the concentration is consistent in the feed, which ensures constant dosage. It has been stated that in order for any probiotic to be effective it should contain a minimum of 10^6 CFU.g⁻¹ of viable microorganisms at the point of consumption (Ouweland and Salminen, 1998, Simon *et al.*, 2005). Based on this, the production of dried probiotic powder concentrates should be formulated at the equivalent cell number to ensure that the minimum concentration and viability is maintained in the feed (Meng *et al.*, 2008). There are no formal guidelines as to what the final spore concentration should be, yet the majority of commercialized probiotic products are formulated to a concentration of $\sim 1 \times 10^9$ CFU.mL⁻¹ (Teo and Tan, 2007, Jeong and Kim, 2014, Kim *et al.*, 2017) which ensures a balance of consistent dispersion, cost effective logistics and easy dosage into premixes and feeds.

1.11 Incorporation of *Bacillus* probiotics during feed manufacturing

The probiotic product is generally incorporated during the feed manufacturing process either directly or through prior inclusion into a feed pre-mix (Simon *et al.*, 2005). Commercial chicken feed is a dry-solid product in mash, crumble or pelleted form. The feed industry requires a convenient product form that must be easily incorporated into existing manufacturing processes. Feed manufacturing involves several mechanically intense processes such as blending, extrusion, pelleting and crushing, which expose the probiotic to high temperatures, pressures and mechanical shear often affecting conventional probiotics negatively (Kosin and Rakshit, 2006). Typical feed for broiler chickens is processed up to 100 °C for 15–20 seconds during extrusion (Kosin and Rakshit, 2006).

The thermostability of *Bacillus* spores in the feed manufacturing process is a major advantage over vegetative cells as they can survive temperature exposures up to 113°C for 8 minutes

(Vasquez, 2016). Additionally, *Bacillus* spores are mechanically stronger than vegetative cells, allowing them to withstand the high pressures and the mechanical shear associated with mixing, extrusion, pelletizing and crushing. Studies regarding the stability of probiotics in poultry feed are limited but it was shown that the recovery of *B. cereus var toyo*i after pelleting at 87 °C was 95%, and after eight weeks in feed storage was 92% (Simon *et al.*, 2005). In an *in vitro* screening study, Chaiyawan *et al.*, (2015) proved that spores were able to survive wet heat at 80 °C regardless of contact time.

Storage of probiotic-augmented feeds are usually in warehouses at fluctuating ambient conditions which affects the viability, especially of conventional probiotics (Markowiak and Ślizewska, 2018). It is generally accepted that the water activity should be below 0.25 and thus moisture content below 5% in order to ensure stability, thus packaging is also an important consideration (Chávez and Ledebøer, 2007). There is very little literature available on the product formulation for *Bacillus* probiotics as this information is generally propriety to industry. Some studies on *Bacillus* spp. have demonstrated improved dry product shelf life of up to 5 years (Laloo *et al.*, 2010a).

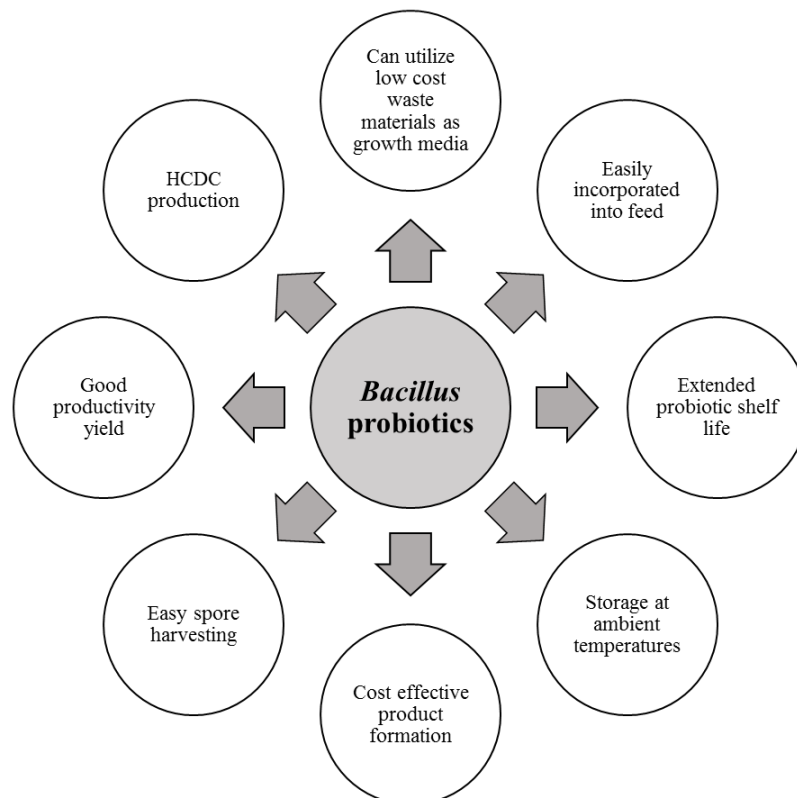


Figure 1.4: Commercial advantages of *Bacillus* spp. as poultry probiotic products.

1.12 Future perspectives

Bacillus spp. are the future of in-feed probiotics. The greatest advantages are in the general ease of isolating suitable candidates, process development, production of spores and cell harvesting. The simplicity in product formulation and their hardy nature makes them ideally amenable to inclusion in poultry feed within current feed manufacturing processes. The shelf life of the probiotic in spore form is also significantly better than conventional probiotics in the vegetative form, under industry relevant storage conditions. Due to future growth in demand for more natural production of poultry, alternatives to AGPs will continue to be an area of interest but the costs and limitations of conventional probiotics remain a challenge to industry. As studies on *Bacillus* spp. increase, there appears to be greater proof of the suitability of this genus as a poultry probiotic. However, more research is required in areas such as strain dependent mechanisms of action, multiple mode probiotic development, consortium studies, individual manufacturing processes, product formulation, stability studies and efficacy studies at commercial scale.

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CHAPTER TWO

ISOLATION, SELECTION AND EVALUATION OF *BACILLUS* SPP. AS POTENTIAL MULTI-MODE PROBIOTICS FOR POULTRY

Uraisha Ramlucken, Yrielle Roets, Santosh O. Ramchuran, Ghaneshree Moonsamy, Christine Jansen van Rensburg, Mapitsi S. Thantsha, and Rajesh Laloo (2019). Isolation, selection and evaluation of *Bacillus* spp. as potential multi-mode probiotics for poultry. *Journal of General and Applied Microbiology* (Accepted).

Isolation, selection and evaluation of *Bacillus* spp. as potential multi-mode probiotics for poultry

Uraisha Ramlucken ^{1,2,3}, Yrielle Roets ¹, Santosh O. Ramchuran ¹, Ghaneshree Moonsamy ¹, Christine Jansen van Rensburg ², Mapitsi S. Thantsha ³, and Rajesh Laloo ^{1*}

¹ The Council for Scientific and Industrial Research, Biosciences, Pretoria, South Africa, 0001

² Department of Animal and Wildlife Science, University of Pretoria, Pretoria, South Africa, 0002

³ Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Pretoria, South Africa, 0002

2.1 Abstract

Bacillus based probiotics are becoming relevant as alternatives to antibiotics used in poultry production and in other animal husbandry. This study describes the isolation of 48 *Bacillus* spp. candidates, from chickens and chicken environments, for use as potential probiotics in poultry production. These isolates, plus a further 18, were tested in a comprehensive *in vitro* screening regime that was specifically designed to select the best isolates that satisfied multiple modes of action desirable for commercial poultry probiotics. This screening programme involved the evaluation of the ability of the isolates to survive and grow in the limiting conditions of the chicken gastrointestinal tract. Only 11 of the isolates fulfilled these criteria, hence they were further evaluated for the ability to adhere to epithelial cells, produce extracellular enzymes and to demonstrate antagonistic activity against selected pathogens of significant importance in poultry production. Of these, a total of six isolates were selected, due to their all-round probiotic capability. Identification by 16S RNA sequencing confirmed these isolates as *B. subtilis* and *B. velezensis*, identities which are generally regarded as safe. The *Bacillus* isolates reported in our study exhibit strong all-inclusive probiotic effects and can potentially be formulated as a probiotic preparation for poultry production.

Keywords: *Bacillus subtilis*; *Bacillus velezensis*; Broiler production; Development of probiotics; Indigenous bacteria; Multi-mode; Probiotics

2.2 Introduction

Consumer demand for poultry products is rapidly increasing due to their affordability and accessibility. The broiler industry must ensure fast growth and high stocking densities to enhance production efficiency (Griggs and Jacob, 2005, Kabir, 2009). These conditions impact negatively on chicken health, driving the indiscriminate use of antibiotics, which leads to an increase in the outbreak of zoonotic diseases due to antibiotic resistance (Martinez and Baquero, 2000, Phillips *et al.*, 2004). As such, in-feed antibiotics have been banned in regions such as Europe, America (Perreten, 2003, Dibner and Richards, 2005) and Scandinavia (Bengtsson and Wierup, 2006, Grave *et al.*, 2006). Global increase in consumer health awareness has also resulted in preferences for poultry products that are free from antibiotics, growth stimulants and other non-natural additives (Griggs and Jacob, 2005, Yiridoe *et al.*, 2005). Therefore, the poultry industry requires new and innovative technologies to address these challenges.

Newer approaches to address these problems include the use organic acids, enzymes, plant derivatives, essential oils and prebiotics, which can all substantially increase the cost of poultry production (Yang *et al.*, 2009). Probiotics are also currently used, the most common being *Lactobacillus* spp. and to a lesser extent others such as *Enterococcus* spp., *Saccharomyces* spp. and *Aspergillus* spp. (Jin *et al.*, 1998b, Kalavathy *et al.*, 2003, Kabir *et al.*, 2004, Kabir, 2009). The main disadvantage associated with most of these probiotics however is their poor or limited survival through the feed production steps and in the chickens' gastrointestinal tract (GIT) (Wolfenden *et al.*, 2010).

These limitations have led to an interest in *Bacillus* based probiotics, due to their spore-forming capabilities, which enables them to resist damage during the feed production process and to also survive the adverse conditions in the GIT such as the presence of bile salts and low pH. *Bacilli* are also relatively easy to produce through conventional fermentation processes and do not require expensive downstream processing to ensure stable commercial products (Cutting, 2011). They are also known for their fast growth rate, the production of a wide array of digestive enzymes and the ability to competitively exclude certain pathogenic bacteria (Hong *et al.*, 2005, Leser *et al.*, 2008, Lee *et al.*, 2012). The positive attributes of this genus offer promise for the development of suitable commercial poultry probiotics.

One concern regarding the probiotic *Bacillus* spp. is their ability to grow under facultative conditions in the chicken GIT, however several studies have shown that *Bacillus* spores can germinate and grow under these conditions (Barbosa *et al.*, 2005, Tam *et al.*, 2006, Wu *et al.*, 2007, Hong *et al.*, 2009). Since then there has been a drastic increase in studies investigating the properties of this species as probiotics for poultry (Teo and Tan, 2005, Wolfenden *et al.*, 2010, Ahmed *et al.*, 2014, Latorre *et al.*, 2014, Chaiyawan *et al.*, 2015, Nguyen *et al.*, 2015, Vasquez, 2016).

Probiotic development requires isolation of potential candidates, followed by the investigation of specific criteria of interest to the poultry industry, such as survival, colonisation and growth within the chicken GIT. Other desirable effects include the production of digestive enzymes, attenuation of disease causing pathogens and immunomodulation (Fuller, 1999, Simon *et al.*, 2001, Kabir, 2009, Lee and Yu, 2013). Establishing the biosafety of the potential probiotic is a critical factor, as this is a major concern of the industry and regulators alike, therefore microorganisms that are generally regarded as safe (GRAS) status are preferred (Fuller, 1992, Lee and Yu, 2013). Since all *Bacillus* strains do not equally possess all probiotic competencies, the proper mathematical quantification of multiple effects to form a multi-functional consortium is critical to delivering a commercially relevant product to the poultry industry (Guo *et al.*, 2006). This integrated approach has not been thoroughly researched, hence our hypothesis that screening, selection and quantitative evaluation based on multiple criteria will result in commercially usable probiotic products, encompassing multiple modes of action.

2.3 Materials and Methods

2.3.1 Sample collection, isolation, purification and storage.

Samples were collected from selected South African chicken (broiler, broiler breeder, egg layer and free range) production farms in the Gauteng region, chosen to ensure a diverse range of bacteria sources. Faecal matter, bedding material and feathers were aseptically collected. Swab samples from the body and foot region of live chickens were also obtained. Fresh samples of the chicken GIT were provided by a commercial abattoir.

Individual samples of feathers, body swabs, faecal matter and bedding material (~ 5 g) were added directly to 100 mL of sterile sporulation media (yeast extract 0.008 g.L⁻¹, MgSO₄.7H₂O 0.5 g.L⁻¹, MnSO₄.4H₂O 0.05 g.L⁻¹ and CaCl₂ 0.1 g.L⁻¹) (Merck, Germany) contained in a Erlenmeyer flask (500 mL) and incubated at 32 °C on a platform rotary shaker (Innova 2300

series, New Brunswick, Canada) at 180 rpm for 7-9 days. A sample from each flask was checked microscopically at 400× magnification (Olympus BX40, Olympus, Japan) to confirm the presence of spores. Chicken GIT samples (~5 g) were aseptically homogenised using a bench top T18 basic homogenizer (Ultra Turrex, IKA, Germany) and treated in a similar manner to other samples. Each spore culture was then treated using an isolation cascade, which comprised a dehydration and a heat treatment step to eliminate non-spore formers (Lalloo *et al.*, 2007).

Samples from each flask (1 mL) were serially diluted in sterile saline and plated on nutrient agar (NA) (Merck, Germany) plates supplemented with 10 mg.mL⁻¹ polymyxin B antibiotic (Sigma-Aldrich, USA) to exclude any gram negative spore forming bacteria. Plates were incubated for 24 hours at 32 °C. Single colonies based on morphology differences were transferred onto new plates until monocultures were obtained consistently through three passages. Each pure colony was subjected to the catalase (Kilian, 2015) and Gram staining (Barile, 2012) tests. The cultures that passed the tests were thereafter cultivated in sporulation medium after which each spore culture was cryo-preserved using 25% v.v.⁻¹ glycerol (Sigma-Aldrich, USA) according to the method outlined by Acosta (2004). These cell banks were then stored at -80 °C in an ultra-freezer (Forma™ 88000 Series, Thermo Scientific, USA). Additionally 18 isolates from an existing in-house *Bacillus* spp. culture collection (CSIR, South Africa) were selected for evaluation of their probiotic potential

2.3.2 Critical screening phase

2.3.2.1 Survival and growth of isolates at pH 3.

Survival of isolates in an acidic environment was tested using a modified and scaled-down method reported by Fuller (1999). The cell concentration of each cryo-culture was measured using a counting slide (Thoma®, Hawskey and Sons, UK), under light microscopy at 400× magnification and cultures were standardised to a cell concentration of 1 × 10⁸ CFU.mL⁻¹ using sterile distilled water. Tryptone soy broth (TSB) (Merck, Germany) was adjusted to pH 3 using 1 M hydrochloric acid (HCl) (Minema Chemicals, South Africa), prior to autoclaving at 121 °C for 15 minutes (Eins Sci Autoclave, Hospi Sterilizers, South Africa) and thereafter cooled and aseptically aliquoted (10 mL) into the wells of a six-well microplate. Each test isolate was inoculated (1% v.v.⁻¹) into the wells in quadruplicate and the microplates were covered using sterile polyester seals (Costar, Corning Incorporated, USA). Microplates were incubated for 4 hours at 42 °C with shaking on a platform rotary shaker (120 rpm). Viable cell counts were

determined using a standard plate count method by serially diluting the samples and spread-plating onto plate count agar (PCA) (Merck, Germany), followed by incubation at 42 °C for 24 hours. Colonies were enumerated using a colony counter (Bibby, Stuart scientific UK). Survival and growth of the isolates was determined using the relative difference of the CFU.mL⁻¹ (Δ CFU.mL⁻¹) between the start (T₀) and completion of the exposure time (T₄). Results were interpreted as the mean of the three most accurate determinations. All isolates that did not survive at pH 3 were eliminated from further screening.

2.3.2.2 Growth of isolates in the presence of bile salts.

Growth in bile salts was determined using the method of Hyronimus *et al.*, (2000) downscaled to a six-well microplate format. Sterile TSB supplemented with 0.3% (wt.v⁻¹) of ox gall bile salts (Sigma-Aldrich, USA) was aliquoted (10 mL) into each well, inoculated in quadruplicate with a cryo-culture of the isolate to be tested and the plates incubated at 42 °C for 24 hours. Growth was measured using the difference in optical density (OD), measured at 660 nm using a micro-plate reader (Synergy HT, BioTek USA), at the start (T₀) and end (T₆) of the cultivation time. The remaining two wells were used as un-inoculated controls which also served as a blank for the OD reading. Results were interpreted as the mean of the three most accurate determinations. All isolates that displayed significantly lower growth ($p < 0.05$) than the mean growth achieved in bile salts, were eliminated from further screening tests.

2.3.2.3 Growth of isolates at intestinal pH extremes.

Growth was evaluated at pH 5 and pH 7 as a representation of the pH extremes of the chicken intestine. As per the growth study at pH 3, sterile TSB medium was adjusted to either pH 5 (HCl) or 7 (NaOH) (Minema Chemicals, South Africa) prior to autoclaving and aseptically aliquoted into six-well microplates. Wells were inoculated with the test isolates, incubated for 4 hours at 42 °C, with growth measurement by optical density at 660 nm. All isolates that displayed significantly lower growth than the mean growth achieved at either pH 5 or 7 were eliminated from further screening.

2.3.3 Secondary screening phase

2.3.3.1 Potential of isolates for production of digestive enzymes.

Isolates that passed the critical screens, that is, those that survived at pH 3, were able to grow in the presence of bile salts and at intestinal pH, were then tested for the production of amylase, cellulase, protease and xylanase enzymes. Each test isolate was inoculated from a cryovial and

grown in flasks containing sterile TSB (100 mL) at 42 °C for 12 h, with agitation at 120 rpm. The resultant culture was standardized to an OD_{660nm} of 2 using sterile distilled water. Triplicate samples (100 µL) were withdrawn and used to inoculate aseptically punched wells in the centre of an agar plate which contained the substrate for the enzyme of interest. Solubilised starch (Sigma-Aldrich, USA) was added for amylase detection (Ibrahim *et al.*, 2012), carboxymethylcellulose (CMC) (Sigma-Aldrich, USA) was added for cellulase detection (Kasana *et al.*, 2008), milk powder and casein (Sigma-Aldrich, USA) was added for protease detection (Kim *et al.*, 2007) and Birchwood xylan (Sigma-Aldrich, USA) was added for xylanase detection (Nair and Shashidhar, 2008). All plates were sealed and incubated at 42 °C for 24 hours (amylase and protease) and 48 hours (cellulase and xylanase). After incubation, the extent of the respective enzyme substrate reactions was visualised using different staining techniques. Grams iodine was used for the detection of amylase and cellulase activity, while trichloroacetic acid (Merck, Germany) (25% v.v⁻¹) was used followed by a 15 minute incubation at 45 °C for the detection of protease activity. A stepwise treatment with a 25% (v.v⁻¹) sodium chloride (Merck, Germany) solution followed by staining of the plate with Congo red (Sigma-Aldrich, USA) was used for the detection of xylanase activity. The diameters of the zones around the wells which were measured using a digital Vernier calliper (Insize, Accu, UK) and were indicative of enzyme activity. The response of each isolate for each enzyme was included in a mathematical matrix evaluation.

