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The effect of Lactobacilli inoculation on organic acid levels in the mucosal film and the small intestine contents in gnotobiotic pigs

Einfluß der Inokulation von Laktobazillen auf die Konzentration organischer Säuren im Schleimhautfilm und im Dünndarminhalt keimfreier Ferkel

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Zusammenfassung: Bei keimfreien Ferkeln wurde der Einfluß der Inokulation von drei Stämmen von Lactobacillus plantarum auf die Konzentration von Milch-, Essig-, Azetessig- und Propionsäure im Schleimhautfilm (F) und im Inhalt des Jejunums und Ileums (O) beobachtet. Im Jejunum der behandelten Ferkel wurden im Schleimhautfilm in Vergleich zum Darminhalt signifikant erhöhte Konzentrationen von Milch-, Propion- und Azetessigsäure sowie insignifikant erhöhte Konzentrationen von Essigsäure gemessen. Im Ileum der keimfreien Ferkel war der Propionsäuregehalt des Schleimhautfilms in Vergleich zum Inhalt signifikant erhöht (21,2 zu 9,5 mmol·l^{-1}, $p < 0{,}05$). Der erhöhte Milch-, Essig- und Azetessigsäuregehalt des Schleimhautfilms war in Vergleich zu dem des Darminhalts insignifikant. Diese Ergebnisse zeigen, daß die signifikant erhöhten Werte der durch die Laktobazillen produzierten organischen Säuren eine effiziente Barriere bei der Inhibition der Adhärenz von Verdauungstraktpathogenen darstellen können.

Summary: The effect of the inoculation of three Lactobacillus plantarum strains upon lactic, acetic, acetoacetic and propionic acid levels in the mucosal film (F) and the jejunal and ileal contents (O) has been investigated in gnotobiotic pigs. In the jejunum of the inoculated animals, the mucosal film revealed significantly increased levels of lactic, propionic and acetoacetic acids when compared to the contents (25.3 vs. 10.8 mmol · l^{-1}, 18.5 vs. 5 mmol·l^{-1} and 29.7 vs. 11.2 mmol·l^{-1}, respectively) as well as insignificantly increased acetic acid levels (11.0 vs. 5.8 mmol·l^{-1}). In the ileum of gnotobiotic pigs, propionic acid levels of the mucosal film were significantly higher than those of the contents (21.2 vs. 9.5 mmol·l^{-1}, $p < 0.05$). In comparison to the contents, the increased lactic, acetic and acetoacetic acid levels in the film proved to be insignificant. The above results suggest that the significantly increased levels of the Lactobacilli-produced organic acids may present an efficient barrier inhibiting the adherence of digestive tract pathogens to the intestinal mucosa.

Introduction

The use of probiotics presents one of the effective methods of preventing and treating digestive tract diseases in farm animals (Watkins et al., 1982). Lactobacilli are the most frequently used microorganisms for probiotic purposes (Fuller, 1989; Jonson and Conway, 1992). The ability of generating organic acids, particularly lactic and acetic acids, presents one of the mechanisms by which lactobacilli perform their inhibitory effect upon pathogens (Piard and Desmazeaud, 1991). With decreasing pH values the inhibitory activity of the above acids increases (Daly et al., 1972), their molecular form being toxic for bacteria. The increased toxicity of acetic acid is attributed to its higher pKa in comparison with lactic acid. Adams and Hall (1988) confirmed Lactic acid lowered the pH of the medium, thus increasing the toxicity of acetic acid.

In attempts to clarify the mechanism of action of probiotics the hypothesis has been postulated according to which a film of metabolites produced by probiotic strains covered the intestinal mucosa and prevented the adherence of pathogens.

It was the aim of the present study to compare lactic, acetic, acetoacetic and propionic acid levels both in the mucosal film and in the jejunal and ileal contents of gnotobiotic pigs inoculated with selected strains of lactobacilli from the viewpoint of their potential inhibitory effect against pathogens.

