

Original Research Article

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## Viability of Spray Dried Probiotics in Crumble Feed during Storage

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### ABSTRACT

A study was conducted to assess the viability of spray dried probiotics in crumble feed during storage. The *Bacillus subtilis* (T1), *Bacillus amyloliquefaciens* (T2), *Bacillus coagulans* (T3), *Bacillus clausii* (T4), *Lactobacillus acidophilus* (T5) and *Bifidobacterium bifidum* (T6) cultures were propagated using skim milk and spray dried by using spray drier. The spray dried probiotic powder was diluted with water and sprayed on broiler crumble feed. After spraying, crumble feed was stored in room temperature (25-33°C) for 0,7 and 14 days and the viability of probiotics was assessed before and after spray drying and during storage with crumble feed. *B. clausii* showed better yield after spray drying while *B. subtilis* showed highest survival rate (83%) among all probiotics studied. At the end of 14 days of storage, the survival rate was maximum in *B. amyloliquefaciens* among spore forming probiotics (95.61%) and among non-spore forming probiotics the *B. bifidum* (85.24%) evinced better survival.

#### Keywords

Probiotics, Skim milk powder, Spray drying, Crumble feed, Viability

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### Introduction

Probiotics are viable microorganisms which when administered in adequate amounts confer a health benefit for all animals and human beings (WHO, 2001). As early as 1910, probiotics were known for their probiotic character in overcoming many gut related problems. When antibiotics came to limelight for countering many pathogens, the use of probiotics dropped during middle of

the 20<sup>th</sup> century. However, the revival of use of probiotics was seen in late 70s in different forms and combinations for larger animals, pigs and poultry. The beneficial and advantage of probiotics are increasingly felt useful when use of antibiotics is gradually opposed for reasons like development of antimicrobial resistance through food producing animals. Further, the role of antibiotics in food producing animals started to diminish when the system of growing

animals and birds was optimal and increasingly banned in many countries. The European Union, in 1999, banned the use of certain major antibiotics as growth promoters to prevent spread of antimicrobial resistance to humans (Casewell *et al.*, 2003).

Probiotic is one of the commonly used and effective alternatives to AGPs and its use is documented since ages. *Lactobacillus* and *Bifidobacterium* are the earliest probiotics used traditionally in various fermented foods (Shortt, 1999). With the feed industry fast changing from mash type to pellet/crumbles, the use of *Lactobacillus* and *Bifidobacterium* in pellet/crumble type of feeds is to be discontinued as both are thermo-sensitive and get destroyed during pelletizing temperature. Alternately, thermo resistant spore forming probiotics, mostly bacillus type of organisms dominated. As reported by Tam *et al.*, (2006) that the bacillus are categorized as part of the gut microflora and by Cartman *et al.*, (2008) that the orally administered spores of *Bacillus subtilis* germinated in the gastrointestinal tracts of chicken, bacillus type organisms were increasingly accepted as alternates to the thermolabile characterized probiotics. In addition, bacillus species has long shelf life and retain its viability during storage and distribution of feeds.

These properties are highly desirable from a commercial perspective and spores based *Bacillus* species were used as direct-fed microbial (DFM) feed supplements. It is identified that about 13 *Bacillus* species with QPS status, including *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. Coagulans* and *B. Megaterium* are used as probiotic candidates in animal feeds (EFSA BIOHAZ Panel, 2013) and these *Bacillus* species were identified as safe mainly due to absence of enterotoxins and emetic toxins. However, FAO (2016) cautioned that the use of *Bacillus* type of probiotics is not risk free,

as some bacillus species are pathogenic to human (Schoeni and Lee Wong, 2005) and went on to comment that *Lactobacillus* and *Bifidobacterium* are probably the safest microorganisms because they are traditionally used in fermented foods (Shortt, 1999). The use of *Lactobacillus* and *Bifidobacterium*, naturally present in age-old conventional and traditional foods, was to be continued in the feed for food animals. It needs data with respect to the longevity of these two thermolabile probiotic candidates when spray-dried and used in pellet/crumble feed for certain duration of storage. These data are currently not available to plan further on the use of them in pellets/crumbles. Hence, an *in-vitro* experiment was designed to find the effect of storage of crumble feeds mixed with spray dried *Lactobacillus* and *Bifidobacterium* microorganisms, which might help to take-up further experimental *in-vivo* trials on the use of *Lactobacillus* and *Bifidobacterium* in feeding the food animals. The newer and recent bacillus types of probiotic candidates are also included in the study for knowledge of getting data by comparison.

