Advantages of the use of Bacillus based probiotics in poultry production

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Bacillus probiotics for poultry production

Summary

Broiler production is one of the most lucrative food industries globally, due to the demand for poultry products. Regulations on the use of antibiotic growth promoters (AGP) in animal husbandry are becoming stricter and have been banned in some countries. As a result, probiotics provide a more suitable alternative as growth promoting agents. Bacillus based probiotics, mostly due to their spore forming ability are attractive alternatives to conventional probiotics. These organisms have shown to elicit a myriad of probiotic effects, which include but are not limited to the reduction in the prevalence of poultry pathogens, aiding in digestion and absorption due to the production of various exogenous enzymes and immunomodulation benefits. Furthermore, there are advantages in the cost and efficiency of the isolation, selection and development of processes. Additionally, many *Bacillus spp*. are safe and the spores are tolerant to the harsh conditions of the GIT. 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 cell density, survive the conditions of downstream processing and retain viability when formulated into probiotic products. In addition, the ability of spores to retain metabolic activity and regenerate upon application allows for stable storage and longer product shelf life.

Key words: Bacillus, probiotics, broiler, production, industrial application

Introduction

The poultry industry is amongst the largest meat industries globally, 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, consequently exposing poultry to stressful conditions, resulting in 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 (phytogenics), enzymes, essential oils, and prebiotics. The benefits of these alternatives are covered extensively in reviews (Gadde *et al.*, 2017a, Huyghebaert *et al.*, 2011, Sethiya 2016). Despite some successes in broiler health and production, these additives contribute considerably to the cost of poultry production, necessitating the need for alternative products (Yang *et al.*, 2009).

Probiotics are an attractive alternative as an 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 utilisation, feed conversion ratio (FCR), reduce the prevalence of disease and improve the holistic health and vigour in poultry. Furthermore, being safe and natural, probiotics do not risk the well-being of poultry or consumers with ongoing use (Ghadban 2002, Kabir 2009, Patterson and Burkholder 2003).

The most abundantly used probiotics in broiler production are *Lactobacillus spp.* and *Bifidobacterium 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, Kabir 2009, Patterson and Burkholder 2003). 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 results in reluctance for 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, due to their attractiveness. 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.

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 ochratoxicosis, coccidiosis and mycotoxins, protection against bacterial infections and are devoid of issues with regards to transmission of antibiotic resistance (Czerucka *et al.,* 2007, Gao *et al.,* 2008, Reddy *et al.,* 2005). *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 (Audisio *et al.,* 2000, Franz *et al.,* 2011, Samli *et al.,* 2007, Wendt *et al.,* 1998). *Bifidobacteria* also indigenous to chickens assist in reducing pathogen transmission and produce beneficial compounds (Baffoni *et al.,* 2012, Jung *et al.*, 2008).

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 has been shown extensively (Haghighi *et al.,* 2006, Jahromi *et al.,* 2016, Jin *et al.,* 1996, Jin *et al.,* 1998, Kabir *et al.,* 2004, Kalavathy *et al.,* 2003, Mookiah *et al.,* 2014, Pascual *et al.,* 1999, Timmerman *et al.,* 2006, Tsai *et al.,* 2005).

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 fatness, all crucial parameters required for probiotic acceptance in the poultry industry. A study by Haghighi *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 as reported by Santini *et al.,* (2010) who demonstrated the *in vitro* survival of only two of 11 different *Bifidobacterium* and Lactobacillus strains tested in a simulated gastric environment. In another 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. Furthermore, a tolerance to bile was shown by *Lactobacillus spp.* however there was low viability in simulated gastric juice (Martin *et al.,* 2018).

Besides issues of viability within the GIT, most conventional probiotics have disadvantages in their production and in subsequent downstream production processes, mainly due to the fragile vegetative state, which is more susceptible to physical parameters such as pH, temperature, pressure, oxygen and mechanical sheer. Feed probiotics need to be produced at much larger quantities than those used for human consumption, as larger quantities are required for animal cultivation, and as a result need 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 (Chin *et al.*, 2015, Doleyres and Lacroix 2005, Lacroix and Yildirim 2007, Saarela *et al.*, 2004).

The poultry industry prefers the use of stable powdered products for various reasons such ease of handling and incorporation into the feed, easier administration to the birds and more 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 dry processing 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.*, *Bifidobacterium* 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 specialised 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 ºC, whereas the tolerant temperature range of some *Lactobacillus spp.* is only 60-65 ºC (Teixeira *et al.*, 1997), which results in the destruction of the majority of viable cells (Kosin and Rakshit 2006). Other classical probiotics such as *Enterococcus spp.* and *Bifidobacterium* have been shown to withstand temperatures

between 50- 60 ºC and are therefore destroyed during the higher temperature processes involved in feed manufacture (Lian *et al.,* 2002, Simon *et al.,* 2005).

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

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 species is their inherent ability to form spores that resume viability under favourable conditions. Bacilli are renowned work horses of industry with applications in almost every sector (Schallmey *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 that they are predominately aerobic, questioning their ability to proliferate within the anaerobic regions of the small intestine (Cutting 2011). To illustrate, the ceaca region of the poultry gut, is predominantly anaerobic, and 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 (in place of oxygen) 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 elucidated that Bacilli are found within the chicken GIT and thereafter a study by Cartman *et al.,* (2008) has proven that *B. subtilis* are able to germinate in the chicken GIT. Furthermore, there have been various other reports of *Bacillus spp.* isolated from the GIT of

chickens (Chaiyawan *et al.,* 2015, Nguyen *et al.,* 2015, Wolfenden *et al.,* 2010), mitigating the reservations of the survival of this species within the gut.

