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To cite this version:
Irene Salinas, José Meseguer, Maria Ángeles Esteban. Antiproliferative effects and apoptosis induction by probiotic cytoplasmic extracts in fish cell lines. Veterinary Microbiology, Elsevier, 2007, 126 (1-3), pp.287. <10.1016/j.vetmic.2007.07.003>. <hal-00532290>

HAL Id: hal-00532290
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Submitted on 4 Nov 2010

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Accepted Manuscript

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PII: S0378-1135(07)00331-8
DOI: doi:10.1016/j.vetmic.2007.07.003
Reference: VETMIC 3754

To appear in: VETMIC

Received date: 14-4-2007
Revised date: 4-7-2007
Accepted date: 5-7-2007

Please cite this article as: Salinas, I., Meseguer, J., Esteban, M.Á., Antiproliferative effects and apoptosis induction by probiotic cytoplasmic extracts in fish cell lines, Veterinary Microbiology (2007), doi:10.1016/j.vetmic.2007.07.003

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Antiproliferative effects and apoptosis induction by probiotic cytoplasmic extracts
in fish cell lines

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Short title: antiproliferative effects and fish probiotics
Abstract

Probiotic bacteria are known to exert a wide range of beneficial effects on their animal hosts. Control of intestinal homeostasis, inflammation suppression and a reduction in the incidence of cancer all rely on the antiproliferative potential of probiotics. In this paper, we assess the antiproliferative activity of probiotics in two teleost fish cell lines SAF-1, a fibroblast cell line and EPC, an epithelioma from carp. Cells were grown in the presence of cytoplasmic extracts obtained from two bacterial strains, Lactobacillus delbrueckii subsp. lactis (LDL) and 51M6. Proliferation and apoptosis were measured after 4, 24, 48 or 72h in culture by the crystal violet or by double staining flow cytometry assays, respectively. Generally, LDL had stronger effects on cell growth than 51M6. Moreover, SAF-1 cells were more susceptible to growth inhibition than EPC cells. Apoptosis took place following growth inhibition, especially when LDL extracts were used. The results are discussed in terms of the biological significance of probiotic bacteria that naturally occur on the fish mucosal surfaces with an emphasis on how dose and species specificity may be determinant factors.

Key words: probiotics; SAF-1; EPC; cell proliferation; apoptosis; teleost
1. Introduction

Probiotic bacteria are symbiotic bacteria that live in most mucosal surfaces of animals and produce a vast range of beneficial effects on the homeostasis of vertebrate animals, which has led them to be used as therapeutic tools in veterinary and human medicine (Isolauri, 2001).

In higher vertebrates, probiotic bacteria suppress intestinal inflammation (Riedel et al., 2006) and down regulate immune responses in the gut (Ait-Belghnaui et al., 2006; Isolauri, 2001). On the other hand, they have been shown to inhibit the proliferation of mononuclear cells (Pessi et al., 1999) and cell lines (Ewaschuk et al., 2006).

Research on probiotics for use in farmed fish began recently and the number of studies that reveal positive effects of probiotic bacteria continues to increase. Furthermore, beneficial bacteria may be used dead or alive and added to the food or directly to the water (Gatesoupe, 1999). However, no information is available regarding the potential for probiotic bacteria to affect fish cells growth and for that reason the present work was carried out. Two fish cell lines, SAF-1 and EPC, were selected. SAF-1 cells are fibroblasts obtained from the seabream (Sparus aurata L.) dorsal fin, which were spontaneously immortalised but lack a tumoral origin (Béjar et al., 1997). Epithelioma papulosum cyprini (EPC) cells, in turn, are derived from carp epidermal herpes virus-induced hyperplastic lesions, (Cyprinus carpio L.) (Fijan et al., 1983).

Similarly, two probiotic bacteria were chosen in order to evaluate their potential capacity for controlling cell proliferation in fish. The first strain was Lactobacillus delbrueckii subsp. lactis (LDL), a lactic acid strain whereas the second strain was a fish-derived probiotic, 51M6, present on seabream skin and capable of inhibiting pathogenic bacteria (Chabrillón et al., 2005). Both strains show in vivo enhancing effects on the seabream innate immunity (Díaz-Rosales et al., 2006; Salinas et al., 2006).
The present work aims to address if probiotic bacteria of two different origins (LDL, and 51M6) have the capacity to inhibit the proliferation of SAF-1 and EPC fish cell lines. Moreover, we aim to assess if apoptosis is induced by these probiotics and if it plays a role in controlling cell proliferation.

