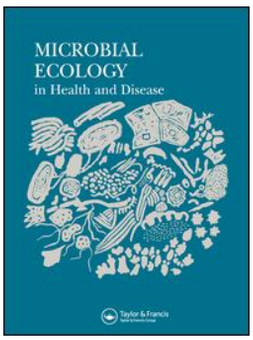

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Effect of Temperature on *in vitro* Adhesion of Potential Fish Probiotics

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As fish are poikilothermic animals, i.e. the temperature of their body is dependent on the external environment, we aimed to investigate the effect of temperature on the *in vitro* adhesive ability of potential fish probiotics. The tested strains were *Bifidobacterium animalis* Bb12, *Lactobacillus rhamnosus* GG, *L. rhamnosus* LC 705, *L. rhamnosus* LCR 1/83 and *Enterococcus faecium* M74. The *in vitro* adhesive ability of the five strains to two types of rainbow trout mucus (skin and intestinal mucus) was determined at temperatures ranging from 4°C to 25°C. Three of the tested strains, *B. animalis* Bb12, *L. rhamnosus* GG and *L. rhamnosus* LCR 1/83, showed high adhesion to both types of mucus at all temperatures studied (19–30% and 11–29% of the added bacteria to skin and intestinal mucus, respectively). The adhesive abilities of the remaining strains were low in comparison with the above-mentioned group at all temperatures tested (3–10% and 2–19% to skin and intestinal mucus, respectively). In the case of skin mucus, three strains showed significant differences in adhesion depending on the incubation temperature, while two strains exhibited significant temperature-related differences in adhesion to intestinal mucus. The quartic polynomial fit was the best model to describe the changes in the adhesive ability related to the temperature changes. In conclusion, in order to optimize probiotic functionality in aquaculture, the dose may have to be adjusted with regard to the water temperature. *Key words:* probiotics, rainbow trout, adhesion, intestinal, skin mucus.

INTRODUCTION

Successful aquaculture takes into consideration the engineering design (water source and water quality, pond and tank containment systems, water filtration and aeration) and the biology of the aquatic species (feeding, water flow and temperature needs, disease prevention and control). For optimal rearing results, the prevention and control of disease is particularly important, currently this is usually done with vaccines and antibiotics. However, the large-scale and sometimes uncontrolled use of antimicrobials for disease control and growth promotion in animals increases the selective pressure exerted on the microbial world and encourages the natural emergence of bacterial resistance (1). There is therefore a growing concern about the large-scale use of antimicrobial drugs not only in human medicine and agriculture, but also in aquaculture. Alternative strategies to the use of antimicrobials in aquaculture have been proposed. The use of probiotics is one of the promising current and potential alternatives. Probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host (2). The research on probiotics for aquatic animals is increasing with the demand for environment-friendly aquaculture and this study is a part of the ongoing studies in this field.

Adhesion is acknowledged as the first step of a microorganism in the process of intestinal colonization. It is therefore important that lactobacilli chosen for probiotic preparations adhere well to the gastrointestinal mucosa of the target host (3–6). In addition, the ability to compete with pathogens for nutrients and adhesion sites is important for potential probiotics. Adherent strains are therefore key candidates as probiotics (1, 7).

Fish are poikilothermic animals, i.e. their body temperature is dependent on the external environment. In addition, it has been reported that human probiotics may have beneficial effects on fish (8–10). Therefore, the aim of the current study was to investigate the *in vitro* adhesion of human probiotics, for potential use as fish probiotics, to intestinal and skin mucus extracted from rainbow trout (*Oncorhynchus mykiss*), and the effect of temperature on this adhesion. The latter is of major importance, as the adhesion and thereby the efficacy of fish probiotics may be affected by the seasonal variations in the temperature of the rearing water. The bacterial strains were chosen based on the hypothesis that they are human probiotics and

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therefore they are safe for human consumption in the final product.