Material and methods

Five gnotobiotic pigs, obtained by open hysterotomy and reared in isolators were included in the experiment. The animals were fed dried whole milk (PMV, Hradec Králové; chemical composition in g·kg^{-1}: protein 273, fat 253, lactose 383 and iron 0.004), at a dilution of 1 : 9, 4 times a day, ad libitum. At 8 days of age the mean daily milk intake was 0.6 l per suckling.

On day 2, 3 and 4 of life the germ-free animals were inoculated by three strains of Lactobacillus plantarum. These were selected from 81 strains isolated from the gut contents of healthy, 7–14-day old sucklings on the basis of the most pronounced in-vitro inhibitory behaviour. Each inoculum contained 1×10^8 of germs and it was administered once daily at a dose of 2 ml. Two and 5 days after inoculation 3 and 2 pigs were slaughtered, respectively. Samples of intestinal contents (O) were taken by slight pushing after transverse section of the vertically positioned gut part. Then the intestine was placed into horizontal position and logitudinally cut. Samples of the mucosal film (F) were taken by a covering slide, very carefully in order not to damage the mucosa. The mucosal film obtained was homogenous, of mucous consistence, without feed particles. Each ml of the intestinal contents and of the mucosal film samples was filled up to 50 ml with saline and homogenized at 3000 rev. per min. Then the samples were filtered through filtration paper and the parameters under observation determined in the filtrate. The leftover parts of the intestine were used in electron microscopic studies. Lactic, acetic, acetoacetic and propionic acids were determined by capillary isotachophoresis. As conducting and finishing electrolytes, 0.001 mmol/l hydro-

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chloric acid (pH 4.25) and 5 mmol/l capronic acid (pH 4.5) were used, respectively. Student's t-test was employed for statistical evaluation.

Results

In the jejunum, the level of lactic acid in the mucosal film (25.3 ± 3.4 $mmol \cdot l^{-1}$) was significantly higher than in the contents (10.8 ± 7.0 $mmol \cdot l^{-1}$; $p < 0.01$). In the ileum this difference was not significant (Fig. 1).

Both in the jejunum and the ileum, acetic acid levels were higher in the mucosal film than in the contents (11.0 ± 7.2 vs. 5.8 ± 1.9 $mmol \cdot l^{-1}$, and 13.1 ± 6.6 vs. 7.1 ± 1.7 $mmol \cdot l^{-1}$, respectively), the differences, however, being insignificant (Fig. 2).

In both sections of the small intestine, the level of acetoacetic acid in the mucosal film was higher than in the contents (Fig. 3), the differences being only significant in the jejunum (film 29.7 ± 5.9 $mmol \cdot l^{-1}$, contents 11.2 ± 8.1 $mmol \cdot l^{-1}$; $p < 0.05$).

In the mucosal film of the jejunum and the ileum, propionic acid levels reached 18.5 ± 6.7 $mmol \cdot l^{-1}$ and 21.2 ± 8.5 $mmol \cdot l^{-1}$, respectively; they appeared to be significantly increased when compared to those in the jejunal (5.5 ± 2.2 $mmol \cdot l^{-1}$) and in the ileal contents (9.5 ± 3.6 $mmol \cdot l^{-1}$), the respective levels of significance being $p < 0.01$ and $p < 0.05$ (Fig. 4).

Discussion

The mechanism of the antibacterial effect of probiotics may be explained by several factors: generation of organic acids (lactic, acetic and formic acids) and decrease in pH (Babel, 1977), generation of hydrogen peroxide or free radicals and their bacteriostatic or bactericide effect (Piard and Desmazeud, 1991), generation of natural antibiotic substances – bacteriocins (Vandenberg, 1993), competitive exclusion (Chauviere et al., 1992) and antienterotoxic activity (Mitchell and Kenworthy, 1976).