2. Materials and Methods

2.1. Bacteria and cytoplasmic extracts

*Lactobacillus delbrueckii* subsp. *lactis* (CECT 287) were grown in MRS (Man, Rogosa and Sharpe) (Laboratorios Conda, Madrid, Spain) (pH 6.2; 37°C) agar plates for 2-3 days. Colonies from cultured plates were then subcultured in MRS broth for 48h at 37°C. Absorbance at 550 nm was measured and bacteria numbers estimated as explained elsewhere (Salinas et al., 2005). 51M6 (genus *Shewanella*) was grown in tubes containing 5 ml of trypticase soya broth (Oxoid) supplemented with 1.5% NaCl (TSBs) at 22°C, with continuous shaking for 18h. Serial dilutions of the suspension were plated in triplicate and the number of colonies counted after 18h in culture. Both strains were washed four times in sterile phosphate buffered saline (PBS, pH 7.4) and adjusted to $10^8$ cfu/ml and kept at 4°C.

The cytoplasmic extracts of the bacteria ($5 \times 10^8$ cfu) were obtained by disruption of cell walls in a French Press and ultracentrifugation at 70000 x g for 30 min. Samples were then sterilized with a 0.2 μm filter and the protein concentrations were estimated by the BCA protein assay reagent (Sigma) using bovine serum albumin (BSA) as a standard. All samples were adjusted to 100 μg/ ml PBS of protein and stored at -80°C until use.

2.2. Cell lines
SAF-1 cell line (European collection of cell cultures, ECACC 00122301, UK), a fibroblast-like culture derived from the marine fish gilthead seabream (*Sparus aurata*) was grown in sRPMI [RPMI-1640 culture medium (Gibco) with 0.35% sodium chloride (to adjust the medium’s osmolarity to gilthead seabream plasma osmolarity, 353.33 mOs), 100 I.U./ml penicillin (Flow), 100 μg/ml streptomycin (Flow), and 10% fetal bovine serum (FBS, Gibco)] at 23°C, 5% CO$_2$ in 25 cm$^2$ culture flasks. Epithelioma papulosum carpio (EPC) cells (ECACC 93120820, UK) were maintained at 23ºC in DMEM 4.5 (Sigma) containing glutamine (2 mM; Gibco), piruvate (1 mM; Gibco), fungizone (2 μg/ml; Gibco) and gentamicine (50 μg/ml; Gibco) and supplemented with 10% FBS. Both cell cultures, SAF-1 and EPC, were trypsinised (0.25% trypsin 0.25% EDTA, Sigma), then washed twice in sRPMI or DMEM (400 g, 10 min, 23ºC), counted in a Neubauer chamber and adjusted to $10^5$ or $2 \times 10^5$ cells/ml, respectively.

One hundred µl of such cell suspensions were dispensed onto 96 flat-bottomed well plates. Initial cell concentration for each cell line was chosen according to ECACC instructions. Additionally, pilot studies were conducted.

One hundred µl of the bacterial cytoplasmic extracts were added in triplicate to the wells to achieve a final concentration of 12.5, 25 and 50 μg/ml in PBS. The range of concentrations was chosen based on previous studies with other cell lines and bacterial strains. Controls consisted of wells with cells to which 100 µl of PBS had been added. Plates were incubated for 4, 24, 48 or 72h at 23°C and 5% CO$_2$.

2.3. Proliferation assay

Cell proliferation was evaluated *in vitro* by means of the crystal violet assay as used in other studies (Pessi et al., 1999). Briefly, 100 µl of culture medium were removed from each well and the same volume of Carnoy fixative was added. After 5 min, the entire volume was removed and 200 µl of Carnoy were added for 10 min, the fixative was
removed and plates were air-dried and stained with 200 µl of 0.1% crystal violet solution (Panreac) for 30 min at room temperature. The plates were washed 3 times under tap water, carefully dried and the dye present inside cells solubilized with 100 µl of a 10% methanol and 5% acetic acid solution. After shaking vigorously for 3 min, absorbance at 570 nm was measured.

