Propionibacteria in the gut: effect on some metabolic activities of the host

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Summary — The development of a dairy Propionibacterium and its establishment in the gut were studied. Mice fed a conventional diet received a suspension of propionibacteria in skim milk provided in their water bottles for 7 d. Counts of propionibacteria in faeces and intestinal sections indicated that the strain used reached significant levels in the gut during treatment. They remained in high number 1 week after cessation of the diet. Their permanence in the gut could be related to an adhesion onto the intestinal mucosae. The presence of these bacteria in the intestine favourably affected the lipid metabolism and the immune system of mice. The effect on the serum lipids level was evaluated after feeding mice with 5 different diets, containing or not a supplementation of milk cream and propionibacteria. Total cholesterol, HDL, LDL and triglycerides were determined. The results showed that this strain tends to reverse the hyperlipemic effect of a diet with high lipid content. An increase both in the phagocytic activity of peritoneal macrophages and in the phagocytic function of reticuloendothelial system was observed on the 7th d of feeding. The activity decreased when mice returned to a conventional diet. The data confirm that some strains of dairy propionibacteria can develop in the gut and exert beneficial effects on the host.

Résumé — Propionibactéries dans l’intestin : effet sur certaines activités métaboliques de l’hôte. Le développement d’une flore de Propionibacterium d’origine laitière et son implantation dans l’intestin ont été étudiés. Des souris nourries avec un régime conventionnel ont reçu une suspension de propionibactéries dans du lait écrémé fournies avec leur alimentation en eau pendant 7 jours. Le nombre de propionibactéries dans les fèces et les sections d’intestin indiquait que la souche utilisée atteignait des niveaux significatifs dans l’intestin durant le traitement. Il restait à un niveau élevé une semaine après cessation du régime. Son maintien dans l’intestin pourrait être dû à une adhésion à la muqueuse intestinale. La présence de ces bactéries dans l’intestin avait une incidence favorable sur le métabolisme des lipides et sur le système immunitaire des souris. L’effet du niveau de lipides sériques était évalué après alimentation des souris avec 5 régimes différents supplémentés ou non avec de la crème laitière et des propionibactéries. Le cholestérol total, HDL et LDL et les triglycérides étaient déterminés. Les résultats ont montré que cette souche a tendance à inverser l’effet hyperlipi-démique d’un régime riche en lipides. Un accroissement à la fois de l’activité phagocytaire des macro-
phages péritonéaux et de la fonction phagocytai re du système réticuloendothélial était observé le 7e jour. L'activité diminuait lorsque les souris revenaient à un régime conventionnel. Les données confirment que certaines souches de propionibactéries laitières peuvent se développer dans l'intestin et exercer un effet bénéfique sur l'hôte.

bactérie propionique / intestin / cholesterol / immunité

INTRODUCTION

In the last decades, many efforts have been made to improve the health of humans and animals by using microbial dietary adjuncts, which beneficially affect the host either by improving its intestinal microbial balance or by affecting the metabolism of indigenous flora.

Lactic acid bacteria, as normal inhabitants of the intestine, are commonly used as dietary adjuncts on the basis of their influence on the intestinal microflora (Sandine, 1979) and their antagonism against pathogenic bacteria (Gilliland, 1979). Lactobacillus bulgaricus and Streptococcus thermophilus, the yogurt starter microorganisms, are also included due to the benefits of yogurt consumption on the bioavailability of Ca$^{2+}$ (Dupuis et al, 1985), absorption of lactose (Gilliland and Kim, 1984), decrease of enzyme activities involved in carcinogenesis (Farmer, 1983) and stimulation of the immune system (Perdigon et al, 1994).

There is evidence that the consumption of milk and certain dairy products fermented with selected cultures of streptococci or lactobacilli may lead to the reduction of serum cholesterol level in humans and animals (Man and Spoerry, 1974; Hepner et al, 1979; Rao et al, 1981; Grunewald, 1982; Fernandes et al, 1987).

In order to produce in vivo beneficial effects, it is generally considered that the bacteria employed as dietary adjuncts must survive and grow within an environment as hostile as the gastrointestinal tract, and remain in a high level in spite of the continuous renewal of the intestinal content. It has been demonstrated that the predominant bacterial species in the gut reach population levels between $5 \times 10^8$ to $1 \times 10^{11}$ g$^{-1}$ of faeces (Raibaud, 1992). Strains that reach the intestine in low level do not have a chance to play a role in the ecosystem.