2.3.3.2 Physical feed break down potential of the isolates.

The determination of the physical breakdown of feed was done to evaluate the effect of each isolate on feed particle size. A commercial grower feed (nominal pellet diameter ~ 3.5 mm and length ~6 mm) obtained from AFGRI (South Africa) was dried at 60 °C for 12 hours. Exactly 2 g of the feed was added to a pre-sterilized nylon sieve (~ 1 mm nominal mesh breakthrough) which was suspended in a sterile, 50 mL falcon tube (TPP, Switzerland) containing 40 mL of tap water. Standardised pre-cultures of each test isolate, prepared as described in the digestive enzyme study, were then added (1% v.v⁻¹) to the suspended feed. All tubes were incubated at 42 °C for 24 hours with gentle agitation (25 rpm) in a rotary shaker. After incubation, the mesh and remaining feed were removed, whereas the fines were harvested by centrifugation (AllegraX-22R, Beckman Coulter, USA) for 30 minutes at 3900 × g and dried at 60 °C overnight. An un-inoculated negative control was treated in the same manner. The weight of the pellet represented the feed that was broken down to below 1 mm and was used to calculate percentage feed breakdown by expressing the percentage ratio of the broken-down feed portion

over the total feed on a dry basis. The percentage feed breakdown was expressed as a comparison to the negative control to mitigate any breakdown that had occurred naturally from the shaking, incubation and submergence in the water.

2.3.3.3 Gut epithelium adhesion assay.

A Caco-2 cell line (University of Kwa-Zulu Natal, South Africa) was used to mimic chicken epithelial cells as previously described (Tsai *et al.*, 2005). Cells tested negative for mycoplasma contamination by the institute it was obtained from. Cells were routinely maintained in Dulbecco's modified eagle medium (DMEM) with antibiotics as outlined by Hsieh *et al.*, (2013). For the adhesion assay, Caco-2 cells (passage 20-22) were washed with pre-warmed (37 °C) phosphate buffered saline (PBS) (Lonza, Switzerland) and then trypsinised by addition of 0.25% (w.v⁻¹) trypsin and 0.1% (w.v⁻¹) ethylenediaminetetraacetic acid (EDTA) at approximately 85-90% confluency. Cells of a standard concentration (1×10^5 cell.mL⁻¹) were aliquoted (500 µL) into wells of a sterile 24 well tissue culture plate, which was then covered (TPP, Switzerland), followed by incubation at 37 °C in a CO₂ incubator (5% CO₂ in ambient air) until a cell monolayer formed and was microscopically observed. The cell culture media (DMEM) was aspirated from each well and discarded. The adhered Caco-2 monolayer was washed once with PBS before their inoculation with the test isolate cultures. *Bacillus* isolates were cultured overnight in flasks containing sterile TSB (100 mL) as previously described in the digestive enzyme study. Thereafter the cells were harvested by centrifugation at $3900 \times g$ for 20 minutes and the pellet was re-suspended in DMEM to achieve a normalised viable cell concentration of 1×10^8 cells.mL⁻¹. The cell suspension of each isolate was added (200 µL) in triplicate into wells containing pre-adhered Caco-2 monolayers. Plates were incubated for 2 hours at 42 °C with gentle agitation (25 rpm) in an orbital shaker (Innova 40R, New Brunswick, Canada). After incubation, free cells in the media were removed by aspiration and collected in a 15 mL sterile falcon tube. The remaining adhered monolayer were washed twice using sterile PBS to remove un-adhered bacteria, which was pooled with the free cell fraction and made up to a total volume of 1500 µL. The adhered portion was trypsinised to release the adhered cells, re-suspended with DMEM and collected in a sterile 15 mL falcon tube (total volume 1500 µL). The total bacterial cells in the free and adhered portion were determined by microscopic cell counting as previously described. The percentage adhesion was determined by calculating the percentage ratio of the total adhered to the total un-adhered cells. The adherence of each isolate was included in a mathematical matrix evaluation.

2.3.3.4 Antagonistic activity of isolates against selected pathogens.

A standard agar well diffusion method (Fijan, 2016) was used to evaluate antagonism against four common chicken pathogens (*Escherichia coli*, *Salmonella enteritis*, *Listeria monocytogenes* and *Clostridium perfringens*). Each test isolate was cultured as previously described in the digestive enzyme study and aseptically added from a culture flask (100 μ L, 1×10^8 cells.mL⁻¹) into pre-made wells on a Tryptone Soy Agar (TSA) (Merck, Germany) plate, previously spread with each of the respective test pathogens. The plates were incubated at 42 °C for 24 hours, after which zones of inhibition were measured using a digital Vernier calliper. The response of each isolate for each enzyme was included in a mathematical matrix evaluation.

2.3.4 Elimination, scoring and selection of isolates.

The data was processed by using statistical clustering and ANOVA (analysis of variance, t-test assuming equal variances). The clusters were based on a standard deviation of ± 0.5 SD (1SD total) of the mean of the data set. Statistical significance of the difference between means of clusters were confirmed by p values < 0.05 . Isolates were only eliminated in the critical screens (growth and survival at pH 3, growth in bile salts and intestinal pH extremes). All isolates that did not survive these tests were eliminated from further testing and selection. In the pH 3 survival test all isolates that survived and those that showed growth were selected. For the studies investigating growth in bile and intestinal pH extremes, isolates that grew slower than the resultant mean growth for the study were eliminated. In the critical screen all isolates that clustered within the normal distribution of the mean were given a score of one. For remaining isolates that clustered above the mean, each data group that was significantly different from each other, was given a unique score, from two upwards. This strategy ensured that only average and above average performers were carried over to subsequent screens.

Using this strategy, a score of zero represented the lowest desirability and the highest score represented the highest desirability to the pre-set criterion for selection of the putative probiotics. Isolates were eliminated from each part of the critical screen if they had obtained a score of zero. In the non-critical screens (ability to produce enzymes, physically breakdown feed, adherence to epithelial cells and demonstration of an antagonistic effect against common chicken pathogens) the data from each response was similarly analysed but those isolates that clustered significantly below the mean were given a universal score of one and those that clustered within the normal distribution of the mean were given a score of two. All remaining

isolates that clustered above the mean were incrementally scored from three upwards. This ensured no isolate was eliminated but scored based on performance for each criterion. The final selection was done using the cumulative scores calculated mathematically for all criteria of the entire screen. The final selection was made based on the desirability of each probiotic on all parameters tested.

2.3.5 Strain identification and biosafety.

Identification of all strains selected as putative probiotics was done by 16S RNA sequence homology executed by Inqaba Biotech™ (South Africa). Genomic DNA from a pure colony of each isolate was extracted using the Bacterial DNA Kit™ (Zymo Research, Cat. No. D6005, USA). Amplification of the 16S target region was performed by using DreamTaq™ DNA polymerase (Thermo Scientific, USA) with two sets of forward and reverse primers (16s - 27 F and 16s -1492 R) which allowed for the sequencing of the gene. The primer sequences were as follows (5' to 3'): AGAGTTTATCMTGGCTCAG and CGGTTACCTTGTTACGACTT respectively. The PCR products were evaluated by gel electrophoresis and the bands were extracted using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Cat. No. D4001, USA). The products were subsequently sequenced in the forward and reverse directions on an ABI PRISM 3500 XL genetic analyser (Thermo Scientific, USA) as per manufacturer's instructions. PCR products were purified using a ZR-96 DNA sequencing clean-up kit (Zymo Research, USA) as per manufacturer's instructions and cycle sequenced on a CLC main workbench 7 (QIAGEN, Germany). The sequence alignments were performed by a BLASTN search (NCBI). Identification was based on the level of confidence of the sequence homology. The BLASTN results correspond to the similarity between the sequence queried and the biological sequences within the NCBI database. (Altschul *et al.*, 1997). Biosafety assessments were based on the strain identification of the putative probiotics of interest and only organisms that were GRAS strains were selected (Wright, 2005).

2.4 Results

2.4.1 Isolation of *Bacillus* spp.

A total of 48 isolates were obtained and successfully purified from poultry rearing environments, of which 15 were obtained from broiler, 16 from free range, 4 from egg laying and 13 from broiler breeder farms. An analysis of the frequency of occurrence of sample groups revealed that the majority of isolates were obtained from the chicken GIT (54%), followed by

faecal matter (17%), body swabs (15%), feathers (8%) and surrounding production environment (6%).

2.4.2 Critical screening phase

2.4.2.1 Evaluation and elimination of isolates against critical survival and growth requirements within the chicken GIT

Figure 2.1 illustrates the results obtained in the critical screen. A further 18 isolates were added from the existing organism database, to the 48 isolates obtained in this study resulting in a total of 66 candidates (Figure 2.1). The additional isolates were included based on historical information related to high digestive enzyme activities or because they were of animal origin. In the pH 3 survival study, 63 isolates were tolerant to pH 3, whilst the three that lost complete viability were eliminated from further testing (Figure 2.1). These 63 isolates were then tested for growth in the presence of bile salts, of which, 19 were unable to grow, 12 grew poorly, whilst the remaining 32 isolates that grew well, were selected for further evaluation of growth at intestinal pH extremes. At pH 5, 20 isolates were eliminated (13 did not grow and seven grew poorly), whilst at pH 7, 12 isolates were eliminated due to poor growth. All isolates resulting in a cumulative desirability co-efficient of zero, were eliminated (Figure 2.2). The remaining 11 isolates that showed significantly higher growth ($p < 0.05$) at both intestinal pH levels were selected and included into the next screening phase.

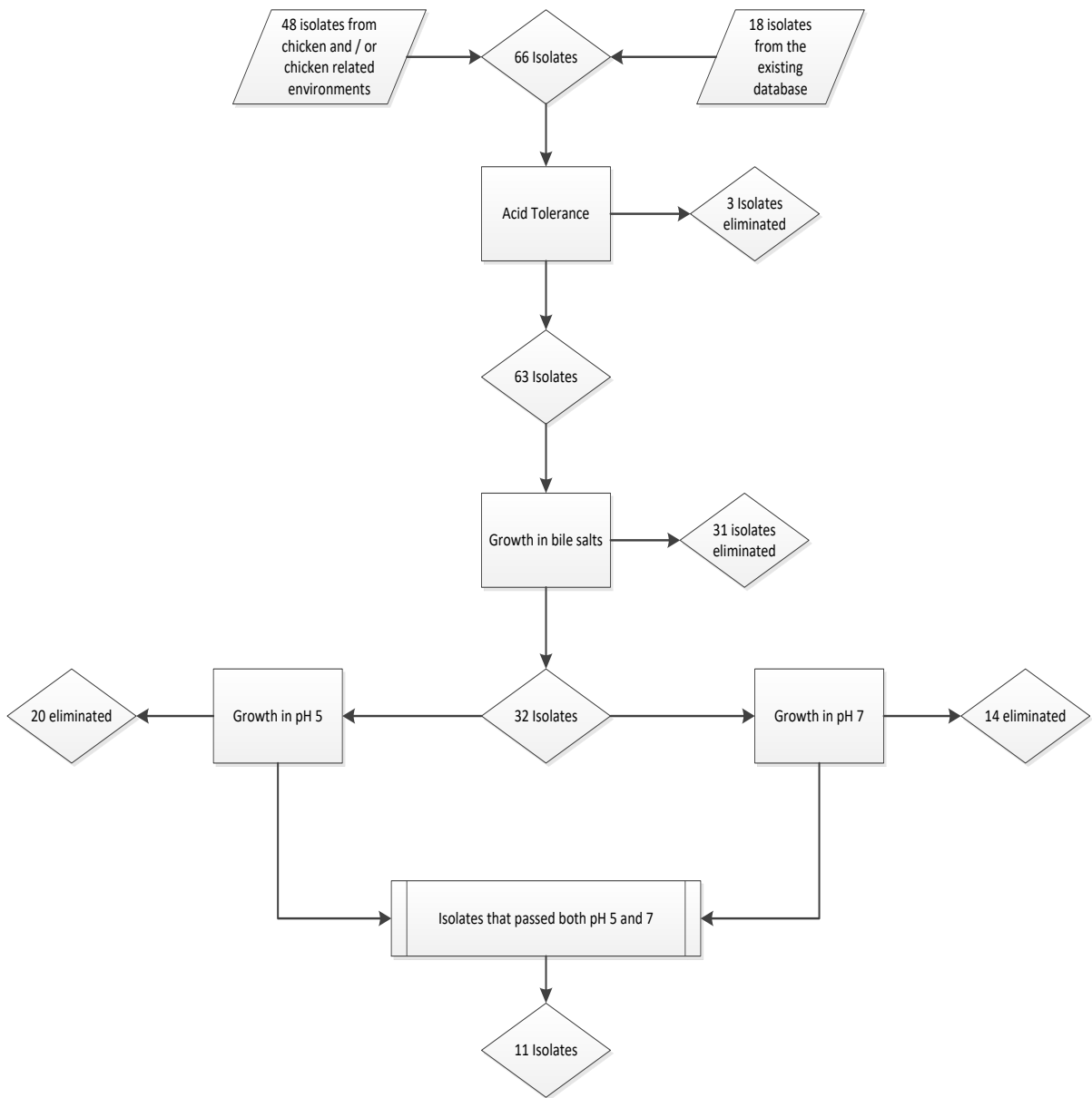


Figure 2.1: Schematic illustration of critical screening steps showing the selection of qualifying isolates. All experiments were triplicated.

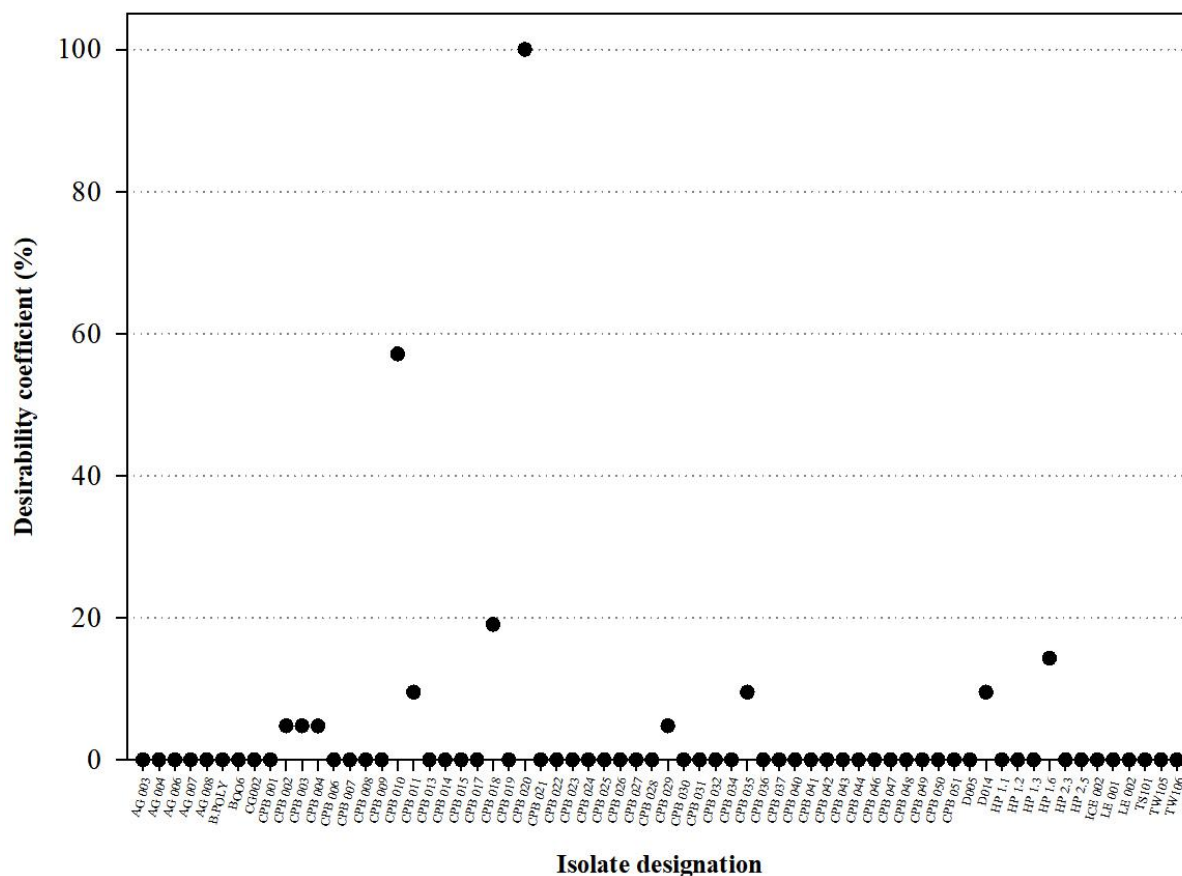


Figure 2.2: Desirability co-efficient (expressed as a percentage relative to the maximum score) showing elimination of undesirable isolates (55) in the critical screen phase.

2.4.3 Secondary screening phase

2.4.3.1 Extracellular enzyme production and the ability to physically break down feed

The 11 isolates carried over from the critical screen to the secondary selection phase were tested for extracellular enzyme production and all isolates produced the four enzymes of interest, but at varying levels (Figure 2.3). In the amylase production test, five isolates clustered significantly above average (CPB 029, CPB 011, D 014, HP 1.6 and CPB 035) ($p < 0.05$). Similarly isolates CPB 011, CPB 029 and CPB 035 were significantly better in the protease test ($p < 0.01$). With regards to the enzymes cellulase and xylanase, three organisms each for cellulase (CPB 003, CPB 029 and D 014) and xylanase (CPB 011, D 014 and CPB 020) performed significantly better respectively ($p < 0.05$ and $p < 0.01$). Four isolates (CPB 029, CPB 035, D014 and HP 1.6) had significantly higher performance in the cumulative scoring rating of all enzymes ($p < 0.01$). In the physical feed breakdown application study, CPB 011,

CPB 035 and D014, were the best isolates and clustered significantly above the average ($p < 0.05$).

