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

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Summary

Bacillus based probiotics are becoming relevant as alternatives to antibiotics used in poultry production and in other animal husbandry. This study describes the isolation of 48 *Bacillus* spp. candidates, from chickens and chicken environments, for use as potential probiotics in poultry production. These isolates, plus a further 18, were tested in a comprehensive *in vitro* screening regime that was specifically designed to select the best isolates that satisfied multiple modes of action desirable for commercial poultry probiotics. This screening programme involved the evaluation of the ability of the isolates to survive and grow in the limiting conditions of the chicken gastrointestinal tract. Only 11 of the isolates fulfilled these criteria, hence they were further evaluated for the ability to adhere to epithelial cells, produce extracellular enzymes and to demonstrate antagonistic activity against selected pathogens of significant importance in poultry production. Of these, a total of 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.

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

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

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and Baquero, 2000; Phillips et al., 2004). As such, in-feed antibiotics (IFAs) have been banned in regions such as Europe, America (Perreten, 2003; Dibner and Richards, 2005) and Scandinavia (Bengtsson and Wierup, 2006; Grave et al., 2006). Global increase in consumer health awareness has also resulted in preferences for poultry products that are free from antibiotics, growth stimulants and other non-natural additives (Griggs and Jacob, 2005; Yiridoe et al., 2005). Therefore, the poultry industry requires new and innovative technologies to address these challenges.

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

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

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

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

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}, $MgSO_4.7H_2O$ 0.5 g.L^{-1}, $MnSO_4.4H_2O$ 0.05 g.L^{-1} and $CaCl_2$ 0.1 g.L^{-1}) (Merck, Germany) contained in a Erlenmeyer flask (500ml) and incubated at 32 ºC 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 Turrex, IKA, Germany) and treated in a similar manner to other samples. Each spore culture was then treated using an isolation cascade, which comprised a dehydration and a heat treatment step to eliminate non-spore formers (Lalloo et al., 2007).

Samples from each flask (1 ml) were serially diluted in sterile saline and plated on Nutrient Agar (NA) (Merck, Germany) plates supplemented with 10 $mg.ml^{-1}$ polymyin B antibiotic (Sigma-Aldrich, USA) to exclude any gram negative spore forming bacteria. Plates were incubated for 24 hours at 32 ˚C. Single colonies based on morphology differences were transferred onto new plates until monocultures were obtained consistently through three passages. Each pure colony was subjected to the catalase (Kilian, 2015) and Gram staining (Barile, 2012) tests. The cultures that passed the tests were thereafter cultivated in sporulation medium after which each spore culture was cryo-preserved using 25 % $v.v.^{-1}$ glycerol (Sigma-Aldrich, USA) according to the method outlined by Acosta (2004). These cell banks were then stored at -80 º C in an ultra-freezer (Forma™ 88000 Series, Thermo Scientific, USA). Additionally 18 isolates from an existing in house *Bacillus* 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 x 10^8 $CFU.ml^{-1}$ 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 ºC for 15 minutes (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 hours at 42 ºC 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 42ºC for 24 hours. Colonies were enumerated using a colony counter (Bibby, Stuart scientific UK). Survival and growth of the isolates was determined using the relative difference of the $CFU.ml^{-1}$ ($\Delta CFU.ml^{-1}$) between the start (T_0) and completion of the exposure time (T_4). 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, Le Marrec, Sassi and Deschamps (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 42ºC for 24 hours. Growth was measured using the difference in optical density (OD), measured at 660 nm using a micro-plate reader (Synergy HT, BioTek USA), at the start (T0) and end (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 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. Similarly to Section 3.2, sterile TSB medium was adjusted to either pH 5 (HCl) or 7 (NaOH) (Minema Chemicals, South Africa) prior to autoclaving and aseptically aliquoted into 6 well microplates. Wells were inoculated with the test isolates, incubated for 4 hours at 42 ºC, with growth measurement by optical density at 660 nm. All isolates that displayed significantly lower growth than the mean growth achieved at either pH 5 or 7 were eliminated from further screening.

Secondary screening phase

Potential of isolates for production of digestive enzymes. Isolates that passed the critical screens, that is, those that survived at pH 3, were able to grow in the presence of bile salts and at intestinal pH, were then tested for the production of amylase, cellulase, protease and xylanase enzymes. Each test isolate was inoculated from a cryovial and grown in flasks containing sterile TSB (100 ml) at 42 ºC for 12 h, with agitation at 120 rpm. The resultant culture was standardized to an OD_{660nm} of 2 using sterile distilled water. Triplicate samples (100 µL) were withdrawn and used to inoculate aseptically punched wells in the centre of an agar plate which contained the substrate for the enzyme of interest. Solubilised starch (Sigma-Aldrich, USA) was added for amylase detection (Ibrahim et al., 2012), carboxymethylcellulose (CMC) (Sigma-Aldrich, USA) was added for cellulase detection (Kasana et al., 2008), milk powder and casein (Sigma-Aldrich, USA) was added for protease detection (Kim et al., 2007) and Birchwood xylan (Sigma-Aldrich, USA) was added for xylanase detection (Nair and Shashidhar, 2008). All plates were sealed and incubated at 42 ºC 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. Grams iodine was used for the detection of amylase and cellulase activity, while trichloroacetic acid (Merck, Germany) (25% v.v^{-1}) was used followed by a 15 minute incubation at 45 ºC for the

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detection of protease activity. A stepwise treatment with a 25% (v.v^{-1}) sodium chloride (Merck, Germany) solution followed by staining of the plate with Congo red (Sigma-Aldrich, USA) was used for the detection of xylanase activity. The diameters of the zones around the wells which were measured using a digital Vernier calliper (Insize, Accu, UK) and were indicative of enzyme activity. The response of each isolate for each enzyme was included in a mathematical matrix evaluation.

