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

HAL Id: hal-00532290

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HAL Authorization

Accepted Manuscript

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

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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-Belgnaoui 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 delbrückii* 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 delbrückii 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 (5×10^8 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% CO₂ in 25 cm² culture flasks. Epithelioma papulosum carpio (EPC) cells (ECACC 93120820, UK) were maintained at 23°C in DMEM 4.5 (Sigma) containing glutamine (2 mM; Gibco), pyruvate (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, 23°C), counted in a Neubauer chamber and adjusted to 10⁵ or 2 x 10⁵ 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 cells 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% CO₂.

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

125 removed and plates were air-dried and stained with 200 μ l of 0.1% crystal violet
 126 solution (Panreac) for 30 min at room temperature. The plates were washed 3 times
 127 under tap water, carefully dried and the dye present inside cells solubilized with 100 μ l
 128 of a 10% methanol and 5% acetic acid solution. After shaking vigorously for 3 min,
 129 absorbance at 570 nm was measured.

130 *2.4. Flow cytometry: viability, apoptosis and necrosis assays*

131 Induction of apoptosis in fish cell lines prompted by the cytoplasmic fractions of the
 132 bacteria LDL or 51M6 was measured by a double staining flow cytometry assay using
 133 fluorescein diacetate (FDA) and propidium iodide (PI) (Salinas et al., 2007). Plates were
 134 centrifuged (400 g, 10 min, 23°C) and culture medium was removed prior to trypsin
 135 treatment for 3 min at room temperature. Trypsinization was stopped by addition of 150
 136 μ l culture medium. Plates were centrifuged as before, the supernatants discarded and
 137 200 μ l fresh culture media was added to each well. Afterwards, 4 μ l of FDA (50 μ g/ml
 138 in sRPMI, Sigma) and 10 μ l PI (400 μ g/ml in PBS, Sigma) were dispensed into each
 139 well. Plates were incubated for 30 min (at 23°C, 5% CO₂), samples collected into FACS
 140 tubes and 300 μ l of cold PBS added. Unlabelled samples were used as controls for
 141 calibration.

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

147 *2.5. Data analysis*

148 The results are expressed as the proliferation, apoptosis or necrosis ratio (mean +
 149 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 $p < 0.05$.

156

157 **3. Results**

158 *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 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$) 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 $\mu\text{g/ml}$ 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

174 $\mu\text{g/ml}$ LDL cytoplasmic extracts, but only the former was statistically significant (Fig.
175 2a).

176 Cytoplasmic fraction of 51M6 only affected EPC proliferation at 4h being the inhibition
177 recorded only mild (proliferation ratios higher than 0.85) and not dose-dependent (Fig.
178 2b). EPC proliferation was also inhibited after 72h in culture regardless of the dose.

179 *3.2. Induction of apoptosis in fish cell lines by cytoplasmic fraction of probiotic bacteria*

180 SAF-1 cells entered apoptosis in a dose-dependent manner after the addition of LDL
181 cytoplasmic fraction (Fig. 3a). There were significantly more apoptotic cells in the
182 samples incubated with the 50 $\mu\text{g/ml}$ dose for 48 and 72h. The intermediate dose (25
183 $\mu\text{g/ml}$) also resulted in apoptosis induction, but the result was only statistically
184 significant after 72h. At this time, the highest dose caused, approximately, 50%
185 apoptosis, which was over 5 times higher than that present in control samples and 2.5
186 times the value recorded for the 12.5 $\mu\text{g/ml}$ treatment. The cytoplasmic extract of the
187 51M6 bacteria did not significantly induce apoptosis in SAF-1 cells. (Fig. 3b).

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

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

194 *3.3 Induction of necrosis in fish cell lines by cytoplasmic fraction of probiotic bacteria*

195 In SAF-1 cultures, necrosis also took place although total numbers of necrotic cells
196 never exceeded those of apoptotic cells. Neither 12.5 nor 25 $\mu\text{g/ml}$ LDL resulted in
197 significant necrosis at any time. 50 $\mu\text{g/ml}$ LDL, in turn, produced a 5-fold increase and
198 6-fold increase in necrosis after 48 or 72h in culture, respectively (Fig. 4a). In total

199 numbers this meant that over 32% of SAF-1 cells were necrotic at the end of the
200 experiment when 25 µg/ml LDL was applied.

201 51M6 protein cytoplasmic fraction, at all the assayed doses, induced necrosis in SAF-1
202 cells after 48 and 72h, when it was between 3 and 4 times higher than in the controls
203 (Fig. 4b). This represented between 13% and 18% of the total cell numbers, a
204 significantly lower value compared with the necrosis rate induced by LDL.

