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Full Length Research Paper

Viability of *Lactobacillus brevis*1, *Lactobacillus plantarum* and *Pediococcus pentosaceus*2 isolated from fish gut after incorporation into the fish feed

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The viability change of the probiotic organisms on the feed was analysed at 5 days interval for a period of 25 days to observe the effect of different storage temperatures on the viabilities change of probiotics (*Lactobacillus brevis1*, *Lactobacillus plantarum, Pediococcus pentosaceus2*) in the feed. The aim of this research was to study the survival of the probiotics during storage. Feed was sprayed with a suspension of 3.2 × 10^8 CFU/ml of probiotics and the viability of the cultured organisms was tested under 4 different temperatures (room temperature (22°C), 0, 4, and 8°C). After spraying, feed was kept at 37°C for 24 h and dried at room temperature prior to the test. The results demonstrated that a refrigeration temperature of 0°C led to highest viability of the organisms but the feed were subjected to chemical damage during freeze-thaw which invariably cause death of some of those organisms. Hence, fish feed incorporated with probiotic should be stored at 4°C. Organisms could not withstand room temperature for more than 15 days after which the viability of the organisms began to drop. Temperature is being considered as a critical factor influencing probiotic viability and survival during storage period.

Key words: *Lactobacillus brevis1*, *Lactobacillus plantarum, Pediococcus pentosaceus2,* probiotics, storage temperature, viability.

INTRODUCTION

Probiotics are described as preparations of living microbial cells that, when ingested in high enough concentration, beneficially affect the host's health by improving the intestinal microbial balance (Fuller, 1989). A good probiotic strain should be able to survive the conditions of handling and storage to be delivered in high concentration to the host. That is especially important when stressful conditions are prevalent in the carrier, for instance in low water content foods like animal feed. Probiotics are usually added to animal feed as freeze-dried cultures which sometimes are mixed with lipids to be added as top dressings in the feed (Robertson et al., 2000; Nikoskelainen et al., 2001). Fatty acids may be also used to encapsulate freeze-dried probiotics to enhance their viability (Siuta-Cruce and Goulet, 2001). They should resist processing and storage conditions and be alive and active even after gastrointestinal passage. They should be safe and impart benefits to the host (Fuller, 1989; Havenaar et al., 1992a). Storage conditions, especially temperature and humidity, represent another important factor affecting microbiological quality of feeds. Improper storage temperature may prolong survival of the micro-organisms present in the feed or even enhance their multiplication and production of toxic substances (Zmyslowska and Lewandowska, 1998). Viability of probiotic bacteria (the number of viable and active cells per g or ml of probiotic

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Table 1. Variation trend of viable and active cells (3.2×10^8 CFU/g) of probiotic fish feed diet at 5-day interval during 25 days of storage period.

Probiotic organism	Storage Temp. (°C)	* Day 0 (CFU/g of feed)	Day 5 (CFU/g of feed)	Day 10 (CFU/g of feed)	Day 15 (CFU/g of feed)	Day 20 (CFU/g of feed)	Day 25 (CFU/ml)
Lactobacillus brevis1	0	3.20	3.20^a	3.20^b	3.11^b	2.90^a	2.81^a
	4	3.20	3.20^b	2.91^a	2.72^b	2.62^a	2.41^a
	8	3.20	3.02^a	2.53^b	2.54^b	2.32^a	2.22^a
	22	3.20	2.81^b	2.44^b	2.22^a	2.01^a	1.81^a
Lactobacillus plantarum	0	3.20	3.20^a	3.20^a	3.20^b	3.20^b	3.16^b
	4	3.20	3.20^b	3.20^b	2.81^b	2.73^a	2.63^a
	8	3.20	3.11^a	2.61^a	2.52^a	2.44^a	2.26^a
	22	3.20	3.02^b	2.53^b	2.30^a	2.32^a	2.16^b
Pediococcus pentosaceus2	0	3.20	3.20^b	3.06^b	2.81^a	2.81^a	2.58^a
	4	3.20	3.03^a	3.03^a	2.73^a	2.51^a	2.33^b
	8	3.20	3.11^b	2.82^b	2.52^b	2.32^a	2.21^a
	22	3.20	2.73^a	2.51^a	2.24^a	1.86^b	1.63^b

* Immediately after incorporation. Means with different superscripts are significantly different ($P < 0.05$).

food product at the time of consumption) is the most critical value for these products because it determines their healthful efficiency (Tamime et al., 2005; Khorbekandi et al., 2011).

