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β-Galactosidase production by *Streptococcus thermophilus* is higher in the small intestine than in the caecum of human-microbiota-associated mice after lactose supplementation

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Transit kinetics and survival rates of a bacterial species from yoghurt (i.e. *Streptococcus thermophilus* strain FBI3) were examined in different digestive compartments of gnotoxenic and human-microbiota-associated mice. The production of the lactose-hydrolysing enzyme (i.e. β-galactosidase) was also investigated within the digestive tract, using a chromosomal reporter system based on luciferase genes from *Photorhabdus luminescens* under the control of the p*lac* promoter. In both mice models, *S. thermophilus* cells transited within 2 h from the stomach to the caecum–colon compartment of the digestive tract where they displayed a survival rate of nearly 100 %. In gnotoxenic mice, luciferase activity was found to increase in the second half of the small intestine and in the caecum–colon compartment when lactose was added to the drinking water provided to the animals. In human-microbiota-associated mice drinking lactose, luciferase activity was similarly increased in the second half of the small intestine but was drastically reduced in the caecum–colon compartment. This feature could be ascribed to the presence of the resident human microbiota.

Digestive tract: Human microbiota: Lactose intolerance: *Streptococcus thermophilus*: Yoghurt

Lactose intolerance is a worldwide problem causing abdominal pain, diarrhoea and flatulence to milk consumers with deficiency of the lactose-hydrolysing enzyme, i.e. β-galactosidase (Bayless & Huang, 1971; Simoons 1978). It is now well established that β-galactosidase-deficient individuals digest lactose more efficiently when they eat fresh yoghurt containing live bacterial cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* than when they eat heated yoghurt or milk (Savaiano *et al.* 1984; Lerebours *et al.* 1989; Marteau *et al.* 1990; Varela-Moreiras *et al.* 1992; Shermak *et al.* 1995; Rizkalla *et al.* 2000). This probiotic effect is believed to be due to the β-galactosidase of microbial origin in the yoghurt culture (de Vrese *et al.* 2001).

In a previous study (Drouault *et al.* 2002), we obtained *S. thermophilus* strains expressing the *Photorhabdus luminescens* luciferase genes under the control of the p*lac* promoter. A good correlation was established between β-galactosidase production and luciferase expression. Using one of these strains, we demonstrated that *S. thermophilus* could produce an active β-galactosidase during its transit in the digestive tract of gnotoxenic mice (G-mice), although it did not multiply. The expression of the enzyme was shown to be enhanced in mice faecal samples when lactose was added to the drinking water provided to the animals. Since β-galactosidase production was examined in the faeces of mice, our results did not make it possible to determine whether the enzyme was active in the digestive compartments where lactose might be fermented. Hence, since using a G-mice model implied that *S. thermophilus* was the unique bacterial strain present in the digestive tract of the animals, our experiments did not consider the possible influence of an autochthonous microbiota on β-galactosidase production.

Human-microbiota-associated mice (HMA-mice) represent an animal model, the faecal microbiota of which displays a bacterial composition similar to that of man (Andremont *et al.* 1985; Hirayama, 1999). In the present study, we take advantage of this point by using this model to investigate the influence of human microbiota on β-galactosidase production by the FBI3 strain of *S. thermophilus* in the digestive tract. Transit kinetics and survival rates of the bacteria are compared in the second half of the small intestine (SI2) and in the caecum–colon (CC) compartment of G-mice and HMA-mice. Furthermore, β-galactosidase production is assessed in both compartments.

Abbreviations: CC, caecum–colon; CFU, colony-forming unit; G-mice, gnotoxenic mice; HMA-mice, human-microbiota-associated mice; SI2, second half of the small intestine.

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Materials and methods

Bacterial strains and culture media

S. thermophilus strain FBI3 is derived from the *S. thermophilus* strain recovered from the S85 symbiotic association of yoghurt (Drouault *et al.* 2002). It contains, integrated in the chromosome, a transcriptional fusion of the p*lac* promoter and the *luxAB* genes from *Photorhabdus luminescens*, as well as a chromosomal gene encoding resistance to erythromycin.

S. thermophilus was grown at 42°C in liquid M17 medium supplemented with glucose or lactose at 0·5 % (w/v). Erythromycin (Merck, Darmstadt, Germany) was added to M17-agar media at a final concentration of 5 μg/ml. Spores of the thermoresistant *Bacillus stearothermophilus* were used as transit markers and were germinated at 60°C on G-spore agar medium (Ducluzeau *et al.* 1970).

