A new probiotic to tackle enteritic problems in a post-antibiotic era

Despite the use of many solutions, clostridiosis is still prevalent globally impairing growth rate and feed conversion and resulting in high condemnation rates at the slaughterhouse. Pathogenic strains of Clostridium perfringens in the gut are responsible for different ailments in poultry and other livestock.

by Dr Kiran Doranalli, Dr Rose Whelan and Dr Stefan Pelzer, Evonik Nutrition and Care GmbH, Germany. www.evonik.com

Among these, it is common to find bacterial enteritis, necrotic enteritis, and colangiohepatitis. Specifically concerning is bacterial and necrotic enteritis in poultry.

Necrotic enteritis (NE) is caused when toxins produced by C. perfringens damage the gut mucosa.

Further, it affects the digestion and absorption of nutrients, most often leading to dysbacteriosis. In addition, gut health issues such as bacterial enteritis or subclinical NE can increase litter moisture and therefore the risk of broilers developing footpad lesions.

The cost of subclinical NE to the global poultry industry is estimated to be $4-6 billion per year, making it a significant issue worldwide.

Dietary inclusion of probiotic bacteria is one effective strategy for preventing intestinal bacterial dysbiosis as is seen in NE.

However, for probiotics to be considered as an effective alternative solution to antibiotic growth promoters (AGP), the mode of action should be proven for different criteria, primarily:

- The inhibition of the target pathogen.
- The modulation of the intestinal microbial population.
- The improvement of animal performance.

Bacterial strains within the species Bacillus subtilis are widely used as direct fed microbials (probiotics).

However, the strains of B. subtilis have unique characteristics that help define their function and efficacy as probiotics. Therefore, mode of action characteristics of a particular strain cannot be generalised to all B. subtilis.

A probiotic based on the spore-forming B. subtilis DSM 32315 strain was selected for its ability to inhibit the growth of enteric pathogens, in particular Clostridium perfringens and support the maintenance of intestinal microbial balance. From a practical standpoint, the efficacy of a probiotic may be limited by certain factors, which can affect its application under commercial conditions.

Therefore, the selection procedure resulting in the final B. subtilis DSM 32315 strain (GutCare) was a multi-parameter step process to ensure the efficacy and activity of the probiotic within the animal.

These factors include:

- Gut fitness.
- Safety assessment.
- Feed processing and storage.
- Inhibition of C. perfringens in vitro and in vivo.
- Broiler performance under NE challenge conditions.
- Microbial modulation within the gut.

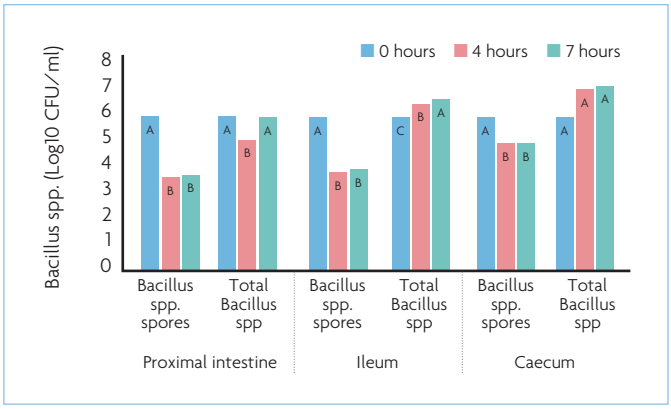


Fig. 2. Enumeration of Bacillus spp. from broiler digesta at different time points after ex-vivo incubation with B. subtilis DSM 32315 spores.

Gut fitness

Bacillus subtilis spores must be able to survive, and germinate into metabolically active vegetative cells in the gastro-intestinal tract (GIT).

In general, B. subtilis probiotics are selected for their ability to withstand low pH and high concentrations of bile acids in the chicken GIT, however this ability may vary by strain.

The effect of bile on probiotic bacteria may include oxidative stress, which damages bacterial proteins and DNA. In this context, two separate studies were conducted to evaluate B. subtilis DSM 32315 spores for bile resistance activity, as well as their ability to germinate and proliferate as vegetative cells in the digesta of broiler chickens.

Continued on page 9

Fig. 1. Growth of B. subtilis DSM 32315 in veal infusion broth (VIB) with or without bile.