Some bacteria, with the ability to adhere to intestinal cells and mucus, have the best chance to colonize the gastrointestinal tract and may be detected for long periods in the host. Different cell wall components may be responsible for adhesion to the intestinal epithelium. The nature of adhesion determinants differs depending on the bacterial cell studied. Since all strains do not possess the ability to adhere to intestinal cells, the adhesion assays should be done before selecting strains for use as dietary adjuncts (Klaenhammer, 1982).

According to Finegold et al (1983), species of the genus Propionibacterium are present in a low percentage of subjects in a very variable range (log_{10}/g of faeces of 4.3 to 12.0). Dairy propionibacteria, as they are present in dairy products, take part in the human diet. Little is known, however, about their ability to develop in the intestine and to exert influences on the metabolic activities of the host.

The cholesterol uptake by strains of Propionibacterium freudenreichii from laboratory media has been recently studied (Somkuti and Johnson, 1990). Furthermore, it has been shown that propionic acid, but not propionate, infused into the stomach or cecum, moderates the increase of choles-
terol concentration in rats fed a casein diet (Ebihara et al, 1993).

There is evidence that the nonspecific stimulation of host macrophages by Propionibacterium acnes inhibits translocation of gram-negative bacteria (Berg, 1992). A similar stimulation would be desirable if it could be produced by dairy propionibacteria.

The present study was undertaken to determine if feeding with dairy propionibacteria increases the intestinal level and affects the lipid metabolism and immune system of the host.

MATERIALS AND METHODS

Animals

The experimental model was BALB/c male albino mice of 22 g average initial weight. For the different experiments, they were identified by ear cut procedures and housed in plastic cages, 5 mice in each cage.

Microorganism

The strain used in this study was Propionibacterium acidipropionici CRL 1198 (Centro de Referencia para Lactobacilos), isolated from Emmental cheese.

Stock cultures were maintained at 4°C in BHI broth. Before use, they were incubated at 37°C and activated by 3 successive transfers every 24 h.

Feeding procedures and assays for determination of propionibacteria in the intestine

Mice were randomly divided into treatment groups with 10 animals in each group. They received throughout 7 d a conventional balanced diet supplemented or not with propionibacteria. Another group of 5 animals were employed as control before feeding.

A 24-h-old culture of the selected strain in BHI broth was harvested by centrifugation, washed in sterile saline solution and suspended in sterile nonfat milk (powder NFM 10%) to the desired concentration. The suspension was added at 3% concentration in the drinking water of the test group of mice (10⁹ bacteria/ml). The same amount of milk was added in the drinking water of the control group. The mice received their assigned diet ad libitum and after 1 week, both groups were returned to a normal diet of chow plus water for another 7 d.

Faeces of mice were collected on the 2nd, 5th and 7th d throughout the treatment and also on the 14th d after cessation of the diet, and counts of propionibacteria, anaerobic and aerobic total flora were determined.

Five animals in each group were sacrificed by cervical dislocation on the 7th d of feeding, and the others on the 14th d of the treatment. Small bowel and cecal contents were separately collected and weighted. Intestinal tissues were washed with PBS (0.01 mol/l phosphate buffered saline, pH 7.2), weighted and disrupted. Counts of anaerobic total flora and propionibacteria in the intestinal contents and propionibacteria in gut walls were determined.

Aerobic total flora was enumerated on BHI agar, incubated 48 h at 37°C. Anaerobic total flora was determined in blood-supplemented BHI agar, incubated 5 d under anaerobic conditions. The number of propionibacteria was determined in a specially developed medium (ECOTEC media patent in process) for its detection in the gut. Lactobacilli, streptococci, lactococci or coliform bacteria were inhibited in that medium.

Feeding procedures and assays for determination of serum lipid level

In order to study the effect on lipid metabolism, 5 different diets were employed (table III). Mice received throughout 7 d a stock diet plus: water (W group), skim milk (SM group), skim milk plus 10⁸ propionibacteria per milliliter (SMP group), skim milk plus 5% milk cream (MC group) and skim milk plus cream and bacteria (MCP group). On the 7th d, mice were returned to a diet of chow plus water for another week. After the 1st and 2nd weeks of feeding, blood samples were drawn.
from the retroorbital venous plexus from 5 mice per group for the determination of the serum total, HDL and LDL cholesterol and serum triglycerides. Cholesterol and triglycerides were determined by enzymatic methods.

Livers were excised and weighted and the ratio liver weight/body weight was calculated.