Animals

Ten-week-old female and male adult mice were used. Animals used in gnotoxenic experiments (G-mice) were C3H/He germ-free mice inoculated with *S. thermophilus* cells and *B. stearothermophilus* spores. For experiments with HMA-mice, C3H/He germ-free mice were first given two weekly inoculations of faecal dilutions of a single healthy human donor as previously described (Oozeer *et al.* 2002). G- and HMA-mice were reared in sterile Trexler-type isolators (La Cahlène, Vélizy, France) fitted with a rapid transfer system in an environmentally controlled room (21°C) with a 12 h light–dark cycle. Mice were given free access to irradiated food (UAR, Villemoisson, France) and sterilised water.

Experimental design

Identical experiments were performed using G- and HMA-mice with animals randomly assigned to one of the two treatment groups. The control group drank water while the second group had free access to water containing 4·5 % lactose throughout the experiment. At time zero, the mice were administered a 0·5 ml orogastric dose containing *S. thermophilus* culture (inoculum of 10^8 colony-forming units (CFU)/ml) mixed with *B. stearothermophilus* spores (inoculum of approximately 10^8 CFU/ml). A tube was passed through the mouth into the stomach, allowing the direct injection of the bacterial suspension. The mice were then killed at various times after inoculation for digestive content analysis. Four to six animals were sampled at each time point.

Sampling and analysis of digestive content

The mice were killed by cervical dislocation and the entire intestine of each mouse was immediately removed. The SI2 and the CC compartment were then isolated and the total content was separately collected, weighed and diluted 10-fold in liquid casein–yeast extract medium.

Appropriate dilutions of each sample were used for plating on selective media. Viable *S. thermophilus* cells were enumerated on selective M17-agar plates containing erythromycin. Using this antibiotic, the autochthonous microbiota do not interfere with the counting of the erythromycin resistant strain FBI3. The *B. stearothermophilus* spores were used as transit markers to follow the inoculum through the digestive tract. Spores that remain totally inert during transit, showing neither growth nor destruction, can be easily counted on agar plates at 60°C, a temperature at which intestinal bacteria are unable to develop.

Luciferase assay

Luciferase activity was determined with a Lumat LB 9507 tube luminometer (Berthold Technologies, Bad Wildbad, Germany). Light emission was measured immediately after the addition of 5 μl decylaldehyde (Sigma Aldrich, France) to the 10-fold-diluted fresh digestive samples, as previously described (Oozeer *et al.* 2004). The results are expressed hereafter as micro-relative light units/CFU.

Results

Transit and survival of Streptococcus thermophilus

In G-mice (Fig. 1(A)), bacterial populations increased between 0·5 and 2·5 h post-inoculation in both the SI2 and the CC compartments. In the meantime, *S. thermophilus* cell density remained similar to that of transit marker spores, indicating a full survival of the bacteria. After 2·5 h, bacterial populations reached a plateau in the CC compartment (between 10^6 and 10^7 CFU/g digestive content), while cell densities started to drop in the SI2 and finally reached undetectable levels after 10 h. The bacteria:spore ratio remained close to 1 in the CC during the plateau period while, in contrast, it progressively declined in the SI2 during the elimination period. *S. thermophilus* survival rate was, hence, near 100 % in the CC throughout the experiment, while it was strongly reduced to less than 0·2 % in the SI2 at 10 h (ratio $<0{\cdot}002$).

With HMA-mice (Fig. 2(A)), bacterial populations in the SI2 were maximal as early as 0·5 h post-inoculation (between 10^7 and 10^8 CFU/g digestive content, as for G-mice) and remained at this level for about 1 h before starting to decrease (cell densities reached undetectable levels after only 6 h). Maximal cell density was obtained about 1 h later in the CC (between 1·5 and 2·5 h) and was maintained at a plateau at about 10^7 CFU/g digestive content. As the bacteria:spore ratios indicated in G-mice experiments, *S. thermophilus* survival was not altered in the CC of HMA-mice, while less than 7 % of survival was observed in the SI2 after 4 h (ratio $<0{\cdot}07$).

In these GF- and HMA-mice experiments, no difference was observed regarding the enumerations of *S. thermophilus* and the bacteria:spore ratios whether lactose was added to the drinking bottle (Fig. 1(A) and Fig. 2(A)) or not (data not shown).

