UDC 579.864.1:612.39
ISSB 1330-9862 *original scientific paper*

(FTB-1019)

Effect of Protectors on the Viability of *Lactobacillus acidophilus* M92 in Simulated Gastrointestinal Conditions

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Received: January 17, 2000
Accepted: May 17, 2000

Summary

L. acidophilus has been considered to be among the predominant lactobacilli in the intestinal tract of healthy humans. The ability of *L. acidophilus* to survive and retain its viability through the gastrointestinal (GI) tract is one of the main characteristics desirable for probiotic activity. The influence of mucin and dietary constituents, such as casein, whey proteins concentrate (WPC) and skim milk, on survival of *L. acidophilus* M92 in the simulated GI conditions, was determined. A washed cell suspension was exposed to pepsin (3 g/L) and sodium chloride (5 g/L) at pH=1.5, 2.0, 2.5 and 3.0, and to pancreatin (1 g/L), sodium chloride (5 g/L) and oxgall (1.5, 2.0, 3.0, 5.0 mg/mL) at pH=8.0, mimicking the GI environment. Much greater tolerance to gastric and small intestinal juice was observed in the presence of mucin and milk proteins, especially with WPC. The influence of WPC, as the best protector, on the cell viability of *L. acidophilus* M92 in simulated gastric and small intestinal juice was confirmed by a mathematical model. Critical points, indicating GI conditions that *L. acidophilus* M92 can tolerate, were established. These observations showed that *L. acidophilus* M92 survives at pH=1.99 in simulated gastric juice for 2 h and at 3.1 mg/mL oxgall in simulated small intestinal juice for 4 h, while in the presence of WPC it tolerates even lower pH (1.77) and higher concentration of oxgall (4.25 g/L). Furthermore, only 15 % of the *L. acidophilus* M92 cells survived the direct transit from simulated gastric to simulated small intestinal juice, but with added WPC, 45 % of the *L. acidophilus* M92 cells survived. These results suggested the addition of WPC as a protector in preparation of *L. acidophilus* M92 for probiotic use.

Key words: L. acidophilus, probiotic properties, survival, simulated gastrointestinal conditions, whey proteins concentrate (WPC)

Introduction

If probiotic bacteria have to survive and be active in the digestive tract, they should be resistant to the defence mechanisms of the host *(1).* The gastrointestinal (GI) tract includes the dynamic physiological and physicochemical processes of the stomach and small and large intestines. The physiological parameters comprise pH, concentrations of gastric and small intestinal enzymes, concentrations of bile salts, and the kinetics of chyme passage through the stomach and intestine *(2).* Although lactobacilli have been isolated from all portions of the human GI tract, the terminal ileum and colon appear to be the preferential sites of colonization of intestinal lactobacilli. Unfortunately, most studies on probiotic actions ignored this fact and, as a result, data on the tolerance of probiotic strains to small intestinal secretions, other than bile, are not available *(3).*

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Although resistance to human gastric transit has been demonstrated *in vivo* for potentially probiotic lactic acid bacteria and constitutes an important *in vitro* selection criterion for probiotic bacteria, a satisfactory *in vitro* method, which closely simulates *in vivo* gastric transit, has not been defined. For this purpose HCl-acidified distilled water, broth and buffers have been widely used as opposed to fresh human gastric juice *(4–6).* Conway *et al. (6)* demonstrated that bacteria survive better in human gastric juice than in buffer at an equivalent pH indicating that studies using buffers probably underestimate survival potential *in vivo*.

In previous papers by Šušković *et al. (7,8)* some important probiotic properties of *Lactobacillus acidophilus* M92 were demonstrated. This strain has shown the antimicrobial activity against enteropathogenic, spore forming and fungal microorganisms which is supposed to be of benefit in competition of *L. acidophilus* M92 with other microorganisms in the intestine *(7).* Furthermore, *in vitro* studies have shown that *L. acidophilus* M92 can assimilate cholesterol in the presence of bile. It is postulated that this strain may help decrease serum cholesterol *in vivo* if it is able to survive GI transit *(8).* This paper presents *in vitro* experiments which attempt to simulate gastric and small intestinal transit of *L. acidophilus* M92 *in vivo*, confirming the probiotic properties of this strain. The influence of mucin and dietary constituents such as skim milk, casein and WPC on the survival of *L. acidophilus* M92 in the simulated GI conditions was also examined. The viability/mortality of *L. acidophilus* M92 in simulated gastric and small intestinal juice with and without protector were calculated.

