ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

# POULTRY SCIENCE

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

International Journal of Poultry Science 9 (4): 317-323, 2010
ISSN 1682-8356


# Evaluation of a Screening and Selection Method for *Bacillus* Isolates for Use as Effective Direct-fed Microbials in Commercial Poultry

R.E. Wolfenden, N.R. Pumford, M.J. Morgan, S. Shivaramaiah,
A.D. Wolfenden, G. Tellez and B.M. Hargis
Department of Poultry Science, University of Arkansas, Fayetteville, AR 72701, USA

**Abstract:** *Bacillus*-based direct-fed microbials may be an effective alternative to antibiotic growth promoters. Environmental samples were pasteurized to remove vegetative cells, plated onto TSA or SPA for 24 or 72 h and overlayed with soft agar containing *S. enteritidis* or *C. perfringens*. Isolates which produced antimicrobial activity against both pathogens were used to inoculate a solid state fermentation media and allowed to sporulate, to numbers greater than $10^9$ spores/g and subjected to *in vivo* testing in both poults and chicks. In exp. 1 chicks fed isolates PHL-RW35 and PHL-RW41, at doses of $10^7$ and $10^5$ spores/g feed respectively, showed significant increases ($p \leq 0.05$) in both Body Weight (BW) and Body Weight Gain (BWG). No significant differences in BW or BWG were noted in poults for any treatment. In this experiment, all groups were challenged with $10^5$ cfu of *S. typhimurium* at day-of-hatch, no significant differences in *Salmonella* were noted between groups. In experiment 2 PHL-RW41 fed at $10^5$ spores/g of feed significantly increased BWG by 8.3 and 11.7% in chicks and poults respectively. Isolate PHL-RW35 also increased BW and BWG in poults. These data indicate this approach for *in vitro* selection may be effective for screening and selection of *Bacillus* direct-fed microbials capable of causing an increase in BW and BWG in commercial poultry.

**Key words:** Screening, *Bacillus* spores, *Salmonella*, *Clostridium*, poultry

## INTRODUCTION

Antibiotics have long been used in veterinary medicine and animal agriculture for therapeutic purposes as well as for growth promotion. In recent years, concerns over antibiotic residues and antibiotic resistance generated by the use of antibiotics in food animals has led to increased pressure to ban or severely limit the use of these chemicals in animal agriculture. The European Union has already banned the use of antibiotics as growth promoters and although the use of antibiotics to promote growth is still legal in the United States, consumer preference toward poultry raised without antibiotics is on the rise. *Bacillus*-based Direct-fed Microbials (DFM) or probiotics may be one viable alternative to growth promoting antibiotics in commercial poultry.

Numerous studies have shown that specific strains or formulations of beneficial bacteria are able to increase production parameters (Torres-Rodriguez *et al*., 2007; Vicente *et al*., 2007; Vila *et al*., 2009) as well as reduce gastrointestinal pathogens such as *Salmonella* sp., *E. coli* and *Campylobacter jejumi* (Snoeyenbos *et al*., 1979; Corrier *et al*., 1993; Mead *et al*., 1996; Hakkinen and Schneitz, 1999; Scharek *et al*., 2007). Most probiotic formulations used in commercial poultry production are defined cultures predominantly made up of *Lactobacillus* and closely related genera of autochthonous intestinal bacteria. The most common route of administration of these probiotic cultures has been through the drinking water, but application using this method can lead to compliance issues and improper administration. One of the biggest disadvantages to applying a probiotic in this manner is that improper administration of this product can result in reduced viability of the culture, which may reduce or eliminate efficacy. As these cultures are made from vegetative cells, the shelf life is often limited to several months under optimal conditions, and temperature fluctuations or high ambient temperatures can further reduce shelf life. Effective DFM formulations with increased stability, long shelf life, and ability to be applied through the feed have advantages over those designed for drinking water administration alone.

The hallmark characteristic of the genus *Bacillus* and related genera is the ability to form environmentally-resistant spores. The spore is the survival form of the *Bacillus* and spores of some *Bacillus* isolates are extremely resistant to heat, chemicals, radiation and desiccation (Setlow, 2006). Additionally, spores can remain viable for years (Nicholson, 2002) and in some cases millennia (Vreeland *et al*., 2000). Most commercial poultry rations are pelleted to increase body weight and feed consumption (Choi *et al*., 1986; Cutlip *et al*., 2006; Cutlip *et al*., 2008). The stability of the

**Corresponding Author:** B.M. Hargis, Department of Poultry Science, University of Arkansas, Fayetteville AR 72701, USA

spore allows it to be more resistant than vegetative bacteria to the temperatures and pressures commonly exerted during pelleting and storage of commercial feed. Several recent studies have shown that some *Bacillus*-based DFM can increase production parameters and decrease pathogens in commercial poultry (Fritts *et al*., 2000; Vila *et al*., 2009). Previous work by Barbosa *et al*. (2005) has focused on the screening for *Bacillus* isolates in the gastrointestinal tract of chickens to identify potential probiotic candidates. Other investigators have also screened for potential probiotic isolates of other bacterial species found in the in the gastrointestinal tract of poultry (Bielke *et al*., 2003; Taheri *et al*., 2009). The most logical place to screen for potential probiotic isolates may be either from the gut or fecal material of healthy chickens or turkeys because it is in the gastrointestinal tract that these bacteria act. While this may be the best place to search for isolates which are obligatorily associated with the gastrointestinal tract of poultry, other potential sources of probiotic bacteria may actually be found elsewhere as outlined below.

