Incorporation of bacteriocin producing lactic acid bacteria in alginate layers as a bio-controller of *Listeria monocytogenes*

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Introduction

Listeria monocytogenes is a foodborne pathogen of considerable health concern, that has been repeatedly isolated from fish products, in particular from cold smoked salmon (Rorvik, 2000; Hu et al., 2006), which is stored under vacuum, at temperatures below 4 ºC. Under these conditions the pathogen can grow and therefore, its number increases during storage. In order to protect the consumer, the FDA in the USA and certain European countries (Italy, France and Austria), have imposed a zero tolerance in ready to eat foods (RTE), such as cold smoked salmon. Other countries (Germany, Sweden and Denmark) have established a limit of 100 cfu/g. Also FAO/WHO experts on risk assessment of *L. monocytogenes* have also considered smoked salmon and undercooked fish, as RTE foods of highest risk to consumers.

To ensure an adequate protection of cold smoked salmon consumer, the salmon processing industry must apply scrupulous good manufacturing practices and proper sanitary programs. On the other hand, the use of chemical preservatives in a food product is not allowed and the use of physical treatments may have adverse effects on the sensory characteristics of the food. Another alternative is biopreservation, which also would satisfy the demand of the consumer for natural products. Bacteriocins or bacteriocin producing lactic acid bacteria (LAB), have long been studied, alone or in combination with other hurdles, as a mean of enhancing food safety against *L. monocytogenes* (Duffes et al., 1999). Various strategies have been developed for the application of the bacteriocin producing LAB on salmon. One approach, is the bioencapsulation in alginate beads and as a more practical approach for the industry, the development of bioactive packaging films, containing the antagonistic microorganisms. The objective of the present study was to design an alginate based film with bacteriocina producing LAB incorporated in the film, as a protective barrier against *L. monocytogenes*, on cold smoked salmon.

Material and Methods

Microorganisms and culture conditions
For the study, two strains of lactic acid bacteria (LAB) producing a bacteriocin like substance (BLS), active against *Listeria monocytogenes*, were used . LAB-A had previously been isolated from cold smoked salmon and LAB-B had been isolated from vacuum packaged meat. The target strain *Listeria monocytogenes* 4/00, was also isolated from cold smoked salmon. All isolations were made at the Instituto de Ciencia y Tecnología de los Alimentos, from the Universidad Austral de Chile. The strains were kept frozen (- 20 ºC) in D-MRS broth with glycerol and tryptic soy broth respectively. Before use, the strains were subcultured twice for 24 h, in each broth.

Antimicrobial film preparation
For the film formation different formulations were used with alginate, glycerol and starch (details are not given because of patent rights). After a thorough homogenization of the alginate with the rest of the ingredients, the mixture was autoclaved at 10 psi for 5 min. To each preparation an equal volume (1:1) of the 24 h bacterial culture was added. The mixture was homogenized and different

volumes (10 ml, 15 ml and 20 ml) were placed in a 15 cm Petri dish. The films were dried at 25 ºC and 37ºC during 12 h, in an incubator oven. For further testing the films were stored at 4º C.

For the experiment three variables were considered for each type of film (with and without starch): a) film with LAB-A, b) film with LAB-B, c) film with LAB-A + LAB-B.

Evaluation of the films

Diffusion of the BLS from the film. In order to study de BLS producing capacity of the LAB strains in the film and its diffusion outside the film, 8 mm diameter coupons were cut from each film with a sterile cork borer. From each treatment 10 coupons were cut and placed in Petri dishes containing tryptic soy agar. Previously each coupon was placed for 20 sec in 70 % ethanol and rinsed in distilled water, in order to eliminate the cells of the LAB strain attached to the outside of the membrane. The plates were placed in a refrigerator at 4 ºC, and after 24 h, 48 h and 72 h of storage, covered with a lawn containing de indicator organism *L. monocytogenes* 4/00, at a concentration of 10^5 cfu/ml. The plates were incubated at 25 ºC during 24 h and the inhibition zones were measured.

Viability of the LAB strains and BLS production after extended storage at 4 ºC. The films were stored during 30 days at 4 ºC and after 1 day, 10, 20 and 30 days of storage, coupons were placed in Petri dishes with trypticase soy agar containing a *L. monocytogenes* 4/00 lawn. The plates were incubated 24 h at 25 ºC and inhibition zones around each coupon were measured, as described previously.