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

209 51M6 treatments had no significant effect on necrosis of EPC cells at any of the assayed
210 doses or times (Fig. 4d).

211

212 **4. Discussion**

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

218 It is known that probiotic bacteria are capable of inhibiting proliferation of mammalian
219 cells both in cell lines and primary leucocyte cultures (Pessi et al., 1999; Lee et al.,
220 2004).

221 For the present work, two cell lines were chosen, one of tumoral origin (EPC) the other
222 (SAF-1) lacking this feature. SAF-1 cells were characterised over a decade ago showing
223 normal karyotype and DNA content (Béjar et al., 1997). EPC cell line was first

224 discovered in the eighties, thus more studies are available than in the case of SAF-1
 225 cells. The epithelioma originated from a hyperplastic lesion in carp skin consequence of
 226 a rhabdovirus infection.

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

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

241 On the other hand, it is clear that the lactic acid strain, LDL, has stronger
 242 antiproliferative properties than 51M6 on both cells lines, since shorter times and lower
 243 doses sufficed to produce a significant inhibitory effect. This is in agreement with
 244 available data from other LAB strains tested in mammalian cell lines which produced a
 245 dose-dependent decrease in proliferation rates (Lee et al., 2004). Since 51M6 is present
 246 on the fish natural environment, it is likely that a higher dose is needed due to
 247 ecological adaptation or tolerance compared with an allochthonous probiotic bacteria.

248 We detected apoptosis in the studied fish cell lines due to probiotic treatment. SAF-1
249 apoptosis occurred after the inhibition of proliferation, as shown in our kinetics study.
250 Whereas the highest LDL dose decreased proliferation from the beginning to the end of
251 the trial, apoptosis was significantly increased only at 48 and 72h. At the end of the
252 experiment over 50% SAF-1 cells were apoptotic. EPC cells, on the other hand, were
253 more resistant, the highest apoptotic rate being 11%. 51M6 did not induce apoptosis in
254 SAF-1 cells although higher doses and longer incubation times may have revealed a
255 similar result as the one found with LDL.
256 Only recently, the induction of apoptosis by conjugated linoleic acid produced by
257 different probiotic strains was demonstrated in HT-29 and Caco-2 mammalian cell lines
258 (Ewaschuk et al., 2006). The exact compounds present in the here assayed probiotics
259 and responsible for the apoptotic effects on fish cells are yet to be elucidated.
260 In conclusion, our study shows that both LDL and 51M6 cytoplasmic extracts have
261 antiproliferative effects on SAF-1 and EPC cells lines, generally in a dose-dependent
262 manner. However, EPC cells were more resistant and required higher doses and longer
263 incubation times than SAF-1 cells. Finally, apoptosis and necrosis were induced, which
264 points them as modes of action by which probiotics suppress cell growth in animal
265 hosts.

266

267 **Acknowledgements**

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

271

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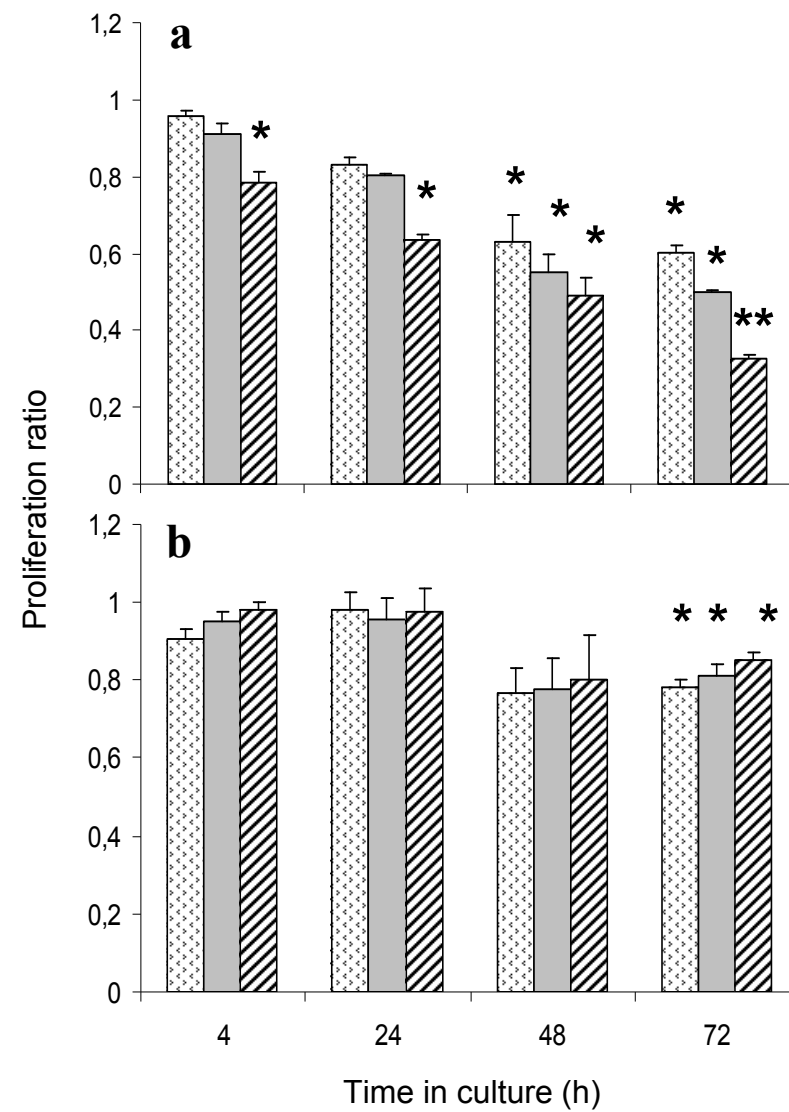
326 **Figure legends**