Luciferase activity in the digestive tract

The luciferase activity was measured for all the samples collected in the digestive compartments and the activity per bacterium was calculated using the bacterial enumerations reported in Fig. 1(A) and Fig. 2(A).

Luciferase activity kinetics in G-mice are described in Fig. 1(B) and Fig. 1(C) for the SI2 and CC, respectively.

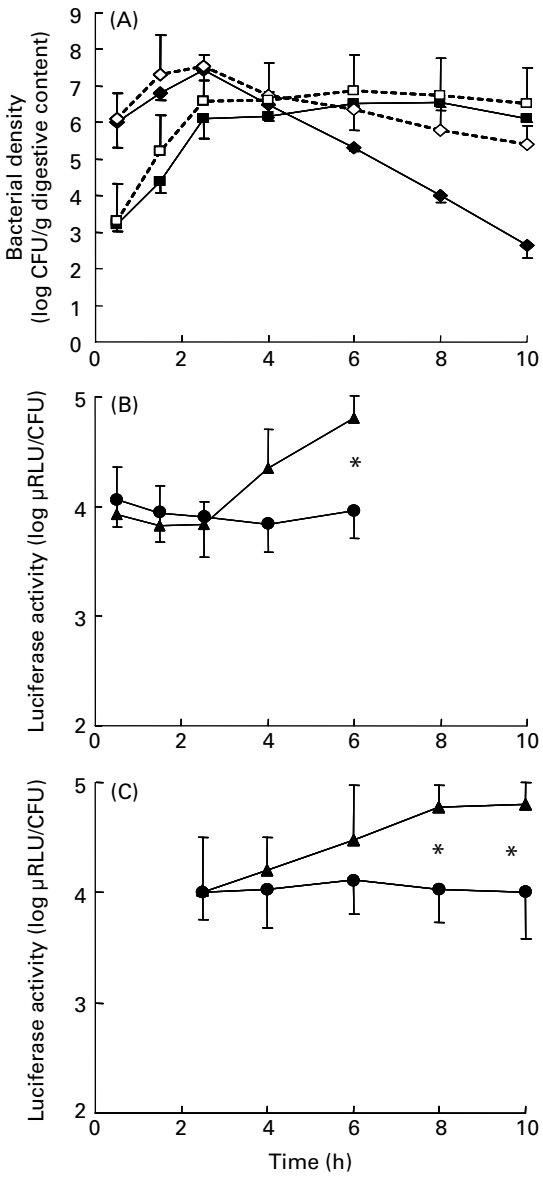

Fig. 1. (A) Transit kinetics of *Streptococcus thermophilus* strain FBI3 in the digestive compartments of gnotoxenic mice (G-mice). Lactose (4·5 %) was present in the drinking water provided to the animals. (◆), *S. thermophilus* cells in second half of the small intestine (SI2); (■), *S. thermophilus* cells in caecum–colon (CC) compartment; (◇), *Bacillus stearothermophilus* spores used as transit markers in CC compartment; (□), *B. stearothermophilus* spores in CC compartment. CFU, colony-forming units. (B,C) Variations in luciferase activity of *S. thermophilus* strain FBI3 in the digestive compartments of G-mice. Luciferase activities in the SI2 (B) and in the CC compartment (C) were expressed as a function of bacterial density. For time points greater than 6 h in (B) and for time point 0·5 h in (C), cell densities were not sufficient to measure significant luciferase activity. (●), No lactose added to the drinking water; (▲), drinking water containing 4·5 % lactose. RLU, relative light units. Values are means (n 3), with their standard deviations represented by vertical bars. * Mean values were significantly different ($P<0·05$; Student's t test).