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

Gut epithelium adhesion assay. A Caco-2 cell line (University of Kwa-Zulu Natal, South Africa) was used to mimic chicken epithelial cells as previously described (Tsai et al., 2005). Cells tested negative for mycoplasma contamination by the institute it was obtained from. Cells were routinely maintained in Dulbecco Modified Eagle Medium (DMEM) with antibiotics as outlined by Hsieh *et al.* (2013). For the adhesion assay, Caco-2 cells (passage 20-22) were washed with pre-warmed (37 ˚C) phosphate buffered saline (PBS) (Lonza, Switzerland) and then trypsinised by addition of 0.25% (w.v^{-1}) trypsin and 0.1% (w.v^{-1}) ethylenediaminetetraacetic acid (EDTA) at approximately 85-90% confluency. Cells of a standard concentration (1×10^5 cell.ml^{-1}) were aliquoted (500 µL) into wells of a sterile 24 well tissue culture plate, which was then covered (TPP, Switzerland), followed by incubation at 37 ˚C in a 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 Section 3.5. Thereafter the cells were harvested by centrifugation at 3900 *x g* for 20 minutes and the pellet was re-suspended in DMEM to achieve a normalised viable cell concentration of 1 x 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 hours at 42 ^{o}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 (*E. coli*, *S. enteritis*, *L. monocytogenes* and *C. perfringens*). Each test isolate was cultured as previously described in Section 3.5 and was aseptically added from a culture flask (100 µL, 1 × 10^8 cells.ml^{-1}) into pre-made wells on a Tryptone Soy Agar (TSA) (Merck, Germany) plate, previously spread with each of the respective test pathogens. The plates were incubated at 42 ºC for 24 hours, after which zones of inhibition were measured using a digital Vernier calliper. The response of each isolate for each enzyme was included in a mathematical matrix evaluation.

Elimination, scoring and selection of isolates. The data was processed by using statistical clustering and ANOVA (analysis of variance). The clusters were based on a standard deviation of ± 0.5 SD (1SD total) of the mean of the data set. P values < 0.05 or 0.01were regarded as statistically different between means using the t-test. Isolates were only eliminated in the critical screens (growth and survival at pH 3, growth in bile salts and intestinal pH extremes). All isolates that did not survive these tests were eliminated from further testing and selection. In the pH 3 survival test all isolates that survived and those that showed growth were selected. For the studies investigating growth in bile and intestinal pH extremes, isolates that grew slower than the resultant mean growth for the study were eliminated. In the critical screen all isolates that clustered within the normal distribution of the mean were given a score of one. For remaining isolates that clustered above the mean, each data group that was significantly different from each other, was given a unique score, from 2 upwards. This strategy ensured that only average and above average performers were carried over to subsequent screens.

Using this strategy, a score of 0 represented the lowest desirability and the highest score represented the highest desirability to the pre-set criterion for selection of the putative probiotics. Isolates were eliminated from each part of the screen if they had obtained a score of 0. In the non-critical screens (ability to produce enzymes, physically breakdown feed, adherence to epithelial cells and demonstration of an antagonistic effect against common chicken pathogens) the data from each response was similarly analysed but those isolates that clustered significantly below the mean were given a universal score of 1 and those that clustered within the normal distribution of the mean were given a score of 2. All remaining isolates that clustered above the mean were incrementally scored from 3 upwards. This ensured 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'): AGAGTTTATCMTGGCTCAG and CGGTTACCTTGTTACGACTT respectively. The PCR products were evaluated by gel electrophoresis and the bands were extracted using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Cat. No. D4001, USA). The products were subsequently sequenced in the forward and reverse directions on an ABI PRISM 3500 XL genetic analyser (Thermo Scienific, USA) as per manufacturer's instructions. PCR products were purified using a ZR-96 DNA sequencing clean up kit (Zymo Research, USA) as per manufacturer's instructions and cycle sequenced on a CLC main workbench 7 (QIAGEN, Germany). The sequence alignments were performed by a BLASTN search (NCBI). Identification was based on the level of confidence of the sequence homology. The BLASTN results correspond to the similarity between the sequence queried and the biological sequences within the NCBI database.

