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Full Paper

Survival of *Lactobacillus plantarum* 44a after spraying and drying in feed and during exposure to gastrointestinal tract fluids in vitro

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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. The aim of this research was to study the survival of the probiotic candidate *Lactobacillus plantarum* 44a after spraying and drying in feed, and during storage and exposure to gastrointestinal tract fluids in vitro. In addition, the viability of the strain during exposure to distilled water and 2% NaCl was studied. Feed was sprayed with a suspension of $\approx 2\times10^{10}$ CFU of *L. plantarum* 44a in 10, 15, 20, 25 and 30% v/w of the feed and dried to constant weight (6% moisture) in a convective oven at 25°C. *L. plantarum* 44a survived 14.67, 36, 51.86, 78.9 and 105.3% respectively in relation to the original % v/w of the feed. After 3 weeks of storage at 25°C, survival was similarly low in all the treatments. *L. plantarum* 44a stored in feed containing 13% moisture, vacuum-packaged and stored in refrigeration, maintained high viability (≈100%) after 1 year of storage. Survival was not affected after feed-containing lactobacilli was exposed to gastrointestinal fluids in a simulation model. Viability of *L. plantarum* 44a as a cell suspension in PBS added directly to distilled water or distilled water with 2% NaCl was maintained up to 48 h; after 72 h, viability started to decline. It is concluded that *L. plantarum* 44a maintained high viability after being dried and stored in feed even after exposure to gastric and intestinal fluids in vitro.

Key Words——dry feed; fish; *Lactobacillus plantarum*; probiotic; survival

Introduction

Probiotics are microorganisms that when ingested in high enough numbers beneficially affect the host's health. To reach these beneficial results, it is necessary to maintain good viability of the probiotic strains during processing, storage and consumption. Stress conditions that usually affect viability of probiotics during production are the following: large concentration of fermentation by-products (e.g., lactic acid in the culture medium), harvesting (centrifugation, ultrafiltration), freezing, and drying (during the freeze-drying process). In addition, the strains encounter the following stress conditions in the gastrointestinal tract: re-hydration in an acidic environment, long exposure time to stomach acidity, sometimes in the presence of antimicrobial compounds (in certain foods) and thereafter the exposure to bile acids (Siuta Cruce and Goulet, 2001).

Probiotics are usually added to animal feed as freeze-dried cultures which sometimes are mixed with

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lipids to be added as top dressings in the feed (Nikoskelainen et al., 2001; Robertson et al., 2000). Fatty acids may be also used to encapsulate freeze-dried probiotics to enhance their viability (Siuta Cruce and Goulet, 2001).

Convective drying has been suggested as another means to preserve lactic acid bacteria starters with less expensive equipment than freeze drying (Kets et al., 1996; Linders et al., 1996) and also seems to be an attractive possibility to dry and preserve lactic acid probiotics added to the feed. However, little information on this topic has been provided.

In order to maintain viability, several parameters in relation to thermal and drying inactivation (fermentative capacity) as well as culture conditions of the strains have to be optimized, to allow cells to adapt physiologically to the dehydration process (Kets et al., 1996; Linders et al, 1996; Wouters et al., 1998). For instance *L. plantarum* cultured at pH 5 had a higher resistance to osmotic pressure than cells cultured at pH 7 (Wouters et al., 1998). Survival of *L. paracasei* increased during spray drying as a result of preadaptation with heat or salt (NaCl) compared with non-treated controls (Desmond et al., 2001). Bacteria also should interact positively with the food or carrier matrix to use (which depends on feed composition, pH, a_w, etc.) and to extrinsic factors like temperature, O_2, relative humidity, etc. (Linders et al., 1997).

The study of survival rate of bacteria when exposed to various levels of stress has led to a definition of the term adaptative response; that is, when cells are exposed to a moderate level of stress, they acquire increased resistance to a subsequent exposure to a more severe level of the same or other stress that otherwise would be lethal (Kim et al., 2001). Adaptative response has not been reported as a mechanism of survival rate of lactic acid bacteria when subjected to dryness at physiological temperature, particularly, when incorporated as probiotics in animal feed, like fish feed.