MATERIALS AND METHODS

Bacteria and culture conditions

The strains, *Bifidobacterium animalis* Bb12 (Chr. Hansen Ltd, Hørsholm, Denmark), *Lactobacillus rhamnosus* GG (Valio Ltd, Helsinki, Finland ATCC 53103), *L. rhamnosus* LC 705 (Valio Ltd.), *L. rhamnosus* LCR 1/83 (Valio Ltd) and *Enterococcus faecium* M74 (Lactiferm®, Helsinki, Finland), were grown under anaerobic conditions in de Man, Rogosa and Sharpe broth (MRS; Merck, Darmstadt, Germany) overnight at 37°C. Ten µl/ml of tritiated thymidine ([methyl-1,2-^{3}H]thymidine; 117 Ci/mmol; Amersham Pharmacia Biotech UK Ltd) were added to the medium to metabolically radiolabel the bacteria. After incubation, the cells were harvested by centrifugation (7 min, $2000 \times g$), washed twice with phosphate-buffered saline (PBS; 10 mM phosphate, pH 7.2), and resuspended in PBS. The absorbance at 600 nm was adjusted to 0.5 ± 0.01 to standardize the number of bacteria (10^7–10^8 cfu/ml). The relationship between absorbance at 600 nm and cfu/ml was established by flow cytometry (11).

Mucus preparation

The rainbow trout (*Oncorhynchus mykiss*) used for the preparation of mucus was obtained from Hanka Taimen OY Fish Farm (Venekoski, Finland). Mucus was isolated from six healthy rainbow trout immediately after sacrifice according to the method of Cohen and Laux (12). The mucus was obtained by gently scraping the mucosal surfaces with a rubber spatula into a small amount of HEPES (10 mM, pH 7.4)-buffered Hanks' balanced salts solution (HH). The skin mucus was collected from the whole body surface. For intestinal mucus, the intestine was separated from the internal organs, cut longitudinally and the mucus was collected as described above. The mucus samples were stored in 1-ml aliquots at −86°C until use. Before use, the protein concentration was determined by a modification of the method of Lowry et al. (13) as described by Miller and Hoskins (14), using bovine serum albumin (BSA; Sigma, St Louis, USA) as a standard. The mucus was used at a protein concentration of 0.5 mg/ml in HH.

In vitro adhesion assay

Adhesion of the radioactively labelled bacteria was determined as described by Kirjavainen et al. (15). In short, mucus was immobilized on microtitre plate wells by overnight incubation at 4°C. Excess mucus was removed by washing with HH. Radioactively labelled bacteria were added, and the wells were incubated for 1 h at 4, 10, 15, 21 or 25°C. Non-bound bacteria were removed by washing with HH. Bound bacteria were released and lysed by incubation at 60°C for 1 h with 1% sodium dodecyl sulphate in 0.1 M NaOH. Adhesion was assessed by quantifying the amount of radioactivity by liquid scintillation and was expressed as the percentage of radioactivity recovered after adhesion relative to the radioactivity in the bacterial suspension added to the immobilized mucus.

Statistical analysis

All results are shown as the average of three independent experiments and each assay was performed in four replicates to correct for intra-assay variation; variation is expressed as standard deviation. The data were analysed by JMP 5.1 SAS Institute Inc. The Tukey HSD test was used to identify the differences in the adhesions at different incubation temperatures in every strain separately (after analysis of variance). Unpaired t-test was used to compare the adhesions to both types of mucus for every strain at every temperature separately.

RESULTS

Adhesion of probiotics:

There were highly significant differences between the adhesive abilities of the bacterial strains when compared at each studied temperature separately. The significant differences were true in the adhesion to skin and to intestinal mucus as well. The potential fish probiotic strains *B. animalis* Bb12, *L. rhamnosus* GG, *L. rhamnosus* LCR 1/83 adhered well to the two types of mucus tested at all different adhesion temperatures studied, adhesion ranged from 19% to 30% for skin mucus and from 11% to 29% for intestinal mucus (Figs. 1 and 2). The strains *L. rhamnosus* LC 705 and *E. faecium* M74 exhibited a low level of adhesion to skin mucus and *L. rhamnosus* LC 705 adhered poorly to the intestinal mucus at all studied temperatures (3–10% and 2–19% to skin and intestinal mucus, respectively).

Effect of mucus types

The unpaired t-test showed a significant difference between the adhesion to skin and to intestinal mucus only with strain *E. faecium* M74 at 15°C ($p < 0.05$) and 25°C ($p < 0.01$) (Figs. 1 and 2).