MATERIALS AND METHODS

Fish samples and bacterial enumeration

Lactobacillus brevis1, Lactobacillus plantarum and *Pediococcus pentosaceus*2 were cultured and isolated from the gut of *Sphyraena afra, Clarias gariepinus* and *Tilapia guineesis* respectively using modified MRS (de Man Rogosa and Sharpe) broth and MRS agar. Biochemical tests were carried out (Gram staining, catalase, endospore and motility test) and identification was based on the characteristics of the lactobacilli as described in Bergey's Manual of Determinative Bacteriology (Azcarate-Peril), fermentation of different carbon sources (API 50 CHL, BioMérieux).

Preparation of experimental diets and incorporation

Three different experimental diets with probiotics supplementation namely *L. brevis*, *L. plantarum* and *P. pentosaceus2* isolated from fish gut were formulated. The probiotic diets were prepared by gently spraying the required amount of bacterial suspension on the commercial diet (10 ml bacterial suspension per kg diet) and mixing it part-by-part in an improvised bowl to obtain a final concentration of 3.2×10^8 CFU ml^{-1} (Ghosh et al., 2008; Merrifield et al., 2010a, b).

Four storage temperature of the feed for fish were taken into account in this study: Room temperature (22°C), 0, 4 and 8°C.

Sample collection and storage

About 5 g of feed diet was taken from each prepared experimental diets containing *L. brevis1*, *L. plantarum* and *P. pentosaceus2* and placed in each of the four storage temperature: Room, 0, 4 and 8°C and stored for a period of 25 days to determine the survival of probiotics incorporated into the feed until the 25th day of storage. The microbiological analysis of the feed was carried out before storage and other examinations were performed at 5-day interval until 25 days.

Survival rate was determined every 5 day for the period of examination. Plate count method was used and 1.0 Mcfarland standard solution was used to adjust the turbidity of bacteria suspension with cell density of approximately 3.2×10^8 CFU ml^{-1} to determine the number of colony forming units (CFU).

RESULTS AND DISCUSSION

Table 1 show the reduction level and variation trend in the microbial cell counts of *L. brevis1, L. plantarum* and *P. pentosaceus2* in fish feed during the storage period at 5- day intervals. No significant difference ($P< 0.05$) was observed between the refrigeration temperature of 0, 4 and 8°C after 5-days of storage. There was rapid decrease of live bacteria numbers and few numbers of live cells were obtained on the 25th day of storage. The feed kept at room temperature (22°C) began to experience reduction in the microbial cell counts of the

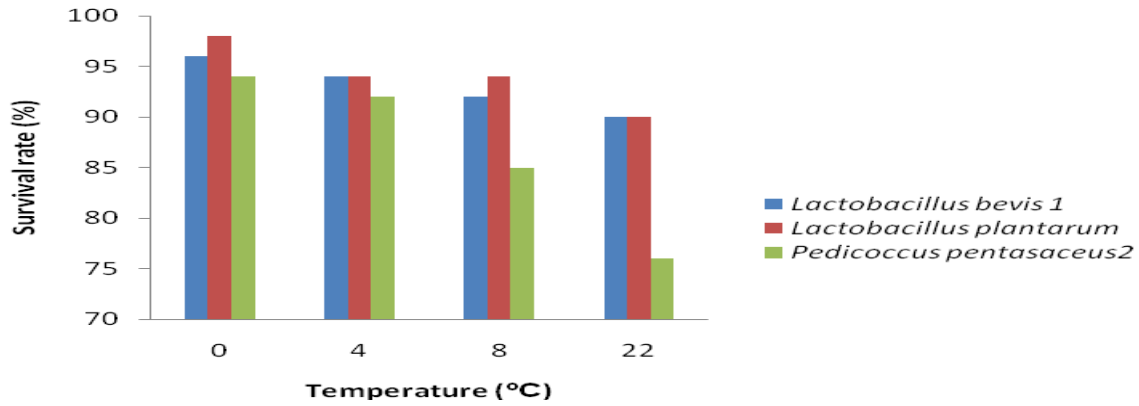

Figure 1. Survival rate of probiotics organism at the 5th day of storage at varying temperature.