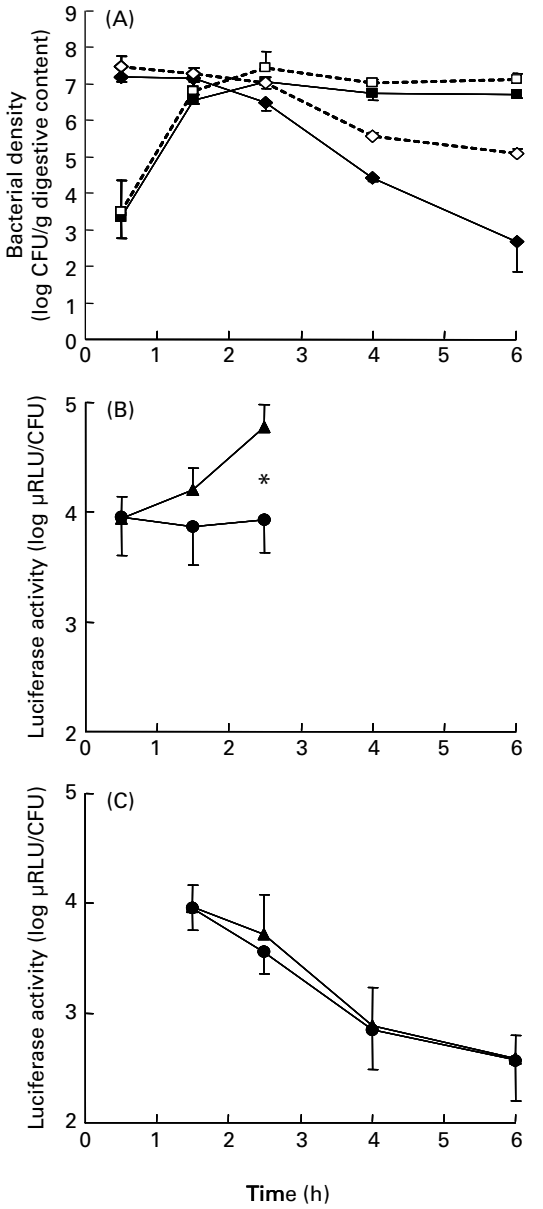

Fig. 2. (A) Transit kinetics of *Streptococcus thermophilus* strain FBI3 in the digestive compartments of human-microbiota-associated mice (HMA-mice). Lactose (4·5 %) was present in the drinking water provided to the animals. (◆), *S. thermophilus* cells in second half of the small intestine (SI2); (■), *S. thermophilus* cells in caecum–colon (CC) compartment; (◇), *Bacillus stearothermophilus* spores used as transit markers in CC compartment; (□), *B. stearothermophilus* spores in CC compartment. CFU, colony-forming units. (B, C) Variations in luciferase activity of *S. thermophilus* strain FBI3 in the digestive compartments of HMA-mice. Luciferase activities in the SI2 (B) and in the CC compartment (C) were expressed as a function of bacterial density. For time points greater than 2·5 h in (B) and for time point 0·5 h in (C), cell densities were not sufficient to measure significant luciferase activity. (●), No lactose added to the drinking water; (▲), drinking water containing 4·5 % lactose. RLU, relative light units. Values are means (n 3), with their standard deviations represented by vertical bars. * Mean values were significantly different ($P<0·05$; Student's t test).

When lactose was not added to the drinking bottle, high and stable luciferase activities (about 10^4 micro-relative light units/CFU) were detected both in the SI2 (Fig. 1(B)) and the CC (Fig. 1(C)). In the presence of 4·5 % lactose, the luciferase activity started to increase 2·5 h post-inoculation whatever the compartment (Fig. 1(B) and Fig. 1(C)).

Luciferase activity kinetics in the SI2 of HMA-mice (Fig. 2(B)) were similar to those of G-mice, except that the increase in activity was observed about 2 h earlier. In contrast, luciferase activity in the CC of HMA-mice (Fig. 2(C)) was

reduced about 1·5 log in a 4 h period while *S. thermophilus* viability remained unchanged. A similar decrease in luciferase activity was observed whatever the degree of presence or absence of lactose (Fig. 2(C)).

Discussion

In a previous study (Drouault *et al.* 2002), we showed that the transcriptional coupling of luciferase genes with the promoter of the *lac* operon was a powerful tool to assess the *in vitro* or *in vivo* production levels of the lactose-hydrolysing enzyme. We actually demonstrated for *S. thermophilus* that the two enzyme activities were linked both in culture media and in G-mice faeces (Drouault *et al.* 2002). The special feature of such a luciferase-based reporter system is that the activity of the enzyme can be detected exclusively in fully viable cells (Corthier *et al.* 1998). Indeed, light emission requires available reduced FMN and this depends on the metabolic state of the cells (Meighen, 1991). Hence, it can be assumed that when luciferase activity is observed, β-galactosidase is produced by living *S. thermophilus* cells and not released by dead ones. Otherwise, several studies showed that distinct species of lactic acid bacteria contained non-limiting amounts of FMN for luciferase production in the digestive tract of G- or HMA-mice (Corthier *et al.* 1998; Drouault *et al.* 2002; Oozeer *et al.* 2002, 2004). From the analysis of the faeces of G-mice, our previous work concluded that *S. thermophilus* can increase β-galactosidase production when lactose was added to the diet of the animals (Drouault *et al.* 2002).