Effect of temperature

There was a significant difference between the adhesive abilities to skin mucus at the different incubation temperatures with *B. animalis* Bb12, *L. rhamnosus* LCR 1/83, and *E. faecium* M74 but not with *L. rhamnosus* LC 705 and *L. rhamnosus* GG. On the other hand, there was a significant difference between the adhesive abilities to intestinal mucus at the different incubation temperatures with strains *L. rhamnosus* GG and *L. rhamnosus* LCR 1/83 but not with *Bifidobacterium animalis* Bb12, *L. rhamnosus* LC 705 and

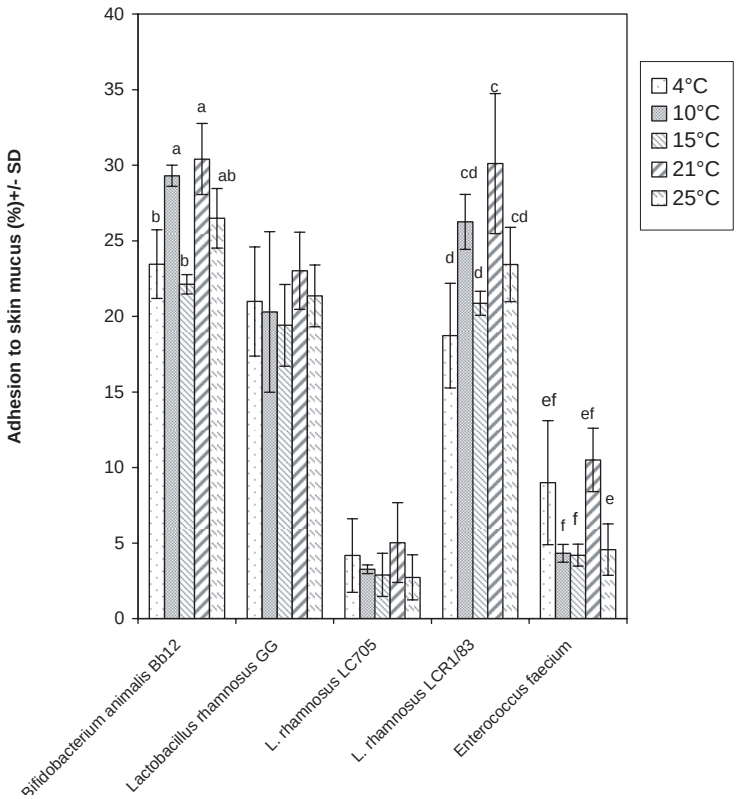

Fig. 1. The influence of incubation temperature on *in vitro* adhesion of potential fish probiotic bacteria to mucus extracted from rainbow trout skin; error bars indicate standard deviations (SD). The bars that share the same letter (or have no letter) are not significantly different (alpha = 0.05).

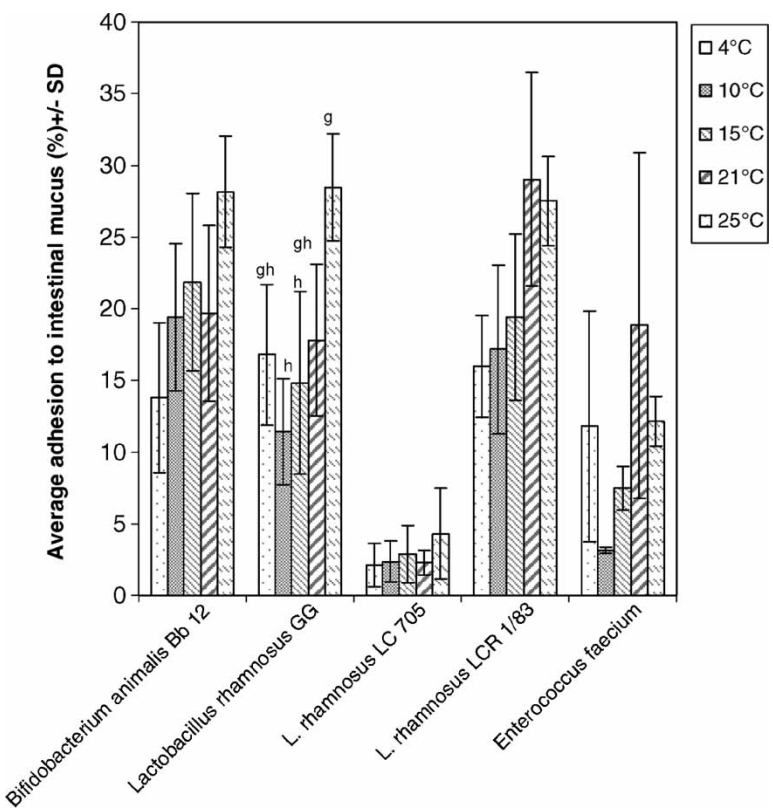

Fig. 2. The influence of incubation temperature on *in vitro* adhesion of potential fish probiotic bacteria to mucus extracted from rainbow trout intestine; error bars indicate standard deviations (SD). The bars that share the same letter (or have no letter) are not significantly different (alpha = 0.05).