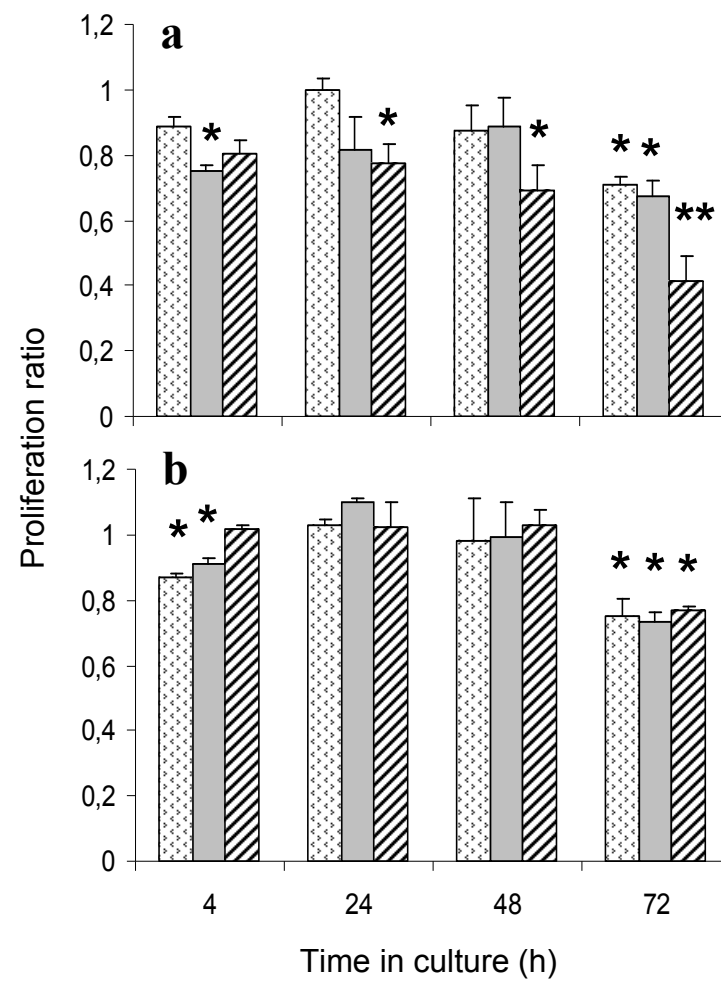
327 **Fig. 1:** Effect of probiotic cytoplasmic extracts (**a** LDL and **b** 51M6) (□ 12.5; ▣ 25;
328 ■ 50 µg protein/ml) on the proliferation of SAF-1 cells. Data are expressed as mean
329 proliferation ratio + standard error. Asterisks denote statistically significant differences
330 (* $p < 0.05$; ** $p < 0.005$).