In the present investigation, we studied the survival rate of *L. plantarum* in function of re-hydration and dehydration in low-moisture feed to try to define some optimal process parameters to maximize viability. The viability of cells in the best treatment was studied during storage and exposure to simulated gastrointestinal tract fluids. In addition, the viability of the strain during exposure to distilled water and 2% NaCl was studied as it is also possible to administer probiotic strains through (drinking) water.

We selected *Lactobacillus plantarum* 44a for our studies because it is a probiotic candidate for warm freshwater fish as it displays inhibitory activities against common fish and human pathogens, and it is tolerant to the intestinal environment, being acid tolerant and fish bile tolerant (Bucio et al., 2004).

Materials and Methods

Bacterial strains. Two strains were used: *L. plantarum* 44a, isolated from the intestinal content of European eel (*Anguilla anguilla*) (Bucio et al., unpublished results) and *Lactobacillus salivarius* (win) supplied by Winclove Bioindustries b.v., Amsterdam, The Netherlands. *L. salivarius* (win) was used as a comparative strain for some experiments because that strain has nearly the same acidification capacity as *L. plantarum* 44a, but differs in its peptidoglycan type (Information from Winclove and Kandler and Weiss, 1986).

Feed. The fish feed was Trouvit-2 (Trouw, Fontaines, Les Vervins, France) manufactured by Nutreco, France, taken from the fish farm De Haar Vissen, belonging to Wageningen University. Feed was prepared from fish meal, soybeans and other grain products. Composition of the feed in (%) is crude protein, 55; crude fat, 16; ash, 12; Vit. A, 10,000 U; Vit. D3, 1,500 U; Vit. E, 250 U and butylated hydroxytoluene (BHT) as an antioxidant.

Volume of the cell suspension (inoculum) in relation to feed weight. *L. plantarum* 44a was grown as a 24 h culture at 30°C in MRS broth (Merck, 1.10661, Darmstadt, Germany), washed 3 times in 0.1 M potassium phosphate buffer (PBS) pH 6 and harvested by centrifugation (2,760×*g* for 15 min). The cells were resuspended in the same buffer. The cell suspension was divided into 5 equal portions which were adjusted with PBS pH 6 to represent 10, 15, 20, 25 and 30% v/w of the final feed (100 g). Cells were sprayed on fish feed and dried at 25°C up to 96 h to completely dry the feed. The relative humidity (RH) in the convective oven was about 40%. Samples were also analyzed after 3 weeks of maintenance in the same oven.

Sampling. Samples of 1 g of feed were collected during drying time or in the final product (dry feed). Enumeration of lactobacilli was performed on MRS agar. Serial dilutions of samples were prepared using Reduced Peptone Solution (Hartemink and Rombouts, 1999). Plates were incubated at 30°C in anaerobic jars

with a mixture of 78% N_2, 10% H_2 and 10% CO_2 using the Anoxomat system (Mart, Lichtenvoorde, The Netherlands). The percent survival at each of the treatments was calculated as follows: % survival$=(A_0/A_1)\times 100$, where A_0 is the number of bacteria per gram of dry matter before drying and A_1 is the number of bacteria per gram of dry matter after drying.

Dry matter. The moisture content and dry matter of the feed before and after drying was determined by grinding an aliquot of about 1 g in a mortar and drying it in an oven at 103°C. The results were expressed in % of the moisture and dry matter content.

Water activity. Feed with the highest viability of bacteria at 24 h was selected to study the kinetics of a_w during the first 24 h of drying time. Water activity was measured using a Novasina a_w box (RS 232, Axair, Pfäffikon, Switzerland).

Comparative survival of L. plantarum stored at 25 and 4°C. Feed with the highest viability of *L. plantarum* 44a after 24 h of drying was selected to study storage life. Feed was placed in closed flasks or in vacuum packages and stored at 25 and 4°C. As a reference to survival in refrigeration, the *L. salivarius* (win) strain was also tested. *L. salivarius* (win) was cultured and harvested as described for *L. plantarum* 44a. *L. salivarius* (win) was also used in lyophilized form, which was prepared by suspending 0.1 g in 30 ml of PBS pH 6. All the cell suspensions were sprayed at 30% of the feed weight, dried for 24 h and stored as described previously.

