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Inclusion methods and storage conditions of commercial probiotics, *Bacillus* sp. in aquafeed

[1,2]Mat N. Noordiyana, [1]Rusli Noraisyah, [1] Zufar S. B. Muhammad, [1]Noor E. Sharifah

[1] School of Fisheries and Aquaculture Sciences, University Malaysia Terengganu, Terengganu, Malaysia; [2] Institute of Tropical Aquaculture, University Malaysia Terengganu, Terengganu, Malaysia. Corresponding author: M. N. Noordiyana, diyananoordin@umt.edu.my

Abstract. A study was conducted to investigate the inclusion methods and storage conditions of a commercial probiotic, containing *Bacillus* sp., in aquafeed. The commercial probiotic was included in aquafeed at 0.3% of diet dry weight via infusion (IB), infusion and evenly coated with molasses (IBM) and direct inclusion in pellet during pellet processing (PB). The diets were then stored in three different conditions: refrigerator (7°C), incubator (37°C) and room temperature (28°C). Between the three inclusion methods, IBM yields the highest growth of *Bacillus* sp. within the first 3 days of inclusion followed by IB and PB. Incubator showed to be the least favorable condition to store tested pellets. The differences were often significant with tested pellets stored in room temperature at day 7 of the experiment ($p<0.05$). Regardless of the storing condition, IBM diets yield the highest growth percentage of *Bacillus* sp. Based on the results, inclusion of the commercial probiotics, *Bacillus* sp. in pellet and further coated with molasses should be one of the considerable methods to be applied in aquafeed industry. In addition, under this experimental condition, pellets with *Bacillus* sp. could be stored in room temperature only, however prolonged storage warrants further study.

Key Words: *Bacillus sp*., aquafeed, inclusion method, storage condition

Introduction. Aquaculture is a fast growing food production sector due to the high market demands but with an inconsistent yield of fisheries capture (Subasinghe 2005). To increase production of cultured fish, aquaculture system has been upgraded from simple extensive culture to intensive enterprises. However, intensification of aquaculture means rearing fish at high stocking density, which is dependent on formulated feed, and all these subsequently leads to poor water quality and disease outbreak (Robertson & Kuenen 1990; Balcazar et al 2006). To overcome this, traditionally, aquaculture has rely on the use of antibiotics as growth promoters and disease inhibitors, however their usage lead to growing concern because of the potential development of antibiotic-resistant bacteria, the destruction of existing microflora in the environment, weaken the aquatic animal's immune system and perhaps residue of antibiotics from the cultured fish could be transferred to human through their diets (Sapkota et al 2008).

One of the strategies to combat disease outbreaks in aquaculture is to use more environmental friendly alternatives like probiotics. Probiotics are a group of beneficial bacteria that when transferred into culture water or given orally to aquaculture species (Irianto & Austin 2002), will act by modifying microbial community associated with the host and will improve the utilization of feed, nutrients absorption and host response towards disease (Mayer 2011). In suitable condition, the probiotics will proliferate and slowly reduce propagation of pathogenic bacteria by consuming available nutrients (Nageswara & Babu 2006). Among many, the most commonly used probiotics in aquaculture includes *Bacillus* sp. in culture water of *Penaeus monodon* (Porubcan 1991), combination of *Bacillus subtilis* and *Saccharomyces cerevisiae* in diets of *Oreochromis niloticus* (Marzouk et al 2008) and *Lactobacillus helveticus* in diets of *Scophthalmus maximus* (Gatesoupe 1999). *Bacillus* sp. is a gram-positive bacteria, which most likely to be rod-shaped and can be found abundantly in environment such as air, soil and in water organisms (Frazier & Westhoff 1988; Wistreich 2007).

Inclusion of probiotics in aquafeed is highly dependent on the methods of inclusion and storage. In aquafeed, it is common to subject the pellet to high temperature (60-120^{o}C) to increase digestibility of starch compounds as well as aiding in the binding process (FAO 1991). Biourge et al (1998) reported that the extrusion-expansion and drying process resulted in 99% loss of *Bacillus cereus* strain spores. In addition, temperature as low as 45^{o}C has been suggested to be able to kill free probiotic cells (Mansouripour et al 2013). Therefore this study was designed to investigate growth percentage of *Bacillus* sp. at different inclusion methods and response to storage at different conditions. *Bacillus* sp. used in the present study was obtained from commercial probiotics supplier in Taiwan.