Other concerns centre around the ability of *Bacillus spp.* to elicit a probiotic effect, as *Lactobacillus spp.* have been considered as the gold standard with regards to beneficial effects not only to poultry applications, but also in humans. Newer information provides evidence of *Bacillus spp.* showing probiotic characteristics in several *in vitro* and *in vivo* studies (Cutting 2011, Grant *et al.,* 2018, Hong *et al.,* 2005). The poultry industry is swiftly moving towards the use of Bacillus based probiotic products, mostly because of its ease of use. Many companies have successfully commercialized Bacillus based poultry products as listed in Table 1, and 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: *Bacillus spp.* probiotics used in the poultry industry

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

Modes of action of *Bacillus spp.*

Bacillus species have a wide range of beneficial features which can be categorised 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 mechanism of action through which *Bacillus sp.* 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).

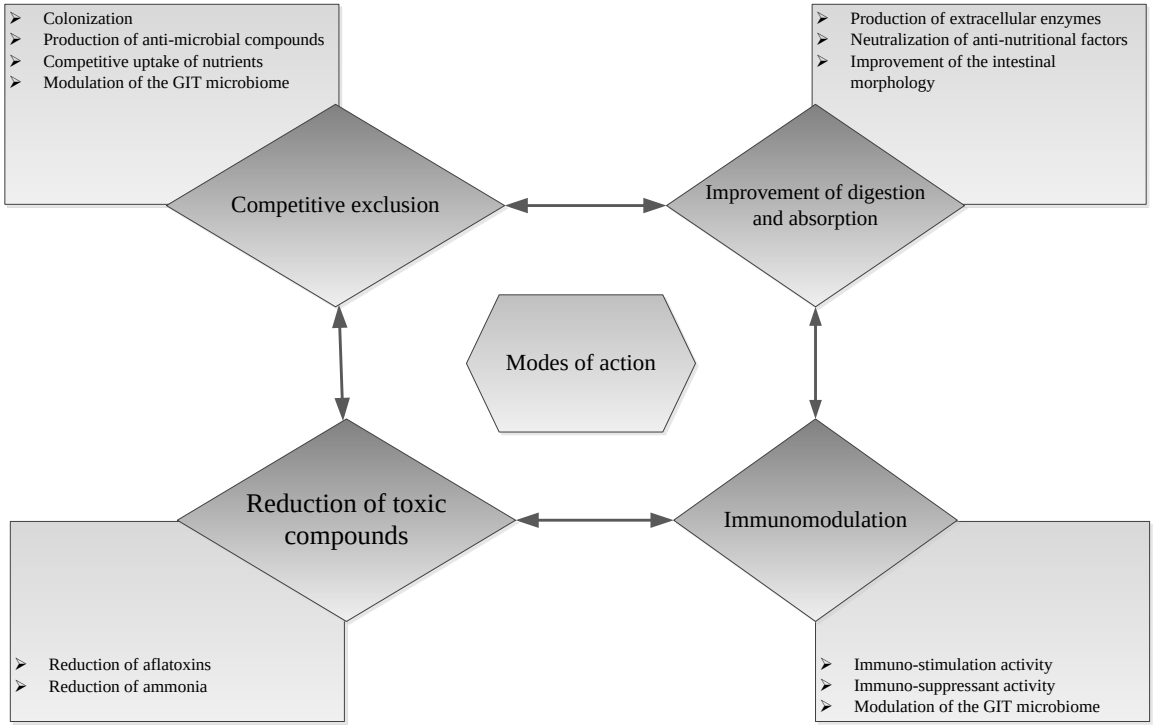

Figure 1: Modes of action (diamonds) of Bacillus probiotics and associated mechanisms of actions (boxes) relevant to the poultry industry

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 and synergistic interactions of two or more of the above mechanisms (Bermudez-Brito *et al.,* 2012, Callaway *et al.,* 2008). 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 as one of the methods 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 colonisation by both commensal and pathogenic bacteria (Hughes 2008). The introduction of probiotics enables colonization of only beneficial bacteria at a young age thereby reducing diseases propensity. *Bacillus spp.* have been shown to populate this niche environment (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 this 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 colonisation 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, Latorre *et al.,* 2016, Prieto *et al.,* 2014).

The colonisation 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 colonisation through various regions of the gut as demonstrated by Aguiar *et al.,* (2013). This study reported on the ability of a Bacillus based probiotic to competitively exclude *Campylobacter jejuni* due to motility of the 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 microorganisms, *Bacillus spp.* have a high organic carbon utilization rate which enables them to outcompete pathogens for specific nutrients (Slepecky and Hemphill 2006). Iron is important nutrient for pathogen growth as it facilitates several vital processes including oxygen binding, catalysis, and gene expression (Patel *et al.,* 2009). The synthesis of siderophores by *Bacillus spp.*, which are low molecular weight chelating compounds that

facilitate competitive uptake of iron and its role in pathogen exclusion was shown (Lalloo *et al.,* (Lalloo *et al.,* 2010b, Patel *et al.,* 2009). 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.* (Ghadban 2002, Jin *et al.,* 1997, Patterson and Burkholder 2003). *Bacillus spp.* are also capable of producing a large number of antimicrobial peptides (AMP) such as lipopeptides, surfactin, bacteriocins and bacteriocin-like inhibitory substances (Baruzzi *et al.,* 2011, Urdaci and Pinchuk 2004). 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 is extensively shown *in vitro* during pathogen inhibition studies (Khochamit *et al.,* 2015, Lim and Kim 2009).

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 (Cotter *et al.,* 2013, Gruenheid and Le Moual 2012).