Materials and Methods

Bacterial culture

The bacterial strain *Lactobacillus acidophilus* M92 was maintained in MRS broth (Difco Laboratories, USA) at 37 °C. After serial transfer in broth, the bacterial cells were collected by centrifugation at 10000 x g for 1 min, washed three times and resuspended in a sterile NaCl solution (0.5 %).

Preparation of simulated gastric and small intestinal juice

Simulated gastric and small intestinal juice were prepared according to Charteris *et al. (5)* with some modifications.

Simulated gastric juice was prepared by suspending pepsin (3 g/L) in a sterile sodium chloride solution (0.5 %) and adjusting the pH to 1.5, 2.0, 2.5 and 3.0 with concentrated HCl. Pepsin (from porcine stomach mucosa) was obtained from Sigma Chemical Co, St. Louis, USA.

Simulated small intestinal juice was prepared by suspending pancreatin (1 g/L) and bile salts (1.5, 2.0, 3.0 and 5.0 mg/mL oxgall) in a sterile sodium chloride solution (0.5 %) and adjusting the pH to 8.0 with 0.1 mol/L NaOH. Pancreatin (from hog pancreas, 165 U/mg) was obtained from Fluka Biochemica, Switzerland, and oxgall (dehydrated fresh bile) from Difco Laboratories, USA.

Survival of L. acidophilus M92 *in the simulated gastric and small intestinal juice*

The washed cell suspensions of *L. acidophilus* M92 (0.2 mL) were vortexed with gastric and small intestinal juice (1 mL), respectively, and 0.3 mL of NaCl (0.5 %). The changes in total viable count were monitored during treatment with gastric (2 h) and small intestinal juice (4 h), respectively, by pour plate method using MRS agar. The samples were taken every hour. Plates were incubated at 37 °C for 48 h. Results of log (CFU/mL) are expressed as the mean of three replicates.

Influence of milk proteins and mucin on survival of L. acidophilus M92 *in the simulated gastric and small intestinal juice*

Skim milk (BBL Microbiology System, Cockeysville, MD, USA) 0.3 mL, casein (Sigma Chemical Co., St. Louis, USA) 0.3 mL, whey proteins concentrate (WPC) (Zdenka d.d., Veliki Zdenci, Croatia) 0.3 mL, and mucin from hog stomach (Fluka Biochemica, Switzerland) 0.3 mL, were prepared by suspending in a sterile sodium chloride solution (0.5 %) at a concentration of 1 g/L and added to simulated gastric and small intestinal juice. Dissolving of casein was accomplished by heating over 60 °C. Survival of *L. acidophilus* M92 was determined as described above.

Cumulative effect of simulated gastric and small intestinal juice on survival of L. acidophilus M92

After exposure to simulated gastric juice (pH=2; residence time 2 h), the bacterial cells were centrifugated at 10000 x g for 1 min and resuspended in simulated small intestinal juice (pH=8; 3 mg/mL oxgall; residence time 4 h). The same procedure was used with addition of WPC as protector. The viability of *L. acidophilus* M92 was determined as described above.

Measurement of L. acidophilus M92 *viability/mortality in simulated GI conditions with/without protector*

A mathematical model for determination of cell viability/mortality in the presence of effectors was used *(9)*. In our case different pH values (1.5, 2.0, 2.5 and 3.0) and four concentrations of oxgall (1.5, 2.0, 3.0 and 5.0 mg/mL) were applied as effectors in simulated gastric and small intestinal juice, respectively. The effect of WPC as protector on *L. acidophilus* M92 viability in simulated GI conditions was also determined. On the basis of total viable count, log (CFU/mL), determined at the beginning and the end of exposure to simulated gastric (2 h) and small intestinal juice (4 h), three parameters that indicate viability and three parameters that indicate mortality of *L. acidophilus* M92 were calculated:

a) Cell viability in simulated gastric juice without effector (pepsin, 3 g/L, in 0.5 % sterile saline), and small intestinal juice without effector (pancreatin, 1 g/L, in 0.5 % sterile saline, pH=8):

$$V/\% = \frac{N_t}{N_0} \times 100 \qquad /1/$$

V cell viability (without effector)