## Materials and Methods

*Bacillus subtilis* (NDRI strain), *Bacillus amyloliquefaciens* (ATCC 23842), *Bacillus coagulans* (ATCC 7050), *Bacillus clausii* (BCL 2B), *Lactobacillus acidophilus* (Russian strain) and *Bifidobacterium bifidum* (HI 48) were selected as probiotics candidates for this study and obtained from National Dairy Research Institute, NDRI, Karnal, Mystical Biotech Private Limited, Hoskote-562114, Karnataka and Anthem Biosciences Pvt, Ltd, Harohalli Industrial Area, Phase-II, Kanakapura Taluk, Ramanagara District-562 112, Karnataka.

*Bacillus* organisms were propagated in nutrient broth and incubated at 39°C for 12

hours (Shearer *et al.*, 2000). *Lactobacillus acidophilus* was propagated in MRS broth, while *Bifidobacterium bifidum* was propagated in modified MRS (supplemented with L-cysteine hydrochloride, 0.5g/L) broth as per the protocol described (Miranda *et al.*, 2014).

Five hundred microlitres of propagated culture of *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus clausii*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum* were added in sterile 1.5 mL tubes containing 500 microlitres of 20% glycerol (v/v) (Pop *et al.*, 2015). The 1.5 mL tubes were stored at -20°C for further propagation.

### **Propagation of probiotic organisms**

Glycerol broth cultures of *B. subtilis*, *B.amyloliquefaciens*, *B.coagulans*, *B.clausii*, *L. acidophilus* and *Bifidobacterium bifidum* were propagated by using growth media and inoculated at the rate of 5 per cent into reconstituted skim milk (20%) and incubated at 39° C for 10-12 hrs for spray drying (Chavez and Ledebouer, 2007 and Ananta *et al.*, 2005).

### **Spray drying and recovery**

The cultures propagated in the skim milk medium were spray dried by using an automatic glass spray dryer (*Milk-Tech Engineers*, Bangalore, India) with a 0.7 mm single fluid nozzle. The solution was sprayed in a co-current flow with air as drying medium. The inlet air temperature used for spray drying of culture ranged from 180°C - 185°C and outlet temperatures was adjusted to 90-95°C for all the cultures used in this study, after initial standardization of optimal outlet temperature for better yield and minimal loss of probiotics. Spray dried powder was collected as fine particles and

transferred into pouches, sealed and stored at 4°C. The yield (%) was calculated as follows

$$\text{Yield (\%)} = (\text{Total solid} - \text{recovered solids}) / \text{Total solids} \times 100$$

Samples of spray dried probiotics were taken for microbiological examination before and immediately after spray drying and number of viable cells were enumerated (Chavez and Ledebouer, 2007).

### **Spraying on crumble feed**

Three grams each of spray dried cultures of *B. subtilis*, *B.amyloliquefaciens*, *B.coagulans*, *B.clausii*, *L.acidophilus* and *B.bifidum* were separately mixed with 40 ml of sterile distilled water and sprayed on 3 kg of sterile broiler crumble feed with 6 replicates each. After spraying, the feed were kept in a sealed HDPE polythene bag in room temperature (Maximum 33°C and Minimum 25°C) for 0, 7 and 14 days.

### **Assessment of viability of probiotics**

The number of viable cells before and after spray drying and during storage (0, 7 and 14 days) in crumble feed was enumerated by using spread plate technique. The probiotic samples and feed were dissolved in autoclaved saline water and serial dilution was prepared for each probiotic. All diluted samples of *Bacillus spp.*, *L. acidophilus* and *B. Bifidum* were spread on nutrient agar, MRS agar and Modified MRS agar (added with 0.05% cysteine) plates respectively. The Nutrient agar plates were incubated at 37°C for 24 h. The modified MRS agar plates and MRS agar plates were incubated anaerobically at 37°C for 24 hour.