Viability of L. plantarum 44a after gastrointestinal simulation. These experiments were carried out using feed inoculated either with *L. plantarum* 44a or *L. salivarius* (win) both in lyophilized form or as overnight cultures. The three kinds of inoculated feed had previously been stored in refrigeration for 3 weeks.

Stomach simulation. For the experiment, 0.5 g of each feed inoculated was weighed and put in flasks containing 25 ml PBS pH 6 and a magnetic rod. After 2 h of agitation in a water bath at 30°C, 0.11 g pepsin (Sigma P6887) previously dissolved in 1 ml 0.5% NaCl was added. Then, the pH was adjusted to 3.0, using 8 ml 0.1 M HCl. After 15 min pH was adjusted again to this value adding 0.5 ml 0.1 M HCl. The suspensions with the cells were kept spinning with magnetic rods for 2 h. After this, 1 ml of the solution was taken for microbiological analysis.

Intestinal simulation. For the intestinal simulation, 10 ml PBS pH 8 was added to the flasks and the pH was adjusted to 7 with 2.5 ml 0.6 M NaOH. Then 0.100 g pancreatin (Sigma P1750) was dissolved in 1 ml 0.5% NaCl and added to the solution. The total volume of the solution was 45.5 ml; then 0.14 g of bile extract (Sigma B-8631) previously dissolved in 1 ml 0.5% NaCl was added to the suspension. The flasks were maintained in the water bath at 30°C for another 2 h, while stirring with magnetic rods. Experiments were done in duplicate.

Viability of L. plantarum 44a in distilled water and distilled water with 2% NaCl. A cell suspension of *L. plantarum* 44a was prepared from a 24-h culture in MRS and washed 3 times in PBS as described previously. One milliliter of the cell suspension was added to 999 ml of distilled water or distilled water containing 2% NaCl. Flasks were maintained at 25°C and viable counts were estimated at 24 h intervals using Reduced Peptone Solution to make dilutions and MRS to determine viability as described earlier.

Results

Optimization of volume of the cell suspension (inoculum) in relation to feed weight

Figure 1 shows the kinetics of the re-hydration and dehydration process of feed sprayed with lactobacilli and the associated viability of the strain for the same intervals of time. It can be seen that a complete dehydration of fluid was achieved after 48 h. *L. plantarum* 44a viability appears to be relatively constant in two of the treatments (treatments with the highest initial moisture content) throughout the drying time (Fig. 1B). *L. plantarum* 44a viability in the other three treatments began to fall after 14 h of drying and appeared to be stabilized after 48 h. Standard deviations of viability were relatively high for some of the treatments, suggesting that lactobacilli were not homogeneously distributed in the feed samples. Despite that variability, the regression line generated with the data plotted in Fig. 2, shows that viability of lactobacilli was directly related to the amount of fluid used to spray the feed and its associated moisture content. After 3 weeks of storage of the dried feed at 25°C and 40% RH, viability decreased to approximately the same percentage for all the treatments (Fig. 2).

Figure 3 shows the kinetics of water activity of the inoculated feed (30% v/w) during drying at 25°C along with the survival of *L. plantarum* 44a. In an amount of

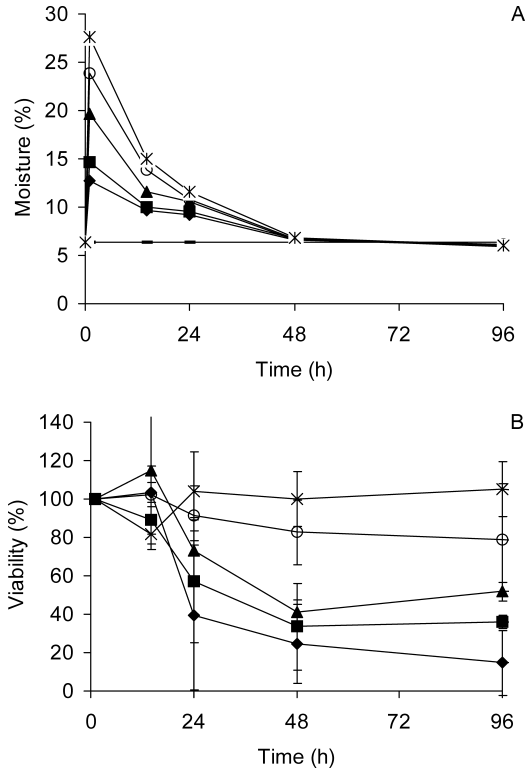

Fig. 1. Variation of moisture (A) and *Lactobacillus plantarum* 44a viability (B) during dehydration at 25°C.