N_t number of viable cells/mL at the end of exposure to simulated gastric/small intestinal juice
N_0 number of viable cells/mL at t=0

b) Cell viability in simulated gastric juice with effector (different low pH values) and small intestinal juice with effector (four concentrations of oxgall):

$$V_E/\% = \frac{N_{Et}}{N_0} \times 100 \qquad /2/$$

V_E cell viability (with effector)
N_{Et} number of viable cells/mL at a certain concentration of effector at the end of exposure to simuated gastric/small intestinal juice
N_0 number of viable cells/mL at t=0

c) Cell mortality in simulated gastric juice without effector (pepsin, 3 g/L, in 0.5 % sterile saline), and small intestinal juice without effector (pancreatin, 1 g/L, in 0.5 % sterile saline, pH=8):

$$M/\% = 100 - V \qquad /3/$$

M cell mortality (without effector)
V see Eq. /1/

d) Cell mortality in simulated gastric and small intestinal transit with effector:

$$M_E/\% = 100 - V_E \qquad /4/$$

M_E cell mortality (with effector)
V_E see Eq. /2/

To eliminate the effect of simulated gastric and small intestinal juice without effectors on the cell viability, the specific cell viability (V_S) and specific cell mortality (M_S) were calculated.

e) Specific cell mortality:

$$M_S/\% = \frac{M_E - M}{V} \times 100 \qquad /5/$$

M_S specific cell mortality
M_E see Eq. /4/
M see Eq. /3/
V see Eq. /1/

f) Specific cell viability:

$$V_S/\% = 100 - M_S \qquad /6/$$

V_S specific cell viability
M_S see Eq. /5/

The intersection of the specific cell viability curve and specific cell mortality curve was defined as a critical point. It represents the concentration of effector where mortality reaches viability. After this crossing point mortality overtakes viability of *L. acidophilus* M92 (Figs. 5 and 6).

Results and Discussion

Probiotic bacteria are commonly used in diet. The potential probiotic strain *L. acidophilus* M92 has shown resistance to lysozyme – the enzyme of the oral cavity *(7)*. However, this strain must be examined for its ability to resist the digestion processes in the stomach and in the intestinal tract, so that it may be considered as candidate for a probiotic strain *(10)*. The survival of *L. acidophilus* M92 in the gastrointestinal tract is essential for exertion of its potential health benefits, such as antimicrobial activity and decrease of cholesterol level *(7,8)*.

The gastrointestinal transit begins by exposing *L. acidophilus* M92 to low pH and pepsin in stomach. When gastric juice is secreted, it has a pH of approximately 2.0 and a salt content of not less than 0.5 % *(11)*. Berrada *et al. (12)* reported the time from entrance to release from the stomach to be about 90 min. Therefore, the survival of *L. acidophilus* M92 in simulated gastric juice at pH=1.5, 2.0, 2.5 and 3.0 during 2 h was investigated (Fig. 1). After 1 h of exposition, the survival in simulated gastric juice has been very high (99 %) at pH=2.0, 2.5 and 3.0, but after 2 h at the same pH values the viable count of *L. acidophilus* M92 was decreased for 49, 39 and 17 %, respectively. These results indicate that *L. acidophilus* M92 exhibited an appreciable level of survival in simulated gastric juice. A decrease of the viable number with the decrease of pH values was expected, but it was rapid only at pH=1.5. In fasting individuals the pH of the stomach is between 1.0 and 2.0 and most microorganisms, including lactobacilli, can survive after a short time under these conditions *(6)*. The examined strain *L. acidophillus* M92 has not survived the extremely low pH =1.5 (Fig. 1). Therefore, it is better to introduce the probiotic strain in a buffered system such as milk, yoghurt or milk protein-based foodstuffs.

The conditions encountered in the duodenum, where bile salts and pancreatic juice are secreted, have a more dramatic influence on bacterial viability, since, according to Drouault *et al. (13)*, at best only 10–30 % of the ingested bacteria survive. According to Charteris *et al. (5)* the majority of the examined *Lactobacillus* and *Bifidobacterium* strains were intrinsically resistant to simulated pancreatic juice (small intestinal juice without bile salts). In this work *L. acidophilus* M92 showed no re-