**Phagocytosis assays in vitro**

Mice were fed as previously indicated for groups W, SM, SMP, MC and MCP. To measure the in vitro phagocytic activity, portions of peritoneal macrophages (1 x 10^6 cells/ml) were incubated for 15 min at 37°C with the same volume of heat killed *Salmonella typhimurium* suspension (1 x 10^7 cells/ml). The incubation was stopped in an ice-cold bath, the tubes were centrifuged (5 min, 1 500 g) and the sediment observed by use of a Zeiss microscope. The percentage of macrophages with ingested bacteria was determined by counting 200 cells.

**Results and Discussion**

**Establishment of propionibacteria in the gut**

The level of total microflora was increased at the beginning of the experiment (fig 1). The change to a diet with more proteins and lactose may be the reason for that effect as flora in the control group was also increased (data not shown).

Anaerobes showed their highest level on the 7th d and remained stable until the end of the experiment. In contrast, aerobes reached their highest number on the 5th d and no significant increases were registered until the end of the experiment. This may be due to the increment of the flora producing volatile fatty acids, since these organic acids are well-known inhibitors of coliforms and aerobes.

Counts of 10^6 propionibacteria per gram of faeces were obtained for mice before treatment. *Propionibacterium acidipropionici* of the diet was detected in a high level in faeces of the test group throughout the 14 d of feeding. Thus, the log_{10} number was sig-
Table I. Anaerobic total flora and propionibacteria in the small intestine of mice fed with Propionibacterium acidipropionici CRL 1198.*

<table>
<thead>
<tr>
<th>Days of treatment</th>
<th>Total anaerobes</th>
<th>Propionibacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Test</td>
<td>Control Test</td>
</tr>
<tr>
<td>Intestinal content</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>9.0 ± 0.2b</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>9.3 ± 0.3</td>
<td>10.1 ± 0.4</td>
</tr>
<tr>
<td>14a</td>
<td>8.9 ± 0.2</td>
<td>9.6 ± 0.6</td>
</tr>
<tr>
<td>Intestinal tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>14a</td>
<td>nd</td>
<td>3.2 ± 0.4</td>
</tr>
</tbody>
</table>

* Test mice were fed with a diet containing a suspension of 10^8 propionibacteria/ml in 3% skim milk. Control mice received the same diet without bacteria. Small intestine was removed on the 7th d from control and test mice. Intestinal contents were collected for determination of total anaerobes and propionibacteria. Intestinal tissue of mice was intensively washed and disrupted before determination of number of propionibacteria. a Mice were fed for 7 d with the indicated diet and then returned to a conventional alimentation until the 14th d of treatment; b data are log_{10} cfu/g of intestinal content and tissue ± SE (n = 5). nd: not determined; -: lower than the limit of detection.

As shown in tables I and II, propionibacteria were detected in the large intestine, but not in the small intestine before treatments. After 1 week of feeding, the number of these bacteria in the control group was not significantly altered. In contrast, propionibacteria in the test group were 8 and 4 log increased in the small and large intestine, respectively. Total anaerobes also reached a higher number in the test group than in the control group.

During feeding propionibacteria and after cessation of the diet, small intestine tissue of the mice in the test group showed a log_{10} number between 3.0 to 4.0. However, these bacteria were not detected in the small intestine tissue of the control group. The cecal tissue of these mice group had a low number of propionibacteria, which increased 2 log during feeding.

Seven days after cessation of the diet, the number of propionibacteria was significantly reduced in the large intestine but not in the small bowel. These results were surprising, taking into account the low growth rate of these bacteria. Some adhesion factors might
Table II. Anaerobic total flora and propionibacteria in ceca of mice fed with Propionibacterium acidipropionici CRL 1198.

Flore totale aérobie et propionibactéries dans le cecum de souris nourries avec Propionibacterium acidipropionici CRL 1198.

<table>
<thead>
<tr>
<th>Days of treatment</th>
<th>Total anaerobes</th>
<th>Propionibacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Test</td>
</tr>
<tr>
<td>Intestinal content</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>9.0 ± 0.1b</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>9.4 ± 0.2</td>
<td>10.0 ± 0.3</td>
</tr>
<tr>
<td>14a</td>
<td>9.1 ± 0.2</td>
<td>9.9 ± 0.3</td>
</tr>
<tr>
<td>Intestinal tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>14a</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

* Test mice were fed with a diet containing a suspension of $10^8$ propionibacteria/ml in 3% skim milk. Control mice received the same diet without bacteria. The ceca was removed on the 7th d from control and test mice. Intestinal contents were collected for determination of total anaerobes and propionibacteria. Intestinal tissue of mice was intensively washed and disrupted before determination of number of propionibacteria. b Mice were fed for 7 d with the indicated diet and then returned to a conventional alimentation until the 14th d of treatment; data are $\log_{10}$ cfu/g of intestinal content and tissue ± SE ($n = 5$). nd: not determined.