Our previous studies (Bomba et al., 1996 a, 1996 b) showed that the competition for adhesion receptors on the intestinal mucosa did not play an important role in the mechanism of inhibiting enteropathogenic *E. coli*. More likely it seemed to be a metabolite-mediated inhibition. The bacteriocins produced by Lactobacilli usually act only within this species. The antienterotoxic activity of Lactobacilli is rather important, but from the viewpoint of the pathogenity of *E. coli* the adhering ability of the latter seems to be the decisive factor.

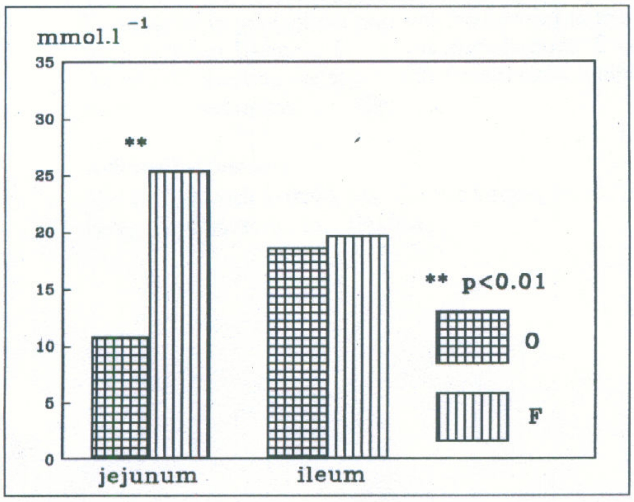

Fig. 1 Lactic acid levels in the mucosal film (F) and in the contents (O) of the jejunum and ileum in gnotobiotic pigs after inoculation with Lactobacillus plantarum

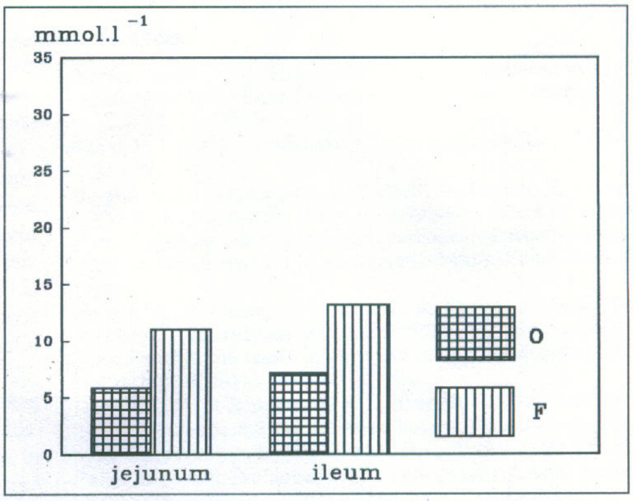

Fig. 2 Acetic acid levels in the mucosal film (F) and in the contents (O) of the jejunum and ileum in gnotobiotic pigs after inoculation with Lactobacillus plantarum

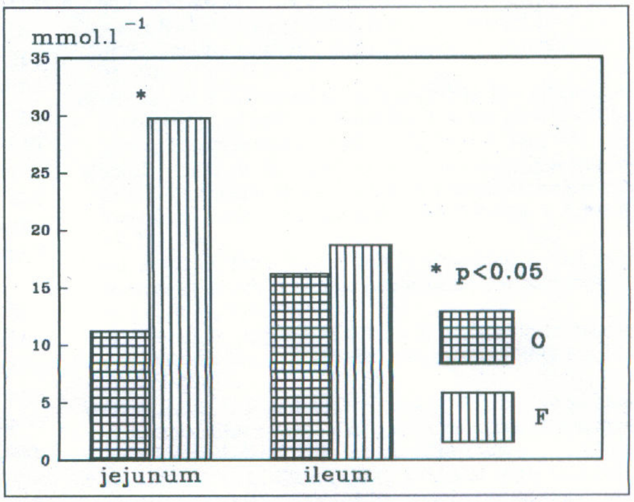

Fig. 3 Acetoacetic acid levels in the mucosal film (F) and in the contents (O) of the jejunum and ileum in gnotobiotic pigs after inoculation with Lactobacillus plantarum