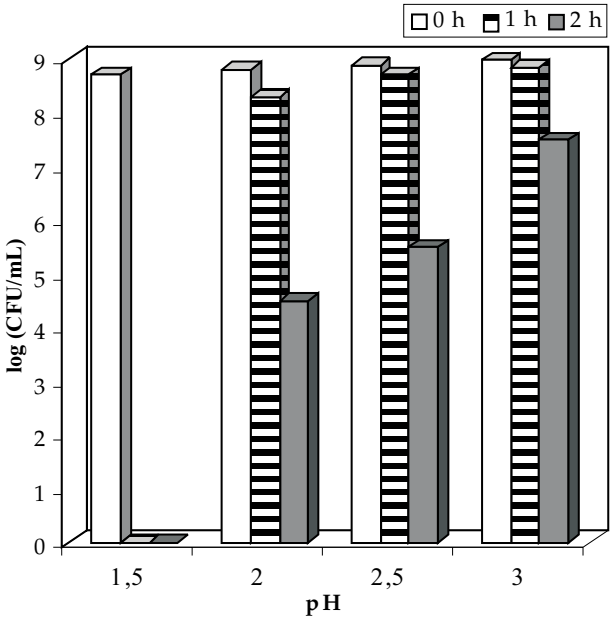

Fig. 1. Survival of *L. acidophilus* M92 during 2 h of exposure to simulated gastric juice (pH range 1.5–3.0)

duction in viability in simulated small intestinal juice without bile salts for up to 4 h (Fig. 2). This result suggests that *L. acidophilus* M92 has an intrinsic resistance to the pancreatic enzymes trypsin, chymotrypsin, carboxy- and amino-peptidases, lipase and ribonucleases. The small intestine contains relatively high concentrations of bile salts, which can inhibit growth or kill many bacteria, including lactobacilli. With addition of bile salts (1.5 and 3 mg/mL oxgall) to pancreatic juice a decrease in viable count was observed (Fig. 2). In earlier experiments *L. acidophilus* M92 survived, but it grew slowly when bile salts were added to the growth medium *(7).* According to Gilliland and Speck *(14)* bacteria from non-intestinal origin such as *L. delbrueckii* ssp. *bulgaricus* and *Lactococcus lactis* are very sensitive to bile: concentrations lower than 0.05 % were inhibitory. On the contrary, probiotic bacteria should be resistant to 0.15–0.3 % of oxgall to be effective *(15). L. acidophilus* M92 has shown a relatively high degree of survival (68 and 57 %) in the presence of 1.5 and 3 mg/mL bile salts, respectively (Fig. 2). It could be ascribed to bile salts hydrolase activity of *L. acidophilus* M92 as the detoxification mechanism *(16).*

An improved *in vitro* methodology is presented which mimics GI environment based on characteristics that can be employed in the selection of species for use in probiotic functional foods and nutraceutical preparations. Furthermore, the influence of casein, skim milk, mucin or whey proteins concentrate (WPC) on the survival of *L. acidophilus* M92 under conditions that mimic the GI environment was examined (Figs. 3 and 4). The milk, WPC and mucin may function as buffering agents and inhibitors of digestive protease activity *in vivo (6).* The exposure of *L. acidophilus* M92 to simulated gastric juice at pH=2 for 4 h demonstrated that *L. acidophilus* M92 survival is better in the presence of WPC (Fig. 3). Also, the addition of hog gastric mucin (as a surrogate for human gastric mucin) to simulated gastric juice increased the survival of *L. acidophilus* M92. Moreover, it is observed that the protecting capacity of all examined protective agents decreased after exposure of 2 h to simulated gastric juice (Fig. 3). Notwithstanding the well-known toxic effect of bile salts, the viable count of *L. acidophilus* M92 after 4 h of incubation retained at a level of 10^5 to 10^6 CFU/mL with 3.0 mg/mL oxgall in simulated small intestinal juice with addition of protective agents (Fig. 4). WPC has shown a slightly better protecting capacity. Additionally, it is well known that WPC added to the fermented dairy products improves its sensorial, textural and nutritive properties *(17).*