After incubation, colonies were enumerated and mean number of bacteria was expressed as cfu/ml. Survival rates were calculated as

follows: Survival (%) =  $N/N_0 \times 100$ , where  $N_0$  and  $N$  represent the number of bacteria before and after spray drying, respectively.

## Results and Discussion

### Yield of probiotics after spray drying

An attempt was made to spray dry the probiotics propagated in nutrient broth. It was found that the nutrient broth got stuck to the walls of drying chamber and impacted the collection of dried powder. The probiotics were spray dried after propagation in reconstituted skim milk (20%) as per the procedure adopted by Ananta *et al.*, (2005) who reported that the skim milk powder based medium provided high yield with better protection for lactobacilli

The yield of the spray dried products is presented in Table 1. The highest yield was observed in *Bacillus clausii* (38%) and lowest was seen in *B. bifidum* (29%). While in this study yield of *B. coagulans* was (33%). Panday and Vakil (2017) found that the biomass of skim milk based *B. coagulans* was 46 %. The bacillus organisms had generally higher yield (31-38%). However, non spore formers had lowest yield of 29-30 %, probably due to their thermolabile characters. Ananta *et al.*, (2005) reasoned that the carrier used in spray drying encapsulated the probiotics and accounted for 10-50% of weight of entire spray dried powder.

### Assessment of viability of probiotics

Viability of spore forming (*B. subtilis*, *B. amyloliquefaciens*, *B. coagulans* and *B. clausii*) and non-spore forming (*L. acidophilus* and *B. bifidum*) probiotics was assessed before and after spray drying and during storage of crumble feed and the data are presented in Table 2 and are also figuratively expressed in Figure 1.

### Loss of viability probiotics before and after spray drying

The initial concentration of *Bacillus subtilis* before spray drying the reconstituted skimmed milk was 12.79 log cfu/ml, which after spray drying was found to be decreased by 17 % to 10.56 log cfu/ml.

The initial count of *Bacillus amyloliquefaciens* before spray drying was 14.78 log cfu/ml, which decreased during spray drying by 3.10 log cfu/ml (21%) to 11.68 log cfu/ml. *Bacillus amyloliquefaciens* spores suspended in mashed carrot was reported to have a viable count of 8 log<sub>10</sub>cfu/ml and after spray drying the count was decreased by 3.1 log cfu/ml (39%) (Castellvi *et al.*, 2010) in an earlier work.

*Bacillus coagulans* population before spray drying was 11.33 log cfu/ml and it drastically decreased during spray drying process to 8.55 log cfu/ml 2.78 log cfu/ml which was 21%. In a recent work, a viability loss of *Bacillus coagulans* was observed in the range of 20-40% (Pandey and Vakil, 2017).

*Bacillus clausii* concentration before spray drying was 15.75 log cfu/ml which after spray drying lost by 3.21 log cfu/ml (20%) to have a final concentration of 12.54 log cfu/ml.

The initial population of *Lactobacillus acidophilus* was 15.24 log cfu/ml before spray drying and there was a huge decline by a margin of more than one third (36%) to 9.73 log cfu/ml after spray drying. A similar viability loss of about 35% was also reported in *Lactobacillus rhamnosus* after spray drying (Ananta *et al.*, 2005) where in however the initial count of *Lactobacillus acidophilus* was only 5.28 log cfu/ml before spray drying.

While *B. bifidum* had a concentration of 10.29 log cfu/ml before spray drying, the decrease

in its microbial population was by 2.14 log cfu/ml (21%) to a final value of 8.05 log cfu/ml after spray drying. In contrast, the viability loss of spray dried *B. bifidum* was reported to be 70% and reduction in viability was due to use of maltodextrin as a carrier during spray drying (Shokriet *al.*, 2015) in earlier works.

Non-spore formers are thermolabile and prone to get destroyed at spray drying temperature. As per the water replacement theory (Crowe *et al.*, 1998), skim milk contains lactose, which replaces the water in the cell membrane that results in direct interaction with the phospholipids of cellular membrane and there by protects the probiotics during spray drying and prolonged storage.