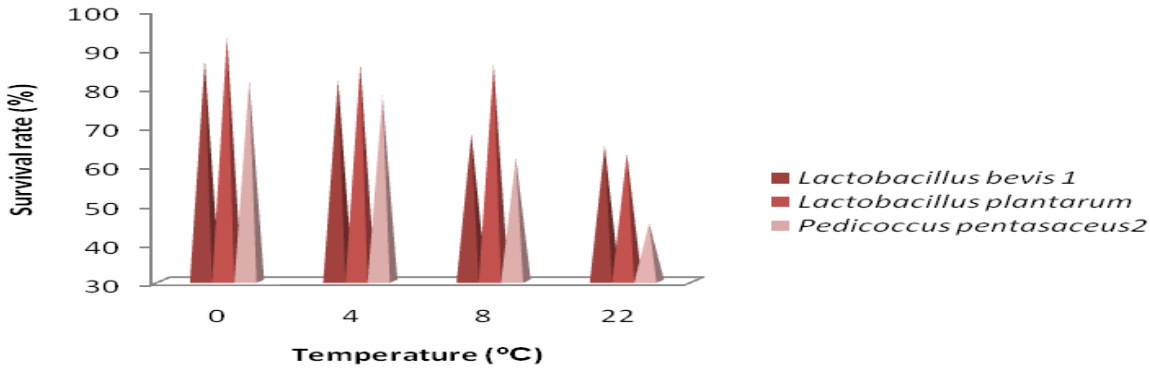

Figure 2. Survival rate of probiotics organism at the 10th day of storage at varying temperature.

organisms after 5-day of storage. High level of viable organism and stability during production and storage are important criteria for the selection of suitable strains for fish feed production. However, *L. plantarum* had the highest viable and active cells from day 0 until the 20th day of storage temperature (0°C) and until the 15th day of storage temperature (4°C). This may be due to relatively high tolerance of the strain of *L. plantarum* used compared with other species or strains.

For probiotics to be functional, they have to be viable and in sufficient dosage levels (Galdeano and Perdigon, 2004). The best storage temperature was observed to be 0°C where highest viability of the organisms was recorded as revealed in microbial cells of the three probiotics, *L. brevis1, L. plantarum* and *P. pentosaceus2*, in fish feed during the storage period but the feed at this temperature was subjected to chemical change during melting (freeze-thaw) which invariably caused death of some of those organisms and they were exposed to some osmotic effect (Jay et al., 2005). Figures 1 to 5 represent the survival rate of probiotics organism during the storage period of 5-days intervals for 25 days. Survival was high till 15 days of storage except for feed kept at room temperature (22°C). Highest survival rates of the probiotic organisms were observed in *L. plantarum*, while the least values were recorded in *P. pentosaceus2* throughout the period of storage. At the 5th day of storage, high survival rates were recorded under storage temperature of 0°C, 4°C, 8°C and 22°C in all the probiotic organisms and no significant difference was observed in the treatments ($P<0.05$). As the storage periods increased, the rate of survival of the probiotic organisms in the fish feed decreased. At the end of the storage, the rate of survival of these viable organisms at 22°C reduced to 3%, 10% and 1% in *L. brevis1, L. plantarum and P. pentosaceus2* respectively. 1% recorded in *P. pentosaceus2* at room temperature (22°C) indicated that few organisms survived at 25 days of storage. Low survival rate of *P. pentosaceus2* in the feed might have been caused by their high susceptibility to external condition and temperature variation. Patent (2013) reported that for a probiotic feed for fish, a concentration of viable probiotic bacteria of 10^7 CFU/g for at least 15 days of storage and 10^6 CFU/g for at least 91 days of storage at ambient temperature would be ideal for management. In this present study, the concentration of

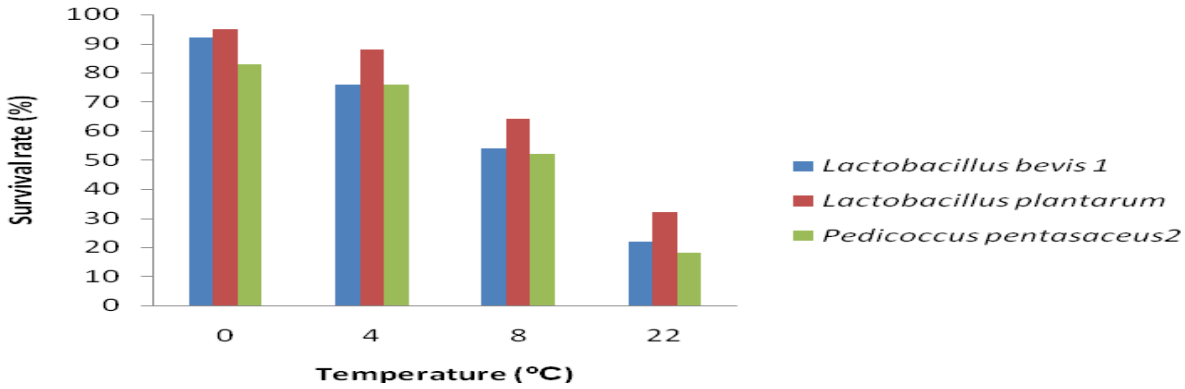

Figure 3. Survival rate of probiotics organism at the 15th day of storage at varying temperature.