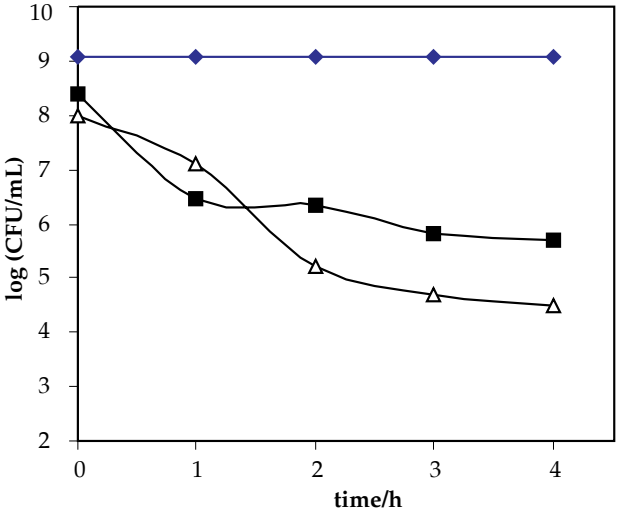

Fig. 2. Survival of *L. acidophilus* M92 in simulated small intestinal juice, in the absence or presence of oxgall; ◆ small intestinal juice without oxgall; ■ small intestinal juice with 1.5 mg/mL oxgall; Δ small intestinal juice with 3 mg/mL oxgall

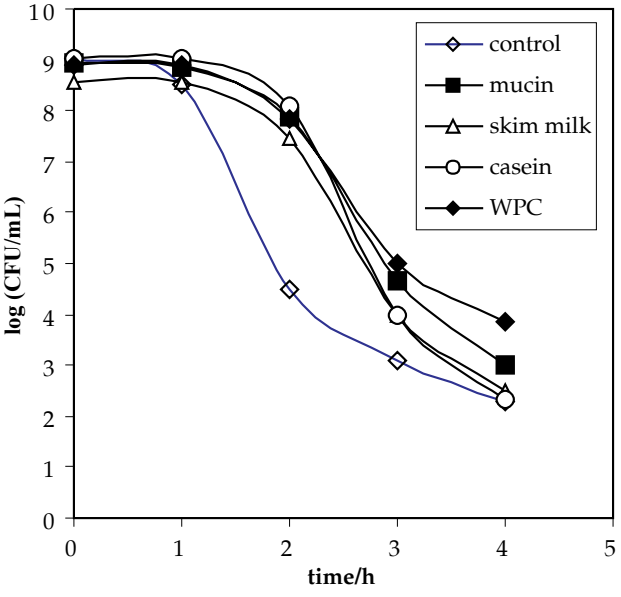

Fig. 3. Effect of mucin, skim milk, casein and whey proteins concentrate (WPC) on survival of *L. acidophilus* M92 in simulated gastric juice (pH=2)

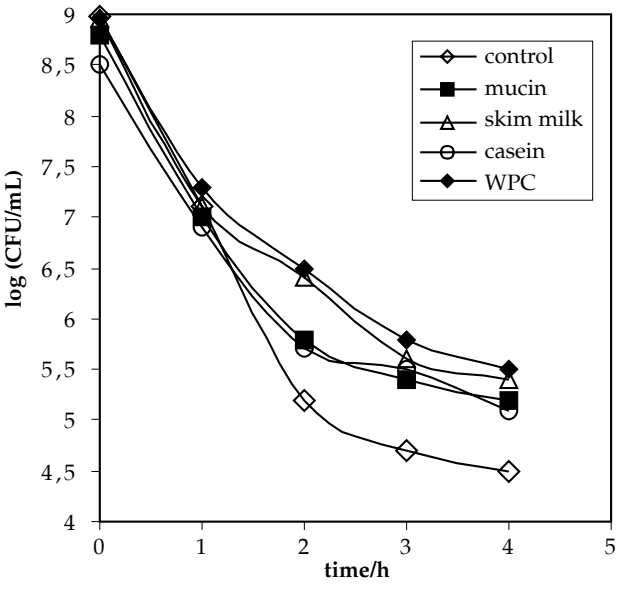

Fig. 4. Effect of mucin, skim milk, casein and whey proteins concentrate (WPC) on survival of *L. acidophilus* M92 in simulated small intestinal juice with 3 mg/mL oxgall

The calculated parameters, indicating cell viability *vs.* mortality in the bacterial population, were determined in the presence of protector and various pH levels and oxgall concentrations (Figs. 5 and 6). According to the presented results, the WPC was the best protector. When WPC was added to simulated gastric juice, the specific cell viability (V_s) was increased (as much as 34 and 37 %, at pH=2 and 2.5, respectively) (Fig. 5). In the presence of 2, 3 and 5 mg/mL oxgall specific cell viability (V_s) was higher (about 10 %) when WPC was added to simulated small intestinal juice (Fig. 6). The application of this model, through plotting V_s and M_s, has shown the influence of WPC addition on the critical points for the pH value (Fig. 5) and oxgall concentration (Fig. 6) which *L. acidophilus* M92 can tolerate. The critical points indicate that *L. acidophilus* M92 may survive in simulated gastric juice (pH=1.99) and simulated small intestinal juice (concentration of oxgall 3.1 mg/mL). In the presence of WPC *L. acidophilus* M92 can tolerate a lower pH (1.77) and a higher concentration of oxgall (4.25 mg/mL) (Figs. 5 and 6). These observations indicate an important role of WPC addition on the viability