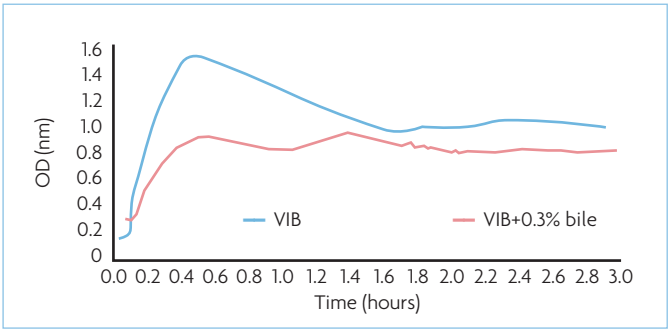
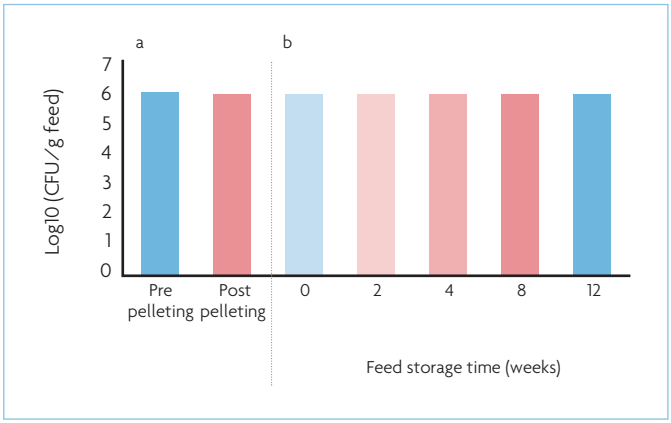


Fig. 3a. Recovery of spore after mixing (n=10) the feed with B. subtilis DSM 32315 spores and after pelleting (n=5) of feed at 85°C. 3b. Recovery of spores after pelleting the feed (n=3) at 85°C and sampling after 0, 2, 4, 8, and 12 weeks of storage at 40°C and 80% humidity.



Well diffusion assay using strains grown in LB Kelly media (mm clearance of pathogen)			
Strains tested	C. perfringens type A (NE outbreak field isolates)		C. perfringens (Library collection)
	α- and netB-toxin	α- and β2-toxin	ATCC 13124
B. subtilis DSM 32315	16.50	18.00	15.00

Table 1. Bacillus subtilis DSM 32315 inhibits pathogenic Clostridium perfringens.

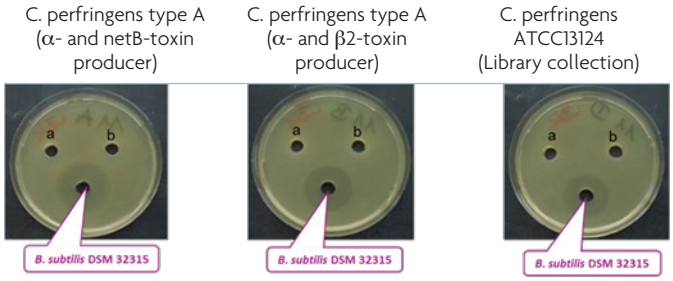


Fig. 4. Inhibition of various pathogenic strains of Clostridium perfringens in well diffusion assay using LB Kelly media (mm clearance if pathogen). a. competitor Bacillus spp. strain and b. negative control.

Continued from page 7

In the first study, B. subtilis DSM 32315 was grown in veal infusion broth (VIB) alone or VIB + 0.3% bile. The growth measured over a three hour period showed that B. subtilis DSM 32315 is able to grow in the presence of bile (Fig. 1).

A germination study was conducted to investigate the ability of B. subtilis DSM 32315 spores to germinate in GIT conditions. B. subtilis DSM 32315 spores were incubated in digesta samples collected from the proximal intestine, ileum and caecum of broiler birds in conditions mimicking the intestinal sections.

Total Bacillus spp. cells significantly increased within four hours of incubation in the digesta from ileum and caecum, while spores of Bacillus spp. decreased significantly in all sections of the intestine (Fig. 2).

This indicates that germination of spores occurred in the digesta from all sections of the gut, but perhaps that proliferation of vegetative cells is optimal in the ileum and caecum.

Safety assessment

For commercial application purposes, it is important to establish the safety of the micro-organism used as a probiotic. The most serious risk posed by probiotic microbes in feed are:

- The transfer of antibiotic resistance due to the presence of transmissible antibiotic resistance genes.
- The presence of toxin genes.
- The presence of plasmids or mobile genetic elements.