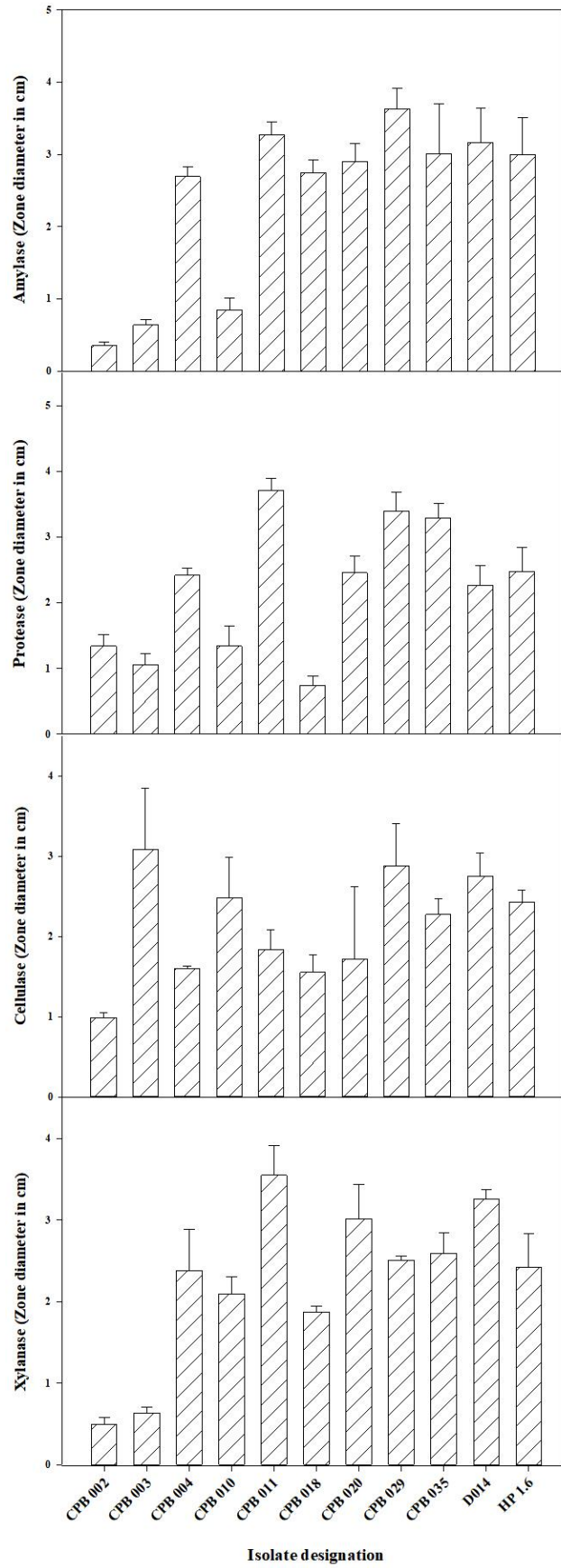


Figure 2.3: Extracellular enzyme activity of putative probiotics measured by the zone diameter (cm). Enzymes evaluated were amylase, protease, cellulase and xylanase. Error bars represent standard deviations, n= 6.

2.4.3.2 Adherence potential to gut epithelium cells

Three isolates (CPB 010, CBP 035 and CPB 029) showed significantly higher adherence (~43% attachment) to the Caco-2 monolayer ($p < 0.05$), of the 11 evaluated. Isolate CPB 010 resulted in the highest adherence (~57%), whilst five isolates were average (~ 37% adherence) and three isolates were poor (~20% adherence) (Figure 2.4).

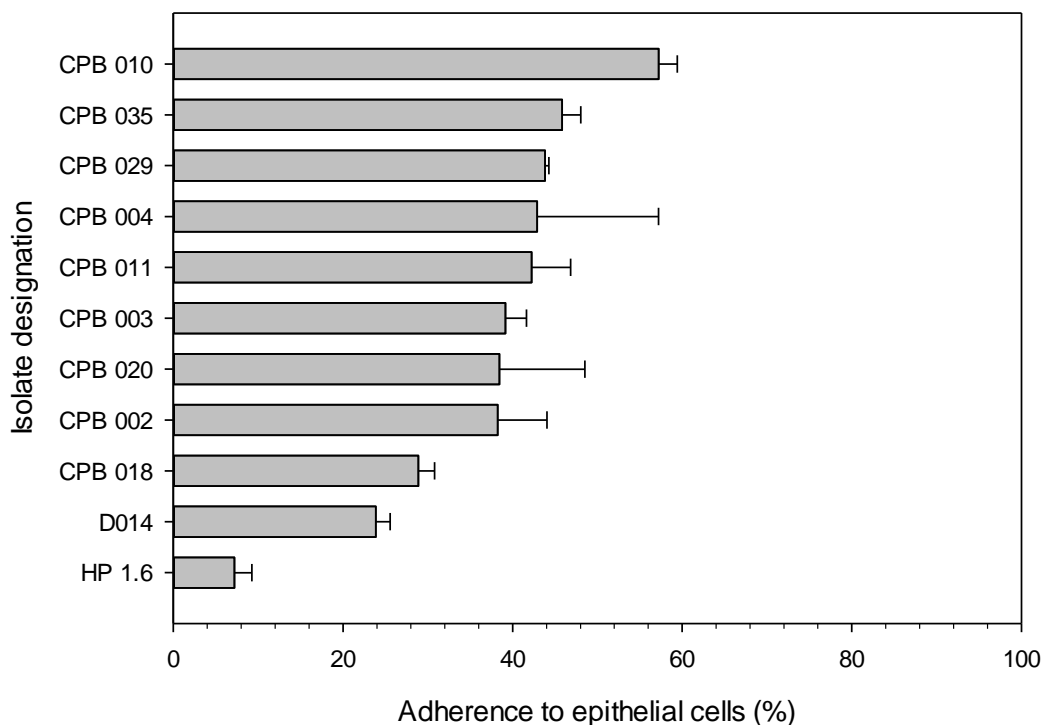


Figure 2.4: Adherence activity of putative probiotics to epithelial cells (Caco-2). Error bars represent standard deviations, n= 3.

2.4.3.3 Antagonistic activity against common poultry pathogens

When, antagonistic activity of the 11 isolates was measured against *E. coli*, CPB 011, CPB 020, CPB 029, CPB 035 and HP 1.6 resulted in significantly better antagonism against the pathogen ($p < 0.01$) (Figure 2.5). Similarly, isolates CPB 011, CPB 029, CPB 035, and HP 1.6 expressed significantly higher antagonism against *S. enteritis* ($p < 0.05$), whereas CPB 011,

CPB 035, HP1.6 and D014 displayed significantly better antagonism ($p < 0.05$) against *L. monocytogenes*. Isolates CPB 011, CPB 003, HP 1.6 and CPB 020 were the best organisms regarding antagonism against *C. perfringens* ($p < 0.01$). When comparing the overall antagonistic activity against all four of the pathogens of interest, isolates CPB 011, CPB 020, CPB 035 and HP 1.6 resulted in the highest scores.

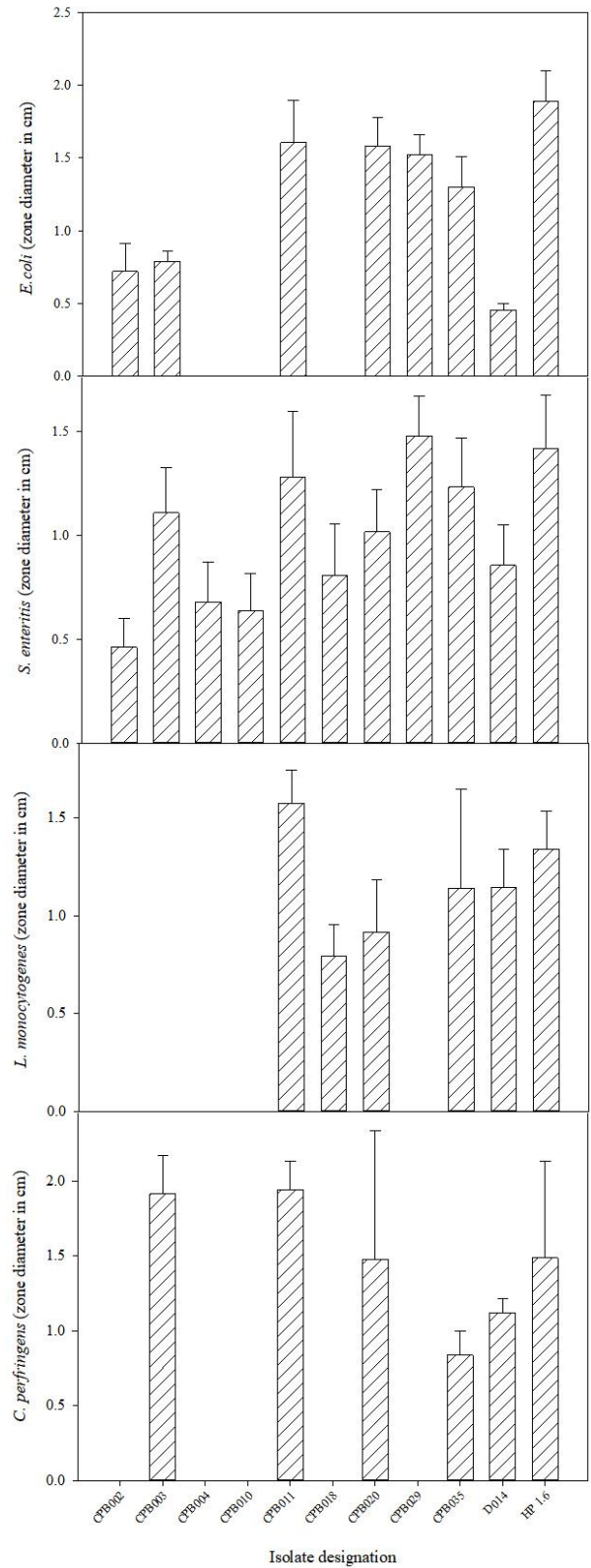


Figure 2.5: The antagonistic activity of isolates against common chicken pathogens namely *Escherichia coli*, *Salmonella enteritidis*, *Listeria monocytogenes* and *Clostridium perfringens*. Competitive exclusion was measured by spatial domination of each isolate (cm). Error bars represent standard deviations, n= 6.

2.4.4 Final selection of putative probiotics

Using the mathematical strategy designed for this study, results showed that isolates CPB 020, CPB 035 and CPB 011 performed significantly better than the average performers, based on the cumulative score ($p < 0.05$) and were therefore selected as the core consortium (Table 2.1). The average performers CPB 010, CPB 029, HP 1.6 and D014 also resulted in a high cumulative desirability co-efficient (80- 90%) and were included as auxiliary isolates (Table 2.1). All seven of the isolates selected were subsequently subjected to a growth suitability evaluation to confirm production potential in industrial media wherein isolate CPB 010 resulted in extremely poor growth (data not shown) and was therefore excluded from selection.

Table 2.1: Desirability co-efficient of each putative probiotics showing suitability to each criteria and cumulative multi-mode performance rating (relative % to maximum).

Organism Designation	Survival and Growth at pH3	Growth in Bile	Growth at intestinal pH	Adherence	Digestive Enzymes	Pathogen Antagonism	Feed Breakdown	Cumulative
Desirability Co-efficient (%)								
CPB 020	100.0	50.0	100.0	50.0	80.0	85.4	50.0	100.0
CPB 035	28.6	50.0	60.0	75.0	93.3	91.7	100.0	96.7
CPB 011	14.3	50.0	100.0	50.0	100.0	100.0	75.0	94.9
<i>CPB 010^d</i>	28.6	100.0	60.0	100.0	73.3	37.5	50.0	87.2
<i>CPB 029</i>	14.3	100.0	40.0	75.0	100.0	66.7	50.0	86.5
<i>HP 1.6</i>	14.3	100.0	80.0	25.0	86.7	100.0	25.0	83.6
<i>D014</i>	14.3	50.0	80.0	25.0	100.0	68.8	75.0	80.1
CPB 018	28.6	50.0	80.0	25.0	66.7	45.8	50.0	67.1
CPB 002	14.3	100.0	80.0	50.0	33.3	39.6	25.0	66.4
CPB 003	14.3	50.0	60.0	50.0	60.0	68.8	25.0	63.6
CPB 004	14.3	50.0	60.0	50.0	73.3	37.5	25.0	60.2

Core isolates in bold (significantly better than auxiliary isolates, $n=3$, $p < 0.05$) and auxiliary isolates in italics (significantly better than the remaining isolates $n=3$, $p < 0.05$).

^dUnable to grow in industrial media

2.4.5 Microorganism identification

Four of the six strains were identified as *B. subtilis* (CPB 011, CPB 029, D014 and HP 1.6), and the remaining two (CPB 020 and CPB 035) were identified as *B. velezensis*.

2.5 Discussion

2.5.1 Isolation of *Bacillus* spp.

The use of indigenous microorganisms from within the host is preferred when developing probiotics, as it not only gives the best chance of surviving and colonizing the intestine but also alleviates many of the challenges associated with introducing foreign bacteria. The result obtained from our isolation programme targeting poultry environments confirm the general expectation that samples associated with the GIT and faeces resulted in a higher yield of putative isolates. Traditionally, *Bacillus* spp. are considered mostly aerobic but our study, similarly to those of other researchers, (Barbosa *et al.*, 2005, Wolfenden *et al.*, 2010, Latorre *et al.*, 2014, Chaiyawan *et al.*, 2015), showed that they can be successfully isolated from the facultative and anaerobic zones of the chicken GIT.

2.5.2 Critical screening phase

In this phase, the core strategy was to eliminate isolates that did not survive or grow under *in vitro* conditions that simulated the chicken GIT because these isolates would not be suitable as probiotics (Fuller, 2001). The screen comprised of the survival in pH 3, growth in the presence of bile salts and growth at intestinal pH extremes.

2.5.2.1 Evaluation and elimination of isolates against critical survival and growth requirements within the chicken GIT.

The first screen was designed to evaluate survival at low gizzard pH wherein 63 of the 66 isolates survived, attributable to the resistant nature of *Bacillus* spores (Spinosa *et al.*, 2000, Cutting, 2011). Of the 63 survivors, the seven that grew were scored higher because growth in the gizzard delivers actively growing cells to the intestine where the probiotic effect is required (Hughes, 2008). Interestingly, all three isolates that did not survive at pH 3, were not obtained from poultry environments, perhaps indicating better adaptation of isolates from the target host (Pan and Yu, 2014).

In the bile salt growth test, isolates that did not survive or grew poorly (31 isolates) were eliminated from further screening of the 63 that were carried over into this test. Eliminated isolates did not qualify as suitable probiotics due to the lack of resistance to the antimicrobial properties of bile salts. It is likely that these organisms do not produce the enzyme bile hydrolase which offers protection from the toxic effects of bile (Begley *et al.*, 2006, Patel *et al.*, 2010). In contrast the 32 isolates that grew well in the presence of bile salts were selected

for the next screen. Similar to our findings, other researchers have also shown the survival and growth of *Bacillus* spp. in the presence of bile (Lee *et al.*, 2012, Menconi *et al.*, 2013). Bile salts can be detrimental to probiotic bacteria as bile is a part of the host's antimicrobial defence. When selecting feed probiotics, the survival in bile salts is therefore considered a minimum requirement for proper functionality in the intestine (Begley *et al.*, 2005, Begley *et al.*, 2006).

As expected, all of the 32 isolates tested grew at pH 7, which is close to the optimum growth pH of most *Bacillus* spp. (Rasko *et al.*, 2005, Stahly *et al.*, 2006). At pH 5, 12 of the 32 isolates tested grew well, indicating their ability to be active in the beginning of the small intestine, which has a lower pH due to acid carryover from the gizzard.

Overall, of the 66 isolates screened, only 11 isolates were selected based on the critical elimination criteria (Figure 2.2), resulting in an 83% elimination efficiency, which enables focus on a smaller number of isolates in subsequent screens.

2.5.3 Secondary screening phase

The 11 isolates remaining were subjected to a secondary screening phase comprising enzyme production, adherence and pathogen antagonism studies. In these tests, all organisms were evaluated and scored, without any elimination, as the objective was to assess cumulative probiotic effects with a view to find a multi-mode probiotic consortium.

2.5.3.1 Extracellular enzyme production and the ability to physically break down feed

Isolates CPB 029, CPB 035, D 014 and HP 1.6 produced all the test enzymes and cumulatively produced the highest level of digestive enzymes of interest to the poultry industry. A total of five of the 11 isolates were above average in the production of amylase, which is an important enzyme for the hydrolysis of complex carbohydrates which is the predominant ingredient in broiler feed. Even though the chicken naturally produces this enzyme, additional production from probiotics enhances the digestion of carbohydrates resulting in improved uptake (Latorre *et al.*, 2016). The best protease producers were isolates CPB 011, CPB 029 and CPB 035. Protease is often supplemented in the diet as it is important for the digestion of complex proteins, improvement in amino acid digestibility, neutralizing of anti-nutritional factors and allergenic proteins as well as the degradation of low-quality proteins, thus making these isolates desirable (Ravindran, 2013). Only three of the 11 isolates were able to produce high levels of cellulase and xylanase. The global trend is moving towards formulating broiler feed to

incorporate more non-starch polysaccharides (NSP), such as wheat and barley to circumvent the increasingly high cost of maize based diets (Latorre *et al.*, 2016). These NSP based diets increase intestinal viscosity, negatively affecting digestibility and absorption of nutrients (Annison, 1993, Choct, 2006, Khattak *et al.*, 2006, Latorre *et al.*, 2016). Therefore probiotics that produce glycosyl hydrolase enzymes are of great interest, furthermore because they are not naturally produced by chickens (Guo *et al.*, 2013).

This enzyme study confirmed that the putative probiotics of interest all produced the four key enzymes of importance to digestibility and feed conversion efficiency (Murugesan *et al.*, 2014). Three out of the four best performing isolates in the enzyme production assays were also the best performers in the feed breakdown test, whilst the worst enzyme producers were poor. The impact of enzymes in increasing surface to volume ratio of the feed particles, which enhances energy conversion efficiency, is often overlooked in screening studies, but we have successfully shown this for our best enzyme producing isolates (Amerah *et al.*, 2007).

2.5.3.2 Adherence potential to gut epithelium cells

The results of our study indicated that our isolates possess moderate adherence capabilities which correlate to the finding of Thirabunyanon and Thongwittaya (2012). Moderate attachment was also realised *in vivo* by Latorre *et al.*, (2014) in a broiler study investigating germination, persistence and distribution of *Bacillus* spores throughout the GIT. Contrastingly higher adherence to epithelial cells (> 70%) was reported by Chaiyawan *et al.*, (2015). However, adherence studies on *Bacillus* spp. remain sporadic in literature preventing validation of acceptable adherence levels in functional probiotics. Adherence to the epithelial cells of the host offers a competitive advantage as the attachment improves the residence time and thus the probiotic effect in the gut (Bernet *et al.*, 1994, Servin and Coconnier, 2003). Attached organisms are beneficial, as the flow of digested feed due to peristalsis, hinders probiotic activity if the cells are not attached, especially because the residence time is relatively short in chickens (Hughes, 2008). Additionally, chicks used for broiler production are hatched in artificial incubators and as such their GIT is pioneered entirely by exogenous organisms (Pan and Yu, 2014). This presents an opportunity for the use of probiotics to colonize the GIT of day-old chicks and reduces the potential for exogenous pathogen attachment. (Ouwehand *et al.*, 1999). As *Bacillus* cells are moderately adherent, transient presence in the GIT of chickens needs to be maintained by continuous administration and higher levels of efficacy.