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* was 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 feacalis* (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 defence (Dhama *et al.,* 2011, Kabir *et al.,* 2004). The optimum functioning of the GIT is of primary interest to the industry because it directly influences the vigour, 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 continuous 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 mechanisms is the enhancement of the epithelial barrier integrity by increasing the regulation of tight junction proteins which bind to one another forming 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. (Keeney and Finlay 2011, Ng *et al.,* 2008). *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 favourable 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 (Lei *et al.,* 2015, Wu *et al.,* 2011). Hosoi *et al.,* (2000) proposed that *B. subtilis* were 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 birds 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, Menconi *et al.,* 2013, Park and Kim 2014, Thirabunyanon and Thongwittaya 2012), *Clostridium spp.* (Abudabos *et al.,* 2013, Jayaraman *et al.,* 2013, Teo and Tan 2005), *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.

Probiotic effect 2: Improvement in digestion and adsorption

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 of soluble and non-soluble Non-Starch Polysaccharides (NSP) (Khattak *et al.,* 2006). These diets usually comprise of maize alternatives such as wheat, oats, barley and rye. These NSP 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-hydrolysed resulting in low feed conversion. Besides the use of NSP ingredients, the use of low grade maize can also contain a high concentration of anti-nutritional components (Cowieson 2005). Additionally water soluble ß-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 is one of the most expensive nutrients in broiler feed and the two most important protein sources in poultry diets are from animal and plant products. 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 (Teguia and Beynen 2005). Soybean meal (SBM) is the preferred protein source used in poultry feed manufacturing, due to its high crude protein content, however it is costly. Furthermore raw and processed soybean contain a high concentration of ANFs such as protease inhibitors (trypsin and chymotrypsin) which effect protein utilization, lectins that effect carbohydrate utilization, glycinin that have goitrogenic activity effecting the thyroid, saponins which effect palatability and phytic acid that complexes with certain minerals (calcium, phosphorus, magnesium copper, iron and zinc) and reduce their bioavailability (Yasothai 2016). These ANFs in soybean meal is often heat treated to neutralize the activity, however this increases cost. SBM it is being replaced by cheaper legume grains (black beans, groundnut and cowpea) which are also high in ANFs such as protease inhibitors and lectins. In the case of fats, to counter act the use of expensive oils, nutritionists utilize alternatives such as coconut oils and other oils rich in in lauric and myristic acid that can negatively affect the intestinal morphology of birds (Zeitz *et al.,* 2015).

The incorporation of 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 enzymes include high cost,

stability at high temperatures and uncertainty of the amount and ration 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 digested monosaccharides thus reducing intestinal digesta viscosity and improving uptake (Latorre *et al.,* 2016). The action of these enzymes also results 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. The production of enzymes by Bacillus based probiotics is an important criteria and is often screened for *in vitro* (Hmani *et al.,* 2017, Latorre *et al.,* 2015, Lee *et al.,* 2012). It was demonstrated that broilers fed with *B.* coagulans NJ0516 showed increased amylase and protease activity which led to enhanced ng 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 prevent precipitation of penta-calcium phosphate, 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 ß-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 wheat digestibility (approximately 48%) *in vitro*.

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 also responsible for epithelial turnover and activation of cell mitosis. As a result, an improvement of these morphologies lead 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 (Abudabos *et al.,* 2013, Al-Fataftah and Abdelqader 2014, Jayaraman *et al.,* 2013, Lei *et al.,* 2015, Li *et al.,* 2018, Ramlucken *et al.,* 2019, Sen *et al.,* 2012).

The impact of Bacillus based probiotics on improvement in digestion due to enzymes and gut morphology are mainly realised *in vivo* through improvement in FCR, as shown by studies on *B. subtilis* (Jacquier *et al.,* 2019, Molnár *et al.,* 2011) and *B. coagulans* (Li *et al.,* 2018), were probiotic addition resulted in FCR improvement of approximately 5%. Several other studies

474 also showed an improvement in FCR due to Bacillus based probiotics (Gil De Los Santos *et*
475 *al.,* 2005, Jeong and Kim 2014, Lei *et al.,* 2015, Park and Kim 2014, Zhang *et al.,* 2013). In
476 our latest study using a multimode Bacillus probiotic, we showed an improvement in FCR due
477 to a combination of enzyme activity and improvement in GIT histomorpholgy (Ramlucken *et*
478 *al.,* 2019).
479
480 **Probiotic effect 3: Immunomodulation**
481 Immunomodulation refers to the alteration of the host's immune response to foreign agents and
482 pathogens either by antibody stimulation (immune-stimulation) or inflammation suppression
483 (immunosuppressant), to maintain the desired level of host immune-protection. (Klasing 2007).
484 Accordingly, the intestinal immune system must trigger a protective immune response against
485 pathogenic microbes while maintaining tolerance to antigens from food and commensal
486 bacteria. Gut-associated lymphoid tissues (GALTs) represent the largest compartment of the
487 immune system, and they are affiliated with the nervous and endocrine systems. Like all other
488 immune systems, a variety of both innate and adaptive immune responses against pathogenic
489 microbes takes place in the intestine (Kim and Lillehoj 2019). Innate immunity refers to non-
490 specific defence mechanisms that come into play quickly in response to antigens, whereas
491 adaptive immunity is more complex dealing with memory that facilitates future responses
492 against specific antigens. Monoclonal antibodies, cytokines, glucocorticoids, macrophages,
493 immunoglobulins, plasmapheresis, and related agents mainly produced by the GALT are
494 known to alter cellular or humoral immunity (Brisbin *et al.,* 2008, Wigley *et al.,* 2014).
495 Although avian cytokines are not as well defined as those of humans, there are studies that have
496 isolated a specific range of cytokines found predominantly in avian species. These include pro-
497 inflammatory cytokines: IL-6, IL-8 and IL-1β, T helper lymphocytes (TH) which include TH1
498 cytokines: IFN-γ, IL-2, IL-18, which induce cell-mediated immunity and TH 3 cytokines: TGF-

β. There are also T- helper cytokines: IL-2 and others such as IFN-α, IFN-β, IL-15, IL-16 and chemokines also 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 (Markowiak and Śliżewska 2018, Ng *et al.,* 2008). 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 (*Eimera spp.* and *C. perfringens*). Several other studies using

Bacillus spp. also demonstrated immunomodulation in chickens (Gadde *et al.*, 2017b, Lee *et al.*, 2011, Lee *et al.*, 2010b, Lee *et al.*, 2013, Rajput *et al.*, 2017, Xu *et al.*, 2012). 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 in *Bacillus spp.* (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.