**Table.1** Yield of probiotics after spray drying

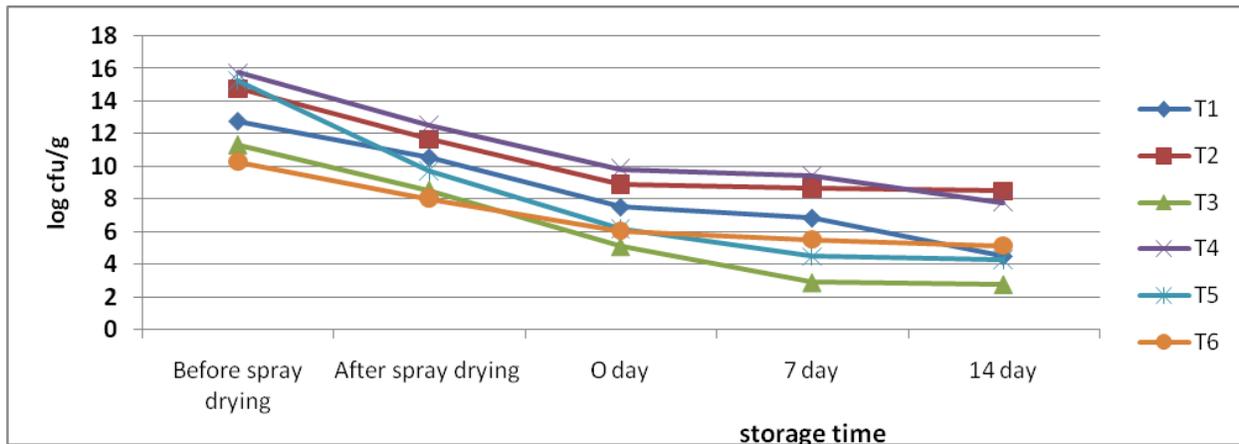
Treatment	Yield of spray dried powder (g)	Recovery (%)
<i>Bacillus subtilis</i> (T1)	72	36
<i>Bacillus amyloliquefaciens</i> (T2)	61	31
<i>Bacillus coagulans</i> (T3)	65	33
<i>Bacillus clausii</i> (T4)	76	38
<i>Lactobacillus acidophilus</i> (T5)	61	30
<i>Bifidobacteriumbifidum</i> (T6)	58	29

**Table.2** Viability of probiotics before and after spray drying and during storage on broiler crumble feed

Treatment	Loss of viability (log <sub>10</sub> cfu/ml)					
	Spray drying			Crumble feed with probiotics		
	Before	After	Loss in viability in absolute values and (%)	Viability immediately after mixing with feed (0 day)	After 7 days of storage (Loss in %)	After 14 days of storage (Loss in %)
<i>Bacillus subtilis</i> (T1)	12.79	10.56	2.23 (17)	7.51	6.83 (9.05)	4.49 (40.21)
<i>Bacillus amyloliquefaciens</i> (T2)	14.78	11.68	3.10 (21)	8.88	8.65 (2.59)	8.49 (4.39)
<i>Bacillus coagulans</i> (T3)	11.33	8.55	2.78 (21)	5.09	2.87 (43.61)	2.75 (45.97)
<i>Bacillus clausii</i> (T4)	15.75	12.54	3.21 (20)	9.86	9.45 (4.16)	7.80 (20.89)
<i>Lactobacillus acidophilus</i> (T5)	15.24	9.73	5.51 (36)	6.20	4.47 (27.90)	4.28 (30.97)
<i>Bifidobacterium bifidum</i> (T6)	10.29	8.05	2.14 (21)	6.03	5.50 (8.79)	5.14 (14.76)

Values in the parenthesis indicate that percentage of survival of probiotics.

**Fig.1** Survival of probiotics before and after spray drying and its spray on broiler crumble feed for 0, 7 and 15 days of storage



In this study, the viability of probiotics was found to decrease after spray drying. The loss of viability of probiotics may be attributed to damage of cytoplasmic membrane, the cell wall, ribosome and DNA at higher spray drying temperature (Gardiner *et al.*, 2000; Meng *et al.*, 2008).

