Full Paper

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

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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 (Dibner and Richards, 2005; Perreten, 2003) 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 of 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., 1998; Kabir, 2009; Kabir et al., 2004; Kalavathy et al., 2003). 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 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 6 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.

Key Words: Bacillus subtilis; Bacillus velezensis; broiler production; development of probiotics; indigenous bacteria; multi-mode; probiotics
probiotics, due to their spore forming capabilities, which enable them to resist damage during the feed production process, and also to 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; Lee et al., 2012; Leser et al., 2008). The positive attributes of this genus offer promise for the development of suitable commercial poultry probiotics.

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

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; Kabir, 2009; Lee and Yu, 2013; Simon et al., 2001). 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 have a generally regarded as safe (GRAS) status are preferred (Fuller, 1992; Lee and Yu, 2013). Since all Bacillus strains do not equally posses 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.

**Materials and Methods**

**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 400x 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 Turrax, 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 h at 32°C. Single colonies based on morphological 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 a 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 (FormaTM 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.

**Critical screening phase.**

Survival and growth of isolates at pH 3: Survival of isolates in an acidic environment was tested using a modified and scale-down method reported in Fuller (1999). The cell concentration of each cryo-culture was measured using a counting slide (Thoma®, Hawskey and Sons, UK), under light microscopy at 400x 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 min (Eins Sci Autoclave, Hospi Sterilizers, South Africa) and thereafter cooled and aseptically aliquoted (10 mL) into the wells of a 6-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 h 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 h. 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 (ΔCFU·mL⁻¹) between the start (T₀) and completion of the exposure time (Tₙ). 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.

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 6-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 h. 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 (T\(_0\)) and end (T\(_6\)) of the cultivation time. The remaining 2 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 a significantly lower growth (p < 0.05) than the mean growth achieved in bile salts, were eliminated from further screening tests.

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 being aseptically aliquoted into 6-well microplates. Wells were inoculated with the test isolates, incubated for 4 h at 42°C, with growth measurement by OD\(_{660\text{nm}}\). All isolates that displayed a significantly lower growth than the mean growth achieved at either pH 5 or 7 were eliminated from further screening.

Secondary screening phase.

Potential of isolates for the production of digestive enzymes: Isolates that passed the critical screens—survival at pH 3, growth in the presence of bile salts, and growth in an 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\(_{660\text{nm}}\) of 2 using sterile distilled water. Triplicate samples (100 \(\mu\)L) 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 h (amylase and protease) and 48 h (cellulase and xylanase). After incubation, the extent of the respective enzyme substrate reactions was visualised using different staining techniques. Gram’s 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 of inhibition were measured using a digital Vernier Caliper (Insize, Accu, UK) and were indicative of enzyme activity. The response of each isolate for each enzyme was included in a mathematical matrix evaluation.

Physical feed breakdown potential of the isolates: The determination of the physical breakdown of feed was carried out 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 h. 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 h 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 min at 3,900 \(\times\) 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 the 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 water.

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 were tested negative for mycoplasma contamination by the Institute it was obtained from. Cells were routinely maintained in Dulbecco 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) (Lanza, Switzerland) and then trypsinised by the 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 \(\times\) 10\(^5\) cell·mL\(^{-1}\)) were aliquoted (500 \(\mu\)L) into wells of a sterile 24-well tissue culture plate, which was then covered (TPP, Switzerland), followed by incubation at 37°C in a CO\(_2\) incubator (5% CO\(_2\) in ambient air) until 80% cell confluence was microscopically observed. The cell culture media (DMEM) was aspirated from each well and discarded. The adhered Caco-2 cells were 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. After this, the cells were harvested by centrifugation at 3,900 \(\times\) g for 20 min and the pellet was re-suspended in DMEM to achieve a normalised viable cell concentration of 1 \(\times\) 10\(^8\)
cells·mL$^{-1}$. The cell suspension of each isolate was added (200 µL) in triplicate into wells containing pre-adhered Caco-2 cells. Plates were incubated for 2 h 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 cells 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.

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 enteritidis*, *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 µL, $1 \times 10^8$ 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 h, after which zones of inhibition were measured using a digital Vernier Caliper. The response of each isolate for each enzyme was included in a mathematical matrix evaluation.

**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. The 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

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**Fig 1.** Schematic illustration of critical screening steps showing the selection of qualifying isolates. All experiments were carried out in triplicate.
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 the 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 the 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 secondary 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 that no isolate was eliminated but scored based on performance for each criterion. The final selection was done using the accumulative 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.

**Strain identification and biosafety.** Identification of all strains selected as putative probiotics was done by 16 S 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′):

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\text{AGAGTTTATCMTGGCTCAG and CGGTTACCTTGTTACGACTT, respectively.}
\]

**Fig. 2.** Desirability co-efficient (expressed as a percentage relative to the maximum score) showing the elimination of undesirable isolates (55) in the critical screen phase.

**Fig. 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, \( n = 6 \).
**Bacillus multi-mode probiotic for poultry**

(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 the manufacturer’s instructions. PCR products were purified using a ZR-96 DNA sequencing clean up kit (Zymo Research, USA) as per the 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).