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 vigour.

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 as a metabolite, which prevents excessive ammonia toxicity arising from

hydrolysed 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 a *B. amyloliquefaciens* probiotic was able to reduce ammonia in the GIT, with the correlation of the reduction directly proportional to the probiotic concentration. Although not clear on the exact mechanism of ammonia reduction, various studies demonstrated a significant decrease in ammonia emissions from the faecal matter of broilers that were fed a *B. subtilis* preparation (Jeong and Kim 2014, Tanaka and Santoso 2000, Zhang *et al.,* 2013).

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 introduced the concept of screening specially for aflatoxin removal when developing novel Bacillus based probiotics and showed it's *in vivo* efficacy in Japanese quails (Bagherzadeh Kasmani *et al.,* 2012).

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 (Damalas and Koutroubas 2016, Gbotosho and Burt 2013) contributes to phosphorus and nitrogen load, which ultimately ends up in natural habitats. Ammonia emissions are one of the most pressing environmental concerns especially with high stocking densities. Although, more prevalent in laying hens due to the age and rearing time, it however, still poses a challenge and is a major concern for 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, however 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 (DebRoy *et al.,* 2013, Kim *et al.,* 2005, Yang *et al.,* 2011) 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 faecal 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*, however 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, however, 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 by high keratinolytic activity 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 application by the industry, could be of significant importance in selecting *Bacillus* based probiotics over other species.

The development of Bacillus based probiotic

The development of chicken feed probiotics requires a methodological and systematic approach. 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. Therefore it is 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 requirement in the development of probiotics, is the validation of its efficacy *in vivo*.

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. Host specific probiotics could be better evolved to elicit desirable probiotic effects, for example, immunomodulation, as their metabolites will be compatible to the specific cytokines produced by the host (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-sporulating bacteria (Cutting 2011), therefore 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, faeces, bedding, feathers and if possible swabs from the chickens (Barbosa *et al.,* 2005, Wolfenden *et al.,* 2010) and 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 which induce vegetative cells to sporulate. This allows for the formation of mature spores in large quantities (Földes *et al.,* 2000). Other procedures that can be applied include elevated temperatures and exposure to ethanol to induce sporulation (Nemutanzhela *et al.,* 2014). Simple sub-culturing procedures on nutrient agar are generally used to purify individual Bacillus cultures and simple verification techniques include microbial procedures such as microscopic morphology, gram stain, catalase reaction and other metabolic tests (Földes *et al.,* 2000, Nemutanzhela *et al.,* 2014).

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 proventriculous, toxic bile concentrations produced by 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 sheer, hydrolysing 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 ability of a probiotic to survive the conditions of the GIT are extremely strain dependant, with some strains surviving, whereas others within the same species, do not. However, the survivability of *Bacillus spp.* seems to be much higher than their non-spore forming equivalents under GIT conditions. The use of the elimination stage in the rationale for development of probiotics is important as it eliminates large numbers of unwanted strains that would not be functional as probiotics.

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. The growth and proliferation under the harsh conditions of the GIT is the first selection criteria to ensure the presence and activity of the probiotic in large numbers in the GIT. The functional aspects also need to be evaluated using appropriate *in vitro* screening

techniques (Harimurti and Hadisaputro 2015). It is important that the tests used for screening be simple, rapid, and comprehensive to select the best strains from a large group of candidates which show the highest levels of probiotic efficacy to the mechanisms of action relevant to poultry production. (Taheri *et al.*, 2009). The two most desirable modes of action from an industrial standpoint is the competitive exclusion of poultry pathogens and the improvement of digestion and absorption of feed.

The mechanisms involved in competitive exclusion can be ascertained by many *in vitro* screens. Generally, the colonisation potential of probiotic candidates can be determined by auto-aggregation, cell surface hydrophobicity and adherence to epithelial cells assays. Auto-aggregation is a quick method applicable to a large number of test strains, and it shows clumping of strains due to high surface hydrophobicity thus inferring adhesion ability to the gut mucus (Garriga *et al.,* 1998). Cell surface hydrophobicity measures the hydrophobic properties of the outermost surface of probiotic cells, by determining the capacity of the bacteria to attach to hydrocarbons (eg. hexadecane, xylene, and toluene) thus reflecting non-specific cell adhesion to the hydrophobic epithelial region (Ehrmann *et al.,* 2002, Papadimitriou *et al.,* 2015). Bacillus spores have been associated with high cell surface hydrophobicity (Thwaite *et al.,* 2009). Other assays include the attachment to commercially available mucin, which are large glycoproteins that strengthen the intestinal mucosal surfaces forming a protective layer (Papadimitriou *et al.,* 2015). The adherence to epithelial cells by probiotics is one of the most direct ways to determine their colonization capacity. Some studies employ the use of type cell cultured epithelial cells or actual epithelial cells obtained from poultry, but both these methods are costly and time consuming (Hmani *et al.,* 2017). An excellent alternative to the use of chicken epithelial cells, is the use of human colon adenocarcinoma cell line (Caco-2 and HT-29) cells, which are readily available and easier to culture. These specific cell lines,

have been used to elucidate adherence activities of *Bacillus spp.* (Chaiyawan *et al.,* 2015, Ozkan *et al.,* 2013). The ability to form biofilms by *Bacillus spp.* may also be screened for to determine the success of persistence in the GIT (Barbosa *et al.,* 2005).