2.4. Flow cytometry: viability, apoptosis and necrosis assays

Induction of apoptosis in fish cell lines prompted by the cytoplasmic fractions of the bacteria LDL or 51M6 was measured by a double staining flow cytometry assay using fluorescein diacetate (FDA) and propidium iodide (PI) (Salinas et al., 2007). Plates were centrifuged (400 g, 10 min, 23°C) and culture medium was removed prior to trypsin treatment for 3 min at room temperature. Trypsinization was stopped by addition of 150 µl culture medium. Plates were centrifuged as before, the supernatants discarded and 200 µl fresh culture media was added to each well. Afterwards, 4 µl of FDA (50 µg/ml in sRPMI, Sigma) and 10 µl PI (400 µg/ml in PBS, Sigma) were dispensed into each well. Plates were incubated for 30 min (at 23°C, 5% CO₂), samples collected into FACS tubes and 300 µl of cold PBS added. Unlabelled samples were used as controls for calibration.

Samples were acquired in a Coulter® Epics® XL™ flow cytometer (Beckman Coulter). Analyses were performed on 5000 cells, which were acquired at a rate of 300 cells/s. Side scatter (SSC, granularity), forward scatter (FSC, size), FL-1 (green fluorescence) and FL-3 (red fluorescence) were represented in dot plots or histograms. Viable (FDA⁺/PI⁻), apoptotic (FDA⁻/PI⁻) and necrotic (FDA⁻/PI⁺) cells were recorded.

2.5. Data analysis

The results are expressed as the proliferation, apoptosis or necrosis ratio (mean ± standard error, SE). Ratios were obtained by dividing each value by the mean control
value at the same sampling point for each measured parameter. Values higher than 1 express an increase and lower than 1 a decrease in each parameter. The data from the flow cytometric assays were analysed using the statistical option of the Lysis Software Package (Becton Dickinson). Data were statistically analysed by one-way analysis of variance (ANOVA) and Tukey’s comparison of means when necessary. Differences were considered statistically significant when \( p < 0.05 \).

3. Results

3.1. Effects of probiotic bacteria cytoplasmic extracts on proliferation of fish cell lines

SAF-1 cells proliferation was significantly inhibited by LDL cytoplasmic fraction in a dose dependent manner. The antiproliferative effect was significant at 4 and 24h when the highest dose of protein cytoplasmic fraction was applied. Longer culture times (48 or 72h) revealed that SAF proliferation was significantly inhibited by all LDL treatments, however 50 µg/ml LDL inhibition was significantly greater than the rest at 72h (Fig. 1a).

Cytoplasmic fraction of 51M6 bacteria had a less marked effect on SAF-1 cell growth compared to those obtained from LDL. In fact, SAF-1 proliferation was inhibited only after 72h in culture regardless of the dose (Fig. 1b).

EPC proliferation was inhibited by LDL cytoplasmic fraction at the highest assayed dose (50 µg/ml) after 24h or more in culture, the greatest inhibition occurring after 72h, when the number of cells present was less than 50% of the controls (mean ratio = 0.48) (Fig. 2a). At this time, 12.5 and 25 µg/ml of the cytoplasmic extract of LDL also significantly inhibited EPC proliferation, but proliferation ratios were about 0.8. A 20% decrease in proliferation was also recorded after 4h in contact with both 12.5 and 25
µg/ml LDL cytoplasmic extracts, but only the former was statistically significant (Fig. 2a).

Cytoplasmic fraction of 51M6 only affected EPC proliferation at 4h being the inhibition recorded only mild (proliferation ratios higher than 0.85) and not dose-dependent (Fig. 2b). EPC proliferation was also inhibited after 72 h in culture regardless of the dose.