In a recent publication, Guo *et al*. (2006) isolated *B. subtilis* strain MA139 from Chinese herbs. From a pool of 750 *Bacillus* strains isolated from both animal and environmental sources, this isolate was selected for *in vivo* testing due to numerous *in vitro* tests to assess the potential of this and other isolates as probiotic candidates. *B. subtilis* strain MA139 was shown to increase body weight and Average Daily Gain (ADG) while decreasing pathogenic *E. coli* in weaned piglets. This clearly demonstrates that potential probiotic *Bacillus* candidates can be isolated from sources outside the gastrointestinal tract of the intended host animal. Since the primary reservoir for *Bacillus* is the soil, the best places to screen for *Bacillus* isolates may be from the soil or plant and animal material closely with the soil. On average, $10^8$ *Bacillus* and related genera are found in each gram of soil (Stein, 2005). Due to the extreme stability of the *Bacillus* spore, they can often lie dormant for weeks or years allowing multiple strains and species to accumulate in a single location (Nicholson, 2002; Felske, 2004; Nicholson, 2004). A single sample of consisting of several grams of soil may therefore potentially yield hundreds or even thousands of individual strains and species of *Bacillus*.

We have recently developed and evaluated a potential screening process for environmental isolates of *Bacillus* for use as a probiotic or DFM. Initial *in vivo* testing confirms that this method is able to produce viable probiotic candidates.

## MATERIALS AND METHODS

**Isolation of aerobic spore formers from environmental samples:** Environmental samples were collected using sterile cotton swabs and placed into a sterile borosilicate tube for transport. The swabs were pasteurized either by immersion in 50% ethanol or by heating to 70°C for 15 min. Swabs were then struck for a lawn of growth on both Tryptic Soy Agar (TSA) (catalog no. 236950, Becton Dickinson, Sparks, MD) and Spizizen Potato Agar (SPA) (Spizizen, 1958) then incubated for 24 h and 72 h respectively at 37°C. The plated samples were overlaid with a TSA soft agar (Tryptic Soy Broth (TSB) (catalog no. 211822, Becton Dickinson, Sparks, MD) 30 g/L, Bacto Peptone (catalog no. 211677, Becton Dickinson, Sparks, MD) 20 g/L, NaCl (catalog no. BDH0286, VWR, West Chester, PA) 0.5 g/L and Bacto Agar (catalog no. 214010, Becton Dickinson, Sparks, MD) 11.6 g/L) containing 25 μg/mL novobiocin (catalog no. N1628, Sigma, St. Louis, MO) and $10^6$ cfu/ml *Salmonella enterica* serovar Enteritidis phage type 13A (SE) originally obtained from the U.S. Department of Agriculture National Veterinary Services Laboratory. After incubation, plates were evaluated and those colonies which produced zones of inhibition were selected for isolation. The selected colonies were pasteurized in 50% ethanol then struck for isolation onto the same medium from which they were selected and incubated for either 24 h (TSA) or 72 h (SPA) at 37°C. Antimicrobial activity was confirmed using a second SE overlay. Those isolates with confirmed *in vitro* anti-SE activity were used to inoculate fresh TSB and incubated overnight, aliquoted and stored with 30% sterile glycerol at -80°C until needed.

**Detection of hemolysis and identification of isolates:** TSA plates containing sheep blood (TSB 30 g/L, Bacto Agar 15 g/L, defibrinated sheep blood (catalog no. R54012, Remel, Lenexa, KS) 50 ml/L) were inoculated with the aerobic spore former isolates and incubated for 24 h at 37°C. The plates were then evaluated and scored for level of hemolysis. All isolates causing alpha or beta hemolysis were not evaluated further. Similarly, further testing of any isolate with colony morphology consistent with that of the *B. cereus* group (*B. cereus*, *B. mycoides*, *B. thurigensis* and *B. anthrasis*) was discontinued. Those isolates with the greatest *in vitro* antimicrobial activity against SE were identified using the bioMerieux API 50 CHB (catalog no. 50430, Biomerieux, Durhum, NC) test kit. Briefly, *Bacillus* and closely related genera are classified by their ability to utilize the 49 different carbohydrates of this test. The results were analyzed using the API Web database (https://apiweb.biomerieux.com) for species level identification.