(Altschul et al., 1997). Biosafety assessments were based on the strain identification of the putative probiotics of interest and only organisms that were generally regarded as safe (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 (Figure 1). The additional isolates were included based on historical information related to high digestive enzyme activities or because they were of animal origin. In the pH 3 survival study, 63 isolates were tolerant to pH 3, whilst the three that lost complete viability were eliminated from further testing (Figure 1). These 63 isolates were then tested for growth in the presence of bile salts, of which, 19 were unable to grow, 12 grew poorly, whilst the remaining 32 isolates that grew well, were selected for further evaluation of growth at intestinal pH extremes. At pH 5, 20 isolates were eliminated (13 did not grow and seven grew poorly), whilst at pH 7, 12 isolates were eliminated due to poor growth. The remaining 11 isolates that showed significantly higher growth ($p < 0.05$) at both intestinal pH levels were selected and included into the next screening phase.

Secondary screening phase

Extracellular enzyme production and the ability to physically break down feed

The 11 isolates carried over from the critical screen to the secondary selection phase were tested for extracellular enzyme production and all isolates produced the four enzymes of interest, but at varying levels (Figure 2). In the amylase production test, five isolates clustered significantly above average (CPB 029, CPB 011, D 014, HP 1.6

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and CPB 035) ($p < 0.05$). Similarly isolates CPB 011, CPB 029 and CPB 035 were significantly better in the protease test ($p < 0.01$). With regards to the enzymes cellulase and xylanase, three organisms each for cellulase (CPB 003, CPB 029 and D 014) and xylanase (CPB 011, D 014 and CPB 020) performed significantly better respectively ($p < 0.05$ and $p < 0.01$). Four isolates (CPB 029, CPB 035, D014 and HP 1.6) had significantly higher performance in the cumulative scoring rating of all enzymes ($p < 0.01$).

In the physical feed breakdown application study, CPB 011, CPB 035 and D014, were the best isolates and clustered significantly above the average ($p < 0.05$).

Adherence potential to gut epithelium cells

Three isolates (CPB 010, CBP 035 and CPB 029) showed significantly higher adherence (~43 % attachment) to Caco-2 cells ($p < 0.05$), of the 11 evaluated. Isolate CPB 010 resulted in the highest adherence (~57%), whilst five isolates were average (~ 37 % adherence) and three isolates were poor (~20% adherence) (Figure 3).

Antagonistic activity against common poultry pathogens

When, antagonistic activity of the 11 isolates was measured against *E. coli,* CPB 011, CPB 020, CPB 029, CPB 035 and HP 1.6 resulted in significantly better antagonism against the pathogen ($p <0.01$) (Figure 4). Similarly, isolates CPB 011, CPB 029, CPB 035, and HP 1.6 expressed significantly higher antagonism against *S. enteritis* ($p < 0.05$), whereas CPB 011, CPB 035, HP1.6 and D014 displayed significantly better antagonism ($p < 0.05$) against *L. monocytogenes.* Isolates CPB 011, CPB 003, HP 1.6 and CPB 020 were the best organisms regarding antagonism against *C. perfringens* ($p < 0.01$). When comparing the overall antagonistic activity against all four of the pathogens of interest, isolates CPB 011, CPB 020, CPB 035 and HP 1.6 resulted in the highest scores.

Final selection of putative probiotics

Using the mathematical strategy designed for this study, results showed that isolates CPB 020, CPB 035 and CPB 011 performed significantly better than the average performers, based on the cumulative score ($p < 00.5$) 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 production potential in industrial media wherein 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 and is a good starting point when developing probiotics, as it not only gives the best chance of surviving and colonizing the intestine but also alleviants many of the challenges associated with introducing foreign bacteria. This was the rational in targeting the various broiler production facilities. The result obtained from our isolation programme confirm the general expectation that samples associated with the GIT and faeces resulted in a higher yield of putative isolates, as the microorganisms are generally associated with the GIT. Traditionally, *Bacillus* spp. are considered mostly aerobic but our study, similarly to those of other researchers, (Barbosa et al., 2005; Wolfenden et al., 2010; Latorre et al., 2014; Chaiyawan et al., 2015), showed that they can be successfully isolated from the facultative and anaerobic zones of the chicken GIT.

Critical Screening Phase

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

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

The first screen was designed to evaluate survival at low stomach pH wherein 63 of the 66 isolates survived, attributable to the resistant nature of *Bacillus* spores (Spinosa et al., 2000; Cutting, 2011). The remaining three isolates were eliminated from further study, as they did not satisfy the minimum survival criteria. Of the 63 survivors, the seven that grew were scored higher because growth at this pH is furthermore highly desirable. Pre-

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germination and growth in the gizzard delivers actively growing cells to the zones within the intestine where the probiotic effect is required, therefore the probiotic activity of these isolates is expected to be better, especially considering the short transient time within the chicken GIT under production conditions (Hughes, 2008). Interestingly, all three isolates that did not survive at pH 3, were not obtained from poultry environments, perhaps indicating better adaptation of isolates from the target host to low pH. The key requirement for this screen was for the probiotic to retain viability after exposure to the acidic conditions and growth is not obligatory in this section of the digestive system (Fuller, 2001), in contrast to the small intestine which is the main site of probiotic activity (Pan and Yu, 2014). In our study, seven isolates actually grew, which indicated exceptional potential as putative probiotics.