Morata et al studied the chemical composition and morphological aspects of the surface of *P. acidipropionici* CRL 1198 (personal communication). They observed, in cells negatively stained with uranyl acetate, a nonhomogeneous cell wall with some components emerging from the peptidoglycan-containing layer. These structures were resistant to washes both with SDS and acids, indicating that they are covalently bound in the cell wall. The presence of these external components may contribute to the adhesion onto the intestinal mucosae (Mukai et al, 1992; Mukai and Arihara, 1994).

The results showed that this strain could develop and remain in almost a stable number in the gut contents and walls after cessation of the diet. Since that strain reached in intestine a level near that of dominant or subdominant flora, it may be likely that propionibacteria affect the intestinal activities and the host metabolism.

**Effect on lipid metabolism**

Propionibacteria were enumerated in faeces after 7 d in order to determine whether...
changes in alimentation affect its presence in the gut. We have seen that there were no significant differences between treatments, when they contained propionibacteria.

A reduction of total cholesterol and triglyceride serum concentration was observed for mice receiving both skim milk and skim milk with bacteria (SM and SMP groups, respectively; table III), on the 7th d of feeding (fig 2). HDL cholesterol was also reduced in both groups, but only the SMP group had a LDL cholesterol value lower than in the control group. One week after cessation of diets, lipid serum concentration was even lower than in the W group (fig 3).

Mice fed with cream supplement (MC group) showed an increase in whole lipid concentration on the 7th d of treatment, with a slow return toward normality upon cessation of diet. In contrast, mice receiving the same diet plus propionibacteria (MCP group) showed lower serum lipid concentration than the MC group on the 7th d of treatment. These values were not significantly different from those on the 14th d, with the exception of LDL concentration, which reached a normal value (fig 3).

Table III. Composition of the diets for the different groups of mice.
Composition des régimes des différents groupes de souris.

<table>
<thead>
<tr>
<th>Feeding groups</th>
<th>Diets</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>Chow + water</td>
</tr>
<tr>
<td>SM</td>
<td>Chow + skim milk</td>
</tr>
<tr>
<td>SMP</td>
<td>Chow + skim milk + propionibacteria(^a)</td>
</tr>
<tr>
<td>MC</td>
<td>Chow + skim milk + 5% milk cream</td>
</tr>
<tr>
<td>MCP</td>
<td>Chow + skim milk + 5% milk cream + propionibacteria</td>
</tr>
</tbody>
</table>

\(^a\) Propionibacteria were administered to mice at a dose of \(10^8\) bacteria/d in SMP and MCP groups.

Table IV. Serum lipids ratio in mice fed diets without or with propionibacteria addition.*
Rapports en lipides du sérum de souris recevant un régime sans ou avec addition de propionibactéries.*

<table>
<thead>
<tr>
<th>Diets</th>
<th>During feeding</th>
<th>Post-feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HDL/total</td>
<td>HDL/LDL</td>
</tr>
<tr>
<td>W</td>
<td>4.50</td>
<td>0.60</td>
</tr>
<tr>
<td>SM</td>
<td>3.06</td>
<td>0.65</td>
</tr>
<tr>
<td>SMP</td>
<td>4.76</td>
<td>0.62</td>
</tr>
<tr>
<td>MC</td>
<td>2.62</td>
<td>0.56</td>
</tr>
<tr>
<td>MCP</td>
<td>2.94</td>
<td>0.64</td>
</tr>
</tbody>
</table>

* Data are the ratio of mean values of HDL, LDL and total cholesterol, determined for each group of treatment.

* Les données sont le rapport des valeurs moyennes de cholestérol HDL, LDL et total, déterminé pour chaque groupe expérimental.
The ratio HDL/ LDL cholesterol, an indicator of atherosclerosis risk, determined for all groups, did not show great differences, however, the relationship HDL/total cholesterol showed similar values to control only when feeding propionibacteria (table IV).

It has been suggested that the hypcholesterolemic effect of milk and dairy products may be due to the presence of organic acids such as uric and orotic acids which inhibit cholesterol synthesis (Richardson, 1978). A less efficient absorption in the intestine may also result in a lower serum cholesterol level. Thus, deconjugation of bile acids by probiotic bacteria (Gilliland and Speck, 1977) and assimilation of cholesterol by lactobacilli and bifidobacteria strains have been suggested as being involved in the hypcholesterolemic effect of fermented milk (Gilliland et al, 1985). Recently, it has been reported that a small quantity of casein hydrolysate peptides enter the circulatory system from intestinal lumen and affect the lipid metabolism (Asato et al, 1994). The different proteolytic activity of dairy bacteria could be the reason for the different effects observed in dairy products.