***In vitro* assessment of antimicrobial activity against *Clostridium perfringens*:** The *Bacillus* isolates identified using the above methods were further screened for *in vitro* antimicrobial activity against *Clostridium perfringens* (CP). A similar overlay method as described above was used, but overlays were incubated anaerobically without novobiocin.

**Determination of spore viability after heat treatment:** Three samples of each of the sporulated candidate cultures were diluted 1:9 in 0.9% sterile saline. Duplicate samples were subjected to room temperature for 30 min or 100°C for 10 min to assess heat stability of the candidate spores. Following the heat treatments the spore solutions were enumerated using 10-fold dilutions and plate-counting following overnight incubation at 37°C on TSA plates.

**Preparation of feed for animal trials:** In an effort to grow high numbers of viable spores, a Solid State Fermentation Media (SS) developed by Zhao *et al.* (2008) was selected and modified for use in these experiments. Briefly a liquid media component was added to a mixture of 70% rice straw and 30% wheat bran at a rate of 40% by weight. The SS media was added to a 250 mL Erlenmeyer flask and sterilized by autoclaving for 30 min at 121°C. Candidate isolates were grown individually overnight at 37°C in TSB, then 2 mL of a candidate culture were added to the prepared SS media. The inoculated flasks were incubated for 24 h at 37°C then incubated for another 72 h at 30°C. The cultures were removed from their flasks, placed onto petri dishes and then dried at 60°C. The cultures were finely ground and held at room temperature. Isolates PHL-RW 41, PHL-RW 35 and PHL-MM03 were selected for further analysis in *in vivo* trials with poults and chicks due to their performance in the *in vitro* tests. An antibiotic-free corn-soy turkey starter diet formulated to meet or exceed the NRC requirements (NRC, 1994) for critical nutrients of young poults and chicks was used as the basal diet for these experiments. Spores from PHL-RW 41, PHL-RW 35 and PHL-MM3 and were added to the basal diet and mixed with a rotary mixer for 15 minutes to distribute spores throughout feed.

***Salmonella:*** A primary poultry isolate of *Salmonella typhimurium* (ST) selected for resistance to Nalidixic Acid (NA) (catalog no. N4382, Sigma, St Louis, MO), was used as the challenge organism for these experiments. The amplification protocol has been described in detail (Bielke *et al*., 2003). Briefly, ST was incubated for 24 h in TSB at 37°C and passaged at eight hour intervals into fresh TSB. The cells from the final passage were then washed three times with 0.9% sterile saline by centrifugation at 1864 x g. The approximate concentration of ST was estimated using a spectrophotometer and actual concentrations were determined retrospectively by serially diluting the stock solution and plating on Brilliant Green Agar (BGA) (catalog no. 228530, Becton Dickinson, Sparks, MD) plates containing 25 µg/mL NO and 20 µg/mL NA.

**Experiment 1:** The purpose of the first experiment was to do a small scale *in vivo* test of isolates PHL-RW 35 and PHL-RW 41 at different inclusion rates in the feed. Commercial female Cobb chicks and commercial female Hybrid turkeys were obtained on day of hatch and orally gavaged with 1.75 x $10^5$ cfu of ST. They were then randomly assigned to one of 7 treatments: negative control (basal diet), PHL-RW 35 at $10^5$ spores/g feed, PHL-RW 35 at $10^7$ spores/g feed, PHL-RW 41 at $10^5$ spores/g feed, PHL-RW 41 at $10^7$ spores/g feed, PHL-RW 35 and PHL-RW 41 at $10^5$ spores /g feed and PHL-RW 35 or PH-RW 41 at $10^7$ spores/g feed. The poults and chicks were then tagged, weighed and placed into the appropriate floor pens by treatment. Twenty poults and 20 chicks were placed into each of the approximately 2.2 square meter pens. The poults and chicks had fresh pine shaving as bedding material and had free access to feed and water throughout the duration of the trial. The environmental conditions were maintained appropriately for the age of the birds. At the conclusion of this 11 day trial, the chicks and poults were weighed and them humanely killed by carbon dioxide asphyxiation. The cecal tonsils were aseptically removed and placed into tetrathionate broth (catalog no. 210420, Becton Dickinson, Sparks, MD). The ceca were homogenized and diluted 1:4 by weight with sterile saline. Total viable counts of ST were determined by making 10-fold dilutions and plating on BGA plates with 25 µg/mL NO and 20 µg/mL NA after 24 h of incubation at 37°C. The plates were examined for the presence of colonies typical of this strain of ST on BGA media.