3.2. Induction of apoptosis in fish cell lines by cytoplasmic fraction of probiotic bacteria

SAF-1 cells entered apoptosis in a dose-dependent manner after the addition of LDL cytoplasmic fraction (Fig. 3a). There were significantly more apoptotic cells in the samples incubated with the 50 µg/ml dose for 48 and 72 h. The intermediate dose (25 µg/ml) also resulted in apoptosis induction, but the result was only statistically significant after 72 h. At this time, the highest dose caused, approximately, 50% apoptosis, which was over 5 times higher than that present in control samples and 2.5 times the value recorded for the 12.5 µg/ml treatment. The cytoplasmic extract of the 51M6 bacteria did not significantly induce apoptosis in SAF-1 cells. (Fig. 3b).

EPC entered apoptosis when 50 µg/ml LDL were added to the cultures. Whereas no effects were significant after 4 h, apoptosis ratios were 2, 4 and 9 after 24, 48 or 72 h, respectively (Fig. 3c). In absolute numbers, however, apoptosis was much lower than in SAF-1 cells; with a peak mean apoptosis value of 11.2% after 72 h.

All three 51M6 treatments resulted in a 2-fold increase in EPC apoptosis after 48 h in culture but the effect was not significant at any other time of the experiment (Fig. 3d).

3.3 Induction of necrosis in fish cell lines by cytoplasmic fraction of probiotic bacteria

In SAF-1 cultures, necrosis also took place although total numbers of necrotic cells never exceeded those of apoptotic cells. Neither 12.5 nor 25 µg/ml LDL resulted in significant necrosis at any time. 50 µg/ml LDL, in turn, produced a 5-fold increase and 6-fold increase in necrosis after 48 or 72 h in culture, respectively (Fig. 4a). In total...
numbers this meant that over 32% of SAF-1 cells were necrotic at the end of the experiment when 25 µg/ml LDL was applied.

51M6 protein cytoplasmic fraction, at all the assayed doses, induced necrosis in SAF-1 cells after 48 and 72h, when it was between 3 and 4 times higher than in the controls (Fig. 4b). This represented between 13% and 18% of the total cell numbers, a significantly lower value compared with the necrosis rate induced by LDL.

There were 4, 7 and 18 times more necrotic EPC cells after 24, 48 or 72h, in culture with the cytoplasmic fraction of LDL (50 µg/ml), respectively (Fig. 4c). In absolute numbers, however, necrosis was always low; reaching a peak value of 6.7% of the total cells at 72h. Lower doses had no effect and did not differ between each other.

51M6 treatments had no significant effect on necrosis of EPC cells at any of the assayed doses or times (Fig. 4d).

4. Discussion

Probiotics have been extensively studied for decades due to the long list of health-related properties prompted by their consumption (Isolauri et al., 2001). The potential of probiotics in farmed aquatic animals was soon realised and, as a consequence, many studies have investigated the effects of probiotic bacteria in disease resistance and immune stimulation in aquatic hosts (Gatesoupe, 1999; Balcázar et al., 2006).

It is known that probiotic bacteria are capable of inhibiting proliferation of mammalian cells both in cell lines and primary leucocyte cultures (Pessi et al., 1999; Lee et al., 2004).

For the present work, two cell lines were chosen, one of tumoral origin (EPC) the other (SAF-1) lacking this feature. SAF-1 cells were characterised over a decade ago showing normal karyotype and DNA content (Béjar et al., 1997). EPC cell line was first
discovered in the eighties, thus more studies are available than in the case of SAF-1 cells. The epithelioma originated from a hyperplastic lesion in carp skin consequence of a rhabdovirus infection.

Additionally, we assayed cytoplasmic extracts from two bacterial strains of different origin, a LAB strain, LDL, and 51M6, a strain from the Vibrioneaceae family present on seabream skin. This choice was made in order to test whether host specific effects can be attributed to probiotic bacteria as suggested by some authors (Saarela, 2000) but refuted by others (Rinkinen et al., 2003; Chabrillón et al., 2005).

Our results show that growth of the two cell lines was differentially inhibited by the probiotic cytoplasmic extracts. Although LDL decreased proliferation and induced apoptosis both in SAF-1 and EPC cells, apoptosis induction was over 5 times higher in SAF-1 cells than in EPC cells. Also, low dose and short incubation times produced a significant effect, which was not observed in EPC cells until 72h in culture. The latter could be due to i) a greater resistance by tumour cells to the action of the cytoplasmic extracts of probiotic bacteria than non-tumoral cells or to ii) differences regarding the fish species of the two cell lines employed (seabream vs. carp), especially because they have very different ecological backgrounds.