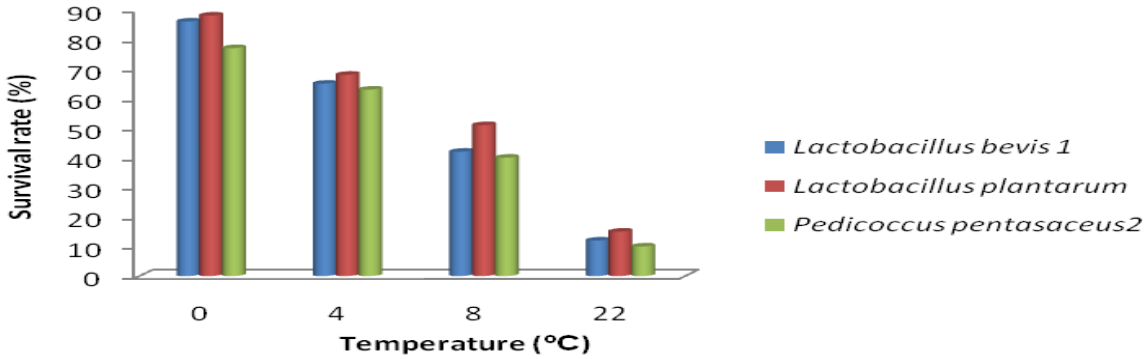

Figure 4. Survival rate of probiotics organism at the 20th day of storage at varying temperature.

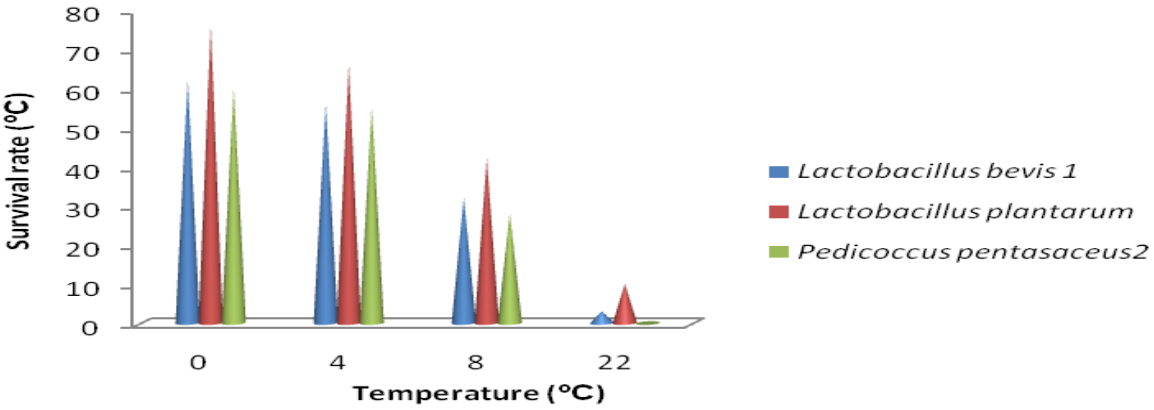

Figure 5. Survival rate of probiotics organism at the 25th day of storage at varying temperature.

viable probiotic bacteria used was 10^8 CFU/g which are of lesser concentration than 10^7 CFU/g and 10^6 CFU/g, and this brought about reduction in the storage period. Increasing the concentration of the viable probiotic bacteria to 10^7 CFU/g and 10^6 CFU/g could bring an increase in the storage period.

Conclusion

The research was conducted to observe and determine the effect of different temperatures on viability/survival of viable bacteria (probiotics) in fish feed throughout the storage period of 25 days. The results revealed that a

storage temperature of 0°C led to the highest viability of *L. brevis1, L. plantarum* and *P. pentosaceus2* throughout 25 days of storage but the feeds kept under this temperature were subjected to chemical change which led to the death of some live bacteria. Hence, the fish feed could be preserved and kept at 4°C if the concentration of viable probiotic bacteria could be increased to 10^6 CFU/g and 10^7 CFU/g during incorporation for the feed to stay for a longer period of storage.

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