Material and Method. The experiment was conducted at the Biosystem and Fisheries Laboratory of School of Fisheries and Aquaculture Sciences, University Malaysia Terengganu. Three diets were prepared with different inclusion methods of *Bacillus* sp. The diets were direct infused with commercial probiotics mix (IB), direct infused with commercial probiotics mix and then evenly coated with molasses (IBM) or direct inclusion of commercial probiotics in powder form in pellet during pellet processing (PB). The amount of commercial probiotics used in all three diets was standardized to 0.3% of the diet dry weight. Commercial probiotics mix was prepared by diluting commercial probiotics powder in 20 mL of fresh water. Each of the treatment were triplicated and then stored in different storage condition: room temperature (28°C), refrigerator (7°C) and incubator (37°C). They were stored for seven days and number of bacteria for each interval days was recorded.

The pellets that were stored in different conditions were first was subjected to the modifying heat treatment of Barbosa et al (2005). The mashed pellet from each treatment was diluted separately in 1 mL of buffered-peptone water in microtube and resuspended by vigorous vortexing until an evenly distributed suspension was obtained. Then, the samples were left incubated at 65°C for 30 minutes. After 30 minutes of heat treatment, the samples undergo a 10-fold serial dilutions in buffered peptone water (up to 10^{-5}) and 0.1 mL of the diluted aliquots were dropped on Luria-Bertani (LB) agar plates according to the drop plate method (Herigstad et al 2001) and incubated for 24 hours at 37°C. The number of *Bacillus* sp. grown was counted and recorded in percentage of *Bacillus* sp. out of whole bacteria count.

% of *Bacillus* sp. = (Number of colonies of *Bacillus* sp. / total number of bacteria) x 100

Data on the percentage of *Bacillus* sp. colonies was analyzed using one-way analysis of variance (ANOVA) and significant differences between treatments were determined using Tukey's test. The differences were deemed significant if the test yield p value of less than 0.05. All statistical analyses were performed using SPSS, version 17 (SPSS, Inc., USA)

Results and discussion. Probiotics in aquaculture industry have been known to assist in degradation of organic matter, improve water quality parameters, increase zooplankton numbers, reduce odors and subsequently achieve its ultimate goal to reduce disease outbreak *e.g.* Vibrio, *Aeromonas* sp. (Sahu et al 2008). However, inclusion of probiotics via diets was dispute to be more effective due to their ability to stimulate fish appetite, detoxification of compound in diets, enhancement of immune response of host species and improve nutrients utilization by production of supplemental digestive enzyme (Sakai et al 1995; Verschuere et al 2000; Carnevali et al 2006). Not many studies have been dedicated on the inclusion of probiotics in aquafeed, thus, the present study aiming the practical application of probiotics, the effect of different inclusions methods and storage conditions of probiotics, *Bacillus* sp. in aquafeed.

Based on the analysis, IBM pellet yield showed the highest percentage of *Bacillus* sp. among the three methods tested (Table 1-3). IBM pellet also showed the highest ability to survive under different condition of storage compare to other inclusion methods. Successful application of probiotic bacteria was often accompanied by addition of carbohydrates (Schutyser et al 2012). Thus, the higher percentage obtained by IBM pellet is perhaps due to composition of molasses that contained up to 50% of glucose, some protein and also trace elements (Olbrich 2006) which would serve as nutrients to multiplication of *Bacillus* sp. In addition, molasses can also act as binders that help to hold the probiotic in pellets as well as to maintain the shape of the pellet.

Table 1

Growth percentage of *Bacillus* sp. in pellets that were directly infused with commercial probiotics mix (IB) at different storage conditions

Day	*Storage condition*		
	Refrigerator (7^{o}C)	*Incubator (37^{o}C)*	*Room temperature (28^{o}C)*
1	-	-	-
3	12.0 ± 2.4^a	11.9 ± 4.1^a	16.6 ± 7.8^a
5	16.5 ± 3.0^{ab}	12.0 ± 0.3^a	22.5 ± 2.6^b
7	15.1 ± 1.7^a	14.4 ± 1.10^a	25.6 ± 2.6^b

The same superscript within the same row means no significant differences ($p>0.05$).
- means no colony was detected.