**Experiment 2:** The second experiment was conducted to better asses the effects of candidate *Bacillus* isolates on body weight and body weight gain in turkey poults and broiler chicks. Commercial male Cobb chicks and commercial female Hybrid turkeys were obtained on day of hatch and orally gavaged with 6.25 x $10^3$ cfu of ST. They were then randomly assigned to 1 of 4 treatments: Negative control (4 pens), PHL-RW41 $10^5$ spores/g of feed (4 pens), PHL-MM03 $10^6$ spores/g of feed (4 pens), or PHL-RW 35 $10^7$ spores/g or feed (3 pens). The poults and chicks were then tagged, weighed and placed into the appropriate floor pens by treatment. The pens had previously been block randomized to reduce variance in the data. Into each of the 2.2 square meter pens 20 chicks and 20 poults were placed. The poults and chicks had fresh pine shaving as bedding material and were given free access to feed and water throughout the duration of the trial. The environmental conditions were maintained appropriately for the age of the birds. The chicks and poults were weighed 9 days post placement and then humanely killed by carbon dioxide asphyxiation.

**Statistical analysis:** Data from these studies were subjected to ANOVA using JMP7 (SAS, 2007). The partitioned means were deemed significant if a p-value of less than or equal to 0.05 ($p \leq 0.05$) was obtained.

## RESULTS AND DISCUSSION

Ideally, a *Bacillus*-based probiotic or direct-fed microbial should be cost effective to produce, have the required stability for in feed application and either control enteric bacterial pathogens or improve flock performance (or both). Four criteria were established for *in vitro* selection of isolates in the present study to meet these standards. The first criterion that candidate isolates had to meet was their ability to kill both *Salmonella enteritidis* and *Clostridium perfringens in vitro*. These bacteria are important in the poultry industry for both animal and human health issues. Additionally as these two pathogens are evolutionarily divergent, isolates that can effectively kill both species of bacteria *in vitro* may have a broad range of antimicrobial activity. Several members of the *Bacillus* genus, most notably *B. antracis* and *B. cereus*, are known human and animal pathogens. As such, the second criterion for further testing was that only known nonpathogenic isolates were retained. The third criterion was the ability of the selected isolates to amplify and sporulate to high numbers in a defined solid state media. For an isolate to be tested further, it had to produce a minimum of $10^9$ spores/g of dried solid state fermentate. The reason for minimum levels of spores in the fermentate are as follows: the estimated inclusion rate of a candidate *Bacillus* probiotic was $10^{11}$ to $10^{13}$ spores per ton of feed, at a yield of $10^9$ spores/g between 100 and 10,000 g of fermentate would be needed per ton of finished feed. Yield below the $10^9$ threshold would make the DFM likely unfeasible for large scale production. As the final criterion, we required that the isolate be highly heat stable. Pelleting temperatures range between 76.7-93.3°C in commercial poultry feed mills (Cutlip *et al*., 2008). As most commercial poultry feed is pelleted, candidate isolates had to be able to withstand high temperature to remain viable after pelleting. All candidate isolates which passed the first three criteria were subjected to 100°C for 10 min. Only candidates which retained viability with less than a 10-fold reduction were selected for further testing.

After our initial screening of environmental samples 2 isolates, designated PHL-RW35 and PHL-RW41, were selected out of an estimated 250,000 isolates screened based on the above *in vitro* selection criteria. These 2 isolates were evaluated in a preliminary experiment (experiment 1) to assess their performance as a probiotic alone or in combination at doses of $10^5$ or $10^7$ spores/g finished feed. After a second round of *in vitro* testing a third isolate, PHL-MM03, was selected using the same *in vitro* selection criteria and evaluated alongside PHL-RW35 and PHL-RW41 in a second experiment (experiment 2).

In experiment 1, *Bacillus licheniformis* isolate PHL-RW41, when added to the feed at a rate of $10^5$ spores/g of finished feed led to a significant increase in Body Weight (BW) and Body Weight Gain (BWG) of broiler chicks as compared to the control group (Table 1). In the same experiment, there were no significant differences between groups of poults, but the group of poults fed PHL-RW41 at the rate of $10^5$ spores/g of feed numerically had the highest BW and BWG when compared to the other groups (Table 2). There were no significant differences between doses of the same isolate or combination of isolates, but there appeared to be possible dose response trends between isolate dosages. Both poults and chicks receiving PHL-RW41 at the rate of $10^5$/g feed were numerically heavier than the group receiving a 100-fold higher dose. The opposite was true of chicks and poults fed *B. licheniformis* isolate PHL-RW35 as the group fed at the rate of $10^7$/g feed was numerically heavier than the group fed the lower concentration of spores. None of the treatments significantly reduced either the percentage of birds colonized by ST in the ceca or the rate of cecal colonization by ST in either chicks or poults (Table 3 and 4).