Feed was sprayed in all the treatments with a suspension of ≈2×10^{10} CFU of *L. plantarum* 44a using PBS in various amounts in relation to feed weight. Symbols: ◆, 10% PBS; ■, 15% PBS; ▲, 20% PBS; ○, 25% PBS; ✳, 30% PBS; –, original moisture content of the feed. Each point represents the mean value of 3 measurements±SD.

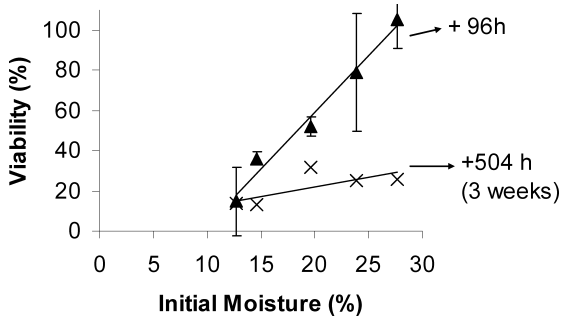

Fig. 2. Regression line between initial moisture and viability after ▲, 96 and ×, 504 h of *L. plantarum* 44a in feed dried to 6% of moisture content.

Vertical lines show standard deviations of each of the means of viability at 96 h.

30% v/w of suspension as compared to the dry feed, the initial a_w of the feed of 0.5 increased to 0.9. After 24 h of drying at 25°C, a_w had dropped to just below 0.6, with a very high survival rate of the inoculum. Although a_w was not measured in the other treatments, it may be inferred that their initial value and final values were lower leading to more severe levels of osmotic stress to the strain.

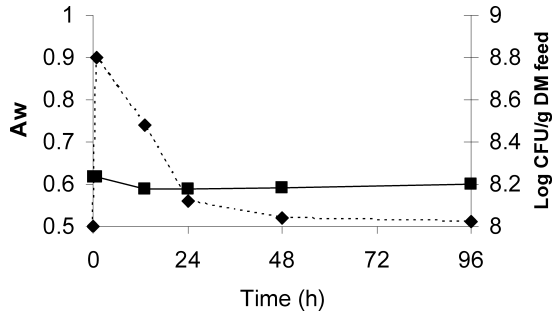

Fig. 3. Water activity and counts of *L. plantarum* 44a during drying at 25°C in duplicate.

Symbols: ◆, water activity; ■, *L. plantarum* 44a counts.

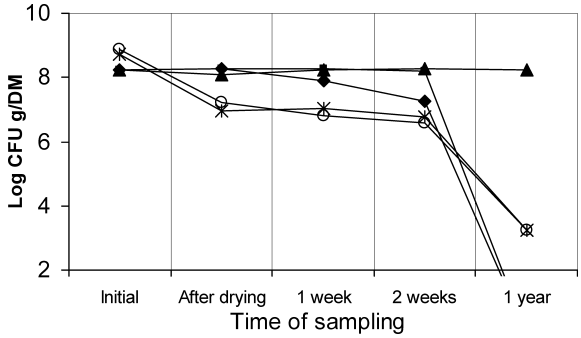

Fig. 4. Survival of *L. plantarum* 44a and *L. salivarius* (win) during drying, and storage in feed.

Symbols: ▲, *L. plantarum* 44a vacuum packaged and refrigerated; ◆, *L. plantarum* 44a stored at 25°C in closed flasks; ○, *L. salivarius* (win) added as fresh harvested, vacuum-packaged and stored in refrigeration; ✳, *L. salivarius* (win), added as a freeze-dried culture, vacuum-packaged and stored in refrigeration. Moisture content for all the samples after drying and during storage ≈13% (a_w 0.55).