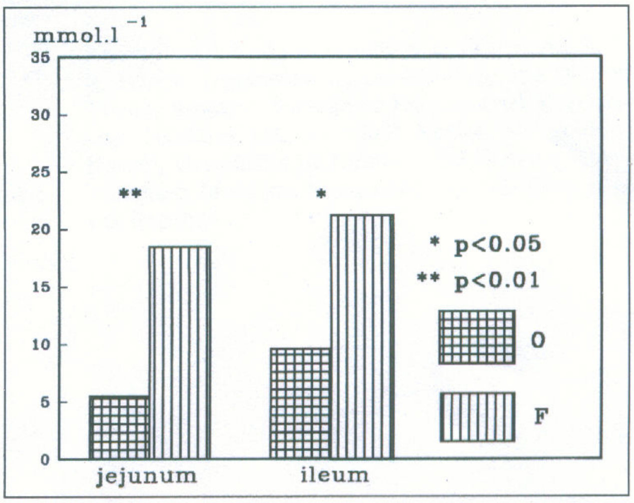

Fig. 4 Propionic acid levels in the mucosal film (F) and in the contents (O) of the jejunum and ileum in gnotobiotic pigs after inoculation with Lactobacillus plantarum

With respect to the above facts our study was aimed at the production of organic acids by Lactobacilli. It was the aim of this work to confirm or disprove the hypothesis about the protective film lactobacilli generate on the intestinal mucosa, thus preventing the pathogens from adhering to the latter. In our experiments on gnotobiotic pigs the levels of all observed organic acids in the mucosal film of the jejunum and the ileum were found to be higher than in the contents. Except for acetic acid the differences showed to be significant. The levels of lactic acid were, at a mean, twice as high as those of acetic acid. This is of importance with respect to the synergistic effect of these acids. Increased lactic acid levels intensify the toxicity of acetic acid (Adams and Hall, 1988).

Similarly to lactic and acetic acids, significantly increased levels were also recorded for acetoacetic and propionic acids. This new knowledge might be used in further investigations in order to explain whether significantly increased organic acid levels produced by Lactobacilli and found in the mucosal film participate in establishing a protective barrier against pathogens.

The levels of lactic and acetic acids determined in the small intestinal contents of gnotobiotic suckling pigs were decreased in comparison to those recorded in our previous study of the interactions between Lactobacillus spp. and enteropathogenic *E. coli* 08 $K88^{+}Ent^{+}$ in gnotobiotic pigs (Bomba et al., 1995 b). Acetic and propionic acid levels in the intestinal contents of gnotobiotic pigs were comparable to those found in gnotobiotic lambs preventively inoculated with *Lactobacillus casei* and subsequently with enteropathogenic *E. coli* 0101 K 99 (Bomba et al., 1995 a), however, lactic acid levels showed to be 4- to 9 times higher.

As to the inhibitory effects of organic acids, maximum effectivity is attributed to acetic acid (Wong and Chen, 1988). Bacterial sensitivity to the latter depends on changes which again depend on the simultaneous effect of other factors such as concentration of salts, water activity, redox potential, etc. Kleter et al. (1984) proved the synergism between acidity and NaCl concentration in the inhibition of *Clostridium tyrobutyricum.*

In this work Lactobacilli were confirmed to produce organic acids that generated an inhibitory barrier against digestive tract pathogens on the mucosa of the small intestine. Our further investigation into the mechanism of the inhibitory effects of Lactobacilli in gnotobiotic pigs will be devoted to the interactions between Lactobacilli and enteropathogenic *E. coli* with the aim of selecting the strain with the strongest inhibitory effects upon pathogens.

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