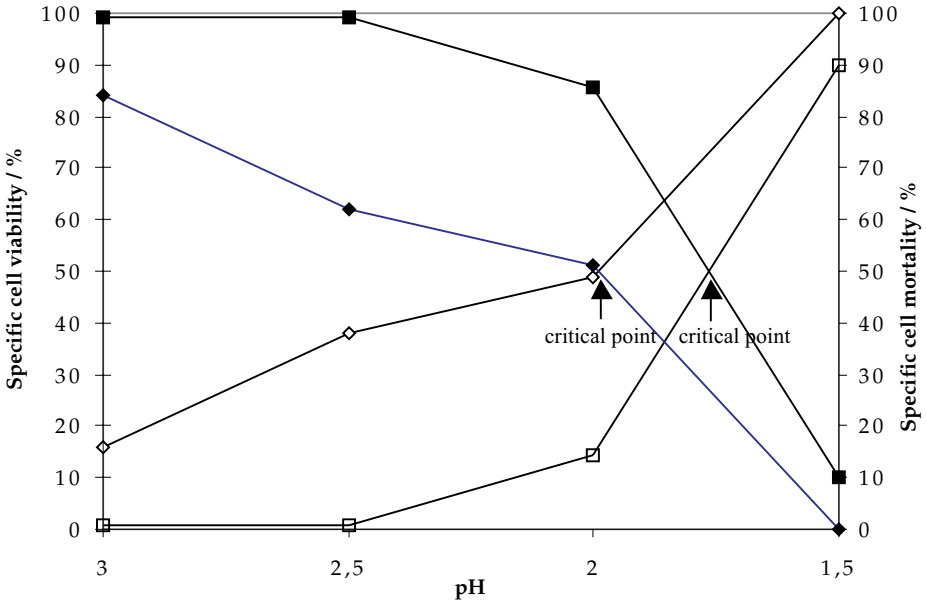

Fig. 5. Specific cell viability and specific cell mortality of *L. acidophilus* M92 in simulated gastric juice with and without addition of whey proteins concentrate (WPC) at different pH values; ◆ specific cell viability in gastric juice; ■ specific cell viability in gastric juice + WPC; ◇ specific cell mortality in gastric juice; □ specific cell mortality in gastric juice + WPC (100 % specific cell viability correspond to 10^9 CFU/mL)

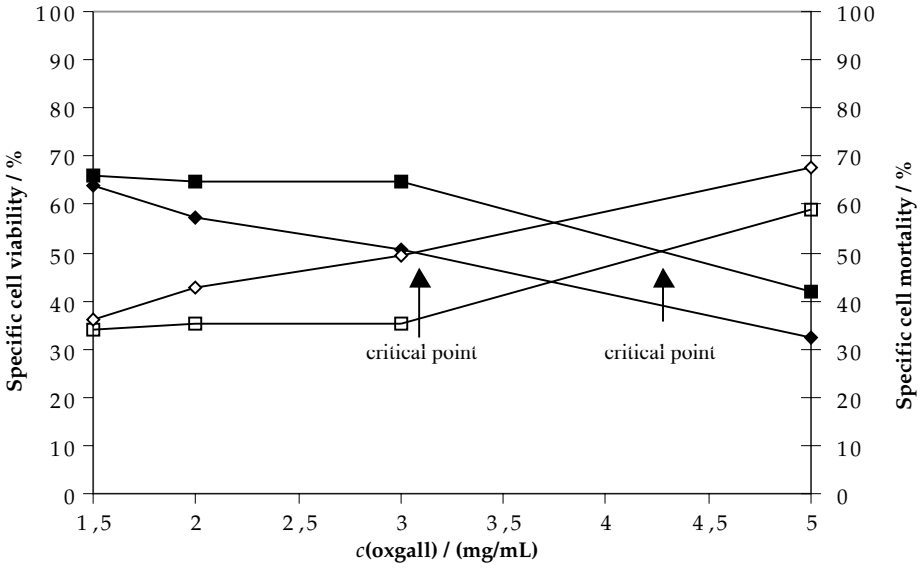

Fig. 6. Specific cell viability and specific cell mortality of *L. acidophilus* M92 in simulated small intestinal juice with and without addition of whey proteins concentrate (WPC) at different concentrations of oxgall; ◆ specific cell viability in small intestinal juice; ■ specific cell viability in small intestinal juice + WPC; ◇ specific cell mortality in small intestinal juice; □ specific cell mortality in small intestinal juice + WPC (100 % specific cell viability correspond to 10^9 CFU/mL)