The mechanisms which enable competitive exclusion of pathogens can be elucidated by various microbial methods, normally targeted against common poultry pathogens such as *E. coli*, *Clostridium spp.*, *Salmonell*a *spp.*, *Campylobacter spp.*, and *Listeria monocytogenes* (Dhama *et al.,* 2013a). The use of co-culturing assays involves the evaluation of competitive growth of the putative probiotic against the pathogen of interest in liquid culture or adhesions studies on epithelial cells (Fijan 2016, Papadimitriou *et al.,* 2015). These approaches can be costly and laborious and is not suitable for screening a large number of isolates against a large battery of pathogens because it requires the counting of both the probiotic and pathogen.

A simpler method to determine antagonistic properties against pathogens involves the use of microbial co-culture plates. These assays involve the co-culture of the probiotic strain and the targeted pathogen on solid agar using different techniques (the cross-streak, the spot-on lawn and well or disc diffusion) (Papadimitriou *et al.,* 2015). In these methods, antagonism by the production of inhibitory compounds against pathogens are defined as a zone of clearing in the solid agar thereby hindering or inhibiting its growth. The degree of clearing is directly proportional to the antagonistic activity of the organism (pathogen or probiotic) (Fijan 2016). With the use of the same methods the mechanism of spatial dominance can be elucidated where there is dominance of probiotic growth over the pathogen as described by Cray et al. (2013).

In order test improvements in digestion and AME usage, the production of key digestive enzymes such as amylase, protease, lipase, cellulase, xylanase and phytase must be evaluated.

Enzyme production is typically assessed using microbial plate assays incorporating the substrate corresponding to the enzyme of interest. These assays typically give a qualitative or semi-quantitative indication of relative enzyme production and enzyme activity between putative probiotics, thus enabling the selection of organisms that have the best enzyme production potential as well as the largest profile of different activities. These assays are quick, easy and cost effective to perform and can handle many target organisms and enzymes of interest.

Other modes of action such as immunomodulation are also of interest in selecting probiotics. Whilst screening for potential immune properties has merit, it is laborious and costly and should be done for probiotics required specifically for immunological benefits. *In vitro* assays used for selection need to be specific for the type of immune response the probiotic is required to achieve (cytokine production, macrophage activation, growth factors etc.). Common methods include bioassays incorporating cell mediated systems with commercially available cells and enzyme-linked immunosorbent assays (ELISA). ELISA measurement of cytokine production is the ideal choice for most laboratories as they are simple to perform, need little specialized equipment and are relatively inexpensive. However, the lack of readily available commercial antibodies to avian cytokines limits these types of tests (Wigley and Kaiser 2003). The use of cell bioassays using chicken spleen cells, closely mimics the *in vivo* model (Papadimitriou *et al.,* 2015) and is a suitable alternative to the more costly chicken lymphocytes (Koenen *et al.,* 2004). Although most accurate for determination of immunomodulatory activity these methods are time consuming and technically difficult requiring cell culture (Wigley and Kaiser 2003). Other molecular techniques include reverse transcriptase PCR (RT-PCR) which allow cytokine production to be detected without the requirement for the protein, just the cDNA. Furthermore, quantitative RT-PCR can allow for cytokines to be quantified in

chicken. This molecular method is ideal for screening this mode of action, as a large number of isolates can be processed relatively quickly. There are continuous efforts in the development of new *in vitro* screens for immunological properties of chicken probiotics (Koenen *et al.,* 2004).

The relevance of *in vitro* test to show immunomodulation is questionable because these tests generally involve only one type of immune cell and ignores the complexity of the *in vivo* communication between different cell types and the other microflora. Other issues with this approach are that it does not differentiate between the innate and adaptive immune system. There is therefore a preference to test this effect *in vivo*, because it indicates more accurately the immune response to a particular challenge. A majority of studies used to determine immune modulation by *Bacillus spp.* were done *in vivo* (Gadde *et al.,* 2017b, Lee *et al.,* 2015, Wang 2017) using already developed probiotics.

The cumulative response of a putative probiotic to each of the screening criteria is a holistic indication of the suitability of each isolate to the predefined criteria of interest to the poultry industry. An elegant approach is to score each response to each of the test criteria, which should ideally be weighted in accordance with the importance of the criteria regarding the probiotic effect. By statistically analysing the data, it is possible to rank candidate probiotics from best to worse based on their significant differences in performance. By using this data, the best candidates with multiple modes of action can be prioritised for selection.

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

Once putative probiotics are prioritised, 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 in databases (Fontana *et al.,* 2013). This bioinformatics approach is more robust as it is based at the genotypic level compared to other tests which are based at the phenotypical and biochemical levels. 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 (Hong *et al.,* 2005, Sanders *et al.,* 2003). *B. anthracis* causes the acute fatal disease anthrax and is a potential biological weapon due to its high toxicity (Helgason *et al.,* 2000). Because of the potential risk of these species, once identified it is almost never applied for use in probiotic applications. *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 are opportunistic pathogens that commonly cause food poisoning (Helgason *et al.,* 2000). However, if isolates belonging to the *B. cereus* group are probiotic candidates, it is imperative that the strains are 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, however, 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 organisation 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). This system uses a rigorous literature screen to determine if a species qualifies to be on the QPS list. In terms of *Bacillus spp.* over 2000 reports were analysed and 14 species were recognised as QPS. These species include *B. amyloliquefaciens, B. atrophaeus, 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 commercialised, thus the probiotic species of choice remains *B. subtilis* and *B. coagulans* (Cartman *et al.*, 2008).