In the bile salt growth test, isolates that did not survive or grew poorly (31 isolates) were eliminated from further screening of the 63 that were carried over into this test. These 31 isolates did not qualify as suitable probiotics mainly due to the lack of resistance to the antimicrobial properties of bile salts and were eliminated from further testing. It is likely that these organisms do not produce the enzyme bile hydrolase which offers protection from the toxic effects of bile (Begley et al., 2006; Patel et al., 2010). In contrast the 32 isolates that grew well in the presence of bile salts (most likely attributable to the production of bile hydrolase) 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 natural defence mechanism and elicits an antimicrobial effect (Begley et al., 2005; Begley et al., 2006). When selecting feed probiotics, the survival in bile salts is therefore considered a minimum requirement for proper functionality in the intestine. In chicken production, bile is constantly produced due to continuous feeding, therefore good growth in the presence of bile is an important requirement (Jin et al., 1998a).

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). However, at pH 5 only 12 of the 32 isolates tested grew well and this is, again, attributable to the resistant nature of the spore state. More than 50 % of the isolates screened were eliminated, indicating the rigor in our elimination procedures. This pH occurs at the beginning of the small intestine due to the acid carried over from the gizzard and growth at this lower pH is important to maximise the probiotic effect.

Overall, of the 66 isolates screened, only 11 isolates were selected based on the critical elimination criteria conferring that approximately 83% of isolates were eliminated. This indicated that the elimination criteria used were sufficiently rigorous to only allow the best candidates that survived the *in vitro* simulated GIT conditions, to be selected for further testing. This screening strategy ensures better potential to find suitable putative probiotics, by eliminating the poor candidates early in the selection process, enabling focus on a smaller number of isolates in subsequent screens. The 11 isolates remaining were then subjected to a secondary screening phase.

Secondary screening phase

The secondary screening phase comprised of the 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 break down feed

Isolates CPB 029, CPB 035, D014 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 make up approximately ~60 % of poultry feed. Even though the chicken naturally produces this enzyme, additional production from probiotics will enhance 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 which are also important in the digestion of complex proteins in the diet (~20%). Although produced by the chicken in smaller quantities, poultry producers supplement this enzyme in their feeding regimes, due to higher levels eliciting multiple benefits, such as, improvement in amino acid digestibility across various protein sources, minimization of the impact of anti-nutritional factors and allergenic proteins in feedstuffs, and augmentation of the degradation of low-quality proteins (Ravindran, 2013).

Only three of the 11 isolates were able to produce high levels of cellulase and xylanase. These isolates are of great interest because these enzymes are not naturally produced by the chicken, therefore their addition in feedstuffs is becoming customary. The global trend is moving towards formulating poultry 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). This change in monogastric animal diets has had a negative impact in growth performance as these NSP based raw materials, increase intestinal viscosity, affecting digestibility and absorption of nutrients by the intestinal surface (Annison, 1993; Choct, 2006; Khattak et al., 2006; Latorre et al., 2016). This requires the addition of NSP-enzymes such as xylanase and cellulase to aid in the digestion of these diets (Guo et al., 2013), but probiotics can serve this function, reducing the need for addition of NSP-enzymes.

This enzyme study allowed us to confirm that the putative probiotics of interest all produced the four key enzymes of importance for 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 the poorest in breaking down feed. This could be expected as the ability to physically break down feed was mainly a function of enzyme activity in our *in vitro* test. A fact often overlooked when screening probiotics, is the impact that enzymes play in the particle size of feed and its role in energy conversion. The ability to physically break down feed by probiotic action within the crop of the chicken, before movement into the gizzard, improves intestinal digestion and absorption because of increased surface to volume ratio of the feed particles. (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 to the finding of Thirabunyanon and Thongwittaya (2012) who demonstrated a range of adherence capabilities from *Bacillus* spp. The moderate attachment abilities of *Bacillus* spp. were corroborated by an *in vivo* study by Latorre *et al*. (2014) which was conducted to ascertain the germination, persistence and distribution of *Bacillus* spores throughout the GIT of broiler chickens. Contrastingly higher adherence to epithelial cells (>70 %) was reported by Chaiyawan *et al*. (2015). However, adherence studies on *Bacillus* spp. remain sporadic in literature preventing validation of acceptable adherence levels in functionalised probiotics but significant information is available for *Lactobacillus* spp. showing relatively higher levels of adherence (Garriga et al., 1998; Ehrmann et al., 2002; Bouzaine et al., 2005; Heravi et al., 2011; Aazami et al., 2014). Adherence to the epithelial cells of the host offers a competitive advantage as the attachment improves the residence time and thus the probiotic effect in the gut (Bernet et al., 1994; Servin and Coconnier, 2003). Attached organisms are beneficial, as the flow of digested feed due to peristalsis, hinders probiotic activity if the cells are not attached, especially because the residence time is relatively short in chickens (Hughes, 2008). Additionally, chicks used for broiler production are hatched in artificial incubators and as such their GIT is pioneered entirely by exogenous organisms (Pan and Yu, 2014). This presents an opportunity for the use of probiotics to colonize the GIT of day-old chicks and reduces the potential for exogenous pathogen attachment. (Ouwehand et al., 1999). As *Bacillus* cells are generally less adherent, transient presence in the GIT of chickens 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 it's infection (particularly the O157:H7 strain) in broilers causes serious commercial losses in poultry production (Kiranmayi et al., 2010). All isolates tested, showed some antagonistic activity against *S. enteritis*, albeit at varying levels. This is an important results as *S. enteritis* is the most prevalent disease causing pathogen in the poultry industry resulting in Salmonellosis (Boyle et al., 2007; Finstad et al., 2012; Dhama et al., 2013a). Our results from this study correlate well with the established research regarding both *E. coli* and *S. enteritis* (Guo et al., 2006; Thirabunyanon and Thongwittaya, 2012; Latorre et al., 2016).