**Results**

**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%).

**Critical screening phase**

**Evaluation and elimination of isolates against critical survival and growth requirements within the chicken GIT.** Figure 1 illustrates the results obtained in the critical screen. A further 18 isolates were added from our existing organism database, to the 48 isolates obtained in this study resulting in a total of 66 candidates (Fig. 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 3 that lost complete viability were eliminated from further testing (Fig. 1). These 63 isolates were then tested for growth in the presence of bile salts, of which, 19 did not grow and 7 grew poorly, whilst the remaining 32 isolates grew well and were selected for further evaluation of growth at intestinal pH extremes. At pH 5, 20 isolates were eliminated (13 did not grow and 7 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 (Fig. 2). The remaining 11 isolates that showed significantly higher growth (p < 0.05) at both intestinal pH levels, were selected for the next screening phase.

Secondary screening phase

Extracellular enzyme production and the ability to physically breakdown 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 (Fig. 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) showed a 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).

Adherence potential to gut epithelium cells. Of the 11 isolates evaluated, 3 isolates (CPB 010, CPB 035 and CPB 029) showed a significantly higher adherence (~43% attachment) to Caco-2 cells (p < 0.05). Isolate CPB 010 showed the highest adherence (~57%), whilst five isolates were average (~37% adherence) and three isolates were poor (~20% adherence) (Fig. 4).

Antagonistic activity against common poultry pathogens.

When the 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 a significantly better antagonism against the pathogen (p < 0.01) (Fig. 5). Similarly, isolates CPB 011, CPB 029, CPB 035, and HP 1.6 expressed a significantly higher antagonism against *S. enteritidis* (p < 0.05), whereas CPB 011, CPB 035, HP1.6 and D014 displayed a 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.

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 their cumulative score (p < 0.05) and were therefore selected as the core consortium (Table 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 1). All seven of the isolates selected were subsequently subjected to a growth suitability evaluation to confirm their production potential in industrial media where upon isolate CPB 010 resulted in extremely poor growth (data not shown) and was therefore excluded from selection.

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*.

Discussion

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, as with those of other

| Organism designation | Survival and growth at pH3 | Growth in bile | Growth at intestinal pH | Adherence | Digestive enzymes | Pathogen antagonism | Feed breakdown | Cumulative |
|----------------------|---------------------------|----------------|------------------------|-----------|-------------------|-------------------|--------------|------------|
| 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        | 94.9       |
| CPB 011              | 14.3                      | 50.0           | 100.0                  | 50.0      | 100.0             | 100.0             | 100.0        | 96.7       |
| CPB 010              | 28.6                      | 100.0          | 60.0                   | 100.0     | 73.3              | 37.5              | 50.0         | 87.2       |
| CPB 029              | 14.3                      | 100.0          | 40.0                   | 75.0      | 100.0             | 66.7              | 50.0         | 86.5       |
| HP 1.6               | 14.3                      | 100.0          | 80.0                   | 25.0      | 86.7              | 100.0             | 25.0         | 83.6       |
| D014                 | 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, auxiliary isolates in italics.

*Unable to grow in industrial media.

Table 1. Desirability co-efficient of each putative probiotics showing suitability to each criteria and cumulative multi-mode performance rating (relative % to maximum).
researchers, (Barbosa et al., 2005; Chaiyawan et al., 2015; Latorre et al., 2014; Wolfenden et al., 2010), showed that they can be successfully isolated from the facultative and anaerobic zones of the chicken GIT.

**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 the survival in pH 3, growth in the presence of bile salts, and growth at intestinal pH extremes.

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

The first screen was designed to evaluate survival at a low gizzard pH wherein 63 of the 66 isolates survived, which can be attributable to the resistant nature of *Bacillus* spores (Cutting, 2011; Spinosa et al., 2000). Of the 63 survivors, the 7 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 a better adaptation of isolates from the target host (Pan and Yu, 2014).

In the bile salt growth test, 31 isolates that did not survive or grew poorly were eliminated from further screening of the 63 that were carried over into this test. The 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). 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, 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 carry over from the gizzard.

Overall, of the 66 isolates screened, only 11 isolates were selected based on the critical elimination criteria (Fig. 2), resulting in an 83% elimination efficiency, which enables focus on a smaller number of isolates in subsequent screens.

**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.

**Extracellular enzyme production and the ability to physically breakdown 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 are 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 (Annisom, 1993; Choct, 2006; Khatattak et al., 2006; Latorre et al., 2016). Therefore probiotics that produce glycosyl hydrolase enzymes are of great interest, more so 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 the 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).

**Adherence potential to gut epithelium cells.** The results of our study indicated that our isolates possess moderate adherence capabilities which correlate with 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 the 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
Attacked 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. As Bacillus cells are moderately adherent, transient presence in the GIT of chicks needs to be maintained by continuous administration and higher levels of efficacy.

**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 (Kiramayi 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; Dham et al., 2013a; Finstad et al., 2012). Results from our study correlate well with other research showing antagonism against *E. coli* and *S. enteritis* (Guo et al., 2006; Latorre et al., 2016; Thirabunyanon and Thongwittaya, 2012).

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 the antagonism of *Bacillus* based probiotics against this pathogen (Dham 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 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 anaerobicosis, and thus provided the best probiotic-to-pathogen challenge conditions (Harwood and Wipat, 1996).

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 an excellent potential to address the commercial challenges of the poultry industry.

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