25.3.3 .Antagonistic activity against common poultry pathogens

Approximately 45% of isolates screened, displayed superior antagonism against *E. coli*, a further 27% produced average activity and the remaining isolates (~ 27%) showed no inhibition and were actually inhibited by the pathogen. It bodes well that the majority of putative probiotics tested showed antagonism against *E. coli*, as its infection (particularly the O157:H7 strain) in broilers causes serious commercial losses in poultry production (Kiranmayi *et al.*, 2010). All isolates tested, showed antagonistic activity against *S. enteritis*, albeit at varying levels, which is important as *S. enteritis* is the most prevalent disease causing pathogen in the poultry industry (Boyle *et al.*, 2007, Finstad *et al.*, 2012, Dhama *et al.*, 2013a). Results from our study correlate well with other research showing antagonism against *E. coli* and *S. enteritis* (Guo *et al.*, 2006, Thirabunyanon and Thongwittaya, 2012, Latorre *et al.*, 2016).

Approximately 54% of the isolates tested were antagonistic towards *L. monocytogenes* and this could be commercially relevant in the reduction of Listeriosis which is becoming a serious threat, as epidemics are occurring worldwide. Currently, it is becoming imperative to screen for antagonism against *Listeria* and our study contributes substantively to the limited information available on antagonism of *Bacillus* based probiotics against this pathogen (Dhama *et al.*, 2013b). Similarly, ~54% of the isolates tested, showed antagonistic activity to *C. perfringens*. Although not detrimental to humans, *C. perfringens* has fatal effects on poultry as it is the cause of clinical necrotic enteritis (NE), (necrosis of the intestine), which is highly infectious and can lead to serious economic losses (Immerseel *et al.*, 2004). Unlike the other pathogens, the *C. perfringens* study was conducted under both aerobic and anaerobic conditions because this organism grows best under obligate anaerobiosis and thus provided the best probiotic-to- pathogen challenge conditions (Rasko *et al.*, 2005, Stahly *et al.*, 2006). The tests confirmed the inhibition of this pathogen under both anaerobic and aerobic conditions.

Based on the overall antagonism results of the 11 isolates against the four pathogens of interest to the poultry industry, CPB 011 resulted in the highest score. Isolates CPB 035, CPB 020 and HP 1.6 also showed promise as antagonistic agents against all pathogens because their antagonistic activity was similar to CPB 011 and clustered in the above average data subset. In contrast, CPB 010, CPB 004, CPB 018 and CPB 002 showed poor antagonism in the overall rating and upon examination of the data, each of these strains were inhibited by at least two of the pathogens, indicating the lack of suitability of these isolates as probiotics.

As antagonism through antibiosis is no better than the addition of commercial antibiotics to poultry feed, especially in organic chicken production, it was essential to infer the mode of action of our putative probiotics against pathogens. For this reason, we further tested each isolate against each pathogen using a cross-streak methodology and confirmed the unlikelihood of antibiotic production because of the absence of zones of inhibition. It is thus likely that the antagonism is due to competitive exclusion of the pathogen related to nutrients or spatial competition. This concept was also described by others where it was shown that aggressive growth, space and nutrients are likely reasons for competitive exclusion (Hibbing *et al.*, 2010, Cray *et al.*, 2013).

2.5.4 Final selection and safety of putative probiotics

The final selection from the 11 candidate probiotics resulted in a core consortium (CPB 011, CPB 020, and CPB 029) and included three ancillary isolates (CPB 035, HP 1.6 and D 014), based on the significant differences between the two groups in overall performance (Table 2.1). Many authors have used similar strategies (Barbosa *et al.*, 2005, Wolfenden *et al.*, 2010, Chaiyawan *et al.*, 2015, Nguyen *et al.*, 2015), but few have used an elimination strategy to circumvent screening a large number of isolates as demonstrated in this study. In probiotic selection, a key factor to consider is that one excellent probiotic characteristic does not surpass the value of a holistic range of probiotic effects (Chapman *et al.*, 2011), especially if a multi-mode product is the end objective. In response to this, our selection strategy yielded a six-strain consortium that comprised the full complement of desirable characteristics and hence offers the poultry industry a convenient single product solution (Fontana *et al.*, 2013). Identification of our consortium revealed four *B. subtilis* and two *B. velezensis* strains with 99% sequence homology, confirming that the consortium selected in our study was safe (Harwood and Wipat, 1996, Fan *et al.*, 2017).

The findings of this study supported the development of a multi-strain probiotic product which was proven to be effective in an *in vivo* broiler field trial (Ramlucken *et al.*, 2019). This multi-mode consortium shows excellent potential to address the commercial challenges of the poultry industry.

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2.6 References

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CHAPTER THREE

PRODUCTION AND STABILITY OF A MULTI-STRAIN *BACILLUS* BASED PROBIOTIC PRODUCT FOR COMMERCIAL USE IN POULTRY

Uraisha Ramlucken, Santosh O. Ramchuran, Ghaneshree Moonsamy, Mapitsi Thantsha, Christine Jansen van Rensburg and Rajesh Laloo (2019). Production and stability of a multi-strain *Bacillus* based probiotic product for commercial use in poultry. *Journal of Industrial Microbiology and Biotechnology* (Manuscript submitted).

Production and stability of a multi-strain *Bacillus* based probiotic product for commercial use in poultry

Uraisha Ramlucken^{1,2,3}, Santosh O. Ramchuran¹, Ghaneshree Moonsamy¹, Christine Jansen van Rensburg², Mapitsi S. Thantsha³ and Rajesh Laloo^{1*}

¹ The Council for Scientific and Industrial Research, Biosciences, Pretoria, South Africa, 0001

² Department of Animal and Wildlife Science, University of Pretoria, Pretoria, South Africa, 0002

³ Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Pretoria, South Africa, 0002

3.1 Abstract

Probiotics can be effective alternatives to the prophylactic use of antibiotic growth promoters (AGPs) in response to industry and consumer concerns around the use of these molecules in poultry production. Studies on the suitability of *Bacillus* based probiotics are emerging and showing positive benefits, but information on the production technology is limited, hampering industry adoption of this technology. In response to this, we developed the production process for a novel multi-strain probiotic product previously shown to be effective in poultry field trials. All strains were cultivated to a spore concentration exceeding 1×10^{10} CFU.mL⁻¹. The spores of each strain were successfully harvested, processed into a powder intermediate and formulated into an end product with an overall recovery of 100% and an actual shelf life stability in excess of 1 year. The probiotic was shown to survive under simulated gut conditions and thereafter successfully incorporated into various types of broiler feed exceeding the desired concentration of 1×10^6 CFU.g⁻¹. Using efficient process technology and lower cost materials, this study presents a commercially relevant case for the potential adoption of probiotic products by the poultry industry.

Keywords: *Bacillus*; Biomass production; Feed-incorporation; Probiotics; Product formulation; Shelf life

3.2 Introduction

Probiotics used in animal rearing benefit animal health and vigour by improving digestion and absorption, reducing the pathogen load, regulating the immune system and generally improving the gastrointestinal tract (GIT) microbiome (Chaucheyras-Durand and Durand, 2009, Chapman *et al.*, 2011, Fijan, 2016, Grant *et al.*, 2018). These benefits have a positive effect on feed conversion efficiency, growth rate and carcass quality, which consequently improves production efficiencies (Bajagai *et al.*, 2016). Probiotics therefore provide a cost-effective alternative to the prophylactic use of antibiotic growth promoters (AGP), thus addressing industry and consumer needs.

We recently developed a multi-strain *Bacillus* probiotic product, which showed excellent performance in a broiler production trial (Ramlucken *et al.*, 2019). However, to ensure the commercial relevance of a probiotic product, the efficiency its production process, which includes high spore concentrations, productivities and high recoveries during the harvesting process at large scale must be proven (Lacroix and Yildirim, 2007, Laloo *et al.*, 2009). For a multi-strain product, it is imperative that the fermentation process requires high cell density cultivation (HCDC) for each strain typically exceeding 1×10^{10} spores.mL⁻¹ (Monteiro *et al.*, 2005) to minimise production cost and account for losses in the downstream processes of cell harvesting and product formulation. The cell harvesting process must also be efficient in terms of throughput and recovery of viable spores and should generally be greater than 80% (Laloo *et al.*, 2009, Laloo *et al.*, 2010a).

Formulation technology for probiotics is normally proprietary to industry, but the main requirement is that the probiotic product must be stable in a powdered form and commonly at a spore concentration of 1×10^9 spores.g⁻¹ (Teo and Tan, 2007, Jeong and Kim, 2014, Kim *et al.*, 2017). The powder formulation process includes mechanically intensive drying and mixing steps, which have potential detrimental effects on probiotic viability (Chávez and Ledebøer, 2007). Therefore the stability of the probiotic product during formulation and under industry relevant storage conditions remains a key requisite for successful commercial application, and the shelf life of such a product should ideally be no less than two years (Mattila-Sandholm *et al.*, 2002, Kosin and Rakshit, 2006).

Before a probiotic product can be included in poultry feed, it is pertinent to test its stability (viability and growth) under simulated GIT conditions, because the harsh conditions of this

environment can negatively affect its optimum functionality (Reuter, 2001, Bernardeau *et al.*, 2017). It is thus important to demonstrate that a probiotic can retain viability within the GIT and grow to a minimum concentration of 1×10^6 CFU.g⁻¹ (Millette *et al.*, 2013).

Lastly, in-feed probiotics must retain maximum viability during feed manufacturing because this process involves high pressures (138 to 552 kPa), high temperatures (76.7 to 93.3 °C) and intensive mechanical shear (Kosin and Rakshit, 2006, Cutlip *et al.*, 2008). Excessive losses in this process reduce the probiotic effect resulting in a negative impact on the cost of inclusion to benefit ratio, desired by the poultry industry. Literature dictates that probiotics must be delivered to the point of consumption at a minimum cell concentration of 1×10^6 CFU.g⁻¹ to elicit the desired probiotic effect (Tripathi and Giri, 2014, Haffner *et al.*, 2016).

Bacillus spp. have been proven to be robust and can be cultivated at high cell densities with excellent sporulation efficiency at industrial scale (Monteiro *et al.*, 2005). The spores of *Bacillus* spp. are resistant to environmental conditions such as extreme temperature, radiation, pH, pressure and toxic chemical agents, thus offering several advantages in process viability and stability (Abel-Santos, 2015). Thus most *Bacillus* based animal feed probiotics are manufactured, packaged, stored and administered as spores (Vasquez, 2016). This paper shows the successful production, harvesting, product formulation and in-feed stability of a multi-strain probiotic product for poultry and further verifies the viability and growth under simulated poultry GIT conditions. The findings of this study are amongst the few to comprehensively quantify the efficiencies in each of the process steps and therefore facilitates the commercial adoption of in-feed *Bacillus* based probiotic products.

3.3 Materials and Method

3.3.1 Culture storage and maintenance

Four *Bacillus subtilis* (CPB 011, CPB 029, D 014 and HP 1.6) and two *Bacillus velezensis* (CPB 020 and CPB 035) strains were individually cryopreserved as described by Acosta (2004) and stored in a -80 °C ultra-freezer (Forma™ 80000 series, Thermo scientific, USA) (Monroy *et al.*, 2004).

3.3.2 Probiotic biomass production using fermentation

3.3.2.1 Inoculum development

A cryo-culture of each isolate was thawed to ambient temperature and separately inoculated into 2 L Fern Bach flasks containing 700 mL of sterile Tryptone soy broth (TSB). Flasks were incubated at 37 °C and 180 rpm on a rotary platform shaker (Innova 2300 series, New Brunswick Scientific, USA) until the culture reached the desired optical density (OD_{660nm} between 4.0 – 5.0), measured using a photometer (Pharo 300 Spectroquant®, Merck, Germany). The purity of the culture was verified by quadrant streaking onto Tryptone soy agar (TSA) plates, which were incubated at 37 °C for 24 hours (which was the standard incubation conditions for all plate cultures, before use as an inoculum) and then the colony viewed under light microscopy.

3.3.2.2 Batch fermentation of strains

The batch fermentation process was performed according to method outlined by Lalloo *et al.*, (2009). Industrial medium, proprietary to the CSIR licensed production technology was used for this study. Media components were sterilized *in situ* in 30 L bioreactors (Braun, Biostat, Sartorius BBI systems, Germany) at 121 °C for 45 minutes prior to inoculation. The working volume of the reactor was ~ 24 L and data points for all process parameters were captured on-line using MFCS software (Sartorius BBI systems, Germany).

For the *B. subtilis* strains the temperature was set to 37 °C, the pH to 6.80, the pressure to 500 mBar and the dissolved oxygen was maintained at a minimum of 30% saturation. Aeration and agitation were ramped up from 1 to 2 $v.v^{-1}.m^{-1}$ and from 200 to 800 rpm respectively, over a period of five hours. The pH was controlled using 10% $v.v^{-1}$ H_2SO_4 or 25% $v.v^{-1}$ NH_4OH . For the *B. velezensis* strains, modified methods reported by Gao *et al.*, (2014) was used. The temperature was maintained at 37 °C, the pH at 7.00, pressure at 100 mBar and dissolved oxygen was maintained above 50% saturation. The aeration was controlled at 1 $v.v^{-1}.m^{-1}$ and agitation between 300 and 500 rpm. The pH was controlled using 10% $v.v^{-1}$ HCL or 25% $v.v^{-1}$ NaOH. The fermentation of each isolate was ended when the sporulation efficiency (SE) and target spore concentration exceeded 80% and 1.0×10^{10} spores.mL⁻¹, respectively. Fermentation broth was harvested into sterile 25 L drums and stored at 4 °C to be further processed within 24 hours.

3.3.3.3 Sampling and analysis

Fermentation samples (50 mL) were collected from the bioreactor at 3 hour intervals for analysis of mono-septic status and calculations of cell concentration (Equation 1) and SE (Equation 2), all using a microscope (Olympus BX40, Olympus, Japan). Cell and spore counts were determined using a counting chamber (Thoma®, Hawksley, UK). Mono-septic status was confirmed by standard streak plating on TSA.

$$\text{Cell conc. (Cell. mL}^{-1}\text{)} = \left(\frac{\text{Average Cell \#}}{\text{Volume}} \right) \times \text{Dilution factor}$$

Equation 1

$$\text{Sporulation efficiency (\%SE)} = \left(\frac{\text{\# of spores}}{\text{\# of spores} + \text{\# of cells}} \right) \times 100$$

Equation 2

The final viable spore concentrations for each strain at the end of fermentation were determined using a standard TSA plate count method and resultant colonies were enumerated using a colony counter (Bibby, Stuart scientific UK).

3.3.3 Cell harvesting post fermentation

The spores for each strain were harvested from the fermentation broth as outlined by Lalloo *et al.*, (2010a). Briefly, the broth was mixed for 1 hour and centrifuged using a disk stack centrifuge (SA1, Westfalia, Germany) operated in continuous mode at $11\,000 \times g$ with a bowl pressure of 80 kPa and a de-sludge time of 2 minutes. The OD_{660nm} of the flow through material was monitored and the feed flow rate appropriately adjusted to maintain the supernatant OD_{660nm} below 3.0, which equated to less than 5% of the spore concentration of the starting material. The resultant spore concentrate at the end of a single pass was re-suspended into stabilization buffer and then washed two more times using the same method. The volumes of the feed, supernatant and spore concentrate fractions were measured and the associated spore concentrations used to determine total spores for each of the three passes through the centrifuge. Viable spore concentration was used to calculate total spores using Equation 3 and subsequent recoveries using Equation 4. Statistical analysis was done using a simple two tailed t-test using equal variances and the 95% confidence level.

$$Total\ viable\ spores = CFU.mL^{-1} \times Volume$$

Equation 3

$$Recovery\ (\%) = \left(\frac{Total\ spores_{out}}{Total\ spores_{in}} \right) \times 100$$

Equation 4

3.3.4 Formulation of the multi-strain probiotic product

The resultant spore concentrates of each strain harvested by centrifugation were processed into a dried product intermediate by mixing the liquid pellet with an inert clay (Biocentric Technologies, South Africa) using a spiral dough mixer (Esmach ISE 30, Labotec, South Africa). This material was subsequently convection dried for 20 hours at 60 °C in an oven (United Scientific, South Africa) and then pin milled (IKA, Germany) into a fine powder (particle size of ~200 µm). The final multi-strain probiotic powder containing all six strains was produced by blending (Lenton, South Africa) the respective mass of each powder intermediate with a calcium carbonate- dextrose carrier (99:1), to achieve a normalised concentration of 1×10^9 CFU.g⁻¹ in the final product, verified by plate counts. Total viable spores, recoveries and statistical analysis was done, as described in Section 3.3.3.

3.3.5 Stability analysis of the probiotic product

The final probiotic product was filled into 1 kg plastic bags (Low density polyethylene, 70 µM), sealed and then 25 bags were packaged into a double walled corrugated cardboard box which was sealed using paper gum tape and stored under ambient warehouse conditions (OptimusBio (Pty) Ltd, South Africa). At production and 12 months later, viable spore concentrations of composite samples randomly extracted from the bags were determined.

3.3.6 Survival and growth of the multi-strain probiotic product in a simulated gastrointestinal tract (GIT) model system

The final probiotic powder product was tested under simulated GIT conditions using filter sterilised simulated gastric fluid (SGF) prepared according to Lian *et al.*, (2003) and sequentially simulated intestinal fluid (SIF) prepared according to United States Pharmacopeial (2005).

Finely crushed commercial broiler feed (50 g) (AFGRI, South Africa) was added to the SGF (50 mL) at a ratio of 1:1 and the mixture homogenized for 5 minutes using a T18 homogeniser (Ultra Turrex, IKA, Germany). Water (100 mL) was added to the resultant mixture at a ratio of 2:1, which represents the fed state within the broiler stomach. The mixture (150 mL) was added to a 250 mL Erlenmeyer flask and the test probiotic powder (0.05 g) was added to the reaction mixture to simulate a starting cell concentration of $\sim 1 \times 10^5$ spores.mL⁻¹ in the stomach. The reaction mixture was then incubated for 4 hours at 42°C on a platform shaker (Innova 2300 series, New Brunswick, Canada) with agitation of 50 rpm. It was subsequently transferred to the Erlenmeyer flask (500 mL) containing 300 mL of SIF adjusted to a pH of 6.8 and agitated at 100 rpm, for a further 12 hours. Samples (2 mL) were withdrawn from the flask at time points 0, 2, 4, 6, 9 and 12 hours for analyses of viability and morphological (spores or vegetative) state of cells.