Approximately 54% of the isolates tested were antagonistic towards *L. monocytogenes* and this could be commercially relevant in the reduction of Listeriosis which is becoming a serious threat, as epidemics are occurring worldwide. Currently, it is becoming imperative to screen for antagonism against this pathogen and our study contributes substantively to the limited information available on antagonism of *Bacillus* based probiotics against this pathogen (Dhama et al., 2013b). Interestingly, more than 40%, of the isolates did not show any inhibition of *L. monocytogenes*, thus the isolates showing inhibition are important in addressing this disease through probiotic technology. Similarly, ~54% of the isolates tested, showed antagonistic activity to *C. perfringens*. Although not detrimental to humans, *C. perfringens* has fatal effects on poultry as it is the cause of clinical necrotic enteritis (NE), (necrosis of the intestine), which is highly infectious and can lead to serious economic losses (Immerseel et al., 2004). Unlike the other pathogens, the *C. perfringens* study was conducted under both aerobic and anaerobic conditions because this organism grows best under obligate anaerobiosis and thus provided the best probiotic-to- pathogen challenge conditions. All putative probiotics performed marginally better in aerobic conditions, but the results were insignificantly different, showing the ability of *Bacillus* spp. to inhibit this pathogen under both conditions even through the preferred growth condition is aerobic (Rasko et al., 2005; Stahly et al., 2006).

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

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

Final selection and safety of putative probiotics

The final selection from the 11 candidate probiotics resulted in a core consortium (CPB 011, CPB 020, and CPB 029) and included three ancillary isolates (CPB 035, HP 1.6 and D 014), based on the significant differences between the two groups in overall performance. The screening rationale used in our study was designed in such a manner as to systematically eliminate isolates with poor potential in the critical screening phase and then to stringently evaluate the putative isolates selected, for probiotics effects. Many authors have used similar strategies (Barbosa et al., 2005; Wolfenden et al., 2010; Chaiyawan et al., 2015; Nguyen et al., 2015), few of which have an elimination strategy to circumvent screening a large number of isolates. Using relative response methodology and by clustering the isolates into sub-populations of significant differences in performance, allowed us to mathematically rate the isolates for each of the criteria. The criteria used were prioritised based on the key requirements for functionality of poultry probiotics (Fontana et al., 2013). Scores were converted to a desirability co-efficient, which served as an overall indicator of the suitability of each isolate across all of the criteria in a

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cumulative manner. In probiotic selection, a key factor to consider is that one excellent probiotic characteristic does not surpass the value of a holistic range of probiotic effects (Chapman et al., 2011), especially if a multi-mode product is the end objective. In response to this, our selection strategy yielded a six-strain consortium that comprised the full complement of desirable characteristics and hence offers the poultry industry of a convenient single product solution.

Identification of our consortium revealed four *B. subtilis* and two *B. velezensis* strains with 99% sequence homology. The latter is closely related to *B. subtilis* and is "generally regarded as safe" (GRAS) by the US Food and Drug Administration (USFDA) (Harwood and Wipat, 1996). The use of *Bacillus* spp. as probiotics has been met with some scepticism due to a few pathogenic species, however the consortium selected in our study has been proven safe.

The findings of this study resulted in development of a multi-strain consortium that in combination, produced extracellular enzymes, adhered to epithelial cells and showed antagonistic activity against common poultry pathogens. The consortium was also successfully shown to survive and grow in the presence of bile and under the range of challenging pH conditions simulating the chicken GIT. This study allowed for the successful selection of a consortium of strains, which was proven to be effective in an *in vivo* broiler field trial (Ramlucken et al., 2019). This multi-mode consortium shows excellent potential to address the commercial challenges of the poultry industry.