Our data suggest that there is a lipid-lowering effect due to milk components, as well as due to the presence of propionibacteria in the gut. The hypolipemic effect is more noticeable in diets with a high lipid content. These results are in agreement with those of other authors who showed that skim milk and skim milk yogurt exerted a hypcholesterolemic effect to a greater extent when cholesterol was added to the diets (Navder et al, 1990).

In our experiments, the liver weight/body weight ratio and the microscopic morphology...
of the livers were not different between treatments, indicating that there was no induction of fatty liver. Thus, the hypolipemic effect of the MCP diet may not be due to a redistribution of lipid from plasma to liver, but instead to a lower intestinal absorption or higher lipid catabolism. The characterization of the mode of action of \textit{P acidipropionici} CRL 1198 is currently in progress.

**Effects on the immune system**

The effects on the immune system of mice receiving propionibacteria in their diet were studied. The \textit{in vitro} phagocytic activity of peritoneal macrophages and the carbon-clearance test were assayed on the 7th d of feeding the 5 diets previously described, and on the 14th d after return to a normal alimentation.

**Table V. Nonspecific immune response in mice fed with propionibacteria.**

<table>
<thead>
<tr>
<th>Feeding groups</th>
<th>Carbon clearance-rate(^{a}) ((t^{1/2}))</th>
<th>Activated macrophages(^{b}) (%)</th>
<th>During feeding</th>
<th>Post-feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>\text{W}</td>
<td>9.90 ± 0.84(^{c})</td>
<td>28.10 ± 3.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\text{SM}</td>
<td>8.50 ± 0.93</td>
<td>32.53 ± 2.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\text{SMP}</td>
<td>1.84 ± 0.54</td>
<td>54.31 ± 4.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\text{MC}</td>
<td>7.80 ± 0.72</td>
<td>36.99 ± 1.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\text{MCP}</td>
<td>1.83 ± 0.40</td>
<td>58.41 ± 1.32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Propionibacteria were administered to mice at a dose of \(10^8\) bacteria/d in SMP and MCP groups.

\(^{b}\) Colloidal carbon was injected into mice on the 7th d of feeding. Blood samples were taken on 0, 1, 2, 3, 6, 9 and 12 min. Carbon clearance-rate \(t^{1/2}\) was calculated by means of the formula: \(t^{1/2} = (t_2 - t_1) \times 1/2 \times O.D. t_1 / \times O.D. t_2 + O.D. t_2) ; \)

\(^{c}\) The macrophages phagocytating bacteria were counted microscopically; \(^{c}\) mean ± SD for each group of mice (\(n = 5\)).

An increase both in the phagocytic activity of peritoneal macrophages and in the carbon-clearance activity was observed in mice fed with \textit{Propionibacterium} (table V).

Carbon-clearance rates \(t^{1/2}\) obtained with \textit{Propionibacterium} diets were lower than those observed in controls, indicating an enhanced phagocytic function of the reticuloendothelial system in mice at the end of the 1st week of feeding.

On the 7th d of feeding, mice receiving SMP and MCP diets showed a phagocytic activity of peritoneal macrophages almost two times higher than the controls (W, SM and MC groups). After returning to a normal diet, the activity was decreased.

It has been demonstrated that some lactic acid bacteria may produce stimulation of the host immune system when administered orally (Perdigon \textit{et al}, 1986). This stimulation may involve macrophages, which play an
important role in the resistance of the host to infections and tumours. The present study shows that the inclusion in the diet of dairy products containing a selected strain of propionibacteria, which can survive and grow in the intestine, may lead to the activation of immunocompetent cells.

CONCLUSION

The development of a dairy Propionibacterium and its establishment in the gut was studied. Counts of propionibacteria in faeces and intestinal sections indicated that the strain used reached significant levels in the gut during treatment and remained in high number 1 week after cessation of the diet. Its permanence in the gut could be related to adhesion onto the intestinal mucosae.

The presence of these bacteria in the intestine favourably affected the lipid metabolism and immune system of the mice. The results showed that this strain tends to reverse the hyperlipemic effect of a diet with a high lipid content. An increase both in the phagocytic activity of peritoneal macrophages and in the phagocytic function of reticuloendothelial system was observed.

We hope that in the future more investigations related to these areas will make it possible to valorize the role of dairy propionibacteria in human health.

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