Viability of L. plantarum 44a during feed storage at two temperatures

Viability of *L. plantarum* 44a in feed stored in refrigeration in vacuum packages was kept constant during 2 weeks, and after 1 year (Fig. 4). When feed was stored in glass flasks at higher temperature (25°C), viability started to decline by the second week (Fig. 4).

Viability of L. salivarius (win) after drying and during feed storage in refrigeration

L. salivarius (win) added to feed either as freeze-dried culture or as cell suspension showed a consider-

able loss in viability after drying in the first 24 h. That viability was maintained during the 15 days of storage in refrigeration, but was almost completely lost after 1 year (Fig. 4).

Viability after gastrointestinal passage simulation

Survival of *L. plantarum* 44a and *L. salivarius* (win) after gastric and intestinal passage simulation was very high (Fig. 5). Practically no reduction was detected. However, because the initial concentration of *L. plantarum* 44a in the feed was higher than *L. salivarius* (win), the counts were higher after the gastrointestinal simulation. *L. salivarius* (win) in freeze-dried form survived less well than freshly cultured forms.

Viability of cells inoculated in water

Figure 6 shows CFU of *L. plantarum* 44a after exposure to distilled water or distilled water with 2% NaCl. There was no statistically significant difference in CFU between cells exposed to distilled water or distilled water with 2% NaCl. CFU remained without changes after 24 and 48 h exposure to the treatments. After 96 h of exposure time, a lower CFU of the cells was observed in both treatments.

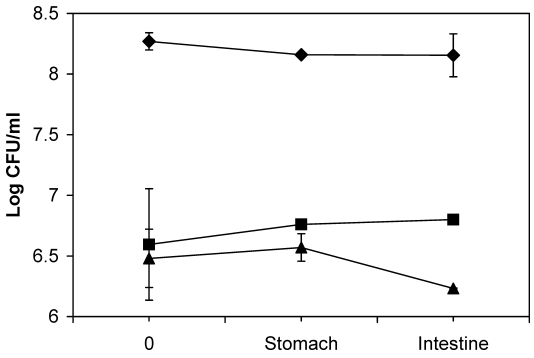

Fig. 5. Counts of *L. plantarum* 44a and *L. salivarius* (win) inoculated in feed, and stored for 3 weeks and before and after 2 h of gastric and 2 h intestinal passage simulation.

Symbols: ◆, *L. plantarum* 44a; ▲, *L. salivarius* (win) inoculated as culture lyophilized; ■, fresh harvested during feed storage in refrigeration. Moisture content ≈13%.

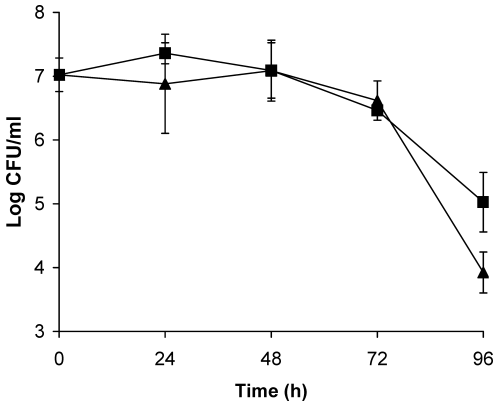

Fig. 6. Counts of *L. plantarum* in distilled water or distilled water with 2% NaCl for up to 96 h.

Symbols: ▲, distilled water; ■, distilled water +2% NaCl. Differences between treatments are not significant according to Student's *t*-test (p=0.15).

Discussion

Maintenance of viability of probiotic products before consumption is a very important aspect to realize the claimed benefits of probiotic supplementation (Havenaar et al., 1992). Major stress factors for probiotic strains in feeds are the high osmotic pressure generated by the low a_w in the feed; the re-hydration in an acidic environment like the stomach and afterwards the exposure to bile and pancreatic fluids.

Fish feed is usually manufactured with a low water activity to prevent microbial deterioration over several months and to confer floatability of the feed in water. Therefore, it is a great challenge to find a suitable method to inoculate the strains without losses of viability, maintaining the physicochemical characteristics of the feed.