In the second experiment PHL-RW35 and PHL-RW41, along with *B. subtilis* isolate PHL-MM03, were mixed with the basal diet at the rate of $10^7$, $10^5$ and $10^6$ spores/g of finished feed respectively. Both chicks and poults fed PHL-RW41 were significantly heavier than the control group (Table 5 and 6). Interestingly, the chicks receiving PHL-RW35 and the poults treated with PHL-MM03

Table 1: Effect of selected direct-fed microbial candidates on chick Body Weight (BW) and Body Weight Gain (BWG) after 11 days of continuous feeding (Day-of-Hatch through end of experiment) (Experiment 1)

| Treatment | Spores/g Feed | BW (g) | BWG (g) | n |
|---|---|---|---|---|
| Negative control | n/a | 236.9±3.3$^{bc}$ | 193.2±3.6$^{bc}$ | 20 |
| PHL-RW35 | $10^5$ | 236.5±6.5$^{bc}$ | 195.6±6.3$^{bc}$ | 20 |
| PHL-RW35 | $10^7$ | 249.6±5.0$^{ab}$ | 206.8±5.1$^{ab}$ | 20 |
| PHL-RW41 | $10^5$ | 256.8±4.6$^{a}$ | 213.7±4.5$^{a}$ | 20 |
| PHL-RW41 | $10^7$ | 246.6±6.5$^{abc}$ | 203.1±6.3$^{abc}$ | 20 |
| PHL-RW35+41 | $10^5$* | 241.7±4.2$^{bc}$ | 198.3±4.1$^{bc}$ | 20 |
| PHL-RW35+41 | $10^7$* | 234.2±6.0$^{c}$ | 190.5±5.9$^{c}$ | 20 |

*Total dose is two fold higher as listed dose is for each individual *Bacillus* isolate. $^{a-c}$Means within a group with different lowercase letters differ ($p \leq 0.05$)

Table 2: Effect of selected direct-fed microbial candidates on poult Body Weight (BW) and Body Weight Gain (BWG) after 11 days of continuous feeding (Day-of-Hatch through end of experiment) (Experiment 1)

| Treatment | Spores/g Feed | BW (g) | BWG (g) | n |
|---|---|---|---|---|
| Negative control | n/a | 233.1±6.6 | 177.5±6.8 | 19 |
| PHL-RW35 | $10^5$ | 227.9±7.0 | 171.2±6.9 | 19 |
| PHL-RW35 | $10^7$ | 227.7±7.1 | 168.5±6.9 | 19 |
| PHL-RW41 | $10^5$ | 241.5±6.4 | 184.4±6.2 | 20 |
| PHL-RW41 | $10^7$ | 235.7±6.6 | 179.5±6.6 | 20 |
| PHL-RW35+41 | $10^5$* | 229.0±6.6 | 174.0±6.4 | 19 |
| PHL-RW35+41 | $10^7$* | 232.5±6.3 | 176.0±6.3 | 20 |

*Total dose is two fold higher as listed dose is for each individual *Bacillus* isolate

Table 3: Effect of selected direct-fed microbial candidates on recovery of *Salmonella typhimurium* from cecal tonsils (*Salmonella typhimurium* ceca positive/total (%)) and ceca ($Log_{10}$ *Salmonella typhimurium* cecal recovery, $Log_{10}$ CFU/g cecal content) of broiler chicks after 11 days of continuous feeding (Day-of-Hatch through end of experiment) (Experiment 1)

| Treatment | Spores/g Feed | *Salmonella typhimurium* ceca positive/total (%) | $Log_{10}$ *Salmonella typhimurium* cecal recovery |
|---|---|---|---|
| Negative control | n/a | 20/20 (100%) | 5.62±0.21 |
| PHL-RW35 | $10^5$ | 20/20 (100%) | 5.77±0.11 |
| PHL-RW35 | $10^7$ | 20/20 (100%) | 5.69±0.17 |
| PHL-RW41 | $10^5$ | 20/20 (100%) | 5.88±0.17 |
| PHL-RW41 | $10^7$ | 20/20 (100%) | 6.03±0.11 |
| PHL-RW35+41 | $10^5$* | 20/20 (100%) | 6.25±0.17 |
| PHL-RW35+41 | $10^7$* | 20/20 (100%) | 6.22±0.18 |

*Total dose is two fold higher as listed dose is for each individual *Bacillus* isolate

Table 4: Effect of direct-fed microbial candidates on recovery of *Salmonella typhimurium* from cecal tonsils (*Salmonella typhimurium* ceca positive/total (%)) and ceca ($Log_{10}$ *Salmonella typhimurium* cecal recovery, $Log_{10}$ CFU/g cecal content) of Turkey poults after 11 days of continuous feeding (Day-of-Hatch through end of experiment) (Experiment 1)

| Treatment | Spores/g Feed | *Salmonella typhimurium* ceca positive/total (%) | $Log_{10}$ *Salmonella typhimurium* cecal recovery |
|---|---|---|---|
| Negative control | n/a | 19/19 (100%) | 4.83±0.16 |
| PHL-RW35 | $10^5$ | 19/19 (100%) | 4.60±0.15 |
| PHL-RW35 | $10^7$ | 19/19 (100%) | 4.34±0.30 |
| PHL-RW41 | $10^5$ | 20/20 (100%) | 3.89±0.25 |
| PHL-RW41 | $10^7$ | 20/20 (100%) | 4.28±0.26 |
| PHL-RW35+41 | $10^5$* | 19/19 (100%) | 4.56±0.23 |
| PHL-RW35+41 | $10^7$* | 20/20 (100%) | 4.49±0.24 |