3.3.7 Survival of the multi-strain probiotic product through the feed manufacturing process

Broiler feeds for different growth phases were produced in eight ton batches, whereby the probiotic powder (1 kg/ton) was added to the feed ingredients of composition described by Ramlucken *et al.*, (2019). The components were mixed for 30 minutes, extruded into pellets at temperatures ranging from 75 to 80 °C and then crumbled to different sizes depending on the feed type. A composite sample of each feed type including associated probiotic negative control feeds was treated by adding 1 g of crushed feed into 9 mL of sterile saline, and homogenised for 5 minutes. The homogenate was heat treated at 60 °C for 15 minutes in a water bath (Labotec, South Africa). Viable spore concentration was determined by the standard plate count method on TSA plates. The negative controls were used to correct for background microbial load from the feed ingredients.

3.4 Results

3.4.1 Probiotic biomass produced through batch fermentation

The overall fermentation performance of each strain is illustrated in Figure 3.1. All the isolates exceeded the target viable spore concentration of 1×10^{10} CFU.mL⁻¹ (based on commercial requirements) (Figure 3.1a). In addition, the cell productivity of each isolate was greater than the target productivity of 1×10^8 CFU.mL⁻¹ per hour (Figure 3.1b). The target sporulation efficiency (> 80%) was also exceeded for all six isolates produced (Figure 3.1c).

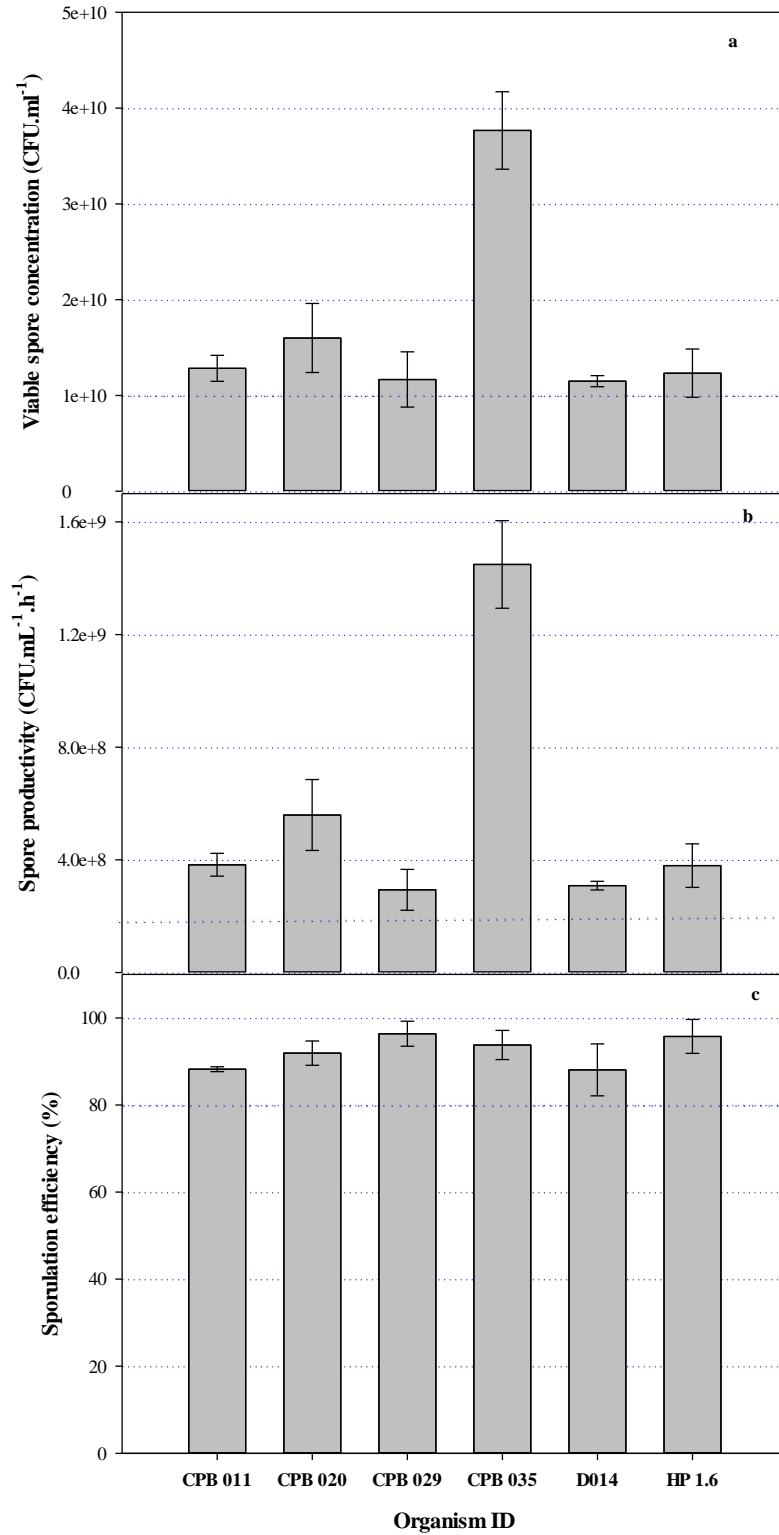


Figure 3.1: Performance parameters of the *Bacillus* strains during fermentation; (a) viable spore concentration, (b) spore productivity (c) sporulation efficiency. The dotted line represents the target performance of each parameter. Error bars expressed as \pm the standard deviation (n=3).

3.4.2 Cell harvesting post fermentation

Table 3.1: Total viable spore count and recovery of each strain.

Strain designation	Total spores <i>in</i> (CFU)	Total spores <i>out</i> (CFU)	p value	Recovery (%)
CPB 011	3.11×10^{14}	3.11×10^{14}	0.11	100
CPB 020	3.40×10^{14}	2.98×10^{14}	0.26	88
CPB 029	3.23×10^{14}	3.19×10^{14}	0.17	99
CPB 035	9.14×10^{15}	8.23×10^{15}	0.49	90
HP 1.6	3.40×10^{14}	3.32×10^{14}	0.12	98
D014	2.79×10^{14}	2.62×10^{14}	0.17	94

All strains except CPB 020 displayed total cell recoveries above 90% through the cell harvesting process unit operation, which resulted in the lowest recovery (88%) (Table 3.1). Nevertheless, this strain still exceeded the minimum threshold recovery of 80%. There were no significant differences ($p > 0.05$) between the total spores into the process and the final spores recovered for all strains during the harvesting process.

3.4.3 Formulation of the product

3.4.3.1 Formulation of powder intermediates for each strain

Table 3.2: Recovery of the viable spores of each strain during powder product intermediate process.

Strain designation	Total spores <i>in</i> (CFU)	Total spores <i>out</i> (CFU)	p value	Recovery (%)
CPB 011	3.11×10^{14}	3.26×10^{14}	0.64	105
CPB 020	2.98×10^{14}	3.50×10^{14}	0.17	118
CPB 029	3.19×10^{14}	3.40×10^{14}	0.16	106
CPB 035	8.23×10^{15}	9.61×10^{15}	0.99	117
D 014	3.32×10^{14}	3.39×10^{14}	0.81	102
HP 1.6	2.62×10^{14}	3.13×10^{14}	0.17	119

No significant differences ($p > 0.05$) were observed between the total spores into and out for each of the intermediate powders produced (Table 3.2). Spore recoveries of all strains through the process exceeded 100%.

3.4.3.2 Blending of the powder intermediates of each strain into a final probiotic product

The final probiotic product containing the powder intermediates of all the six strains had a spore concentration of 1.45×10^9 CFU.g⁻¹, which exceeded the minimum threshold target concentration of 1×10^9 CFU.g⁻¹.

3.4.4 Stability of the multi-strain probiotic product

The probiotic product retained a cell concentration of $1.9 \times 10^9 \pm 8.12 \times 10^8$ CFU.g⁻¹ after a period of 12 months post production, which was not significantly different ($p > 0.05$, $n = 9$) to the concentration of the fresh product which had a viable spore concentration of 1.45×10^9 CFU.g⁻¹.

3.4.5 Survival of the multi-strain probiotic product in a simulated GIT model system

The initial cell concentration of the product at T₀ was $\sim 1 \times 10^5$ CFU.mL⁻¹ to simulate the approximate concentration present in the stomach of the chicken. Microscopic evaluation revealed that the probiotic remained dormant and in a spore state in the simulated gastric environment (0-4 hours), followed by rapid growth in the intestinal environment between 4 and 6 hours (Figure 3.2). In the initial stage of the intestine, the cells were confirmed to be actively vegetative, followed by a transition from vegetative cells back into spores (6 to 12 hours). The maximum viable cell concentration reached was $\sim 1 \times 10^7$ CFU.mL⁻¹ resulting in an approximate 100-fold increase in viable cells from the initial probiotic dose.

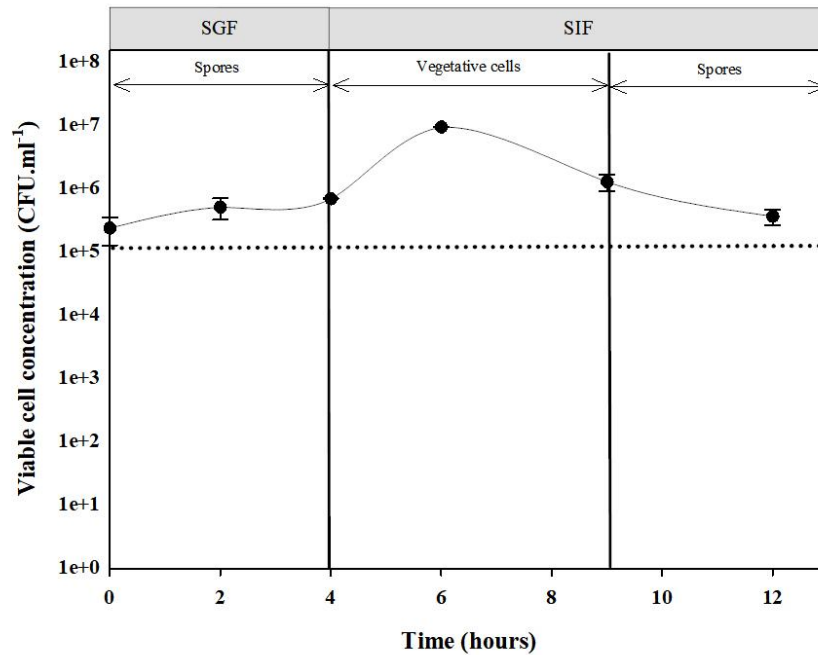


Figure 3.2: The survivability of the probiotic product under simulated GIT conditions. Time period 1 to 4 hours indicated simulated stomach conditions and 4 to 12 hours indicated simulated intestinal conditions. Error bars represent the standard deviations (n =5).

3.4.6 Survival of the multi-strain probiotic product through the feed manufacturing process

The initial and final viable spore count during the feed manufacturing process showed no-significant difference ($p > 0.05$) in any of the feed types produced. The resultant final viable spore concentration in all the feed produced exceeded the minimum threshold concentration of 1×10^6 CFU.g⁻¹ (Figure 3.3).

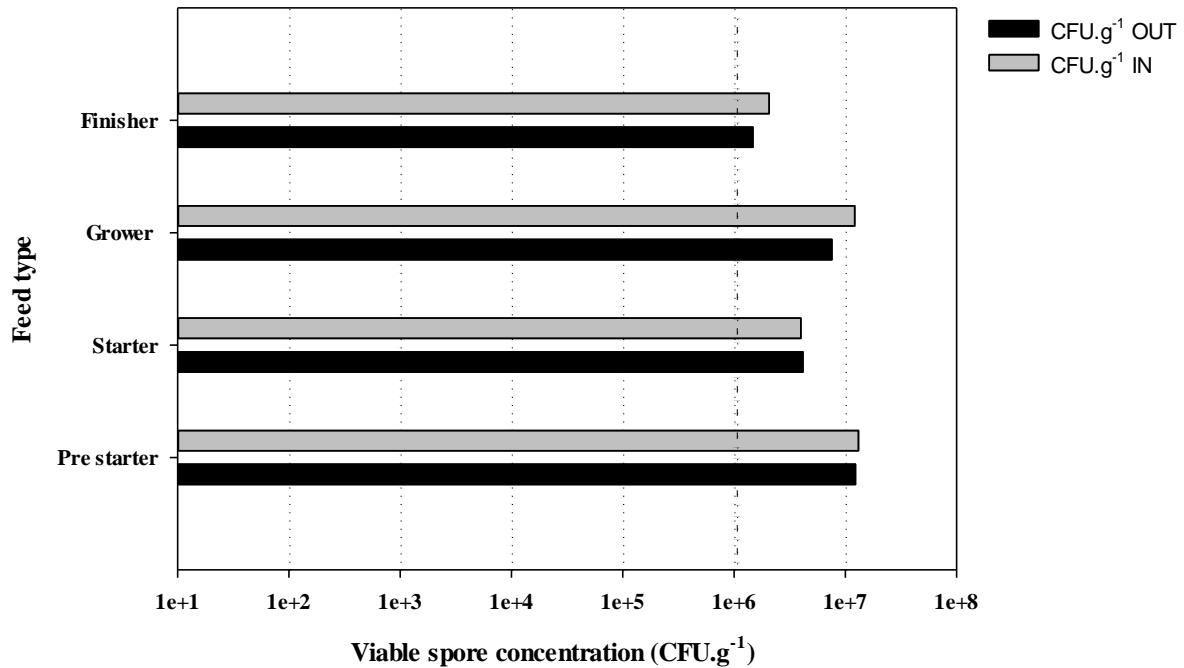


Figure 3.3: The viable spore concentration (n=5) of the probiotic product during the manufacturing of different feed types (pre starter, starter, grower and finisher). The dotted line represents the target cell concentration (1×10^6 CFU.g⁻¹) of the probiotic in the feed.

3.5 Discussion

3.5.1 Production of the probiotic strains in fermentation

The spore concentration, productivity and SE for each individual strain achieved at the end of the fermentation process exceeded the targeted minimum threshold of 1×10^{10} CFU.mL⁻¹, 2.5×10^8 CFU.mL⁻¹.h⁻¹ and 80% respectively (Figure 3.1). The performance thresholds were based on previously reported production of *Bacillus* spp. (Monteiro *et al.*, 2005, Lalloo *et al.*, 2009), together with consideration of the techno-economic targets for acceptance by the poultry industry.

High spore concentrations and productivities are important as they impact on the cost of production, capital utilization efficiency and furthermore, accommodates for any losses in the subsequent downstream processes such as cell harvesting, probiotic product formulation and feed production. Strain CPB 035 resulted in the highest spore concentration and productivity, followed by CPB 020 (both *B. velezensis* strains), in contrast to the other four strains which were all *B. subtilis*, due to slower growth (longer time to end of exponential vegetative growth) of the latter species. There were no significant differences in spore concentration or

productivity between any of the *B. subtilis* strains ($p>0.05$), mainly due to similar growth rates (Sueoka, 1997).

It is important to achieve high SE in the fermentation process as the spore-form is the desired state regarding product stability and survival through the GIT. All strains in our study exceeded 80% SE (Figure 3.1c) under production conditions, indicating minimum loss of viability in the transition phase from vegetative cells to spores (Posada-Urbe *et al.*, 2015). There were no significant differences in the SE between the *B. velezensis* and *B. subtilis* strains ($p>0.05$). We were able to achieve high production performance in a batch process, which is simpler and less capital intensive than fed-batch processes typically used to attain such high cell densities (Elisashvili *et al.*, 2019). All strains were successfully produced under intensive industry conditions which influences the uptake of the technology (Amer and Utkhede, 2000, Keller *et al.*, 2001), especially in the poultry sector, which operates at high volume and low margin. Furthermore, our batch process was able to achieve this performance using lower cost media, thus increasing its attractiveness for industry adoption (Singh *et al.*, 2017).

Information regarding the production processes for *Bacillus* spp. as poultry probiotics is limited, however the production performance achieved in our study correlates well with other reports of high density cultivation of *Bacillus* spp. (Monteiro *et al.*, 2005, Lalloo *et al.*, 2009, Chen *et al.*, 2010).

3.5.2 Cell harvesting post fermentation

There were no significant differences ($p>0.05$) between the total spores into the centrifugation process and the total spores recovered in the spore concentrate, for any of the strains. Furthermore, spore recovery exceeded 80% for all strains (Table 3.1). Although cell harvesting by continuous flow centrifugation is the most preferred method by industry (Moodley *et al.*, 2014), there is still a degree of physical stress, such as pressure and mechanical shear exerted on the probiotic bacteria, due to the centrifugal force and pressure drop during desludging of the spore concentrate. These can have a negative effect on viability (Lange *et al.*, 2001). However, the results obtained during this study shows the robustness of the *Bacillus* spores as loss in viability was minimal. The high recovery of *Bacillus* spp. spores could be attributed for its ability to withstand pressures up to ~50 mPa (Aoyama *et al.*, 2005) and shear stress up to 500 Pa (Faille *et al.*, 2013) A similarly high recovery of *B. cereus* spores was previously demonstrated by Lalloo *et al.*, (2010a).

Downstream product processing is a key factor to consider during the production of biological products and it is essential to ensure that recovery steps are developed to minimize losses and process time (Moodley *et al.*, 2014). The efficient harvesting of cells has a major effect on economic feasibility of implementation of probiotic products (Brar *et al.*, 2006). With regards to production of probiotics for poultry production, there is limited information available on their survivability during the cell harvesting process but our study shows that this can be done successfully.

3.5.3 Formulation of the product

Each of the strains that were processed from the liquid spore concentrates into powder intermediates, resulted in no significant differences ($p > 0.05$) between the total spores added and the total spores in the dry powder product intermediates (Table 3.2). This is attributable to the robustness of *Bacillus* spores, which offers protection from the heat and mechanical shear, conferred by the spore exosporium and small acid-soluble DNA binding proteins, which are characteristic of this genus (Setlow and Setlow, 1995, Lalloo *et al.*, 2010a). The retained viability of spores during the powder intermediate manufacturing process lead to a viable spore recovery marginally exceeding 100% for each strain as the recovery was calculated on an as is basis (Table 3.2).

The final blended probiotic product was produced by incorporating the normalised concentration corrected mass of each of the individual probiotics powder intermediates to achieve a theoretical target of 1.0×10^9 CFU.g⁻¹, which was based on input from the feed and premix industry as well as specifications from other commercial probiotic products (Teo and Tan, 2007, Jeong and Kim, 2014, Lin *et al.*, 2017). The final viable spore concentration achieved in our probiotic product was 1.45×10^9 CFU.g⁻¹, which correlated well with the theoretical target concentration. A slightly higher final product spore concentration is desirable to circumvent the losses that occur during storage and eventual incorporation into the feed manufacturing process (Brar *et al.*, 2006).