B. subtilis DSM 32315 was analysed for toxin related genes and was shown to contain no haemolytic enterotoxin genes (nheABC), no haemolysin genes (hblCDAB), no cytotoxin gene (cytK), and no cereulide gene (cesA).

In addition, B. subtilis DSM 32315 was tested for antibiotic resistant genes. No plasmids were identified on a gene level which was validated by culturing the probiotic in the presence of antibiotics deemed important for medical use. From this profiling, it is concluded that B. subtilis DSM 32315 is safe to be added in the food chain.

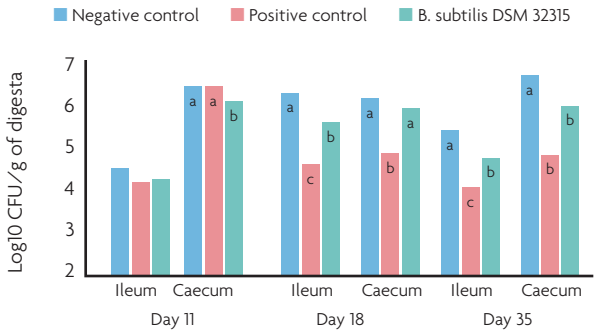


Fig. 6. Effect of B. subtilis DSM 32315 (GutCare) on C. perfringens populations in ileum and caecum of broiler chickens.

Feed processing and storage

In order to verify the potential of B. subtilis DSM 32315 as a probiotic for in feed applications, the ability of B. subtilis DSM 32315 spores to remain viable after pelleting and feed storage was tested. There was no significant loss of B. subtilis DSM 32315 spores in broiler feed during feed pelleting (Fig. 3a).

Additionally, B. subtilis DSM 32315 spores showed excellent mixability in the feed. There was also no significant loss of B. subtilis DSM 32315 spores in pelleted broiler feed stored for up to 12 weeks at high temperature and humidity (Fig. 3b).

Inhibition of Clostridium perfringens

An in vitro study was conducted to test the inhibition of pathogenic strains of C. perfringens. Three strains of C. perfringens that are capable of producing α, β2, and netB toxins were used. Growth of all C. perfringens strains tested were inhibited by adding supernatant from the culture of B. subtilis DSM 32315 (Table 1 and Fig. 4).

Furthermore, by applying a multi-step fractionation approach, the distinct fractions of the supernatant with C. perfringens inhibiting activities were identified (Fig. 5).

Structure elucidation of the fractions with nuclear magnetic resonance spectroscopy confirmed that two compounds were responsible for the inhibition activity the bacteriocin Subtilosin A and Surfactin C. These results were successfully validated in experiments using novel knock-out Bacillus strains where strains missing the genes for Subtilosin A and/or Surfactin C had impaired ability to inhibit C. perfringens growth (data not shown).

Several studies were conducted to validate the ability of B. subtilis DSM 32315 in broiler feed to inhibit C. perfringens induced NE.

In one study, the NE challenge was induced in each bird with an oral inoculation of Eimeria maxima oocysts at 12 days of age as a predisposing factor for the inoculation of the birds with a pathogenic field strain of C. perfringens at 16 days of age. The challenged broilers were

Continued on page 11

Fig. 5. Isolation and structure elucidation of active principle in B. subtilis DSM 32315 inhibiting C. perfringens.

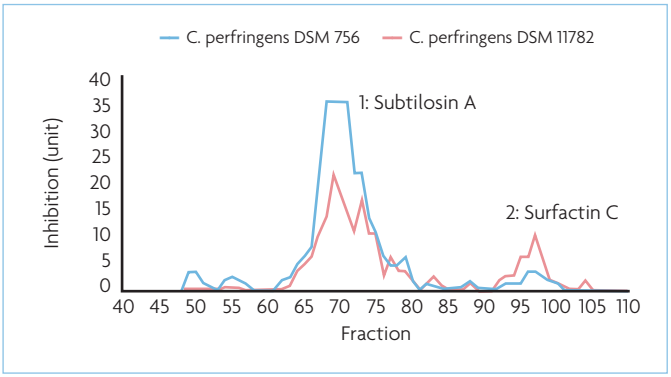
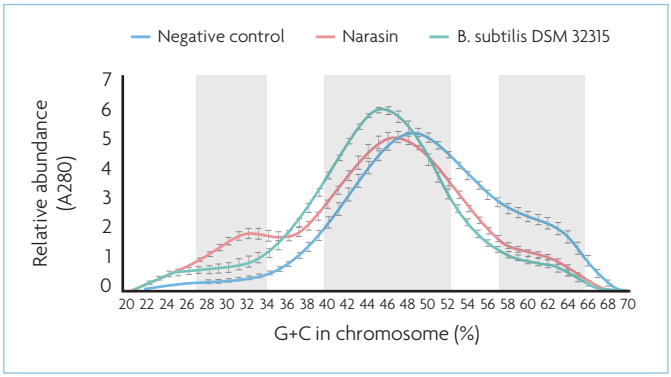