Verification of probiotic functionality

Once putative probiotic strains have been deemed "safe" for use, their functionality must be verified in order to finally select the required commercial strains. Generally, for a multi-mode probiotic, a consortium of strains are preferred instead of an individual strain, because it allows for a holistic probiotic effect and strains can compensate for the lack of effects from other

strains (Chapman *et al.,* 2011). If a consortium is to be used, then it is imperative to test the population dynamics of the individual strains to ascertain if all strains selected can coexist. Candidate strains that do not grow adequately or inhibit the growth of other strains within the consortium should not be selected as a probiotic.

The survival and proliferation of the probiotic consortium should be verified using *in vitro* simulated GIT models (Millette *et al.,* 2013), as it enhances the chances of success. Because these studies are costly, it is generally avoided in the early stages of probiotic development, due to the large number of isolates to be tested. It is however prudent to perform these tests on the final consortium to verify germination, growth and survival of the Bacillus spores under complete GIT conditions, as this gives a true indication of probiotic functionality. Vegetative cells are reported to be very susceptible to gastric acid and bile salts, while spores are generally resistant to both conditions (Barbosa *et al.,* 2005), therefore, studies in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) are important in verifying the usefulness of a probiotic consortium. (Mingmongkolchai and Panbangred 2018).

Even though a rational approach to probiotic development, results in the selection of the best strains, functionality *in vitro* does not always correlate to the *in vivo* efficacy. Therefore, before a probiotic can be commercialised, efficacy must be proven in controlled experimental field trials, with specific effects such as health and productivity evaluated using commercially relevant measures. The validation of selected probiotics *in vivo* following a rationale screening process, has been the approach followed in several studies involving *Bacillus spp.* (Menconi *et al.,* 2013, Nguyen *et al.,* 2015, Wolfenden *et al.,* 2010).

The manufacturing of *Bacillus spp.* probiotics

849 There is immense effort going into probiotic development for use in the poultry industry mainly
850 focused on screening and efficacy. However, a critical aspect of development and which is
851 often neglected is the production of selected probiotics at industrial scale. Commercially viable
852 strains must show attractive techno-economic properties in the production process (Lacroix
853 and Yildirim 2007). Some of the key consideration in ensuring a commercially acceptable
854 production process, includes storage of strains in validated master and working cell banks, an
855 inoculum train that delivers a proper quality and quantity of cells for the fermentation process,
856 a high performance fermentation processes, the efficient harvesting of the probiotic cells, and
857 the formulation into a stable product ready for easy incorporation into premixes or feeds. This
858 facilitates the commercial roll-out of probiotic products, which is largely dependent on the
859 efficiency and cost of the production process at industrial scale to deliver shelf stable product
860 in sufficient quantity (Amer and Utkhede 2000, Patel *et al.*, 2004).
861
862 **Fermentation and cell separation of Bacillus probiotics**
863 **Cell storage and inoculum train**
864 For the commercial production of probiotics, it is important to have a stable culture that is
865 appropriately preserved. *Bacillus spp.* can be stored in spore form with better stability and
866 viability in contrast to vegetative cells (Gao *et al.*, 2007, Monroy *et al.*, 2004). This ensures a
867 consistent starter culture, which impacts on the characteristics of the end product. Cell banks
868 must be validated in terms of stability, purity and cell concentration, preferably greater than 1
869 $\times 10^6$ CFU.mL^{-1} to ensure a robust inoculum (Monroy *et al.*, 2004).
870
871 The inoculum train can have a substantial impact on process performance in terms of
872 productivity, profitability, and process control. It is understood that a well-characterized
873 inoculum train is essential for the bulking of the initial culture into a suitable inoculum for the

main production fermentation (Meyer *et al.,* 2016, Okonkowski *et al.,* 2005). *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, under high cell density cultivation (HCDC) (Lalloo *et al.,* 2009, Monteiro *et al.,* 2014, Monteiro *et al.,* 2005).

Fermentation

Fermentation industries are focussed on HCDC to ensure economic feasibility. 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). The growth medium used can be either defined or undefined , the latter usually applied in industrial processes, based on its lower cost (Prescott *et al.,* 2005). Nutrient sources, specifically carbon and nitrogen, play a dominant role in the efficiency of the production process, since they supply nutritional and growth factors that are directly linked

899 with the formation of viable cells (López *et al.,* 2003). Conventional probiotics often require
900 more expensive complex nutrients such as tryptones, peptones and yeast extracts, but the cost
901 at commercial scale can be prohibitive (Zhang and Greasham 1999).
902
903 *Bacillus spp.* have been shown to grow efficiently on lower cost, locally available waste
904 substrates (Lalloo *et al.,* 2009, Singh *et al.,* 2017), but information specifically related to the
905 production of poultry probiotics is limited. *Bacillus spp.* have been shown to grow on several
906 agricultural and industrial wastes either as is or as hydrolysates, such as molasses, corn steep
907 liquor, soybean, or wheat (Chang *et al.,* 2008, Chen *et al.,* 2010, Lalloo *et al.,* 2009, Prabakaran
908 *et al.,* 2007). A study by Khardziani *et al.,* (2017) showed the growth of *B. amyloliquefaciens*
909 B-1895 in various lignocellulosic materials at concentration of 40 $g.L^{-1}$ yielded a cell
910 concentration of 1×10^{10} $spores.ml^{-1}$.
911
912 HCDC of *Bacillus spp.* is preferably done in fed-batch fermentation because the concentration
913 of the limiting substrate, can be maintained at a low level, thus avoiding the repressive effects
914 of high substrate concentration (Shiloach and Rinas 2010). In this way there is some control
915 over the organism's growth rate and oxygen demand thus ensuring oxygen sufficiency
916 (Elisashvili *et al.,* 2019, Shiloach and Rinas 2010). The use controlled feeding of glucose
917 maximised vegetative cell growth to 1.3×10^{10} $CFU.mL^{-1}$, whilst avoiding premature
918 sporulation (Monteiro *et al.,* 2014). Using a similar glucose fed-batch procedure coupled with
919 the manipulation of carbon to nitrogen ratio, Panday (2016) was able to produce the probiotic
920 *B. coagulans* at a concentration 3.8×10^{11} $cells.mL^{-1}$.
921
922 The efficient production of *Bacillus spp.* is often reflected in the quantity and quality of spores
923 harvested at the end of the fermentation. Therefore, cultivations should be optimized to achieve