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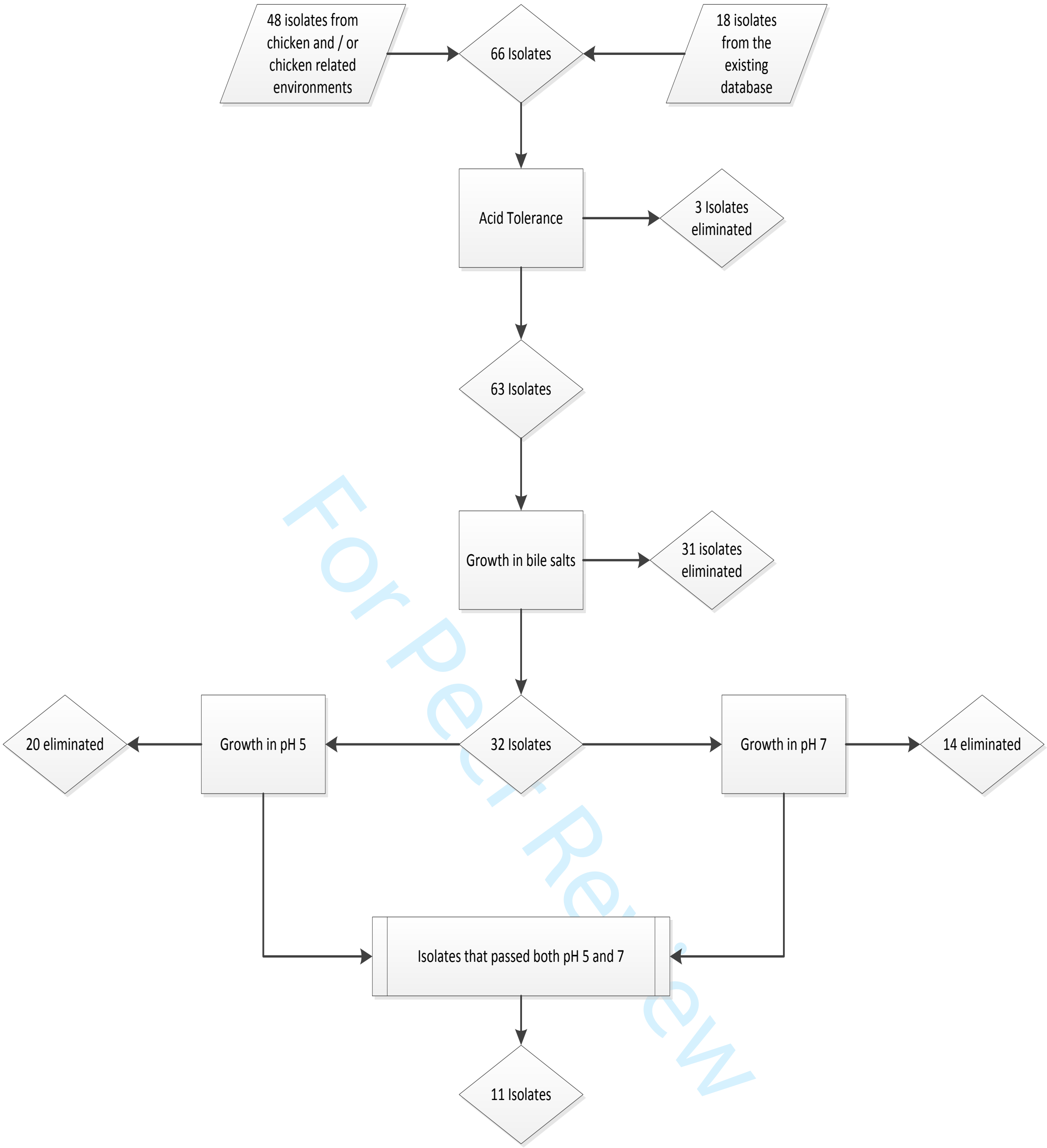