*Total dose is two fold higher as listed dose is for each individual *Bacillus* isolate

Table 5: Effect of direct-fed microbial candidates on chick Body Weight (BW) and Body Weight Gain (BWG) after 9 days of continuous feeding (Day-of-Hatch through end of experiment) (Experiment 2)

| Treatment | Spores/g Feed | BW (g) | BWG (g) | n |
|---|---|---|---|---|
| Negative control | n/a | 166.7±2.8$^b$ | 125.8±2.8$^b$ | 78 |
| PHL-RW35 | $10^7$ | 176.9±3.2$^a$ | 135.8±3.0$^a$ | 60 |
| PHL-RW41 | $10^5$ | 178.4±3.2$^a$ | 137.2±3.1$^a$ | 77 |
| PHL-MM03 | $10^6$ | 162.7±3.3$^b$ | 123.1±3.2$^b$ | 78 |

$^{a,b}$Means within a group with different lowercase letters differ ($p \leq 0.05$)

Table 6: Effect of direct-fed microbial candidates on poult Body Weight (BW) and Body Weight Gain (BWG) after 9 days of continuous feeding (Day-of-Hatch through end of experiment) (Experiment 2)

| Treatment | Spores/g Feed | BW (g) | BWG (g) | n |
|---|---|---|---|---|
| Negative control | n/a | 161.0±2.6$^c$ | 107.7±2.5$^c$ | 79 |
| PHL-RW35 | $10^7$ | 164.1±3.0$^{bc}$ | 111.5±2.9$^{bc}$ | 59 |
| PHL-RW41 | $10^5$ | 175.3±2.5$^a$ | 121.9±2.9$^a$ | 79 |
| PHL-MM03 | $10^6$ | 170.3±2.5$^b$ | 117.0±2.5$^{ab}$ | 80 |

$^{a-c}$Means within a group with different lowercase letters differ ($p \leq 0.05$)

were also significantly heavier than the untreated control, while the poults fed PHL-RW35 and chicks fed PHL-MM03 were not (Table 5 and 6).

These data indicate that selection of heat tolerant, avirulent *Bacillus* isolates capable of killing SE and CP *in vitro* and sporulating to high numbers can result in selection of candidate DFM capable of stimulating an increase in BW and BWG in chicks and poults. This is the first report we are aware of which uses these selection criteria to select for potential *Bacillus* DFM capable of increasing BW and BWG in commercial poultry. Since all 3 isolates tested produced a significant increase in BWG and BW in at least 1 species, it may seem tempting to conclude that the addition of any *Bacillus* isolate to the feed at rates similar to those used in this study will produce an increase in BW and BWG. However, unpublished research in our laboratory using other *Bacillus* isolates has shown some isolates repeatably depress BW in similarly designed experiments. Furthermore, the mechanism of growth promotion is in question for these studies. *Bacillus* isolates were selected based on in vitro antimicrobial activity for use in these experiments and yet no reduction in *Salmonella* infection rates was observed (Table 3 and 4).

Isolate PHL-RW41 significantly increased both BW and BWG in both chickens in both experiments and turkeys in experiment 2. In experiment 2 this isolate improved BWG by 8.3 and 11.7% in chicks and poults respectively (Table 5 and 6). As noted previously, it is interesting that isolates PHL-RW35 and PHL-MM03 seem to have some specificity in terms of probiotic effects for chickens and turkeys respectively, while isolate PHL-RW41 seems to work equally well in either avian species. This may indicate that there is a level of adaptation by certain isolates of *Bacillus* to the environment of the gastrointestinal tract of specific avian species. Unfortunately, not enough evidence is accumulated here to speculate about the mechanisms by which these

probiotic candidates cause as increase in BW and BWG or the reason for the species specificity.

Recent research by other investigators has shown that *Bacillus*-based probiotics can increase production parameters (Fritts *et al*., 2000; Vila *et al*., 2009) as well as decrease specific pathogens (Fritts *et al*., 2000; Newaj-Fyzul *et al*., 2007; Vila *et al*., 2009) in food animals. Some *Bacillus* DFM have been shown to be effective in cattle (Wehnes *et al*., 2009), swine (Zani *et al*., 1998; Guo *et al*., 2006), fish (Newaj-Fyzul *et al*., 2007) and poultry (Fritts *et al*., 2000; Vila *et al*., 2009). While a number of studies have been done to validate the beneficial effects of existing commercial products, relatively few studies have evaluated methods to screen and evaluate novel *Bacillus* isolates and even fewer have concentrated on finding isolates beneficial for commercial poultry (Barbosa *et al*., 2005; Teo and Tan, 2005).

Teo and Tan (2005) and Barbosa *et al*. (2005) isolated *Bacillus* strains from the gastrointestinal tract of chickens then subjected these isolates to *in vitro* screening against common enteric pathogens. Although both studies did extensive *in vitro* testing, no *in vivo* testing was conducted to determine the true probiotic effects of the tested isolates.