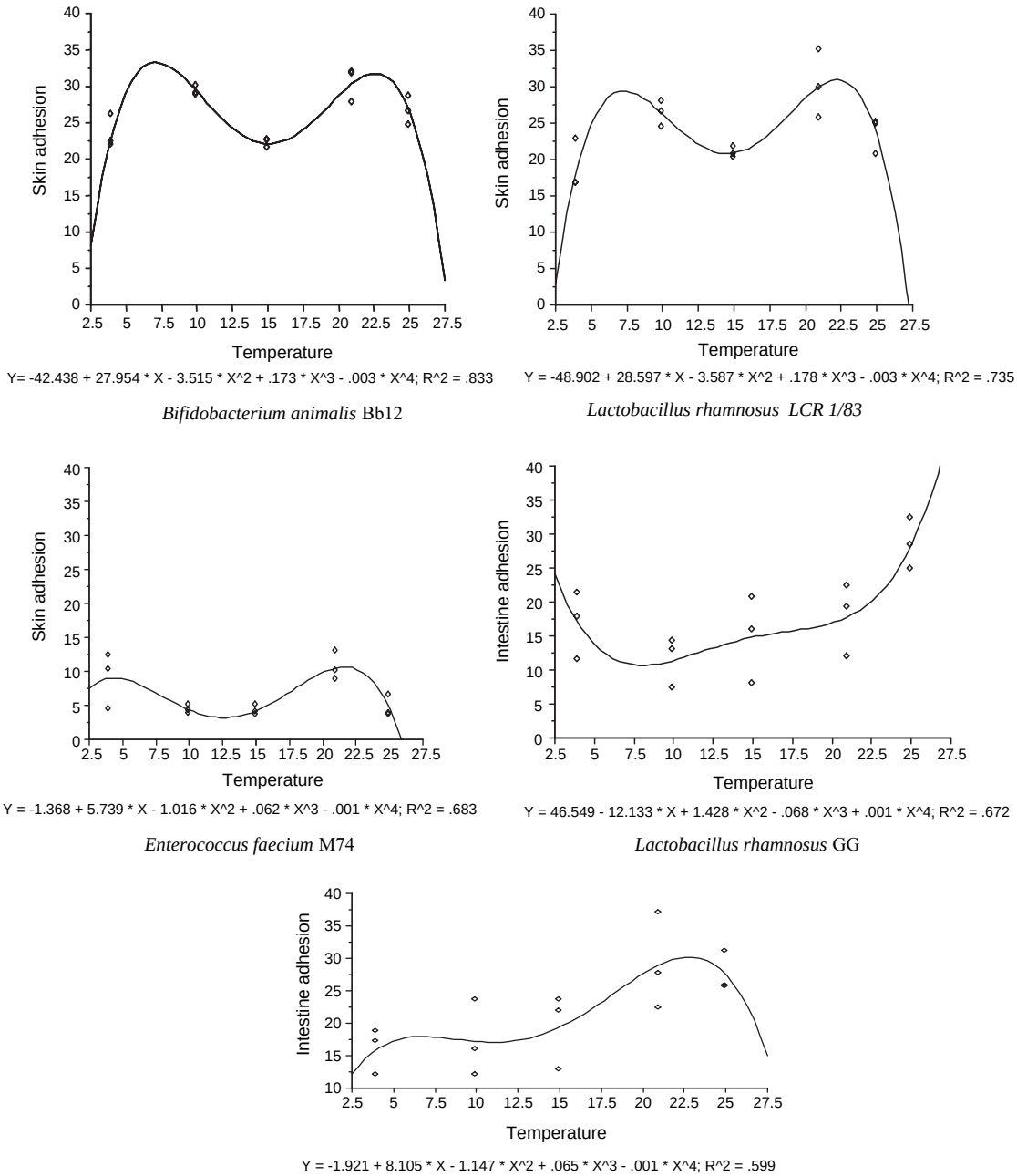

Fig. 3. The polynomial quartic (=4 degree) fit for changes in the adhesive abilities of four bacterial strains over a temperature range (4–25°C) to mucus extracted from rainbow trout skin and intestine.