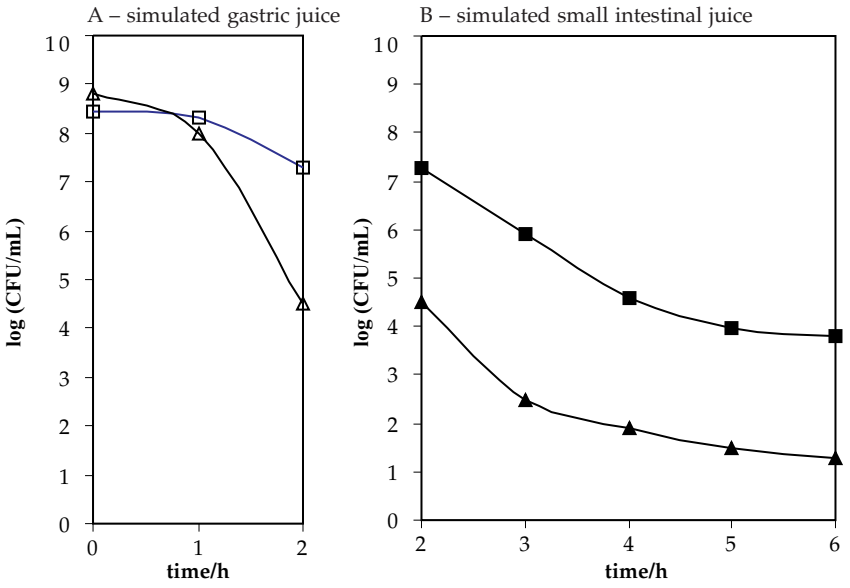

Fig. 7. Effect of whey proteins concentrate (WPC) on the survival of *L. acidophilus* M92 in: A – simulated gastric juice (pH=2; residence time 2 h) and B – simulated small intestinal juice (pH=8; 3 mg/mL oxgall; residence time 4 h); Δ gastric juice; ❑ gastric juice + WPC; ▲ small intestinal juice; ■ small intestinal juice + WPC

of *L. acidophilus* M92 in simulated gastric and small intestinal juice, which were also confirmed by the cumulative effect of both GI compartments, including direct transit from simulated gastric juice (pH=2) to simulated small intestinal juice (pH=8; 3 mg/mL oxgall) as shown in Fig. 7. Only 15 % of the *L. acidophilus* M92 cells survived a direct transit from simulated gastric juice (Fig. 7A) to simulated small intestinal juice (Fig. 7B), but with added WPC, 45 % of the *L. acidophilus* M92 cells survived. These results suggest that the addition of WPC as protector in a probiotic preparation of *L. acidophilus* M92 can improve gastric and small intestinal transit tolerance. The method presented in this study may be useful for ensuring selection of potential probiotic bacteria capable to survive gastrointestinal passage under conditions prevalent *in vivo*.