Several *ex vivo* tests have been developed to evaluate probiotic bacteria in the laboratory, but the staggering complexity of the intestinal milieu makes it difficult to apply data gathered from *in vitro* testing toward selection of candidate isolates. Due to these issues, the selection criteria used in the present study were greatly simplified. Isolates which were able to kill both SE and CP *in vitro* were deemed potential candidates. Species not know to cause disease in vertebrate animals which were shown to be able to produce antimicrobials effective against these divergent bacterial species *in vitro*, were chosen for further testing because these *Bacillus* isolates should have the best chance at competing with other bacteria in the highly competitive environment of the avian gastrointestinal tract.

A unique aspect of the present study was the high throughput screening of environmental samples. As soil is known to contain an average of $10^8$ *Bacillus* and closely related organisms per gram (Stein, 2005), an enormous number of isolates can be identified from a relatively small number of samples. By using the overlay screening method, many strains of *Bacillus* were quickly and efficiently screened prior to isolation of a particular isolate from the sample. This makes it possible to screen more potential isolates in a shorter period of time.

The other selection criteria (heat stability, growth and sporulation, and isolate identification) were to remove any potential pathogens and to determine if a given candidate would be potentially viable for large scale implementation has not previously reported. Additional testing after these *in vivo* experiments to assess the ability of these isolates to kill ST *in vitro* using the same overlay method used for SE, revealed that these isolates did not have the same antimicrobial effect on ST (unpublished observation). This observation may show the need to screen for additional pathogens *in vitro* prior to *in vivo* testing. Serotype-specificity for antimicrobial activity against *Salmonella* from these *Bacillus* isolates was not anticipated in the present study and could be responsible for the failure to reduce ST *in vivo*.

While more strains of pathogens may need to be screened *in vitro* prior to testing in live animals in future studies, the data here show these methods are effective for screening for probiotic isolates of *Bacillus* from environmental samples. The data presented here demonstrate an initial effort to pre-select using sequential *in vitro* screening for testing *Bacillus* DFM candidates for use in commercial poultry with partial success. Ongoing studies will further refine this process by evaluation of other criteria such as the ability of selected *Bacillus* isolates to colonize within the intestinal tract of poultry, along with more stringent screening against multiple isolates of each type of intestinal pathogen.

## ACKNOWLEDGMENT

This work was supported by the Food Safety Consortium Grant.

## REFERENCES

Barbosa, T.M., C.R. Serra, R.M. La Ragione, M.J. Woodward and A.O. Henriques, 2005. Screening for bacillus isolates in the broiler gastrointestinal tract. Appl. Environ. Microbiol., 71: 968-978.

Bielke, L.R., A.L. Elwood, D.J. Donoghue, A.M. Donoghue, L.A. Newberry, N.K. Neighbor and B.M. Hargis, 2003. Approach for selection of individual enteric bacteria for competitive exclusion in turkey poults. Poult. Sci., 82: 1378-1382.

Choi, J.H., B.S. So, K.S. Ryu and S.L. Kang, 1986. Effects of pelleted or crumbled diets on the performance and the development of the digestive organs of broilers. Poult. Sci., 65: 594-597.

Corrier, D.E., D.J. Nisbet, A.G. Hollister, C.M. Scanlan, B.M. Hargis and J.R. Deloach, 1993. Development of defined cultures of indigenous cecal bacteria to control salmonellosis in broiler chicks. Poult. Sci., 72: 1164-1168.

Cutlip, S., J. Hott, N. Buchanan and J. Moritz, 2006. Effect of steam conditioning practices on pellet quality and growing broiler nutritional value. Poult. Sci., 85: 51-52.

Cutlip, S.E., J.M. Hott, N.P. Buchanan, A.L. Rack, J.D. Latshaw and J.S. Moritz, 2008. The effect of steam-conditioning practices on pellet quality and growing broiler nutritional value. J. Applied Poult. Res., 17: 249-261.

Felske, A., 2004. Ecology of *Bacillus* species in soil. In Bacterial Spore Formers Probiotics and Emerging Applications. Ezio Ricca, Adriano O. Henriques and Simon M. Cutting, Eds. Horizon Bioscience, Great Britain, pp: 35.

Fritts, C.A., J.H. Kersey, M.A. Motl, E.C. Kroger, F. Yan, J. Si, Q. Jiang, M.M. Campos, A.L. Waldroup and P.W. Waldroup, 2000. Bacillus subtilis C-3102 (Calsporin) improves live performance and microbiological status of broiler chickens. J. Applied Poult. Res., 9: 149-155.

Guo, X., D. Li, W. Lu, X. Piao and X. Chen, 2006. Screening of Bacillus strains as potential probiotics and subsequent confirmation of the *in vivo* effectiveness of Bacillus subtilis MA139 in pigs. Antonie Van Leeuwenhoek, 90: 139-146.