E. faecium M74. However, the difference in the adhesive abilities of strain *L. rhamnosus* LCR 1/83 was not enough to appear in the conservative multiple comparison test ($p = 0.04$).

With skin mucus, all strains reached their maximum adhesions at 21°C (Fig. 1). Meanwhile, three strains reached their maximum adhesive abilities at 25°C and two strains reached their maximum adhesion at 21°C with intestinal mucus (Fig. 2).

The adhesive ability of bacteria tended to increase with the increase of incubation temperature to a maximal level at 21°C in case of skin mucus and at 25°C in case of intestine mucus as can be seen from Figs. 1 and 2.

A polynomial quartic fit (degree =4) was found to be the best model to explain the changes in the bacterial adhesive ability with temperature in the range from 4°C to 25°C (Fig. 3). This fit was significant in the case of skin mucus with the strains *B. animalis* Bb12 ($p < 0.01$, $R^2 = 0.83$),

L. rhamnosus LCR 1/83 ($p < 0.01$, $R^2 = 0.73$), *E. faecium* M74 ($p < 0.05$, $R^2 = 0.68$).

In the case of intestinal mucus, the polynomial quartic fit was significant with *L. rhamnosus* GG ($p < 0.05$, $R^2 = 0.67$), and *L. rhamnosus* LCR 1/83 ($p < 0.05$, $R^2 = 0.60$).

DISCUSSION

Several strains of the *L. rhamnosus* species, one strain of *Bifidobacterium* and one strain of *E. faecium* have been found to be tolerant for rainbow trout bile and to inhibit some fish pathogens in co-culture and challenge trials (8, 9). The adhesion of probiotic to the intestinal mucosa is considered a prerequisite for transient colonization (16), stimulation of the immune system (17) and for antagonistic activity against enteropathogens (18). The adhesive ability to the intestinal mucosa is one of the most important selection criteria for probiotics (19). As the body surface of fish is one of the potential portals of entry for pathogens (20), colonization resistance and competitive exclusion of pathogens are important for the dermal mucosa.

The effect of incubation temperature on the adhesive ability of probiotics has not been reported previously. Current probiotic adhesion studies have used one incubation temperature, usually corresponding to the host body temperature or room temperature. The body temperature of fish changes according to the temperature of the rearing water. Therefore, in the study of probiotics for fish, it is important to take into account the effect of the environmental temperature on adhesion.

Three of the tested strains, *B. animalis* Bb12, *L. rhamnosus* GG and *L. rhamnosus* LCR 1/83, showed good adhesion to the two types of mucus at all temperatures studied (19–30% of the added bacteria to skin mucus and 11–29% to intestinal mucus) and the results reflect their reported good adherence properties in other systems (8).

The remaining strains, *L. rhamnosus* LC 705 and *E. faecium* M74, showed relatively poor adhesive ability to the skin mucus and low adhesive ability to the intestinal mucus at most temperatures studied (3–10% to skin mucus and 2–19% to intestinal mucus). It is also worth pointing out that *E. faecium* M74 exhibited a relatively high level of adhesion to intestinal mucus at 21°C. The adhesive ability of the tested strains could be put in decreasing order as follows: *B. animalis* Bb12, *L. rhamnosus* LCR 1/83, *L. rhamnosus* GG, *E. faecium* M74 and *L. rhamnosus* LC 705. It is worth noting that all strains were in the above-mentioned order of the adhesive abilities at most incubation temperatures with skin and intestinal mucus. This indicates that the binding is strain-dependent rather than mucus type-dependent. The observed level of adhesion was similar to that reported earlier for probiotic strains in fish (8) and the values are in agreement with earlier studies in other models (21).

All strains, except *E. faecium* M74, did not show preferential binding to the skin mucus over intestinal mucus or vice versa at all incubation temperatures. Although the mechanism of binding was not investigated in this study, this observation might indicate non-specific adhesion. These results are similar to others' findings where the adhesion of probiotic bacteria to rainbow trout mucus was tested (8).

The effect of temperature was evidenced with three strains of bacteria in the case of skin mucus and with two strains using intestinal mucus. The significant quartic fit found with four strains at the temperature range from 4°C to 25° motivates further research with larger sample size considering the temperature factor. It can be concluded that the temperature effect on adhesive ability of different probiotic species should be considered, particularly in poikilothermic animals. Probiotic dosing may need to be adjusted according to seasonal water temperature. These findings warrant further *in vivo* investigations on the influence of the water temperature on the efficacy and transient colonization of potential probiotics in fish.

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