The viability loss was found to be highest in *L. acidophilus* (36%) and lowest in *B. subtilis* (17%) after spray drying. Generally, non-spore forming probiotics are thermolabile and sensitive to high temperatures. However, the viability of *L. acidophilus* NCIMB 701748 post-spray drying was 7.37 log cfu/ml after cruising an inlet temperature of 160°C and outlet temperature of 91.5°C, with 30% loss of viability. The stationary phase bacterial cultures are more resistant to heat compared to bacterial cells in log growth phase (Jobbehdar *et al.*, 2013). Bacteria that enter into stationary phase develop a general stress resistance than bacteria in the log-phase due to exhaustion nutrients that induce stress factors to allow survival of probiotics (Morgan *et al.*, 2006; Vande Guchte *et al.*, 2002).

#### Viability loss of spray dried probiotics on crumble feed during storage

As seen in the Table 2, the viability of

*Bacillus subtilis* decreased during 14 days of storage from 7.51 log<sub>10</sub>cfu/ml (0 day) to 4.49 log<sub>10</sub>cfu/ml (day 14) in sterile broiler crumble feed stored at room temperature. While the loss of viability was only 9.05 % during the first 7 day storage, the loss was higher during the second week storage (31.16 %). The loss increased quickly after 7 days of storage in crumble feed.

The loss of viability with respect to *Bacillus amyloliquefaciens* was very low. The decrease during the span of 14 days was only 4.39 % (from 8.88 log<sub>10</sub>cfu/ml, 0 day) to 8.49 log<sub>10</sub>cfu/ml). *Bacillus amyloliquefaciens* was found to withstand the storage time of 14 days without much loss as observed in *B. subtilis*.

While the viability loss of *Bacillus coagulans* in sterile broiler crumble feed due to storage of 14 days was found to be very higher (45.97 %; from 5.09 log<sub>10</sub>cfu/ml to 2.75 log<sub>10</sub>cfu/ml), it can be observed that the loss at the 7 day storage was 43.61 %. The loss in viability started as early as from 7 days of storage in the case of *B. coagulans*.

The viability of spore forming *B. clausii* in sterile broiler crumble feed was found to be in decreasing trend during the span of 14 days of storage from 9.86 log<sub>10</sub>cfu/ml (0 day) 7.80 log<sub>10</sub>cfu/ml which was 20.89 % loss. Not like

*B. coagulans*, the viability loss was lower in the first 7 days (4.16 %) but there after it increased sharply between 7 and 14 days storage.

The viability of non-spore forming *L. acidophilus* in sterile broiler crumble feed decreased during storage the first 7 days was fairly faster by 27.90 % (from 6.20 log<sub>10</sub>cfu/ml on day 0 to 4.47 log<sub>10</sub>cfu/ml) and thereafter the loss was minimal (from 4.47 log<sub>10</sub>cfu/ml on day 7 to 4.28 log<sub>10</sub>cfu/ml on day 14. In a similar work, the viability loss of lactic acid producing bacteria was reported to be higher (1.01 to 1.51 log cfu/ml) in first week than second week (<0.5 log cfu/ml) of storage period (Olnood *et al.*, 2015).

The count of non-spore forming *B. bifidum* in sterile broiler crumble feed decreased during the span of 14 days of storage from 6.03 log<sub>10</sub>cfu/ml (0 day) to 5.50 log<sub>10</sub>cfu/ml (7 day) and to a final value of 5.14 log<sub>10</sub>cfu/g (14 day) which was equivalent to 14.76%, while the % loss on day 7 was 8.79. The loss was progressively gradual on day 7 and 14 in case of *B. bifidum*.

The viability of probiotics was influenced by the storage environment, length of storage of the feed (Pollmann and Bandyk, 1984).

In conclusion, this study demonstrated that the reconstituted skim milk can be used as medium for spray drying of probiotics. The survival rate of spore forming and non-spore forming probiotics was above 60 % after spray drying. In this study, *Bacillus clausii* gave better yield after spray drying. Survival rate of *Bacillus subtilis* was highest after spray drying amongst the probiotics studied. *Bacillus amyloliquefaciens* among the spore forming bacteria and *B. bifidum* among the non-spore forming bacteria survived better in the crumble feed during storage of 14 days. After 14 days of storage in feed, the overall

viability loss was lowest in *Bacillus amyloliquefaciens* followed by *Bifidobacterium bifidum*, *Bacillus clausii*, *Lactobacillus acidophilus*, *Bacillus subtilis* and *Bacillus coagulans*.

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