References

1. E. Jonson, P. Conway: Probiotics for Pigs. In: *Probiotics, The Scientific Basis*, R. Fuller (Ed.), Chapman & Hall, London (1992) pp. 259–316.
2. M. Alander, I. de Smet, L. Nollet, W. Verstreaete, A. von Wright, T. Mattila-Sandholm, *Int. J. Food Microbiol. 46* (1999) 71–79.
3. P. Marteau, J. C. Rambaud, *FEMS Microbiol. Rev. 12* (1993) 207–220.
4. J. Šušković, B. Kos, S. Matošić, V. Marić, *Food Technol. Biotechnol. 35* (1997) 107–112.
5. W. P. Charteris, P. M. Kelly, L. Morelli, J. K. Collins, *J. Appl. Microbiol. 84* (1998) 759–768.
6. P. L. Conway, S. L. Gorbach, B. R. Goldin, *J. Dairy Sci. 70* (1987) 1–12.
7. J. Šušković, B. Brkić, S. Matošić, V. Marić, *Milchwissenschaft, 52* (1997) 430–435.
8. J. Šušković, *Ph.D. Thesis*, Faculty of Food Technology and Biotechnology, University of Zagreb, Croatia (1996).
9. P. Raspor, M. Batič, P. Jamnik, *Food Technol. Biotechnol. 37* (1999) 81–86.
10. R. Havenaar, B. Ten Brink, J. H. J. Huis in't Veld: Selection of Strains for Probiotic Use. In: *Probiotics – The Scientific Basis*, R. Fuller (Ed.), Chapman and Hall, London (1992) pp. 209–221.
11. M. J. Hill: Factors Controlling the Microflora of the Healthy Upper Gastrointestinal Tract. In: *Human Microbial Ecology*, M. J. Hill, P. D. Marsh (Eds.), CRC Press, Boca Ratton, Florida (1990) pp. 57–85.
12. N. Berrada, J. F. Lemeland, G. Laroch, P. Thouvenot, M. Piaia, *J. Dairy Sci. 74* (1991) 409–413.
13. S. Drouault, G. Corthier, S. D. Ehrlich, P. Renault, *Appl. Environ. Microbiol. 65* (1999) 4881–4886.
14. S. E. Gilliland, M. L. Speck, *J. Food Protect. 40* (1977) 760–765.
15. B. R. Goldin, S. L. Gorbach: Probiotics for Humans. In: *Probiotics – The Scientific Basis*, R. Fuller (Ed.), Chapman and Hall, London (1992) pp. 355–376.
16. J. Šušković, B. Kos, S. Matošić, *Sixth Symposium on Lactic Acid Bacteria: Genetics, Metabolism and Applications*, Book of Abstracts, Veldhoven, The Netherlands (1999) p. J3.
17. U. Svensson: Industrial Perspectives. In: *Probiotics. A Critical Review*, G. W. Tannock (Ed.), Horizin Scientific Press, U.K. (1999) pp. 57–64.

Utjecaj protektora na preživljavanje *Lactobacillus acidophilus* M92 u simuliranim uvjetima gastrointestinalnog trakta

Sažetak

Bakterija *Lactobacillus acidophilus* je najbrojnija vrsta roda *Lactobacillus* u intestinalnom traktu zdravih ljudi. Jedno je od bitnih svojstava bakterije *L. acidophilus*, kao probiotičkog soja, preživljavanje u nepovoljnim uvjetima probavnog sustava. Ovim se radom želio odrediti utjecaj mucina i dijetetskih sastojaka, kao što su kazein, koncentrirani proteini sirutke (WPC) i obrano mlijeko, na preživljavanje *L. acidophilus* M92 u simuliranim uvjetima gastrointestinalnog (GI) trakta. Pripremljena suspenzija stanica podvrgnuta je djelovanju pepsina (3 g/L) i natrijeva klorida (5 g/L) pri pH=1,5; 2,0; 2,5 i 3,0, te pankreatina (1 g/L) i goveđe žuči (1,5; 2,0; 3,0 i 5,0 g/L) pri pH=8, simulirajući gastrointestinalne uvjete. Preživljavanje *L. acidophilus* M92 u simuliranim uvjetima gastrointestinalnog trakta bilo je kudikamo bolje u prisutnosti mucina i proteina mlijeka, posebno koncentrata proteina sirutke. Utjecaj koncentrata proteina sirutke, kao najboljeg protektora na preživljavanje *L. acidophilus* M92 u simuliranom želučanom soku i soku tankog crijeva, potvrđen je primjenom matematičkog modela. Izračunate kritične točke definirale su uvjete gastrointestinalnog trakta u kojima *L. acidophilus* M92 može preživjeti. Utvrđeno je da *L. acidophilus* M92 preživljava 2 sata pri pH = 1,99 u simuliranom soku želuca, te 4 sata u simuliranom soku tankog crijeva s 3,1 g/L goveđe žuči, a u prisutnosti koncentrata proteina sirutke čak pri pH = 1,77, te s 4,25 g/L goveđe žuči. Nadalje, pri izravnom prenošenju *L. acidophilus* M92 iz simuliranog soka želuca u simulirani sok tankog crijeva preživjelo je 15 % stanica, dok je uz dodatak koncentrata proteina sirutke preživjelo 45 % stanica. Prema dobivenim rezultatima koncentrat proteina sirutke trebalo bi dodati u pripravi *L. acidophilus* M92 za probiotičku primjenu.