high sporulation efficiencies. Different culture media for Bacillus sporulation have been reported, where each particular strain had preferential requirements (Cho *et al.,* 2009, Flores *et al.,* 1997, 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 and therefore the media must have a balance of cheaper basic and supplemented components to ensure optimal vegetative cell growth and sporulation. (Posada-Uribe *et al.,* 2015, Sonenshein 2000). For example, Monteiro *et al.,* (2005) observed that an increase in glucose concentration up to 5 $g.L^{-1}$ led to an increase in the vegetative cell and spore concentration of *B. subtilis*, while higher sugar concentrations inhibited sporulation, showing preference towards fed batch fermentation. Monteiro *et al.,* (2014) further showed increases in spore concentration by supplementation with ammonium sulphate, ammonium hydrogen phosphate and calcium.

Physical parameters such as temperature, pH, agitation and aeration have a critical impact on successful spore production (Xiang *et al.,* 2013). These parameters influence the performance, reproducibility and consistency of production process (Tavares *et al.,* 2013). Bacilli being ubiquitous in nature, are able to grow under various conditions, but the physical parameters must be optimised for the strain being produced, to maximise growth and sporulation (Posada-Uribe *et al.,* 2015). Several studies have evaluated the effect of different physical parameters such as temperature (20-30ºC), pH (5.0–9.0) aeration rates (0.5–2.0 vvm) 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 (Chen *et al.,* 2010, Posada-Uribe *et al.,* 2015, Tzeng *et al.,* 2008).

Most microorganism in the vegetative state are temperature sensitive, which also applies to Bacilli with regards to growth and replication, subsequently effecting production efficiency. A study by Lalloo *et al.,* (2008) showed the effect of temperature on growth rate, cell concentration and spore germination of *B. cereus* NRRL 100132. In their study it was showed that low temperatures significantly lower growth and germination and that *B. cereus* has an optimum temperature between 25-30 ºC. Their study also showed that there is no significant effect of pH ranging from 6-9 on growth rate of *B. cereus* which is in accordance with Monteiro *et al.,* (2005), who reported that the sporulation efficiency for *B. subtilis* was found to be independent of the pH values within the range of 6.9-9.0. Contrastingly Posada-Uribe (2015) reported that a pH variation within 5.5-7.0 affected cell concertation and sporulation efficiency. In a review by Elisashvili *et al.,* (2019) it was postulated that neutral pH favours Bacillus growth while the medium acidification suppresses growth, and decreases sporulation efficiency, whilst an alkaline pH promotes sporogenesis.

Agitation, aeration and pressure primarily influence mixing and mass transfer, which affects spore production of different *Bacillus spp.* (Feng *et al.,* 2003). Posada-Uribe *et al.*,(2015) concluded that spore concentration was increased by increasing agitation and aeration, wherein the 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 on agitation and aeration generated higher biomass in different *Bacillus spp.* (Feng *et al.,* 2003, Yeh *et al.,* 2006). This is indicative that sporulation is highly related to oxygen supply and that non-limited oxygen conditions during the growth phase are important to realise high spore yields (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 maximised to minimise, labour, utility and capital utilization costs. *Bacillus spp.* have been shown to replicate rapidly, which maximises 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} within40 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 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 and substrate utilization in studies conducted by Lalloo *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 therefore the sporulation efficiency is critical for Bacillus production. The development of spores from active cells is a result of pathway changes, which involves the phosphorylation of the Spo0A transcriptional factor, which is predominantly induced by the depletion of carbon, nitrogen or essential micronutrients (Fujita and Losick 2005, Tan and Ramamurthi 2014). Sporulation efficiencies over 90% have been reported for *B. cereus* and *B. subtilis* strains (Lalloo *et al.,* 2009, Posada-Uribe *et al.,* 2015). Furthermore *Bacillus spp.* have been proven to yield high spore densities between 1×10^{9} and 1×10^{10} cells.ml^{-1} (Chen *et al.,* 2010, Khardziani *et al.,* 2017, Lalloo *et al.,* 2009, Monteiro *et al.,* 2005). Panday (2016)

reported the highest spore concentration of 1.9×10^{11} spores.mL^{-1}. The studies on high density cultivation of Bacillus spores, although not directly poultry probiotic related, shows great promise for commercialization in the poultry industry. The main technological advantages of this genus as poultry probiotics is illustrated in Figure 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 such as pressure and mechanical sheer, typical in cell harvesting processes. A good harvesting technique should have a minimal number of unit operations to reduce the overall process and validation costs (Brar *et al.*, 2006). Cell harvesting process options such as flocculation and ultrafiltration are costly, therefore 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% (Lalloo *et al.*, 2010a, Villafaña-Rojas *et al.*, 1996). An added advantage is that the centrifugation process can be continuous, resulting in improved process through-put, while maintaining high cell recoveries (Lalloo *et al.*, 2010a, Zamola *et al.*, 1981).