Fig. 7. The average %G+C profiles from the caecal microbiome of broilers fed diets either without additive (negative control), positive control (Narasin) or supplemented with B. subtilis DSM 32315 (GutCare). Vertical columns show the fractions of the curve low (27.0-34.5%), mid (40.5-54.0%) and high (59.0-68.9%) where significant differences (p>0.05) in relative abundance were determined between.



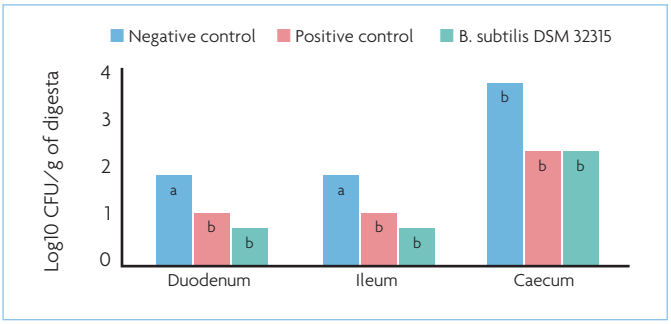


Fig. 8. Effect of B. subtilis DSM 32315 (GutCare) on C. perfringens populations in duodenum, ileum and caecum of broiler chickens.

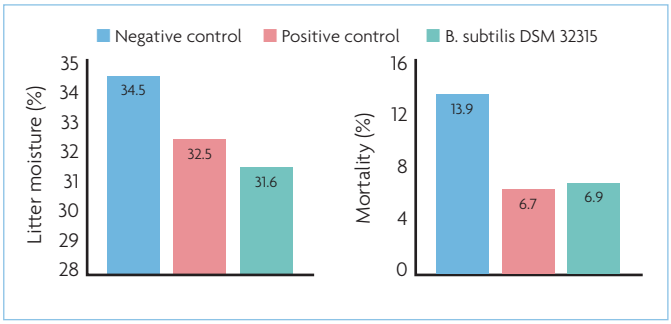


Fig. 10. Feeding B. subtilis DSM 32315 (GutCare) decreases litter moisture and mortality in broiler chickens.

Continued from page 9

either fed a control diet or a diet supplemented with B. subtilis DSM 32315 (GutCare) at 500g/MT (1x10^{6}CFU/g feed) for 35 days. Narasin (650g/ton of feed) was used as a positive control.

Molecular analysis of microbial populations in the ileum and caecum of birds at different time points showed that feeding B. subtilis DSM 32315 consistently and significantly (P<0.05) decreased populations of C. perfringens (Fig. 6).

Caecal digesta samples from day 18 were utilised to investigate the effects of dietary B. subtilis DSM 32315 on the microbiome of broilers during the peak of the NE challenge.

For this purpose the %G+C profiling method was utilised which differentiates microbiome samples based on two essential points;

- Each bacterial species has a characteristic guanine-cytosine (G-C) content in its chromosomal DNA.
- G-C -rich DNA is denser than adenine-thymine (A-T) –rich DNA.

The different bacterial chromosomes within a sample can be separated based on these density differences with high speed centrifugation and relative abundance of chromosomes with different %G+C determined from the fractionated samples.

Comparison of the resultant profiles from individual samples with statistical analysis reveals microbial population changes between treatments. The exact families and species of bacteria contributing to the profile changes can then be identified using sequencing techniques.

This technique identified significant (p<0.05) differences between dietary treatments on the caecum microbiome %G+C profiles in three regions; a low (27.0-34.5%), mid (40.5-54.0%) and high (59.0-68.9%) region (Fig. 7). These regions were then fractionated and chromosomal DNA collected for next generation sequencing to determine the treatment effects on specific families and species of bacteria represented in these fractions.