In the present study, we used the same luciferase approach to examine the production of β-galactosidase from the *S. thermophilus* FBI3 strain in distinct digestive compartments of G- and HMA-mice. The goal of comparing the two animal models was to examine the influence of a human microbiota on the enzyme production in the digestive tract. The transit of the bacteria was monitored over time by selective enumeration of cells recovered from the digestive contents of the SI2 and the CC compartments. As previously described (Drouault *et al.* 2002), *B. stearothermophilus* spores were used as transit markers and were co-inoculated with *S. thermophilus* at the same cell density. In this way, variations in the bacteria:spores ratio could account for the survival of *S. thermophilus* cells in the different compartments.

For a given compartment, the general patterns of transit kinetics and survival rates were similar in G- and HMA-mice; *S. thermophillus* density increased to a maximum level in the SI2 before an elimination phase occurred and the survival rate dropped, while bacterial density reached a plateau in the CC where the survival rate stabilised at nearly 100 %. These patterns were not affected by the presence or absence of lactose in the animal diet, indicating that lactose did not modify the transit or the survival of *S. thermophilus* strain FBI3. The difference between the two animal models may lie in variations in transit times, suggesting that G-mice exhibited delayed transit as compared with HMA-mice. Interestingly, survival rates found in our mice models contrast with generally lower survival capacities of yoghurt bacteria reported in clinical trials, although results in human subjects are rather conflicting and seem to depend on the methodological approach (Mater *et al.* 2005).

Our data on luciferase activities show that the enzyme was expressed at relatively high levels although the animals did not drink lactose. These results are consistent with our previous observations suggesting that the promoter of the *lac* operon of *S. thermophilus* is leaky and functional even in the absence of lactose in the drinking water (Drouault *et al.* 2002). In G-mice drinking lactose, luciferase activity measurements indicate that β-galactosidase production starts during transit through the SI2 and continues in the CC. These results reflect the induction of the operon into the digestive tract of G-mice. In the SI2 of HMA-mice, supplementing the drinking water with lactose also leads to the induction of the *lac* operon of *S. thermophilus*. Although luciferase activity is similar to that of G-mice, the enhancement of β-galactosidase production is quicker in HMA-mice than in G-mice. Since luciferase activities were expressed as a function of bacterial density, this observation cannot be ascribed to the variations in the transit times described earlier. In contrast, it could be explained by differences between environmental conditions in the SI2 of HMA- and G-mice. The resident microbiota present in the ileal compartment may, especially, play a role and modify the genetic expression features of *S. thermophilus*.

In the CC compartment of HMA-mice, β-galactosidase production was shown to be drastically reduced whether lactose was provided or not to the animals. Thus, lactose had no influence on luciferase expression in the CC content of HMA-mice. In the meantime, bacterial cells were kept in perfect living conditions, as confirmed by survival rate data. This observation thus suggests that the resident microbiota can significantly affect *S. thermophilus* physiology but does not alter cell viability. Oozeer *et al.* (2002) did not observe a similar effect of the human microbiota on *Lactobacillus casei* cells which initiated new protein synthesis under the same conditions. The influence of microbiota on cell physiology might thus be genus- and even species-specific.

Yoghurt consumption is known to enhance lactose digestion in β-galactosidase-deficient individuals (Savaiano *et al.* 1984; Lerebours *et al.* 1989; Marteau *et al.* 1990; Varela-Moreiras *et al.* 1992; Shermak *et al.* 1995; Rizkalla *et al.* 2000). Several authors have shown that lactose concentration decreases in the small intestine, thus preventing further sugar fermentation by the colonic microbiota (Lerebours *et al.* 1989; Marteau *et al.* 1990). In the present study, the results demonstrate that in mice harbouring such a human microbiota, live *S. thermophilus* can produce β-galactosidase in the small intestine but not in the CC segment. These results strongly support the idea that live bacterial strains from yoghurt can degrade lactose as of the small intestine, although lactose digestion was not measured in the present study. The present study also emphasises the importance of performing studies with HMA-animal models (in addition to gnotoxenic ones) to take the modulation of bacterial physiology by the autochtonous microbiota into account. Clinical investigations are now needed to confirm our observations using human ileal fluid samples.

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