Assessment of safety and heat stability under commercial conditions of the probiotic strain *Bacillus amyloliquefaciens* CECT 5940 (Ecobiol®)

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Direct-Fed Microbials (DFMs) have been an important functional feed additive in the Veterinarian's and Nutritionist's "toolbox" for the maintenance of intestinal health in poultry. One critical aspect pertaining to the use of DFMs is the close attention that must be given to the entire process of selecting a strain, which encompasses the knowledge on the strain safety and genetic assessment, antimicrobial susceptibility, application potential, survivability, functionality under research and commercial conditions, and different modes of action. Under commercial conditions, each one of the above-mentioned aspects must fall in place to assure the delivery and functionality of the live beneficial microorganisms chosen for that specific poultry flock.

Although several species of *Bacillus* used as DFMs are generally recognized as safe, individual strains could contain genes conferring resistance to antibiotics and/or have the potential for toxin production and hemolytic effects (Marteau, 2001; Pedersen et al., 2002; Doron and Snydman, 2015). Consequently, it is crucial to assess and validate the safety and characteristics of strains considered for use in livestock production. In addition to safety related factors, the stability and survival rate of strains can vary among the same species, which may influence commercial feeding applications. Therefore, the purpose of this paper is to describe safety and antimicrobial susceptibility related studies, and, as the next step into commercial testing, to demonstrate the stability of the strain of Ecobiol (*Bacillus amyloliquefaciens* CECT 5940) under commercial feed manufacturing processes.

ASSESSMENT OF STRAIN SAFETY

A bioinformatic study based on the whole genome sequence (WGS) was conducted to investigate the absence of genes encoding enterotoxins (*nhe, hbl*, and *cytK*) and cereulide synthase (*ces*) in *B. amyloliquefaciens* CECT 5940. Briefly, the genome sequencing of the above-mentioned strain was performed using the whole genome shotgun method. Data generated by Illumina and SMRT sequencing was used to acquire the complete circular genome sequence of *B. amyloliquefaciens* CECT 5940. A BLAST analysis was used to exclude the presence of the following genes encoding relevant toxins: *cytK* (single protein cytotoxin K), *hblA, hblB, hblC,* and *hblD* (hemolysin BL, four protein components), *nheA, nheB,* and *nheC* (non-hemolytic enterotoxin, three protein components) and *cesA* (cereulide synthetase gene cluster). The screening for potential presence of toxin related genes was conducted using *Bacillus cereus* reference genes (EFSA, 2014). Results have shown that the *B. amyloliquefaciens* CECT 5940 strain does not contain hemolytic enterotoxin (*nheABC*), hemolysin (*hblABCD*), cytotoxin (*cytK*), or cereulide (*cesA*) genes.

Following the screening for potential presence of toxin related genes, a cytotoxicity study was conducted to evaluate any potential production of cyclic lipopeptides with surfactant properties. The cytotoxicity study was conducted according to the "Guidance on the characterization of microorganisms used as feed additives or as production organisms" (EFSA, 2018). In short, the toxigenic potential of *Bacillus amyloliquefaciens* CECT 5940 strain was tested on Vero C1008

epithelial cells and measurement was collected by a fluorescence-based cellular test system. Vero cells were treated with aliquots of culture supernatant of the above-mentioned bacterial strain (considered as "test item"), and *Bacillus cereus* ATCC 14579 (DSMZ DSM-31; considered as "positive control").

No cytotoxicity was observed for the negative control and for the *B. amyloliquefaciens* CECT 5940 strain. In contrast, the positive control (supernatant of the *B. cereus* strain) showed an increase in the fluorescence signal, indicating pronounced cytotoxicity even when applied at the lowest concentration on the Vero cells.

Thereby, the study concluded that the DFM strain *Bacillus amyloliquefaciens* CECT 5940 does not have toxigenic potential.

ASSESSMENT OF ANTIMICROBIAL SUSCEPTIBILITY AND POTENTIAL ANTIMICROBIAL RESISTANCE GENES

In addition to the safety studies, a phenotype test determining the minimum inhibitory concentration (MIC, EFSA 2012) for a group of antimicrobials and analyses of the WGS for the presence of known antimicrobial resistance (AMR) genes were performed on the strain *Bacillus amyloliquefaciens* CECT 5940.