The results of the %G+C profiling and subsequent sequencing revealed that dietary inclusion of B. subtilis DSM 32315 in broiler diets significantly altered the intestinal microbiome, increasing bacterial taxa with potential health benefits, such as specific Lactobacillaceae species, while decreasing potentially detrimental populations in the families Lachnospiraceae and Ruminococcaceae.

The results lead to additional hypotheses to be tested regarding the potential for B. subtilis DSM 32315 stabilisation of the broiler intestinal microbiome to subsequently inhibit other bacterial infections of broilers (salmonellosis, colibacillosis) and reduce reservoirs of foodborne pathogens (campylobacter spp.).

In another study, NE challenge was induced with an inoculation of each bird at nine days of age with sporulated Eimeria spp. oocysts as a predisposing factor, followed by the inoculation of a pathogenic strain of C. perfringens on days 14, 15 and 16. Zinc-Bacitracin (20g/ton of feed) was used as a positive control. Feeding B. subtilis DSM 32315 significantly (P<0.05) decreased populations of C. perfringens in the duodenum, ileum and caecum, at day 35 (Fig. 8).

Performance of broilers under NE challenge

Probiotics have been reported to have the ability to improve intestinal microbial balance in broilers, resulting in growth performance improvements.

More specifically, the efficacy of Bacillus-based probiotics in face of a challenge is related to several factors such as the metabolic activity of specific strains, survivability and persistence in the gastrointestinal tract, and concentration administered.

The B. subtilis DSM 32315 strain is a patented probiotic produced by Evonik (GutCare). This strain has shown in vitro and in vivo activity against toxin producing strains of CP and the proposed mechanism of action for such inhibition is the inherent potential to express different secondary metabolites and lactic acid, which in turn, favours the growth of beneficial bacteria present in the intestinal microflora.

In total, five trials were conducted under NE challenge (Table 2). The severity of challenge was different across the trials, however all models were successful in inducing the enteritis challenge. Across the trials, feeding the B. subtilis DSM 32315 strain improved body weight by 5.3% and decreased FCR by 4.3% over the negative control (Fig. 9).

Even the mildest form of clostridiosis can lead to microbial imbalance in the gut resulting in diarrhoea and wet litter, which in turn leads to associated conditions like foot-pad dermatitis.

These conditions can further impair the growth rate and feed conversion causing economic losses to poultry producers.

GutCare has also been shown to support good litter quality and livability (Fig. 10) in broiler chickens, which indirectly improves growth performance (Fig. 9).

Conclusions

- GutCare is a superior probiotic product consisting of bacterial spores that are stable during feed processing and storage.
- It may reduce the threat of pathogenic bacteria colonisation of the gut, especially C. perfringens, resulting in a more balanced intestinal microbial population and improved growth performance.
- GutCare offers an effective and sustainable replacement to AGPs in the maintenance of healthy flocks and the optimisation of performance in poultry.

References are available from the authors on request

Fig. 9. Feeding B. subtilis DSM 32315 (GutCare) improves performance of broilers chickens under NE conditions across the world.

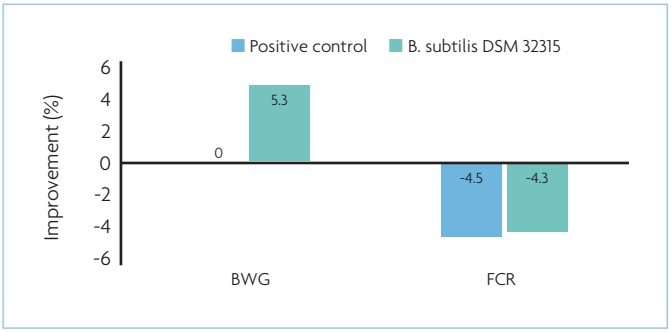


Table 2. Brief description of trial set up at five stations.

Research station	Breed	Challenge model/ Positive control	Duration (days)
Alimetrics Ltd, Finland	Ross 308	11 reps, 14 birds/rep Narasin	35
Animal Research and Consultant Co, Samutprakan, Thailand	Ross 308	8 reps, 12 birds/rep Zn Bacitracin	35
Virginia Diversified Research, Corp. Harrisonburg, USA	Cobb 500	12 reps, 30 birds/rep BMD	42
Southern Poultry Research Inc, Athens, Georgia, USA	Cobb 500	10 reps, 8 birds/rep BMD	28
Northwest A&F University, Xi'an, China	Arbor acres	10 reps, 12 birds/rep BMD	42