A powder form of poultry probiotics is preferred by end users as it has advantages such as uniformity, stability, longer shelf life, ease of packaging and transportation, and easy incorporation into current feed manufacturing processes (Lalloo *et al.*, 2010a, Bhat *et al.*, 2013, Markowiak and Śliżewska, 2018). In our study we used calcium carbonate and dextrose as

carriers which are also used by other commercial probiotic products. Sugars such as dextrose have been known to be an effective protectant against oxidation damage (Schutyser *et al.*, 2012) whilst calcium carbonate helps the probiotic bacteria adhere to the intestinal wall and assists in lowering the acid content, thus increasing probiotic activity in the GIT (Zhao *et al.*, 2009, Trivedi *et al.*, 2015).

The formulation method developed in this study involved lower drying temperatures (~ 60 °C) and low shear blending, which preserved viability loss, in contrast to the higher losses using spray drying which causes more severe heat stress and dehydration (Chávez and Ledebøer, 2007, Yadav *et al.*, 2009). The high temperatures used in spray drying result in high mortality and inactivation of microorganisms, thus damaging the cytoplasmic membranes, cell walls, ribosomes and DNA (Chávarri *et al.*, 2012, Dong *et al.*, 2013), which can also occur even with heat resistant *Bacilli* spores (Zhou *et al.*, 2008). The use of our simple processing technique circumvents the conventional challenges associated with more costly carriers, protective agents and capital. There is limited information regarding the formulation of feed probiotic products as this is usually proprietary to industry, thus our findings contribute to this knowledge gap.

3.5.4 Stability of the multi-strain probiotic product

A shelf life study of the six-strain probiotic product, stored for 12 months under industry conditions, did not result in any measurable decline in spore concentration. The change in spore concentration between the initial and final samples showed no significant difference (1.45×10^9 vs 1.9×10^9 CFU.mL⁻¹, $p > 0.05$, $n=9$) indicating the stability of the spore product. These results show that since the probiotic product was stored at ambient temperature, it mitigates the use of storage at low temperatures which adds cost to the logistics (Vasquez, 2016). One of the technological requisites for feed probiotics, and a serious limiting factor of most conventional probiotics (Mattila-Sandholm *et al.*, 2002), is the ability to be stored under warehouse conditions (Markowiak and Śliżewska, 2018). Since our study mimicked typical industrial storage conditions, the stability of the probiotic product was verified and we could predict a shelf life of up to 5 years, using accelerated stability kinetics for *Bacillus* spp. (data not shown). It has been reported that free spores have a lower shelf life (Sorokulova *et al.*, 2008), but our product formulation approach successfully addressed shelf life constraints. Furthermore Mizak *et al.*, (2012) stated that probiotics in feed and premixes must be active for at least 4 months, a criteria that our probiotic has substantially exceeded.

3.5.5 Survival of the multi-strain probiotic product in a simulated GIT model system

The novel multi-strain probiotic product (in a spore state) was introduced to the simulated GIT model at a concentration of 1×10^5 CFU.mL⁻¹ to simulate the actual concentration of the probiotic when it reaches the stomach (Figure 3.2). The probiotic did not grow within the simulated stomach conditions (SGF), mainly due to low pH, but the spores retained full viability. When the spores were transferred into simulated intestinal conditions (SIF), the spores germinated at high efficiency into vegetative cells, which replicated rapidly and resulted in a ~2 log increase (Figure 3.2). The germination of spores could be attributable to both the favourable conditions of the SIF and the rich nutrients from the feed. These results infer that the probiotic product could survive the stomach conditions within the host and grow in the intestine, which is where probiotic needs to be metabolically active to elicit its beneficial effect (Clavel *et al.*, 2004, Bernardeau *et al.*, 2017).

Two hours after peak growth, the vegetative cells progressively reverted into the spore state, possibly due to nutrient depletion since the SIF was only dosed once with feed. The trend also showed a loss in viability, which is typical when vegetative cells transition into sporulation. Additional factors that could have contributed to decrease in viability include the antibacterial effect of bile salts or the enzymes (trypsin and pepsin) present in the SIF (Begley *et al.*, 2005, Tam *et al.*, 2006). The spore viability however did not decline to lower than the initial dosage concentration of 1×10^5 CFU.mL⁻¹ (Figure 3.2). The results obtained in this study are consistent with the gastrointestinal life cycle of *Bacillus* as reported by Bernardeau *et al.*, (2017). This suggests that the probiotic needs to be continuously administered within the feed to confer maximum benefit (Latorre *et al.*, 2014).

3.5.6 The survival of the multi-mode probiotic product through the feed manufacturing process

The probiotic was successfully incorporated into the different types of feed, and results show that all feeds exceeded the target cell concentration of 1×10^6 CFU.g⁻¹ (Figure 3.3). This target is based on literature which states that in order to be effective, probiotics must be delivered to the point of consumption at a minimum cell concentration of 1×10^6 CFU.g⁻¹ (Tripathi and Giri, 2014, Haffner *et al.*, 2016) but higher dosages normally result in excessive costs of inclusion (Gomes and Malcata, 1999). Furthermore, spore recovery in the feed manufacturing process exceeded 70%, indicating the survivability of *Bacillus* spp., attributable to the thermostability, shear and pressure resistance of spores (Cutting, 2011). Because of the intensive physical stress

involved in poultry feed manufacturing, some degree of loss is inevitable, as shown in our data where there was a less than 30% loss, but we were still able to meet the minimum threshold of 1×10^6 CFU.g⁻¹ (Kosin and Rakshit, 2006, Bora *et al.*, 2009, Bhat *et al.*, 2013). The feed manufacturing process is a critical limiting factor for most conventional in-feed probiotics, thus hampering implementation in the poultry industry, but our results show potential for the feasible inclusion of *Bacillus* based probiotics in feed as was also previously reported by Simon *et al.*, (2005).

3.6 Conclusion

The results of this study endorse the use of *Bacillus* based in-feed probiotics as it confirms that all strains could be produced at high efficiency, were stable with minimal losses during the various processing steps and were successfully incorporated into various types of broiler feed. The study furthermore concluded the survival and growth of the strains under GIT simulated conditions. In a separate broiler field study the actual functionality of the probiotic was verified, showing improved feed efficiency, weight gain and gut morphology (Ramlucken *et al.*, 2019). We further demonstrated excellent product stability under industry relevant storage conditions, therefore comprehensively showing the suitability for commercial adoption by the poultry industry, of this novel multi-strain probiotic product.

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CHAPTER FOUR

THE EFFICACY OF THE PROBIOTIC PRODUCT WHEN TESTED IN A BROILER FIELD STUDY

Uraisha Ramlucken, Santosh O. Ramchuran, Ghaneshree Moonsamy, Rajesh Lalloo, Mapitsi S. Thantsha, and Christine Jansen van Rensburg (2019). A novel Bacillus based multi-strain probiotic improves growth performance and intestinal properties of *Clostridium perfringens* challenged broilers Poultry Science 99: 331-341. DOI:10.3382/ps/pez496.

A novel *Bacillus* based multi-strain probiotic improves growth performance and intestinal properties of *Clostridium perfringens* challenged broilers

Uraisha Ramlucken^{1, 2, 3}, Santosh O. Ramchuran¹, Ghaneshree Moonsamy¹, Rajesh Lalloo¹, Mapitsi S. Thantsha³ and Christine Jansen van Rensburg^{2*}

¹ The Council for Scientific and Industrial Research, Biosciences, Pretoria, South Africa, 0001

² Department of Animal and Wildlife Science, University of Pretoria, Pretoria, South Africa, 0002

³ Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Pretoria, South Africa, 0002

4.1 Abstract

There is a necessity for the implementation of in-feed probiotics in the poultry production industry, following strict regulations around the use of antibiotic growth promoters (AGP). *Bacillus* spp. are becoming an attractive alternative because of their functionality and stability. This study aims to evaluate the effect of a novel multi-strain *Bacillus* based probiotic on growth performance and gut health in male Ross 308 broiler chickens challenged with *Clostridium perfringens* Type A. Broilers on a four phase feeding program were fed diets containing either a standard metabolizable energy (ME) (100%) or a reduced ME (98%) level. The test probiotic was compared to an un-supplemented negative control and a commercial benchmark product as positive control over a 35 day feeding trial, using a 2 × 3 factorial experimental design. Chicks were inoculated with a once-off dose of *C. perfringens* on day 14. Growth performance was measured weekly to calculate body weight (BW), feed intake (FI) and feed conversion ratio (FCR). Villi histomorphology, gut lesions and liver weight were assessed at day 35. Broilers fed the reduced ME diet with the test probiotic achieved higher final body weights ($p = 0.037$) and FCR ($p = 0.014$) than the negative control. Broilers fed the standard ME diet with the test probiotic showed improved ($p = 0.001$) FCR than the negative control from day 21 onwards. Increased duodenal villi height ($p = 0.012$) and villi height to crypt depth ratio (VCR) in the duodenum ($p < 0.0001$) and jejunum ($p = 0.0004$) were observed in broilers fed the reduced ME diet containing the test probiotic. Additionally, the test probiotic resulted in significantly reduced relative liver weights in both ME groups. Consequently, the results suggest that the novel multi-strain *Bacillus* based probiotic enhanced broiler performance and improved gut health and is thus attractive as an alternative to AGP's in broiler production.

Key words: *Bacillus subtilis*; *Bacillus velezensis*; Broiler production; Probiotic

4.2 Introduction

Probiotics are becoming increasingly relevant in the poultry industry, due to the phasing out of antibiotic growth promoters (AGP) worldwide. Probiotics are “live microbes that, when administered in adequate amounts, confer a health benefit to the host” (FAO/WHO, 2002). Probiotics support a healthier digestive tract, due to varying modes of action, such as improving digestion, regulating intestinal microflora, improving gut barrier function, preventing gastrointestinal diseases and modulating the immune system (Edens, 2003, Kabir, 2009, Gaggia *et al.*, 2010). The genus *Bacillus* confers several advantages such as survival in the harsh feed manufacturing processes, longer shelf life, and robustness to the fluctuating conditions within the chicken gastrointestinal tract (GIT) (Cutting, 2011, Vasquez, 2016, Grant *et al.*, 2018, Mingmongkolchai and Panbangred, 2018).

It has been shown that *Bacillus* spp. resulted in improved broiler growth rate and feed utilization efficiency (Gil De Los Santos *et al.*, 2005, Sen *et al.*, 2012, Harrington *et al.*, 2016), They also enhance immune response (Yurong *et al.*, 2005, Huang *et al.*, 2008), confer protection against pathogens (La Ragione *et al.*, 2001, La Ragione and Woodward, 2003, Teo and Tan, 2005, Jayaraman *et al.*, 2013), colonize the GIT of chickens (La Ragione and Woodward, 2003) and improve the histomorphology of intestinal villi (Samanya and Yamauchi, 2002, Al-Baadani *et al.*, 2016). These effects endorse research into their use as natural alternatives to AGP products.

The ban on the use of AGPs in poultry production, has challenged the industry need to prevent poultry diseases. Although zoonotic diseases, such as salmonellosis and listeriosis, have negative effects on the industry, losses in broiler production is most severe due to *Clostridium perfringens* infections, which causes acute necrotic enteritis (NE), resulting in production losses (Immerseel *et al.*, 2004). Casewell *et al.* (2003) correlated an increased incidence of NE with the banning of AGPs. This pathogen also causes sub-clinical necrotic enteritis (SNE), presenting as necrotic dermatitis, cholangiohepatitis, gizzard erosion and lower production efficiency (Lovland and Kaldhusdal, 2001, Hafez, 2011, M'Sadeq *et al.*, 2015).

Feed contributes up to 70% of broiler production costs, therefore nutritionists continuously strive to decrease feed costs by incorporating cheaper raw materials with lower ME values (Świątkiewicz and Koreleski, 2008, Mendes *et al.*, 2013). To address this challenge, exogenous enzymes are added to feed to improve digestibility (Choct, 2006) with varying degrees of success (Leeson *et al.*, 1996). Probiotics may provide a solution to this problem by enhancing the digestibility of lower ME feeds (Torres-Rodriguez *et al.*, 2005).

When developing probiotics, the use of microorganisms indigenous to poultry is preferred, as it not only gives the best chance of probiotic survival and colonization of the GIT but also alleviates many of the challenges associated with the inclusion of foreign bacteria (Dunne *et al.*, 2001, Schrezenmeir and de Vrese, 2001). This study evaluates the *in vivo* efficacy of a novel multi-strain probiotic comprising of *Bacillus* spp. on growth performance and intestinal histomorphological parameters of broilers fed diets containing two ME levels. The broilers were exposed to *C. perfringens* at sub-clinical levels to challenge the birds such that the probiotic effect could be thoroughly evaluated.

4.3 Materials and Methods

4.3.1 Ethics approval

This study was approved by the Animal Ethics Committee of the University of Pretoria (South Africa) (EC005-18). Further approval from the Council of Scientific and Industrial Research (CSIR) (Pretoria, South Africa) Research Ethics Committee (248/2018) was granted prior to the commencement of the trial.

4.3.2 Bird husbandry

A total of 1,656 Ross 308 broiler chicks (males) were obtained from a commercial hatchery where they were vaccinated against New castle disease. Chicks were delivered to the 96-pen broiler test facility (Hillcrest, University of Pretoria, South Africa) the morning after hatched (day 1). The temperature and ventilation of the broiler test facility were constantly regulated by an automatic climate controller (Model DOL 539, Skov, Denmark) for the duration of the trial. Environmental temperatures were gradually decreased weekly from day 0 to day 21 and then maintained according to breeder guidelines (Aviagen, 2007) and litter temperature was checked twice daily. Each pen (2.25 m²) was covered with clean pine shavings and fitted with one tube feeder and five nipple drinkers. Chicks had easy access to feed and potable water *ad libitum* throughout the entire trial period. They were exposed to 23 hours of light up to 7 days

of age, then from there onwards they received 8 hours of darkness continuously in a 24-hour period. All birds orally received booster vaccines against New castle disease and Gumboro respectively, at day 14 and day 21 of age.

4.3.3 Experimental design

A 35-day trial was conducted under challenged conditions by infection with *C. perfringens*. Upon arrival at the test facility, broiler chicks were randomly allocated to six treatment groups, with 12 pen replicates for each treatment and 23 chicks per pen. Pen replications were arranged according to a randomized complete block design, with each of the 12 blocks containing one replicate per treatment. The average body weight of chicks at day 0 was 40 g and the weight did not differ significantly between any of the experimental groups.

4.3.4 Subclinical necrotic enteritis (SNE) challenge

All broilers were subjected to conditions previously described (Pedersen *et al.*, 2008, Lensing *et al.*, 2010, M'Sadeq *et al.*, 2015) for creating SNE. At an age of 10 days, the broilers received a coccidial vaccine containing live, attenuated oocysts of *Eimeria acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella* at 10 times the dosage prescribed by the manufacturer (Paracox-8, Schering-Plough, Kenilworth, NJ). On day 14, all birds were orally administered 1 mL liver broth containing 1×10^8 CFU.ml⁻¹ *C. perfringens* Type A. The *C. perfringens* culture was obtained from Thermo Fischer Scientific (USA) and grown at a biosafety level II facility at Deltamune (Centurion, South Africa).

4.3.5 Probiotic and dietary treatments

A six-strain probiotic containing four *Bacillus subtilis* (CPB 011, CPB 029, HP 1.6 and D 014) and two *Bacillus velezensis* (CBP 020 and CPB 035) strains was previously developed and provided by the CSIR (Biosciences, Pretoria, South Africa). The *Bacillus* spp. were selected for inclusion in the test probiotic product on the basis of their overall performance with regards to chicken probiotic attributes including growth and survival in the pH of the proventriculus and of the intestine, survival in bile salts, production of extracellular enzymes (amylase, protease, cellulase and xylanase), the adherence to epithelial cells and possession of antagonistic activity against selected common poultry pathogens (*Escherichia coli*, *Salmonella enteritis*, *Listeria monocytogenes* and *C. perfringens*). The selection criteria for the microorganisms contained in the test probiotic was based on a comprehensive review of previous studies (Ehrmann *et al.*, 2002, Barbosa *et al.*, 2005, Taheri *et al.*, 2009, Wolfenden *et*

al., 2010). The test probiotic efficacy was compared to a commercially available product containing a *B. subtilis* strain supplied by the manufacturer. The probiotics were added in powdered form to the mash feed at 50 g and 100 g per ton of feed for the commercial probiotic (specified as 2×10^9 CFU.g⁻¹) and test probiotic (specified as 1×10^9 CFU.g⁻¹), respectively. The viability of the *Bacillus* spp. in mixed feed was assessed using the standard plate count method. Prior to analysis, the feed samples were first subjected to heat treatment (55 °C for 20 minutes) followed by homogenization using a bench top homogenizer (T 25 digital ULTRA-TURRAX[®], IKA, Germany) to ensure that only *Bacillus* spores were counted.

This study was conducted on broilers reared on rations containing two different levels of ME, and either no probiotics or one of the two probiotic products, in a 2 x 3 factorial arrangement. ME content of the standard diet was formulated to meet or exceed recommendations for Ross 308 broilers. The reduced ME diet contained 98% ME of that of the standard diet. Probiotics were included in the diets from commencement to termination of the trial.

A four phase feeding program was followed: pre-starter (fed from day 1 to 7), starter (fed from day 7 to 14), grower (fed from day 14 to 28) and finisher (fed from d 28 to 35). The feed ingredients and formulated nutrient composition of the diets are shown in Table 4.1. The treatments were as follow: 1) Reduced ME diet with no probiotic (negative control), 2) Reduced ME diet + commercial probiotic (positive control), 3) Reduced ME diet + test probiotic, 4) Standard ME diet with no probiotic (negative control), 5) Standard ME diet + commercial probiotic (positive control) and 6) Standard ME diet + test probiotic.

Table 4.1: Feed ingredient (%) and calculated nutrient composition (%) of the basal diets.