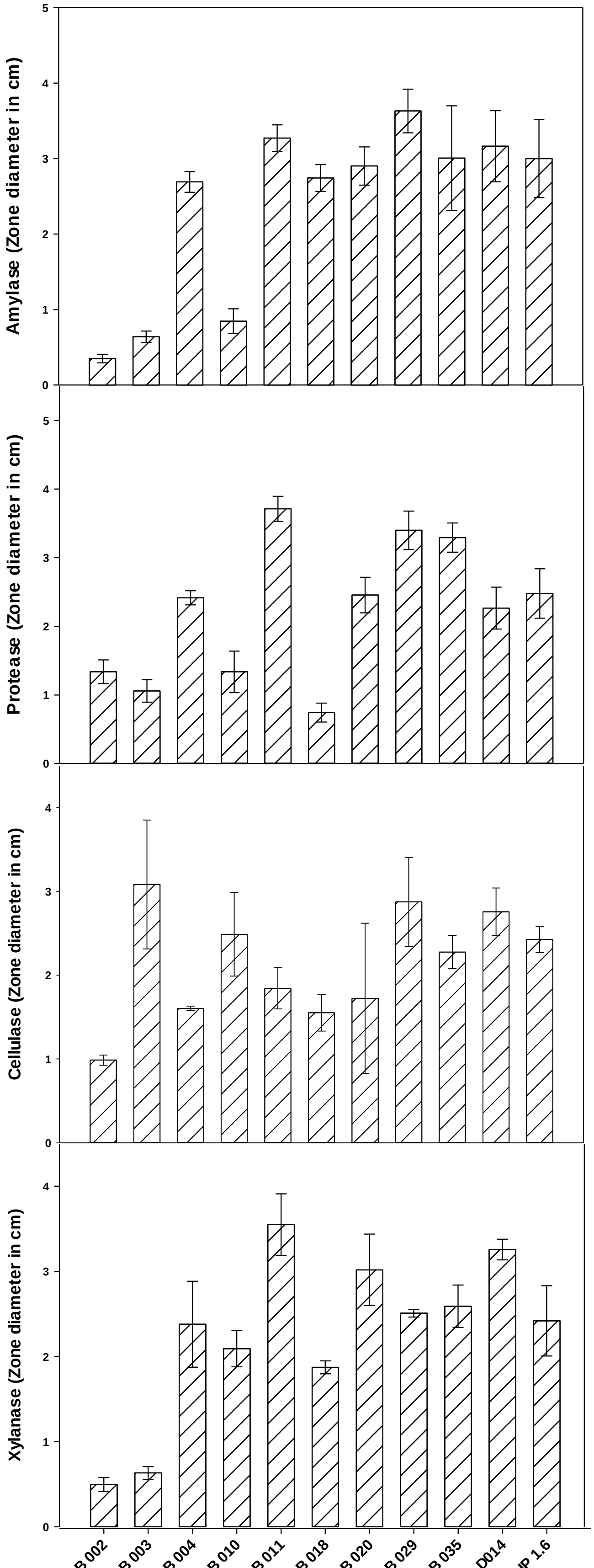
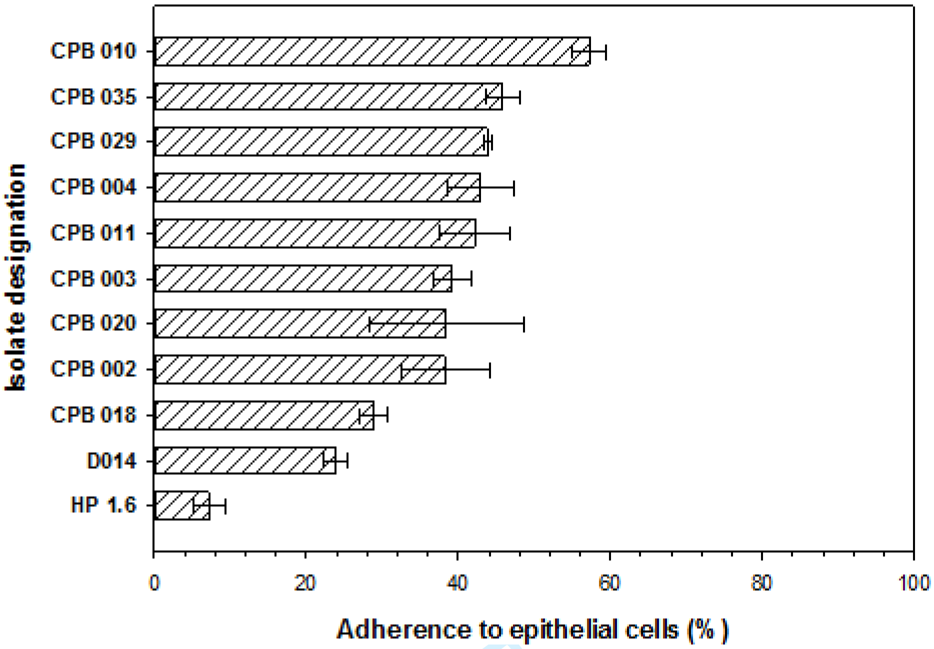