The susceptibility of the strain was tested against ampicillin, streptomycin, kanamycin, neomycin, gentamicin, chloramphenicol, tetracycline, erythromycin, vancomycin, trimethoprim, ciprofloxacin, linezolid, rifampin and clindamycin. Tests were made in microtiter plates containing serial dilutions of the antibiotics according to the Clinical and Laboratory Standards Institute (2006). In addition, the existing WGS of the strain was compared with references in the comprehensive antibiotic resistance database (CARD).

To assess the phenotypic antimicrobial resistance of the *B. amyloliquefaciens* CECT 5940, the MIC values for a selected group of antimicrobials were determined in triplicate measurements. The cut-off values for *Bacillus* published by EFSA (2018) served as reference. Reference cut-off values for antimicrobials not listed by EFSA were taken from the recommendations for *Bacillus* published by the Scientific Committee on Animal Nutrition (SCAN 2001). As result, *B. amyloliquefaciens* CECT 5940 showed susceptibility to all antibiotics tested, with MIC values lower than the reference values. The bioinformatic evaluation showed that the strain *Bacillus amyloliquefaciens* CECT 5940 does not carry any AMR genes for antimicrobials considered as critically important antimicrobials in humans and animals.

ASSESSMENT OF HEAT STABILITY UNDER COMMERCIAL FEED PROCESSING

Pelleting involves a mechanical process in which small feed ingredient particles are agglomerated into larger particles by applying moisture, heat, and pressure. There are several nutritional and performance benefits associated with pelleting; nevertheless, this process can be detrimental to certain feed ingredients (Thomas et al., 1998) and feed additives such as DFMs. Spore-forming *Bacillus* spp. DFMs have the ability to withstand high temperatures, chemicals, and drying processes. Although *Bacillus* spp. spores are generally resistant, testing is important to demonstrate stability throughout feed processing since this characteristic is strain specific.

The stability of *B. amyloliquefaciens* CECT 5940 strain at different pelleting temperatures was demonstrated. For this test, spores of *B. amyloliquefaciens* CECT 5940 were mixed into a broiler starter feed for a final expected concentration of 1×10^6 CFU/g feed for every feed batch. Mash feed was conditioned for six minutes at 190 ºF (87.8 ºC) and 195 ºF (90.5 ºC) and then pelleted at the same temperatures. The pelleted feeds were then dried and cooled under ambient conditions estimated at 70 ºF (21 ºC) and retention time of 15 minutes. Total spores were counted from 10 mash feed samples obtained immediately after mixing and then from another 10 samples obtained after the mash feed had been conditioned and subjected to the different pelleting temperatures. Feed samples were analyzed for spore counts and results were reported as Log_{10} CFU/g feed. Results were analyzed by JMP® v13.1.0 and reported as means and standard deviation (SD). Additionally,

TABLE 1. *Bacillus amyloliquefaciens* CECT 5940 (Ecobiol®) spore enumeration and percent recovery rate in feed before (control mash) and after pelleting at different temperatures.

Feed Sample	Samples, n	Spore Count, Mean $\log_{10}$ CFU/g ± SD	Recovery Rate, %
Control Mash	10	5.91 ± 0.04	98.6
Pellet at 190°F	10	5.80 ± 0.06	96.6
Control Mash	10	6.04 ± 0.34	100.7
Pellet at 195°F	10	5.59 ± 0.08	93.1

recovery rate was estimated based on a target level of 6 $\log_{10}$ CFU/g.

The spore count and recovery rate of *Bacillus* spp. spores are reported in Table 1. Results indicated that spores of *B. amyloliquefaciens* CECT 5940 can survive up six minutes of conditioning time and up to 195 ºF (90.5 ºC) pelleting temperatures. A high recovery rate during commercial feed processing ensures that an adequate number of viable spores is present in the finished feed and are delivered to the animals.

CONCLUSIONS

The unique strain of *Bacillus amyloliquefaciens* CECT 5940 has proven:

1) To be safe for use in animal feeding, since it does not contain toxins, does not possess phenotypic resistance, and does not carry AMR genes against antimicrobials considered relevant to humans and animals.
2) A high survival rate during commercial feed processing of up to 195 ºF (90.5 ºC) with a conditioning time of six minutes.

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