Hakkinen, M. and C. Schneitz, 1999. Efficacy of a commercial competitive exclusion product against *Campylobacter jejuni*. Br. Poult. Sci., 40: 619-621.

Mead, G.C., M.J. Scott, T.J. Humphrey and K. McAlpine, 1996. Observations on the control of *Campylobacter jejuni* infection of poultry by competitive exclusion. Avian Pathol., 25: 69-79.

Newaj-Fyzul, A., A.A. Adesiyun, A. Mutani, A. Ramsubhag, J. Brunt and B. Austin, 2007. Bacillus subtilis AB1 controls Aeromonas infection in rainbow trout (Oncorhynchus mykiss, Walbaum). J. Appl. Microbiol., 103: 1699-1706.

Nicholson, W.L., 2004. Ubiquity, Longevity and Ecological Roles of Bacillus Spores. In Bacterial Spore Formers Probiotics and Emerging Applications. E. Ricca, Henriques, A.O. and Cutting, S.M., Eds. Horizon Bioscience, Great Britain, pp: 1.

Nicholson, W.L., 2002. Roles of Bacillus endospores in the environment. Cell Mol. Life Sci., 59: 410-416.

NRC, 1994. Nutrient Requirements of Poultry. 9th Rev. Edn., Natl. Acad. Press, Washington, DC.

SAS, 2007. JMP, Version 7. SAS Institute Inc., 1989-2007. SAS Institute Inc., Cary, N.C.

Scharek, L., B.J. Altherr, C. Toelke and M.F.G. Schmidt, 2007. Influence of the probiotic *Bacillus cereus* var. toyoi on the intestinal immunity of piglets. Vet. Immunol. Immunopathol., 120: 136-147.

Setlow, P., 2006. Spores of Bacillus subtilis: Their resistance to and killing by radiation, heat and chemicals. J. Appl. Microbiol., 101: 514-525.

Snoeyenbos, G.H., O.M. Weinack and C.F. Smyser, 1979. Further-Studies on competitive exclusion for controlling Salmonellae in chickens. Avian Dis., 23: 904-914.

Spizizen, J., 1958. Transformation of biochemically deficient strains of bacillus-subtilis by Deoxyribonucleate. Proc. Natl. Acad. Sci. U.S.A., 44: 1072-1078.

Stein, T., 2005. Bacillus subtilis antibiotics: Structures, syntheses and specific functions. Mol. Microbiol., 56: 845-857.

Taheri, H.R., H. Moravej, F. Tabandeh, M. Zaghari and M. Shivazad, 2009. Screening of lactic acid bacteria toward their selection as a source of chicken probiotic. Poult. Sci., 88: 1586-1593.

Teo, A.Y.L. and H.M. Tan, 2005. Inhibition of clostridium perfringens by a novel strain of Bacilius subtilis isolated from the gastrointestinal tracts of healthy chickens. Appl. Environ. Microbiol., 71: 4185-4190.

Torres-Rodriguez, A., A.M. Donoghue, D.J. Donoghue, J.T. Barton, G. Tellez and B.M. Hargis, 2007. Performance and condemnation rate analysis of commercial turkey flocks treated with a *Lactobacillus* sp.-based probiotic. Poult. Sci., 86: 444-446.

Vicente, J.L., L. Aviña, A. Torres-Rodriguez, B. Hargis, and G. Tellez, 2007. Effect of a *Lactobacillus* Sp.-based probiotic culture product on broiler chicks performance under commercial conditions. Int. J. Poult. Sci., 6: 154-156.

Vila, B., A. Fontgibell, I. Badiola, E. Esteve-Garcia, G. Jimenez, M. Castillo and J. Brufau, 2009. Reduction of *Salmonella enterica* var. Enteritidis colonization and invasion by Bacillus cereus var. toyoi inclusion in poultry feeds. Poult. Sci., 88: 975-979.

Vreeland, R.H., W.D. Rosenzweig and D.W. Powers, 2000. Isolation of a 250 million-year-old halotolerant bacterium from a primary salt crystal. Nature, 407: 897-900.

Wehnes, C., K. Novak, V. Patskevich, D. Shields, J. Coalson, A. Smith, M. Davis and T. Rehberger, 2009. Benefits of supplementation of an electrolyte scour treatment with a bacillus-based direct-fed microbial for calves. Probiotics and Antimicrobial Proteins, 1: 36-44.

Zani, J.L., F.W. da Cruz, A.F. dos Santos and C. Gil-Turnes, 1998. Effect of probiotic CenBiot on the control of diarrhoea and feed efficiency in pigs. J. Appl. Microbiol., 84: 68-71.

Zhao, S., L. Deng, N. Hu, B. Zhao and Y. Liang, 2008. Cost-effective production of Bacillus licheniformis using simple netting bag solid bioreactor. World J. Microbiol. Biotechnol., 24: 2859-2863.