Table 2

Growth percentage of *Bacillus* sp. in pellets that were directly infused with commercial probiotics mix then evenly coated with molasses (IBM) at different storage conditions

Day	*Storage condition*		
	Refrigerator (7^{o}C)	*Incubator (37-38^{o}C)*	*Room temperature (27-28^{o}C)*
1	-	-	-
3	20.0 ± 5.1^a	17.5 ± 3.2^a	37.2 ± 12.7^a
5	33.9 ± 7.3^a	18.0 ± 1.7^a	41.0 ± 7.5^a
7	24.4 ± 1.0^a	19.8 ± 1.7^a	33.6 ± 4.5^b

The same superscript within the same row means no significant differences ($p>0.05$).
- means no colony was detected.

The growth of the *Bacillus* sp. was the lowest when the commercial probiotics powder was included during diet processing (PB) (Table 3). Following the standard protocol for preparation of formulated diet, the PB pellet was oven dried at 60^{o}C for at least 24 hours (FAO 1991). Hirose et al (2006) stated that temperature do plays an important role in the stability of probiotics. Although *Bacillus* sp. are among the most stable bacteria as they can morphed to spore forms and thus are heat stable and have extended shelf-life at room temperature (Cutting 2011). The formulated pellet contains less than 10% moisture that may hinder *Bacillus* sp. growth rate. Other treatments contained significant amount of moisture due to the infusion method of commercial probiotics and further addition of molasses in IBM treatment.

The most desirable storage among all three inclusion methods was at room temperature followed by refrigerator and incubator (Table 1-3). There were no significant differences ($p>0.05$) in the growth percentage of *Bacillus* sp. stored in the refrigerator, incubator and room temperature during the first three days of storage, however, in day seven, the growth percentage of *Bacillus* sp. was significantly higher when the pellets were stored in room temperature ($p<0.05$) (Table 1-3). The significant differences were not apparent between the diets that were stored in refrigerator and incubator ($p>0.05$). Mansouripour et al (2013) stated that during heating process of pelletization, temperatures above 45°C may kill free probiotics cell. In present study, the incubator was set to be at 37°C and the refrigerator at 7^{o}C. This can be considered as suboptimal temperature that can deprive the growth of *Bacillus* sp.

Table 3

Growth percentage of *Bacillus* sp. in pellets that contained commercial probiotics in powder form at different storage conditions

Day	*Storage condition*		
	Refrigerator (7^{o}C)	*Incubator (37-38^{o}C)*	*Room temperature (27-28^{o}C)*
1	-	-	-
3	10.1 ± 0.6^a	9.3 ± 2.2^a	8.4 ± 4.9^a
5	8.3 ± 0.9^a	12.9 ± 2.0^a	12.6 ± 1.9^a
7	7.1 ± 1.0^a	10.5 ± 2.9^a	18.8 ± 2.6^b

The same superscript within the same row means no significant differences (p>0.05).
- means no colony was detected.

Conclusions. Probiotics have been extensively studied and explored commercially, however, their use in aquafeed is still scarce and still being under study. The present study was conducted to identify the effect of different inclusions method and storage of commercial probiotics, *Bacillus* sp. in aquafeed. From this study, it can be concluded that storage condition with different temperature and inclusion methods in fish pellets has a significant relationship to the growth percentage of *Bacillus* sp. Thus it is possible to improve their survival by the usage of coating material or perhaps a combination of different material. However more research is needed to provide the possibility of adding probiotics prior to heating process in aquafeed.

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Authors:
Mat Noordin Noordiyana, University Malaysia Terengganu, School of Fisheries and Aquaculture Sciences, Malaysia, Terengganu, Kuala Terengganu, 21030; University Malaysia Terengganu, Malaysia Institute of Tropical Aquaculture, Malaysia, Terengganu, Kuala Terengganu, 21030, e-mail: diyananoordin@umt.edu.my
Rusli Noraisyah, University Malaysia Terengganu, School of Fisheries and Aquaculture Sciences, Malaysia, Terengganu, Kuala Terengganu, 21030, e-mail: syahrusli12@gmail.com
Zufar Safwan Borsra Muhammad, University Malaysia Terengganu, School of Fisheries and Aquaculture Sciences, Malaysia, Terengganu, Kuala Terengganu, 21030, e-mail: zufarsafwan@yahoo.com
Noor Emilia Sharifah, University Malaysia Terengganu, School of Fisheries and Aquaculture Sciences, Malaysia, Terengganu, Kuala Terengganu, 21030, e-mail: emilia@umt.edu.my

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