In this study, we can see the importance of temporarily increased initial moisture content of the feed to maintain the viability of the strain, likely due to both a reduction in the initial osmotic pressure in the feed, and probably to an adaptative response of the strain to the progressive reduction in water content. That practice allowed the achievement of a high survival during the drying process, with a final moisture content close to the original values of the feed. Probiotic strain viability thus could be maintained in low water content feed during storage without experiencing considerable additional reduction of viability.

Several adaptation mechanisms to withstand the osmotic stress have been described for *L. plantarum*. One of the most important is the flexibility of the cell wall to stretch as water flows into the cell and to shrink when water flows out of the cell (Delcour et al.,1999; Poolman and Glaasker, 1998). This elasticity and rigidity of the cell wall has been associated with the peptide cross-links between the glycan threads and between the concentric glycan layers (Delcour et al., 1999). Other adaptation mechanisms of *L. plantarum* to with-

stand the osmotic stress consist of the accumulation or release of compatible solutes i) to counteract the extracellular osmotic pressure; ii) to stabilize proteins (Glaasker et al., 1996, 1998; Kets et al., 1996) and iii) probably to maintain integrity of biological membranes (Kets and de Bont, 1997). *L. plantarum* has a limited capacity to synthesize compatible solutes (Glaasker et al., 1996; Kets and de Bont, 1997); however, it can accumulate them by specific transport systems activated by osmotic shock in response to osmotic sensing mechanisms (Konings, 2002). Some compatible solutes are potassium, trimethyl amino acids like betaine and carnitine or some amino acids like proline, which are widely distributed in feedstuffs from animal and plant origin (Kets and de Bont, 1997) and probably were present in the feed used in the experiments, which is composed mainly of fish meal, soy meal, and wheat meal.

Another adaptation of *L. plantarum* to resist the osmotic pressure is due to the phospholipids in the lipid bilayer of the cell membrane which maintains fluidity upon dehydration, in the presence of some carbohydrates like some maltodextrines present in the feed (Linders et al., 1997). Fluidity perhaps could also be maintained by the presence of some triacylglycerols present in the feed. Triacylglycerols can act as protectants of *Lactobacillus* against osmotic pressure (Siuta Cruce and Goulet, 2001).

The presence of meso-diaminopimelic acid as the third residue of the oligopeptide cross-link of *L. plantarum* glycan might be related to its high resistance to the osmotic pressure. On the other hand, the presence of lysine as the third residue of the oligopeptide cross-link of *L. salivarius* (win) glycan might be related with its low resistance to the osmotic pressure. Lysine requires less energy to be hydrolyzed from the cell wall than the mesodiaminopimelic acid (Kandler and Weiss, 1986) and is likely more labile to osmotic pressure.

Viability of *L. plantarum* 44a was maintained in refrigeration for up to 1 year. Feed is generally stored in refrigeration several weeks before use. Other studies have also shown higher survival rates at lower storage temperatures (Gardiner et al., 2000). At a low temperature, such as in a refrigerator, bacterial metabolism decreases, and the accumulation of toxic wastes from the metabolism is likely minimized. However, feed may be exposed for some time to room temperature without compromizing viability (Fig. 4).

Another observation that makes *L. plantarum* 44a suitable as a probiotic is that after being stored in refrigeration, *L. plantarum* 44a can survive with little reduction the stresses found in the gastrointestinal tract, like low pH and pepsin, and subsequently to the bile presence and pancreatin.

It is apparent from the results of this study that *L. plantarum* 44a and the method to inoculate and to store feed, makes this strain suitable to survive the technological barriers that have been reported to reduce viability in probiotic preparations. However, certain constraints exist for industrial adoption of a process of re-hydration and dehydration of lactobacilli in feed, which need to be addressed in current and future research in the area. Adding large amounts of fluid is costly; however, the probiotic may be added to the feed when the ingredients have been pelleted and are being dried. However, temperatures for water removal should be reduced to ambient temperatures, to minimize losses of viability. Since osmotic dehydration is an inherently slow process, several improvements need to be done to increase the rates of osmotic mass transfer without comprising the viability of the probiotic strains.

The results also showed that this strain had a high likelihood to survive stress as in the gastrointestinal tract like low pH and pepsin, and bile presence and pancreatin. Moreover, *L. plantarum* 44a can also survive to tap water or salty water, being suitable as a probiotic strain using water as a vehicle of inoculation.

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