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 the stabilization process such that it results in a useable spore suspension for later end product formulation (Brar *et al.*, 2006, Schisler *et al.*, 2004). Stabilizing spores involves the use of buffers, preservatives and the manipulation of pH but this strategy must take into consideration cost and further downstream impacts such as the safety and suitability of the ingredients.

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, allow for the ease of application and 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 often administered in the potable water fed to chickens; however, special supply chain limitations needs to be considered for this product format. For instance, liquid products require large storage areas and higher costs of shipment. Other factors include refrigeration or freezing of liquid products, in order to maintain stability and viability which is costly (Lacroix and Yildirim 2007). Thus, 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 Śliżewska 2018). In contrast to human probiotics, the poultry industry cannot absorb the high cost of encapsulation, therefore, spray-drying, and bulk drying techniques to form probiotic powders are preferred (Moënne-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 sheer powder blending. Transforming liquid product intermediates of spore concentrates into a dried product requires drying at high temperature (~60 ºC), 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 (Brar *et al.,* 2006, Moënne-Loccoz *et al.,* 2001, Wiwattanapatapee *et al.,* 2004). 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 (Ouwehand and Salminen 1998, Simon *et al.,* 2005). Therefore 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, however, the majority of commercialized probiotic products are formulated to a concentration of ~ 1×10^9 CFU.ml^{-1} (Jeong and Kim 2014, Kim *et al.,* 2017, Teo and Tan 2007), which ensures a balance of consistent dispersion, cost effective logistics, and easy dosage into premixes and feeds.

Storage conditions for probiotic-augmented feed are usually in warehouses at ambient temperature. Furthermore, these storage spaces are exposed to many environmental factors such as humidity, extreme heat and cold, which could affect the viability of probiotics (Markowiak and Śliżewska 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 and prevent cross contamination (Chávez and Ledeboer 2007). Dried products must be stored in conditions that allow for the protection from heat, light and moisture. Furthermore proper packaging material must be selected accordingly (Chávez and Ledeboer 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 (Lalloo *et al.,* 2010b, Sorokulova *et al.,* 2008, Yadav *et al.,* 2009).

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 the 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 pelleting, extrusion and other complementary processes that require high temperatures and pressures which may affect the viability of probiotics (Kosin and Rakshit 2006). Typical feed for broiler chickens is processed at about 75–85 °C for 15–20 s with a moisture content of 15 % before pelleting (Kosin and Rakshit 2006). The manufacturing process of poultry feed generally starts with the blending of the dry ingredients to produce a mash, which is where probiotics are usually added. The mash-feed is subjected to extrusion and pelletizing. These processes involve heating of the mash and forcing it through a circular die at pressure to form an extrudate of a specific diameter, which is then formed into pellets. Production of crumble feed, typically used during the pre-starter and starter phases of poultry rearing, requires an additional pellet-grinding step usually done by large rollers that could damage the viability of probiotics.

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 sheer 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 toyoi* after pelleting at 87°C was 95 % and after 8 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 ºC regardless of contact time. Studies have also shown that Bacillus spores can be stable in dry products exceeding 2 years (Lalloo *et al.*, 2010a).

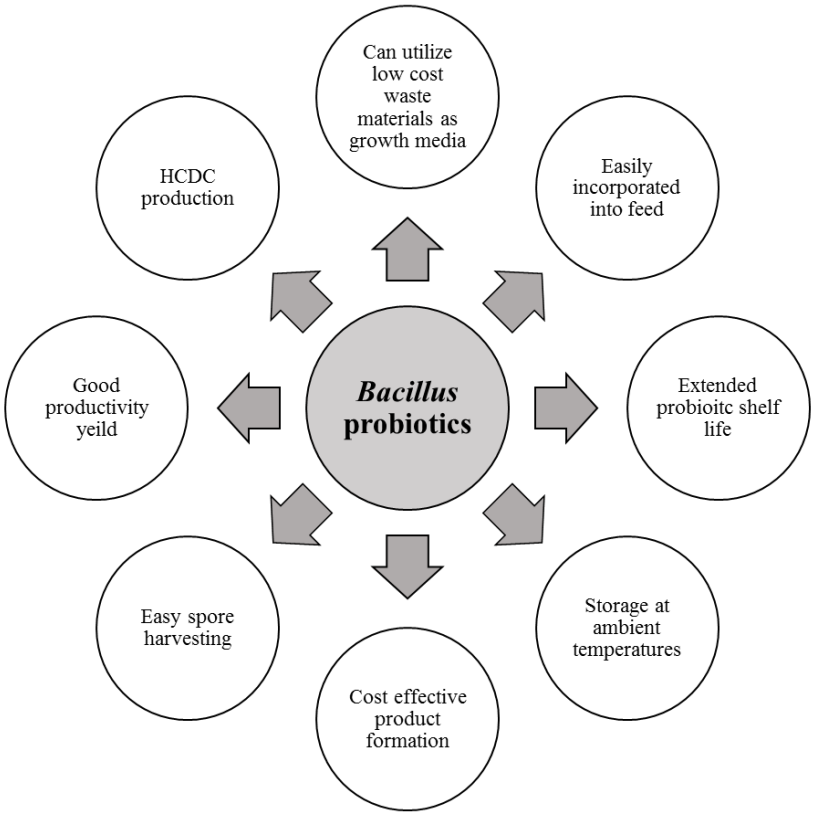

Figure 2: Commercial advantages of *Bacillus spp.* as poultry probiotic products

Future perspectives

Bacillus spp. are the future of in feed probiotics. The greatest advantages are in the general ease of isolating suitable candidates, screening for industry relevant desirable characteristics, 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 AGP's 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 dependant 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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