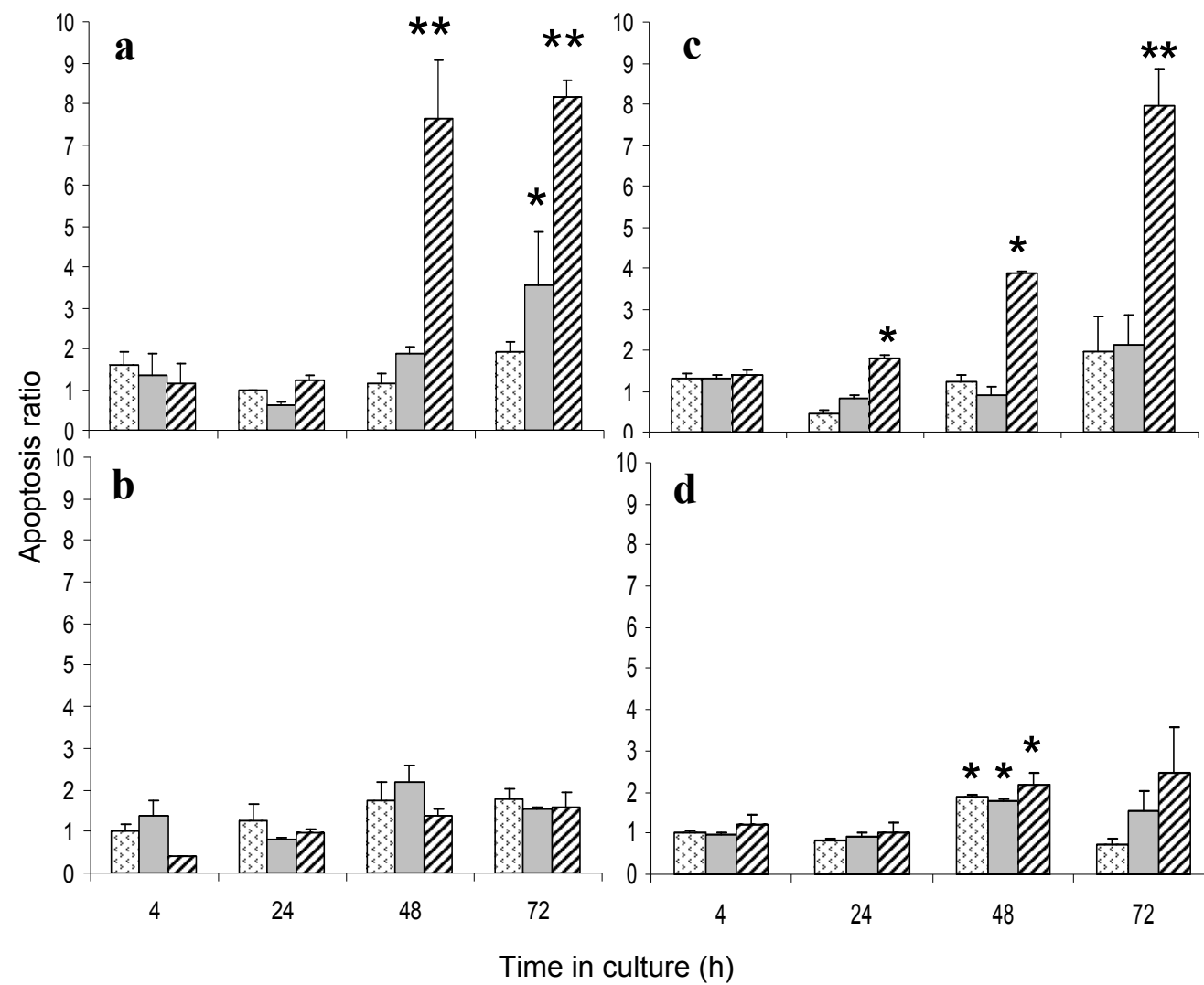
331 **Fig. 2:** Effect of probiotic cytoplasmic extracts (**a** LDL and **b** 51M6) (□ 12.5; ▣ 25;
332 ■ 50 µg protein/ml) on the proliferation of EPC cells. Data are expressed as mean
333 proliferation ratio + standard error. Asterisks denote statistically significant differences
334 (* $p < 0.05$; ** $p < 0.005$).