Feed ingredient	Pre-starter		Starter		Grower		Finisher	
	Standar d ME	Reduce d ME	Standar d ME	Reduced ME	Standar rd ME	Reduce d ME	Standar rd ME	Reduce d ME
Yellow maize	60.7	60.8	61.7	63.4	67.8	68.3	71.1	71.7
Soya oil cake (46.5%)	30.3	29.2	30.6	29.7	24	21.3	19.8	17.4
Sunflower oil cake (36%)	3	4	2.5	3.2	4	7.2	5	7.9
Lysine (78%)	0.276	0.293	0.189	0.204	0.201	0.246	0.314	0.353
Methionine (98%)	0.261	0.257	0.213	0.209	0.173	0.164	0.199	0.19
Threonine (98%)	0.06	0.062	0.018	0.02	0.006	0.011	0.051	0.055
Soya oil (Degummed)	1.061	0	1.492	0	1.046	0	1.091	0
Wheat bran	1	2.2	0	0	0	0	0	0
Limestone	1.64	1.64	1.64	1.64	1.44	1.43	1.29	1.28
Mono-Di-Calcium Phosphate	0.81	0.79	0.82	0.81	0.51	0.5	0.31	0.3
Salt (Fine)	0.335	0.326	0.366	0.359	0.183	0.163	0.143	0.126
Sodium Bicarbonate	0.18	0.193	0.137	0.147	0.322	0.35	0.379	0.404
Axtra Phy10000 P (100 g/t)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Salinomycin (12%)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Vitamin and mineral premix	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Calculated nutrient levels								
Dry matter	89.22	89.13	89.22	89.06	89.03	89.00	88.97	88.92
ME Poultry (ckal/kg)	2849	2763	2894	2808	2930	2844	2971	2885
Crude protein	20.28	20.32	20.01	20.00	18.01	18.01	16.89	16.91
Crude fat	4.20	3.16	4.63	3.19	4.25	3.19	4.32	3.22
Crude fiber	4.24	4.50	4.08	4.22	4.20	4.70	4.26	4.72
Ash	5.60	5.61	5.59	5.57	4.70	4.68	4.16	4.15
Calcium	0.92	0.92	0.92	0.92	0.79	0.79	0.70	0.70
Phosphorous (total)	0.52	0.53	0.52	0.52	0.44	0.45	0.39	0.40
Sodium	0.18	0.18	0.18	0.18	0.16	0.16	0.16	0.16
Chloride	0.30	0.30	0.30	0.30	0.20	0.20	0.20	0.20
Potassium	0.85	0.85	0.84	0.84	0.74	0.73	0.68	0.67
Lysine	1.25	1.25	1.18	1.18		1.04	1.03	1.03

4.3.6 Feed manufacture and analysis

Feed was manufactured by SimpleGrow Agricultural Services (Pretoria, South Africa). The official methods of analysis (AOAC, 2000) were followed for analyses of dry matter (no. 934.01), ash (no. 942.05), crude protein (Leco-Dumas Method no. 986.06), crude fiber (no. 962.09), ether extract (number 920.39), calcium (no. 935.13) and phosphorus (no. 965.17) of the two basal diets from each of the three feeding phases. The nutrient composition of the feed is shown in Table 4.2. The apparent metabolizable energy (AME) was calculated using the following equation which was described by Alvarenga et al. (2015) (Equation 1):

$$\begin{aligned}
 AME_n(DM \text{ basis}) & \\
 &= 4.164.187 + 51.006EE(DM \text{ basis}) - 197.633ash \\
 &\quad - 35.689CF(\% \text{ inDM basis}) - 20.593NDF(DM \text{ basis})
 \end{aligned}$$

Equation 1

Table 4.2: Feed ingredient (%) and calculated nutrient composition (%) of the basal diets.

	Pre-starter		Starter		Grower		Finisher	
	(Standard ME)	(Reduced ME)	(Standard ME)	(Reduced ME)	(Standard ME)	(Reduced ME)	(Standard ME)	(Reduced ME)
Dry matter	89.23	89.33	89.55	88.65	88.73	88.58	88.60	88.25
AMEn (kcal/kg)	2,600	2,482	2,649	2,555	2,754	2,658	2,831	2,750
Crude protein	20.94	21.61	20.83	20.77	18.59	18.71	18.09	18.72
Crude fat	3.23	2.45	4.10	2.47	3.35	2.20	3.52	2.55
Crude fiber	4.71	4.78	4.76	4.29	4.34	4.86	4.05	4.55
Ash	5.30	5.65	5.30	5.28	4.43	4.45	4.08	4.03
Calcium	0.733	0.763	0.717	0.727	0.683	0.655	0.535	0.485
Phosphorous (total)	0.527	0.528	0.550	0.527	0.460	0.478	0.396	0.412

¹ Max CV < 20% between analyzed and calculated nutrient composition.

² AMEn = Nitrogen-corrected apparent metabolizable energy (as is basis)

4.3.7 Broiler performance

Body weight (BW) and feed intake (FI) were recorded weekly (day 0, 7, 14, 21, 28 and 35) as an average per pen. Weights of all the birds that died throughout the trial period were recorded. Feed conversion ratio (FCR) adjusted for mortalities, was calculated for each pen as feed intake (g) / BW gain (g) over a specified period of time. Mortality rate per treatment was calculated for the entire trial period.

4.3.8 Liver weights, lesion scoring and histomorphology of the gastrointestinal tract

All birds were weighed individually on day 35 and then two birds with a BW closest to the average of their respective pen were selected for sampling. Birds were sacrificed humanely by cervical dislocation and the GIT and liver were carefully removed from each bird for further investigation.

4.3.8.1 *Relative liver weight*

The livers of the birds were weighed on a precision balance (Model 1502e, Adam Equipment PGW, United Kingdom). Liver weight was expressed as the percentage of the birds' BW, and a pen average was calculated.

4.3.8.2 *Lesion scoring*

The entire GIT was carefully removed and opened by longitudinal incision. It was then examined for lesions possibly caused by NE and subsequently scored based on the severity of the lesions presented, using the guidelines described by Shojadoost et al. (2012).

4.3.8.3 *Histomorphology of the gastrointestinal tract*

Samples (2 cm in length) were taken from the duodenum, jejunum and ileum. Each section was carefully rinsed with 10% (v.v⁻¹) phosphate buffered formalin (Merck, Germany) to remove digesta and stored in sample bottles containing formalin. Samples were embedded in paraffin (Merck, Germany) and transverse sections of approximately 4 to 5 µm in thickness were cut and every 10th section was collected. These sections were transferred to microscope slides and stained with haematoxylin and eosin (Merck, Germany). Slides were viewed at a 5 × magnification with an AXIO Imager M2 light microscope (Zeiss, Germany). Images of 10 randomly selected intact villi per section were taken and villi height and crypt depth measured using Image J software. Villus height was measured from the tip of the villus to the villus-crypt junction, and crypt depth was defined as the depth of the invagination between adjacent villi. Villi height (µM) to crypt depth (µM) ratio (VCR) was calculated.

4.3.9 Statistical analysis

Statistical analysis was performed using the statistical analysis system (SAS) statistical software program (Statistical Analysis System, 2014). The significance between treatments was determined by an analysis of variance (ANOVA) with the general linear model. Means and standard error of means for the different treatments were calculated and differences ($p < 0.05$)

between means were determined by Fischer's test (Ryman, 2006) at the 95% confidence level. In all cases the level of statistical significance was $p < 0.05$. Differences between treatments for mortalities were calculated with a chi square.

4.4 Results

4.4.1 Analysis of feed

The nutrient analyses of the feeds produced for this study are presented in Table 4.2. The resultant nutritional values for all parameters were similar to the theoretically calculated values as per Table 4.1 (max CV < 20%).

4.4.2 Broiler performance

Within the treatment groups that received the standard energy diets there were no significant differences observed with BW and FI (Table 4.3). However, the broiler groups supplemented with either the commercial or the test probiotics resulted in significantly improved FCRs compared to the un-supplemented group (without probiotics) after day 35. Furthermore, the group supplemented with the test probiotic resulted in significantly improved FCR over the un-supplemented control group, consistently from 21 days onwards.

Within the treatment groups that received the reduced ME diets, the test probiotic treatment resulted in a significantly higher final BW (day 35) compared to the negative control ($p = 0.03$) and was 4.1% higher than the commercial probiotic, but this difference was not significant (Table 4.3). FCR within the reduced ME diets were positively influenced by supplementation of the test probiotic from day 28 onwards, resulting in significantly better FCR than the negative control ($p < 0.05$). The FCR of the test probiotic was also 4.3% better than the commercial probiotic but the difference was not significant.

The benefit of probiotic supplementation on broilers fed with the reduced ME diet is illustrated in Figure 4.1. In all of the responses (BW, FI and FCR), the test probiotic supplementation resulted in improved performance than the un-supplemented and commercial probiotic treatment groups, but was only significantly better than the un-supplemented group for final BW and FCR.

The total mortality rate for all six experimental groups was only 3.7% for the duration of the trial and no effect of treatment could be correlated to mortality.

A significant effect of probiotic treatment was noted for FCR at day 35 (Table 4.3). Irrespective of probiotic supplementation, a suppressing effect on FI from day 21 onwards was observed, when broilers were fed a reduced ME diet in comparison to the standard ME diet ($p < 0.0001$). No significant interaction effects between ME level and probiotic treatment were observed.

Table 4.3: Growth performance of broilers fed diets containing either a standard (100%) or reduced (98%) metabolizable energy level with or without *Bacillus* spp. based probiotics.

	Standard ME			Reduced ME			SEM	Probiotic Treatment	P value ME	Energy × Probiotic Treatment
	Without Probiotics	Commercial Probiotic	Test Probiotic	Without Probiotics	Commercial Probiotic	Test Probiotic				
Body weight (g)										
Day 1	40.87	40.69	40.5	41.05	41.05	40.65	0.23	0.242	0.233	0.883
Day 7	177.8	178.9	179.5	177.6	177.1	177.4	1.75	0.904	0.340	0.844
Day 14	448.5	451.3	450.7	443.1	442.6	446.8	5.04	0.845	0.152	0.885
Day 21	827.4	820.5	821.5	818.1	803.1	817.4	9.54	0.635	0.122	0.756
Day 28	1,366 ^{ab}	1,363 ^{ab}	1,388 ^a	1,349 ^{ab}	1,324 ^b	1,367 ^{ab}	18.01	0.170	0.087	0.808
Day 35	1,906 ^{ab}	1,940 ^{ab}	1,957 ^{ab}	1,881 ^b	1,892 ^{ab}	1,974 ^a	30.66	0.065	0.470	0.564
Feed intake (g/bird)										
Day 0-7	174.4	176.5	172.7	177.7	180.6	174.9	2.78	0.239	0.167	0.941
Day 0-14	522.1	520.2	514.3	527.5	530.3	526.1	6.46	0.963	0.488	0.771
Day 0-21	1,222 ^a	1,164 ^b	1,160 ^b	1,116 ^b	1,126 ^b	1,121 ^b	19.7	0.166	0.001	0.312
Day 0-28	2,218 ^a	2,163 ^a	2,181 ^a	2,021 ^b	2,026 ^b	2,050 ^b	30.5	0.682	<0.0001	0.505
Day 0-35	3,203 ^a	3,144 ^{ac}	3,176 ^{ad}	2,992 ^b	2,977 ^b	3,065 ^{bcd}	43.9	0.393	<0.0001	0.526
Feed conversion ratio (g: g)										
Day 0-7	1.27 ^{ab}	1.28 ^{abc}	1.22 ^b	1.30 ^{ac}	1.33 ^c	1.28 ^{ac}	0.02	0.023	0.005	0.732
Day 0-14	1.58 ^{abc}	1.53 ^{ab}	1.50 ^a	1.59 ^{bc}	1.62 ^c	1.58 ^{abc}	0.03	0.757	0.043	0.599
Day 0-21	1.72 ^b	1.62 ^a	1.63 ^a	1.60 ^a	1.61 ^a	1.58 ^a	0.03	0.074	0.137	0.120
Day 0-28	1.78 ^d	1.72 ^{cd}	1.69 ^{bc}	1.64 ^{ab}	1.65 ^{ab}	1.63 ^a	0.02	0.142	<0.0001	0.099
Day 0-35	1.78 ^c	1.69 ^b	1.68 ^b	1.69 ^b	1.68 ^{ab}	1.61 ^a	0.02	0.002	0.0003	0.263

^{1. a to d} Values without a common superscript in the same row differ significantly (p< 0.05, n=12)

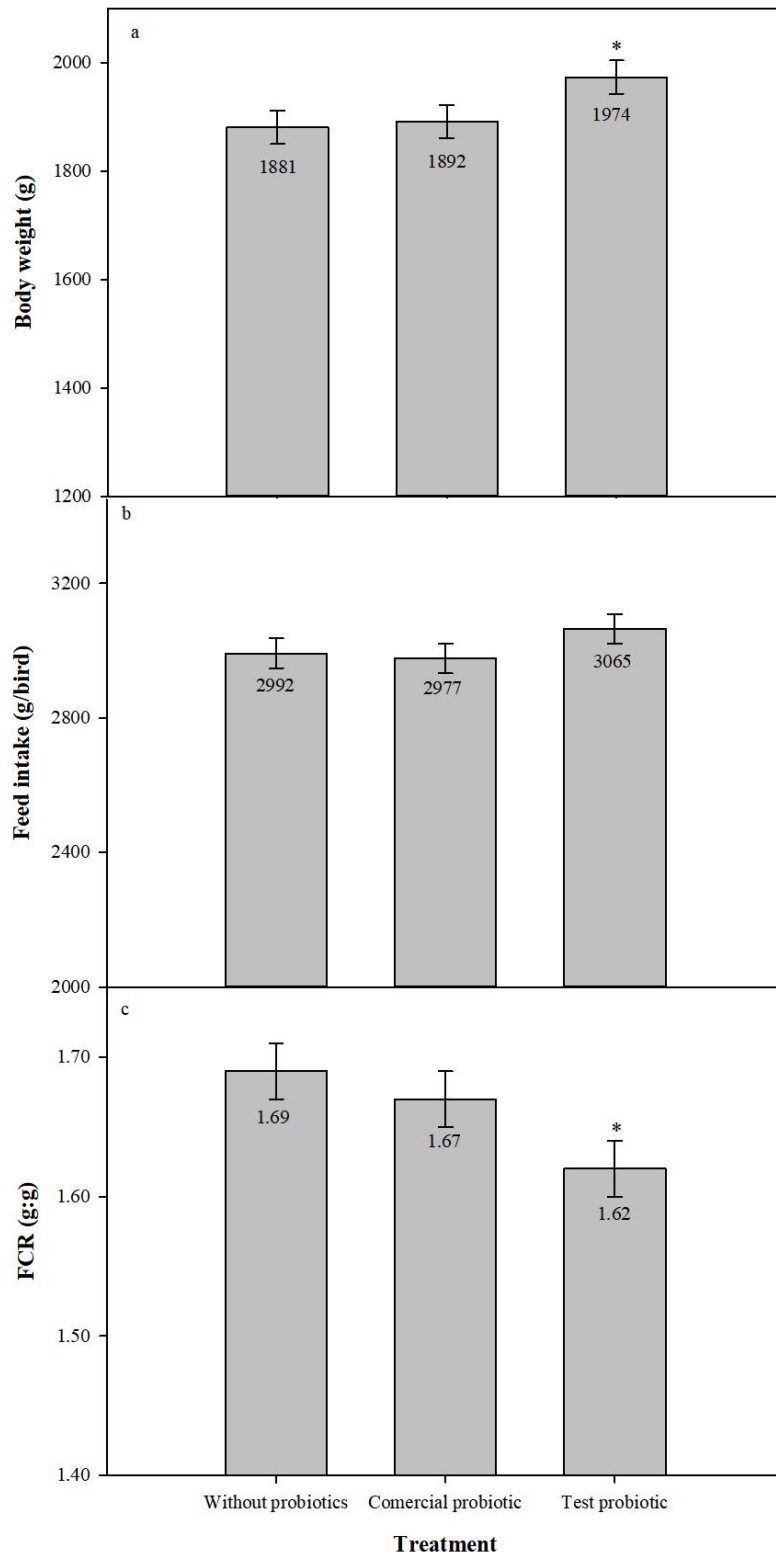


Figure 4.1: The broiler performance of birds fed the reduced ME diet supplemented with or without probiotics. (a) The body weights of birds at day 35 (b) The cumulative feed intake from day 0 - 35 and (c) The cumulative mortality adjusted FCR from day 0 - 35. * are represented as significant difference ($p < 0.05$) over the control.

4.4.3 Lesion scoring and liver weight

In broilers fed the standard ME diets, the cumulative lesion scores across the three regions of the small intestine (duodenum, jejunum and ileum) were 22, 17 and 19 for the un-supplemented control, the commercial probiotic and the test probiotic treatments respectively. Similarly, in the reduced ME diets the cumulative lesion counts were 32, 12 and 26 respectively. No significant differences were found in the numbers and severity of lesions between any of the treatment groups.

For both levels of dietary ME, the livers of broilers that received the test probiotic weighed significantly less than those of both the commercial probiotic and un-supplemented groups (Table 4.4). Irrespective of probiotic treatment, the ME content of the diets had a highly significant ($p < 0.0001$) effect on the liver weight of broilers when expressed as a percentage of BW. The livers of broilers that received the reduced ME diets were heavier than those fed the standard ME diet.

4.4.4 Histomorphology of the gastrointestinal tract

In broilers fed the standard ME diet, there was no significant difference in villi height or crypt depth of the small intestine between any of the treatments (Table 4.4). However broilers fed the standard ME diet supplemented with the commercial probiotic resulted in significantly higher villi height to crypt depth ratios (VCR, $\mu\text{M}:\mu\text{M}$) within the duodenum and ileum when compared to the negative control. Similarly, the test probiotic treatment also resulted in higher ratios, but the differences were not significant.

Broilers fed the reduced ME diets containing the test probiotic, displayed significantly longer duodenal villi when compared to both the un-supplemented ($p = 0.01$) and the commercial probiotic ($p = 0.03$) groups (Figure 4.2). Both duodenal and jejunal crypt depths of broilers fed either of the probiotics supplemented in the reduced ME diets, were significantly shallower than in the un-supplemented treatment group. Additionally, the VCR in both the duodenum and the jejunum were significantly higher for the broilers that received either one of the probiotic treatments under reduced ME conditions (Table 4.4 and Figure 4.2).

Table 4.4: Intestinal villi histomorphology and liver weight of broilers fed diets containing either a standard (100%) or reduced (98%) metabolizable energy level with or without *Bacillus* spp. based probiotics.

	Standard ME			Reduced ME			SEM	Probiotic Treatment	P value ME	Energy × Probiotic Treatment
	Without Probiotics	Commercial Probiotic	Test Probiotic	Without Probiotics	Commercial Probiotic	Test Probiotic				
Duodenum										
Villi height (μM)	1,634 ^b	1,834 ^{ab}	1,807 ^{bc}	1,664 ^b	1,711 ^b	1,936 ^{ac}	73.9	0.015	0.844	0.238
Crypt depth (μM)	195.3 ^b	184.0 ^{bc}	200.2 ^b	252.6 ^a	165.4 ^c	201.8 ^b	9.43	<0.0001	0.088	0.0006
Villi: crypt (μM: μM)	8.430 ^c	10.24 ^{ad}	9.138 ^{cd}	6.713 ^b	10.52 ^a	9.763 ^{ac}	0.49	<0.0001	0.499	0.042
Jejunum										
Villi height (μM)	1184 ^{ab}	1166 ^{ab}	1,275 ^a	1,093 ^b	1,179 ^{ab}	1,243 ^{ab}	59.7	0.126	0.459	0.684
Crypt depth	174.1 ^b	182.9 ^b	195.5 ^{ab}	219.0 ^a	133.5 ^c	171.1 ^b	11.9	0.007	0.325	0.0006
Villi: crypt (μM: μM)	6.853 ^a	6.883 ^a	6.524 ^a	5.108 ^b	8.925 ^c	7.477 ^a	0.45	0.0003	0.258	0.0003
Ileum										
Villi height (μM)	775.3 ^{ab}	801.1 ^{ab}	872.2 ^a	758.5 ^b	818.3 ^{ab}	857.0 ^{ab}	39.4	0.054	0.880	0.889
Crypt depth (μM)	176.8	151.9	179.1	195.9	158.2	190.1	17.6	0.144	0.401	0.936
Villi: crypt (μM: μM)	4.437 ^{bc}	5.493 ^{ad}	4.953 ^{cd}	3.915 ^{bc}	6.133 ^a	4.895 ^{bd}	0.36	0.0002	0.946	0.281
Liver weight (%)	2.32 ^b	2.44 ^{bd}	2.06 ^c	2.61 ^{ad}	2.68 ^a	2.35 ^b	0.06	< 0.0001	<0.0001	0.930

^{1. a to d} Values without a common superscript in the same row differ significantly (p < 0.05).