Effectiveness of selected films on cold smoked salmon. The film that showed the most effective results in the foregoing experiments was used. Pieces of cold smoked salmon (size 4,0 cm x 4,0 cm), were spray inoculated with a 24 h culture of *L. monocytogenes*, at a final concentration of 10^3 cfu/cm^2. Each piece was wrapped in the film and then placed individually in vacuum bags. After vacuum packaging, the samples were stored at 4 ºC during 15 days, simulating the storage conditions of commercial salmon. Growth of *L. monocytogenes* 4/00 was controlled on OXA agar, at day zero and after 7 and 14 days of storage. The salmon pieces were inoculated with different treatments: a) strain LAB-A, b) strain LAB-B, c) strain LAB-A + LAB-B, d) control *L. monocytoges* 4/00.

Results and Discussion.

Biofilms construction with alginate and other substances resulted in a dry layer that was applied on top of smocked salmon to inhibit *L. monocytogenes* (Figure 1).

Figure 1 Biofilm layers constructed with alginate and starch as a protective barrier against *L. monocytogenes*.

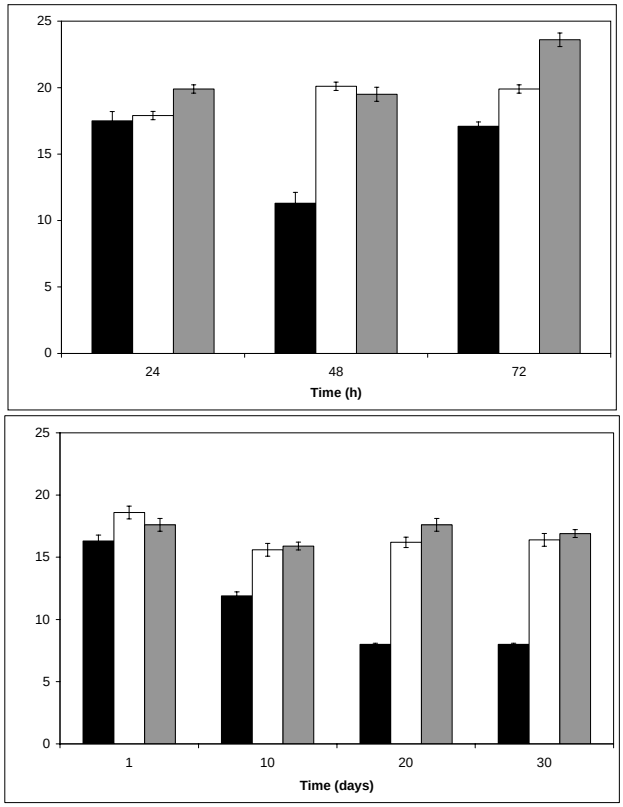

Figure 2 Results for the formulation of biofilm with starch (thickness 0.13mm, 0.19mm, 0.28mm) active against *L. monocytogenes* . The upper figure shows the diffusion of the BLS from the film, with a combination LAB A+B. The lower part shows antilisterial activity of the film stored during 30 days, with the combination of LAB strains.

The combination of alginate glycerol starch was more effective for the protection of the LAB cultures. The same results were observed when more alginate was used for the layer production, i.e. in the presence of 0.19 mm and 0.28 mm layer films, the inhibition of *L. monocytogenes* was more effective, than with the 0.13 mm film (upper Fig.1). Also de "shelflife" of the films stored at 4 ºC during 30 days was improved when the thicker films were used (lower part Fig. 1)

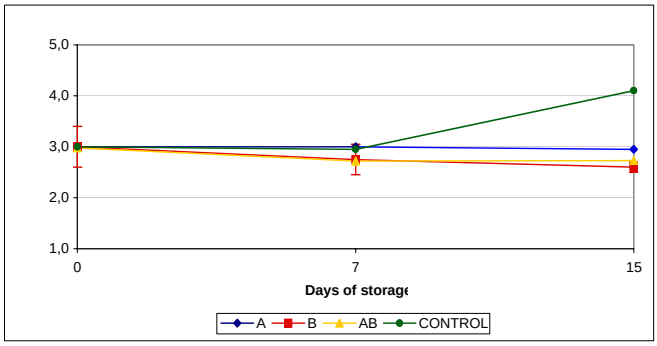

Figure 3 Bacteriostatic effect on the growth of *L. monocytogenes* inoculated on smoked salmon, covered with alginate layers containing a combination of strains LAB A and LAB B.

The combination of strains LAB A and LAB B and each strain alone, had the same inhibitory effect on *L. monocytogenes*. After 7 days of storage on the vacuum packaged salmon at 4º C (Fig. 3), *L. monocytogenes* started growing in the control, while on the salmon containing the LAB strains counts remained at the starting inoculation level. This experiment will be continued with the salmon stored during 30 days at 4 ºC.

Conclusions

Biopreservation with antimicrobial substances produced by two lactic acid bacteria, included in alginate layers with starch, were effective for the inhibition of *L. monocytogenes.* This technique can be a novel approach to avoid this pathogenic agent to growth on foods.

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