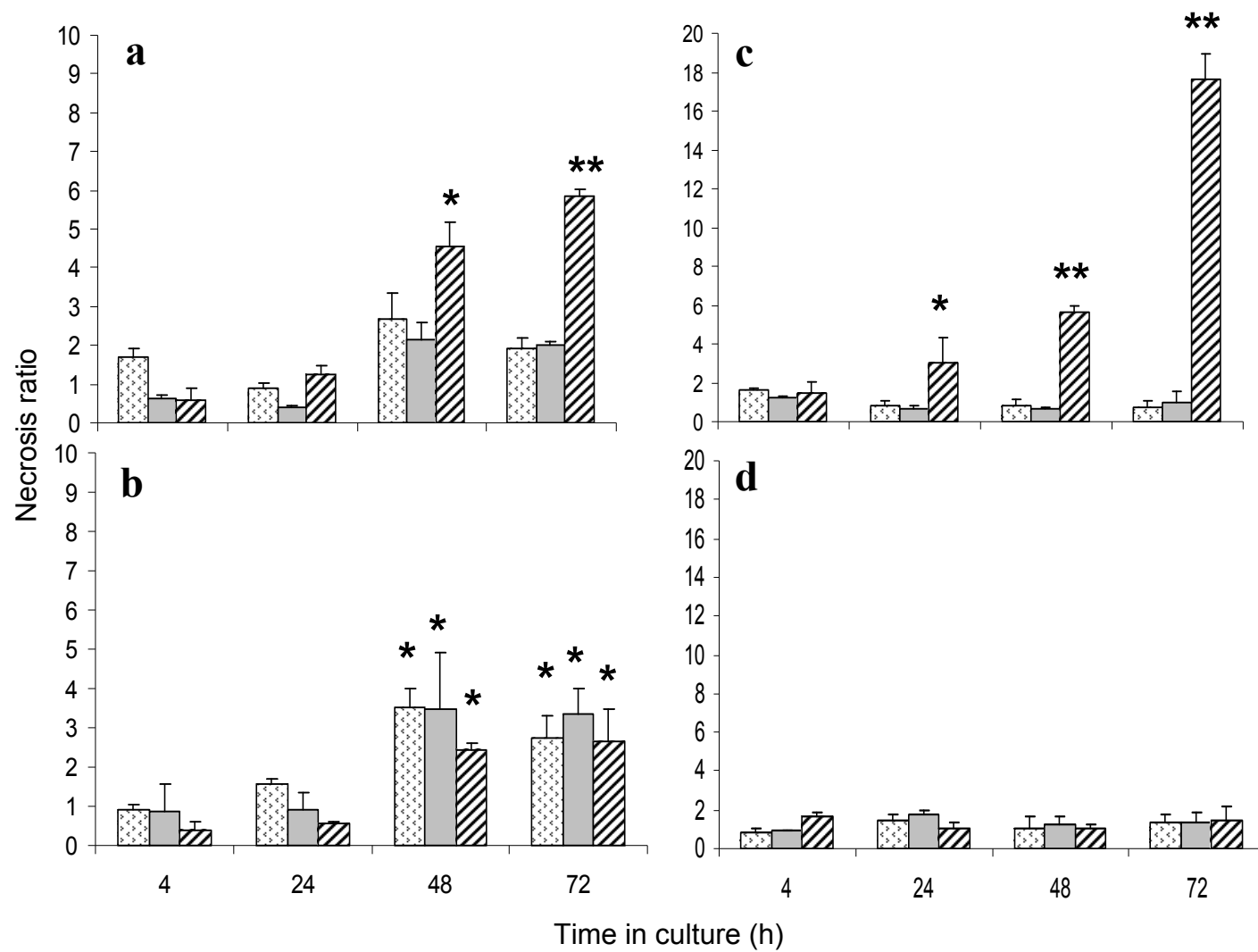
335 **Fig. 3:** (a-b) Effect of probiotic cytoplasmic extracts (**a** LDL and **b** 51M6) (□ 12.5; ▣
336 25; ■ 50 µg protein/ml) on the apoptosis of SAF-1 cells. (c-d) Effect of probiotic
337 cytoplasmic extracts (**c** LDL and **d** 51M6) (□ 12.5; ▣ 25; ■ 50 µg protein/ml) on the
338 apoptosis of EPC cells. Data are expressed as mean apoptosis ratio + standard error.
339 Asterisks denote statistically significant differences (* $p < 0.05$; ** $p < 0.005$).

340 **Fig. 4:** (a-b) Effect of probiotic cytoplasmic extracts (**a** LDL and **b** 51M6) (□ 12.5; ▣
341 25; ■ 50 µg protein/ml) on the necrosis of SAF-1 cells. (c-d) Effect of probiotic
342 cytoplasmic extracts (**c** LDL and **d** 51M6) (□ 12.5; ▣ 25; ■ 50 µg protein/ml) on
343 the necrosis of EPC cells. Data are expressed as mean necrosis ratio + standard error.
344 Asterisks denote statistically significant differences (* $p < 0.05$; ** $p < 0.005$).

**Fig. 1**

**Fig. 2**

**Fig. 3**

**Fig. 4**