² Data represents means based on 12 replicates per treatment.

³ Villi: crypt = villi height to crypt depth ratio.

⁴ Liver weight expressed as percentage of body weight

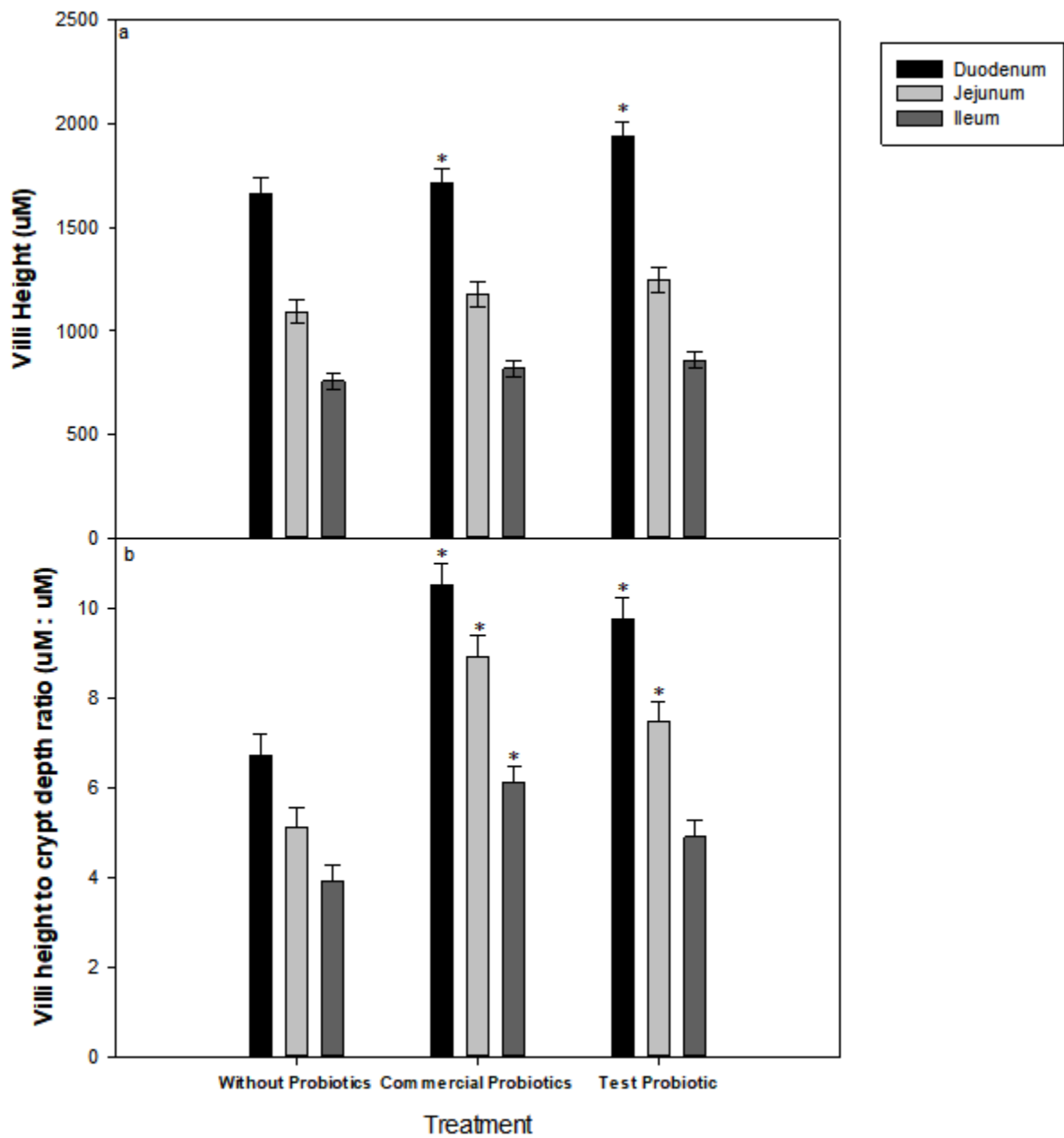


Figure 4.2: The histomorphology of the small intestine of broilers fed the reduced ME diet supplemented with or without probiotics, showing effects on the duodenum, jejunum and ileum. (a) Represents the villi height (b) the villi height to crypt depth ratio. * represents significant difference ($P < 0.05$) over the control.

4.5 Discussion

The main aim of the present trial was to evaluate a novel multi-strain *Bacillus* based probiotic in an AGP-free environment and to compare the efficacy to an un-supplemented control. Furthermore a commercially available *Bacillus* based benchmark product, was used as a positive control.

When the test probiotic product was evaluated within standard ME diets against the un-supplemented control, the test probiotic resulted in a significantly improved FCR ($p < 0.001$). Although not significantly different from the commercial probiotic, the absolute FCR value for the test probiotic was also better (Table 4.3). The improved FCR in both the test and commercial probiotic supplemented groups, was ascribable predominantly to higher body mass gain when compared to the un-supplemented control, indicating improved nutrient and energy utilization due to probiotic supplementation. Other researchers have also shown the positive effect on FCR, when studying *Bacillus* based probiotics supplemented into standard ME basal diets (Mountzouris *et al.*, 2010, Liu *et al.*, 2012, Zhang *et al.*, 2013, Zaghari *et al.*, 2015, Li *et al.*, 2016).

Interesting, this study showed a significant improvement in FCR, but the differences in FI and BW were insignificant, possibly due to the nutrient sufficiency of the standard ME diet coupled to the optimal environmental conditions of the field trial. These test conditions potentially attenuate the full probiotic effects, which are more likely to be realized under challenging commercial broiler production conditions. Infante-Rodriguez (2016) published similar findings, showing that broilers receiving a standard energy diet, under ideal growth conditions, showed a significant improvement in FCR compared to diets with a moderate decrease in ME, but not in BW, for similar reasons.

In the groups fed the reduced ME diets, the test probiotic performed better (BW and FCR, Table 4.3) than within standard ME diets, when compared to the un-supplemented control. There was no significant difference in the FI between any of the treatments ($CV < 1.5\%$ at 35 day), indicating that the main contributor to enhanced FCR was actually BW gain. This can thus infer that the test probiotic enhanced the efficiency of nutrient adsorption and utilization, predominantly ascribable to the production of exogenous enzymes by the test probiotic. The test probiotic comprised of strains that displayed a high diversity and production level of digestion enzymes (amylase, protease, cellulase and xylanase) (U. Ramlucken, CSIR, Pretoria,

Gauteng, personal communication). *Bacilli* are known to produce numerous digestive enzymes which allow for the efficient breakdown of feed into smaller molecules thus improving the efficiency of absorption and assimilation (Latorre *et al.*, 2016). Another probiotic characteristic that may have attributed to improved performance, could have been superior survival and persistence in the GIT, which enhances the probiotic effect.

When comparing different ME diets, the probiotic effects on broiler performance, was enhanced in the reduced ME diets. The reasons for this remain unclear, however, one of the probable causes is the greater impact of exogenous enzyme activity in lower energy diets, compared to diets which provide sufficient nutrients. Goodarzi Boroojeni *et al.* (2018) also concluded that the probiotic effects were more pronounced in nutrient-deficient diets, when testing a *B. subtilis* probiotic on broilers. Furthermore, the observations were consistent with previous findings by Harrington *et al.* (2016), who also reported that broilers supplemented with *B. subtilis*, resulted in improved final BW and FCR in reduced ME diets (2%). In their study, an economic saving of \$0.018/kg BW gain was calculated when broilers were fed a *B. subtilis* probiotic incorporated into a reduced ME diet (2% ME reduction). Knap *et al.* (2011) showed that *B. subtilis* supplementation significantly improved FCR, but not BW, in reduced ME diets (4% ME reduction). This study further contributes to the existing body of evidence indicating commercial attractiveness of probiotic supplementation particularly in reduced ME diets.

In reduced ME diets, the duodenal villi height was significantly greater ($p < 0.05$) in broilers fed diets containing the test probiotic compared to the un-supplemented control. The resultant greater villi height, ascribable to probiotic treatment, increased the intestinal surface area and enabled more efficient absorption of available nutrients (Figure 4.2). This is potentially one of the contributing factors to the improved broiler performance (BW and FCR) (Figure 4.1). The improvements in gut morphology and exogenous enzyme activity, are likely to be the major contributing factors to the improved FCR due to the test probiotic supplementation.

VCR was also significantly improved in the duodenal and jejunal compartments by supplementation of both the test and commercial probiotic when compared to the un-supplemented control. Longer villi and a greater VCR are indicators of a healthy GIT (Xu *et al.*, 2003, Lei *et al.*, 2015). Besides increasing the surface area for optimal nutrient absorption, these indicators of gut morphology are also directly associated with improved epithelial

turnover and activation of cell mitosis (Samanya and Yamauchi, 2002). The crypt is responsible for the synthesis of villi and a deeper crypt indicates a higher demand for tissue turnover, which is an energy consuming process, resulting in poorer energy efficiency. Shorter villi and deeper crypts lead to poor nutrient absorption, presence of toxins, an increase of mucus secretion in the GIT, reduced disease resistance and overall decreased broiler performance (Sen *et al.*, 2012). The results from our study indicate improved gut health and contributing to enhanced broiler growth performance. These results correlate with other studies (Samanya and Yamauchi, 2002, Sen *et al.*, 2012, Lei *et al.*, 2015, Li *et al.*, 2018) who also reported similar gut morphology observations due to supplementation with *Bacillus* based probiotics.

Depression of broiler performance due to *C. perfringens* infection was not demonstrated in this study because inclusion of an uncontaminated control group in the same facility was not possible. However, judged by poor overall performance of all broilers challenged with this pathogen compared to breeder standards (Aviagen, 2007) and previously recorded performance for this genetic strain at the same facility, *C. perfringens* infection and its consequent SNE posed a mild challenge. Besides a reduction in broiler performance, subclinical *C. perfringens* infection may also cause enlargement and inflammation of the liver, typically associated with hepatitis or cholangiohepatitis (Hafez, 2011). Increased liver weight may be indicative of the presence of subclinical infection (Lovland and Kaldhusdal, 2001, Klaric *et al.*, 2018). The smaller livers in broilers fed diets supplemented with the test probiotic may indicate a higher resistance of these birds to the *C. perfringens* challenge. However, due to a lack of liver histopathology data in this study, we cannot, based solely on the liver weight of the birds, infer that the test probiotic reduced *C. perfringens* infection, but that there are potential health benefits indicated by significantly reduced liver weight in birds treated with the test probiotic. A coccidiostat was added to all treatments to safe guard against severe coccidiosis. In South Africa, the broiler industry currently supplements most broiler feed with both an AGP and a coccidiostat. For the purpose of this study we only replaced the AGP with the novel probiotic to keep the test feed as close to the commercial feed as possible. Future trials will evaluate the probiotic effect on exclusion of both AGP and coccidiostat.

The purpose of the study was not to specifically investigate the response of broilers to dietary ME concentration, nevertheless, some interesting effects of ME level on broiler performance were observed. Analyzed crude fat content followed the same pattern as formulated values for crude fat (Table 4.2), which confirmed the feeding of the respective groups with the intended

ME levels. The standard ME diet was supplemented with soya oil to create the differences in dietary energy levels. Since, diets were formulated on an iso-protein basis, a higher CP: ME (g: kJ) ratio was expected for the reduced ME diets. Lower ME level resulted in a significantly higher FI from day 21 to slaughter. According to the theory of feed intake and growth proposed by Gous *et al.*, (1999), broilers would attempt to grow at their genetic potential, and therefore would consume a given feed at a level that would allow them to grow to that genetic potential. It appears as if the standard ME diet with the lower CP: ME ratio was somewhat limiting in CP, which restricted growth rate to below genetic potential during the last two to three weeks of the feeding trial, thus resulting in the difference observed. Burnham *et al.* (1992) showed that broilers increase FI in response to a limiting nutrient (CP) in the feed, in an attempt to obtain more of the limiting nutrient. The broilers in the standard ME (100%) group thus consumed higher levels of feed and thus energy, possibly causing higher levels of lipid deposition (not measured).

Reduced dietary ME, resulted in higher relative liver weight % (of bird body weight) in comparison to the standard ME diets. The lower oil content in the reduced ME diet could have been a further contributory factor for liver enlargement and increased incidence of SNE. In contrast, the soya oil added to the standard ME diet could have somewhat protected the broilers against *C. perfringens* proliferation and restricted liver enlargement. Also in the standard ME diet, higher lipid intake may have contributed to the increased production of bile acids (Smits *et al.*, 1998, Staessen *et al.*, 2019), which can elicit an antibacterial effect in the small intestine, thus attenuating the challenge from *C. perfringens* (Inagaki *et al.*, 2006, Geng and Lin, 2016).

4.6 Conclusions

The multi-strain *Bacillus* probiotic product improved growth performance and generally had a positive effect on *C. perfringens* challenged-broiler well-being, indicated by gut and liver health observations. Furthermore, its probiotic effect appeared to be better than the commercial probiotic product. Some of the key driving factors for industry adoption include improved performance in cheaper reduced ME diets and better resistance to *C. perfringens* challenge. Our results further substantiate the attractiveness of multi-strain *Bacillus* probiotics as a replacement to other undesirable in-feed growth promoting and antibacterial additives. Future considerations include testing the novel probiotic under commercial conditions, as well as elucidating the modes of action of its functionality.

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CHAPTER FIVE

GENERAL CONCLUSIONS, IMPACT AND FUTURE RECOMENDATIONS

5.1 General conclusions

The following conclusions were drawn from the research findings

- A total of 48 isolates belonging to *Bacillus* spp. was successfully obtained from various poultry related environments, providing adequate probiotic candidates for further study. All isolates obtained were verified as *Bacillus* spp. confirming the suitability of the isolation protocols. Four of the six strains were identified as *B. subtilis* and the remaining two as *B. velezensis* at the genome level. The results also confirm the association of *Bacillus* species with poultry and poultry production environment. Since it has proven that the more efficient probiotics are those isolated from the host or its environment, there is potential that the strains will deliver the envisaged benefits in poultry production.
- A six-strain consortium developed satisfied multiple industry relevant criteria, including digestive enzyme production, commercial feed breakdown, adherence to gut epithelial cells and antagonism against key poultry pathogens.
- All six strains were successfully produced at high cell density exceeding 1×10^{10} CFU.ml⁻¹ at industrial scale. Sporulation efficiency and productivity exceeded 80% and 2.5×10^{10} CFU.ml⁻¹.h⁻¹ respectively for all strains, resulting in a successful commercial production process based on industry measures. All strains were harvested at a recovery exceeding 80% confirming an efficient cell harvesting process. Strains were successfully formulated into a powdered product at a concentration exceeding 1×10^9 CFU.g⁻¹ with a mathematically predicted shelf life exceeding 5 years under normal industry storage conditions. This product and process development research yielded a probiotic product that is potentially attractive to the poultry feed and premix industries. The ability to be produced at industrial scale is an important technological criterion for feed probiotics as it critical limitation for many probiotics. The data obtained indicated that the novel probiotic is suitable for industry adoption. Additionally, the extended shelf life of the probiotic makes it feasible for use by industry.
- The probiotics were able to survive and grow under simulated GIT conditions, indicating the potential viability and thus functionality within the host. This is an important prerequisite study before embarking on *in vivo* testing because if the product does not germinate and grow in these conditions it will not survive in the host thus not be functional as probiotics.

- The probiotic product was successfully incorporated into various types of broiler feeds, under existing feed manufacturing conditions. The final probiotic concentration in all of the feed types exceeded the industry specification of 1×10^6 CFU.g⁻¹. Feed manufacturing is a critical limitation of most probiotics due to the harsh physical conditions of the process. The novel probiotic displayed robustness through this process therefore proving their suitability as in-feed probiotics.
- The efficacy of the probiotic was successfully demonstrated in a broiler field trial that confirmed different probiotic effects with regards to performance and health. The probiotic performed best in reduced ME diets resulting in significant improvements in body weight and FCR by ~4.7%. This is desirable as most poultry producers want to reduce cost of feed and utilize lower ME diets. Furthermore, the probiotic supplementation resulted in significantly improved gut morphology, indicated by increased duodenal and jejunal villi height of 14 and 12% respectively which enhances gut health and nutrient uptake efficiency. The villi height to crypt depth ratio was also significantly improved by 31% in probiotic-treated broilers, further confirming improved gut health.

5.2 Impacts of the study

- The isolation screening and selection procedures used in this study provides a successful rational for other research into *Bacillus* based probiotics.
- The product and processes developed fills a significant gap in the limitations of information available around the commercial production of *Bacillus* based poultry probiotics.
- This research project resulted in 4 publications disseminating important applied information to the both the biotechnology and poultry industries.
- The study has furthermore resulted in a commercial royalty bearing technology licence to a commercial producer of biologicals, who are in the process of registering the products and collaborating with probiotic suppliers.
- The product is being evaluated in field studies with local and international partners, as a means to potential commercial roll-out of the product.

5.3 Future recommendations

- Further sampling based on wider biodiversity of birds, including wild bird populations, should be considered to yield the next generation of probiotics.
- The exact modes of action of probiotic strains needs further research to enhance existing knowledge and understanding of the mechanisms involved.
- The evaluation of the auxiliary effects such as degradation of aflatoxins in feed, and ammonia in the gut, should be studied using the probiotic to explore additional industry relevant benefits.
- The probiotic production processes need to be scaled to industrially relevant production volumes.
- The probiotics showed *in vitro* antagonism against *E. coli*, *S. enteritis* and *L. monocytogenes* and *C. perfringens* but this was not confirmed *in vivo*. Further broiler studies are required in this regard.
- Testing of the probiotic product in the emerging field of *in ovo* stimulation of the microbiome should be explored.
- Since field testing in this study was done under controlled experimental conditions, the probiotic product should be further tested at industrial scale with actual production rearing conditions.
- The probiotic product should be tested in associated avian species to explore potential value in a wider range of birds
- The probiotic could potentially be beneficial to other monogastric animals and this should be evaluated in future studies.