48 isolates from chicken and / or chicken related environments
66 Isolates
18 isolates from the existing database
Acid Tolerance
3 Isolates eliminated
63 Isolates
Growth in bile salts
31 isolates eliminated
20 eliminated
Growth in pH 5
32 Isolates
Growth in pH 7
14 eliminated
Isolates that passed both pH 5 and 7
11 Isolates


Amylase (Zone diameter in cm)
Protease (Zone diameter in cm)
Cellulase (Zone diameter in cm)
Xylanase (Zone diameter in cm)
0
1
2
3
4
5
CPB 002
CPB 003
CPB 004
CPB 010
CPB 011
CPB 018
CPB 020
CPB 029
CPB 035
D014
HP 1.6
Isolate designation


CPB 010
CPB 035
CPB 029
CPB 004
CPB 011
CPB 003
CPB 020
CPB 002
CPB 018
D014
HP 1.6
Isolate designation
0
20
40
60
80
100
Adherence to epithelial cells (%)

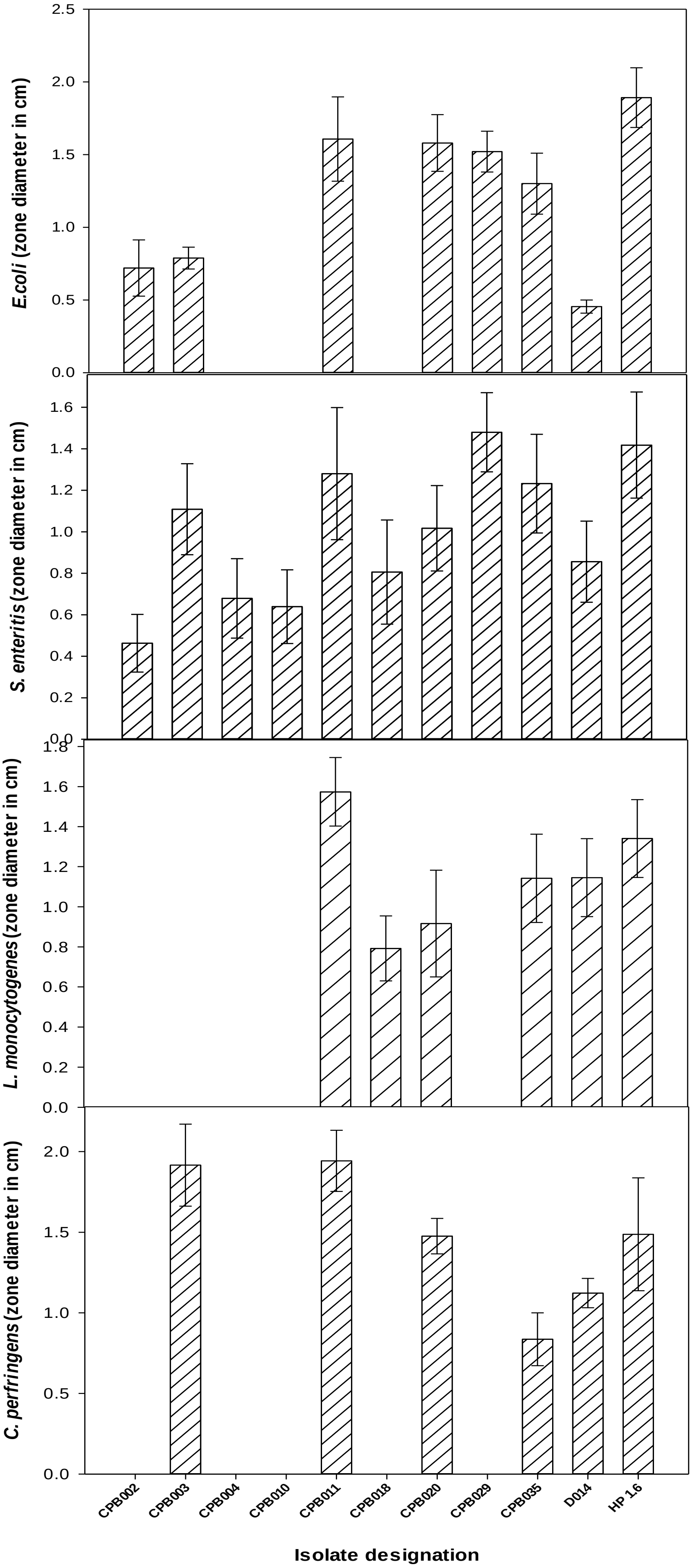

E.coli (zone diameter in cm)
S. enteritis (zone diameter in cm)
L. monocytogenes (zone diameter in cm)
C. perfringens (zone diameter in cm)
0.0
0.5
1.0
1.5
2.0
2.5
0.2
0.4
0.6
0.8
1.2
1.4
1.6
1.8
CPB002
CPB003
CPB004
CPB010
CPB011
CPB018
CPB020
CPB029
CPB035
D014
HP 1.6
Isolate designation

Table 1 Desirability co-efficient of each putative probiotics showing suitability to each criteria and cumulative multi-mode performance rating (relative % to maximum)

Organism Designation	**Survival and Growth at pH3**	**Growth in Bile**	**Growth at intestinal pH**	**Adherence**	**Digestive Enzymes**	**Pathogen Antagonism**	**Feed Breakdown**	**Cumulative**
				Desirability Co-efficient (%)				
CPB 020	**100.0**	**50.0**	**100.0**	**50.0**	**80.0**	**85.4**	**50.0**	**100.0**
CPB 035	**28.6**	**50.0**	**60.0**	**75.0**	**93.3**	**91.7**	**100.0**	**96.7**
CPB 011	**14.3**	**50.0**	**100.0**	**50.0**	**100.0**	**100.0**	**75.0**	**94.9**
CPB 010[d]	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

[d] Unable to grow in industrial media

Fig. 1 Schematic illustration of critical screening steps showing the selection of qualifying isolates. The critical screen consisted of the survivability and growth of isolates at pH 3, in the presence of bile salts and in the pH extremes of the intestine. All experiments were representative of three repeats and all results were determined as mean ± standard deviation.

Fig. 2 Extracellular enzyme activity of putative probiotics measured by the zone diameter (cm) on a substrate dependant medium. Enzymes evaluated were amylase, protease, cellulase and xylanase. Error bars represent standard deviations, n= 6 replicates, p-values (two-tailed), $p < 0.05$.

Fig. 3 Adherence activity of putative probiotics to epithelial cells (Caco-2). Isolates were assessed on their ability to adhere to an epithelial monolayer for two hours after rigorous washing. Error bars represent standard deviations, n= 3 replicates p-values (two-tailed), $p < 0.05$.

Fig. 4 The antagonistic activity of isolates against common chicken pathogens namely *E. coli*, *S. enteritis*, *L. monocytogenes* and *C. perfringens*. Competitive exclusion was measured by spatial domination of the pathogen. Error bars represent standard deviations, n= 6 replicates p-values (two-tailed), $p < 0.05$.