On the other hand, it is clear that the lactic acid strain, LDL, has stronger antiproliferative properties than 51M6 on both cells lines, since shorter times and lower doses sufficed to produce a significant inhibitory effect. This is in agreement with available data from other LAB strains tested in mammalian cell lines which produced a dose-dependent decrease in proliferation rates (Lee et al., 2004). Since 51M6 is present on the fish natural environment, it is likely that a higher dose is needed due to ecological adaptation or tolerance compared with an allochtonous probiotic bacteria.
We detected apoptosis in the studied fish cell lines due to probiotic treatment. SAF-1 apoptosis occurred after the inhibition of proliferation, as shown in our kinetics study. Whereas the highest LDL dose decreased proliferation from the beginning to the end of the trial, apoptosis was significantly increased only at 48 and 72h. At the end of the experiment over 50% SAF-1 cells were apoptotic. EPC cells, on the other hand, were more resistant, the highest apoptotic rate being 11%. 51M6 did not induce apoptosis in SAF-1 cells although higher doses and longer incubation times may have revealed a similar result as the one found with LDL.

Only recently, the induction of apoptosis by conjugated linoleic acid produced by different probiotic strains was demonstrated in HT-29 and Caco-2 mammalian cell lines (Ewaschuk et al., 2006). The exact compounds present in the here assayed probiotics and responsible for the apoptotic effects on fish cells are yet to be elucidated.

In conclusion, our study shows that both LDL and 51M6 cytoplasmic extracts have antiproliferative effects on SAF-1 and EPC cells lines, generally in a dose-dependent manner. However, EPC cells were more resistant and required higher doses and longer incubation times than SAF-1 cells. Finally, apoptosis and necrosis were induced, which points them as modes of action by which probiotics suppress cell growth in animal hosts.

Acknowledgements

Authors wish to thank Dr. M.A. Morinigo from University of Malaga, (Spain) for kindly donating the 51M6 strain. I. Salinas is a Fundación Séneca scholar. This work was partially funded by the Petri project PTR 95-0943.OP.
References


Figure legends

**Fig. 1:** Effect of probiotic cytoplasmic extracts (a LDL and b 51M6) (□ 12.5; ■ 25; ■ 50 µg protein/ml) on the proliferation of SAF-1 cells. Data are expressed as mean proliferation ratio ± standard error. Asterisks denote statistically significant differences (* p<0.05; ** p<0.005).

**Fig. 2:** Effect of probiotic cytoplasmic extracts (a LDL and b 51M6) (□ 12.5; ■ 25; ■ 50 µg protein/ml) on the proliferation of EPC cells. Data are expressed as mean proliferation ratio ± standard error. Asterisks denote statistically significant differences (* p<0.05; ** p<0.005).

**Fig. 3:** (a-b) Effect of probiotic cytoplasmic extracts (a LDL and b 51M6) (□ 12.5; ■ 25; ■ 50 µg protein/ml) on the apoptosis of SAF-1 cells. (c-d) Effect of probiotic cytoplasmic extracts (c LDL and d 51M6) (□ 12.5; ■ 25; ■ 50 µg protein/ml) on the apoptosis of EPC cells. Data are expressed as mean apoptosis ratio ± standard error. Asterisks denote statistically significant differences (* p<0.05; ** p<0.005).

**Fig. 4:** (a-b) Effect of probiotic cytoplasmic extracts (a LDL and b 51M6) (□ 12.5; ■ 25; ■ 50 µg protein/ml) on the necrosis of SAF-1 cells. (c-d) Effect of probiotic cytoplasmic extracts (c LDL and d 51M6) (□ 12.5; ■ 25; ■ 50 µg protein/ml) on the necrosis of EPC cells. Data are expressed as mean necrosis ratio ± standard error. Asterisks denote statistically significant differences (* p<0.05; ** p<0.005).
Figure 1

![Graph showing proliferation ratio over time in culture (h) for two conditions labeled a and b. The graph includes error bars and asterisks indicating statistical significance.](image)
Fig. 2
Fig. 3
Fig. 4