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Lactobacillus casei Is Able To Survive and Initiate Protein Synthesis during Its Transit in the Digestive Tract of Human Flora-Associated Mice

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Live *Lactobacillus casei* is present in fermented dairy products and has beneficial properties for human health. In the human digestive tract, the resident flora generally prevents the establishment of ingested lactic acid bacteria, the presence of which is therefore transient. The aim of this work was to determine if *L. casei* DN-114 001 survives during transit and how this bacterium behaves in the digestive environment. We used the human flora-associated (HFA) mouse model. *L. casei* DN-114 001 was genetically modified by the introduction of *erm* and *lux* genes, encoding erythromycin resistance and luciferase, respectively. For this modified strain (DN-240 041), light emission related to luciferase expression could easily be detected in the contents of the digestive tract. When inoculated into the digestive tract of HFA mice, *L. casei* (DN-240 041) survives but is eliminated with the same kinetics as an inert transit marker, indicating that it does not establish itself. In pure culture of *L. casei*, luciferase activities were high in the exponential and early stationary growth phases but decreased to become undetectable 1 day after inoculation. Viability was only slightly reduced even after more than 5 days. After transit in HFA mice, luciferase activity was detected even when 5-day-old *L. casei* cultures were given to the mice. In culture, the luciferase activity could be restored after 0.5 to 7 h of incubation in fresh medium or milk containing glucose, unless protein synthesis was inhibited by the addition of chloramphenicol or rifampin. These results suggest that in HFA mice *L. casei* DN-240 041, and thus probably *L. casei* DN-114 001, is able to initiate new protein synthesis during its transit with the diet. The beneficial properties of *L. casei*-fermented milk for human health might be related to this protein synthesis in the digestive tract.

Fermented milk containing live lactic acid bacteria shows probiotic effects (8, 35): yogurt with living *Lactobacillus bulgaricus* and *Streptococcus thermophilus* is far more efficient to prevent lactose intolerance than the same product with heat-killed bacteria (33). Live *Lactobacillus casei* reduces diarrhea (26, 27) and appears to modify the digestive microflora (9, 18) and to enhance the immune system during its transit in the digestive tract (DT) (25, 28). The question raised here is how the probiotic bacterium ingested with food survives and adapts to the environmental change from fermented milk to the DT.

The animal model we used is, from a bacteriological point of view, close to the human DT: germfree mice associated with a human flora (2, 20). The bacterial genera that are dominant in humans remain dominant in such mice, in contrast with conventional mice in which lactobacilli are dominant (12).

To study adaptation of *L. casei* in the DT, we developed a genetic approach successfully used with other lactic acid bacteria, *Lactococcus lactis* and *S. thermophilus* (6, 10, 11). It consists of transcriptional coupling of known promoters of the bacteria with the luciferase genes from *Vibrio harveyi* or *Photorhabdus luminescens* (13, 14). Using this approach it was demonstrated (6) that *L. lactis* can respond to a dietary stimulus (malate) in the DT and that *S. thermophilus* was able to produce beta-galactosidase during its transit in the DT (10).

The purpose of this work was to characterize survival during intestinal transit of *L. casei* DN-114 001 and a possible change(s) in *L. casei* DN-114 001 physiology during the environmental shift from fermented milk to the DT of human flora-associated (HFA) mice.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains and plasmids used in this study are presented in Table 1. *Escherichia coli* strain DH5α was grown aerobically at 37°C in Luria-Bertani medium. *L. casei* strain DN-240 041 was derived from the strain DN-114 001 (CNCM number, I-1518). The *L. casei* strains were grown at 37°C in MRS medium (7) or glucose-milk medium (GM medium; 174 g of milk powder/liter of water, autoclaved for 15 min at 110°C, 5% glucose). When required, the concentrations of antibiotics used were 50 μg of ampicillin or 100 μg of erythromycin per ml to select *E. coli* transformants and 5 μg of erythromycin or 5 μg of chloramphenicol per ml for *L. casei*. Chloramphenicol (ICN) and rifampin (Merck, Darmstadt, Germany) were used at 100 μg/ml for inhibition of translation and transcription studies, respectively. Thermoresistant *Bacillus subtilis* spores were used as transit markers and activated at 60°C in G-spore medium according to the method described for enumeration (4).

DNA manipulations. Purification of genomic DNA from *L. casei* was performed with the DNeasy tissue kit (Qiagen, Courtaboeuf, France). Restriction endonucleases, T4 DNA polymerase, T4 DNA ligase, and *Taq* DNA polymerase were obtained from Roche Diagnostics (Roche Diagnostics GmbH, Mannheim, Germany) and were used according to the instructions of the manufacturers. *L. casei* was transformed by electroporation with a Gene-Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) as previously described (31). Plasmid DNA extractions were performed by a method adapted from that of Sambrook et al. (34).

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristic(s)	Reference or source
Strains		
E. coli DH5α	F⁻ *lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA endA hsdR17 phoA supE* λ⁻ *thi-1 gyrA relA*	Life Technologies
Lactic acid bacteria		
JIM4930	*L. lactis* strain IL1403 containing the plasmid pJIM2530	32
DN-114 001	*L. casei* Danone strain	
DN-240 013	*L. casei* strain DN-114 001 containing the plasmid pJIM2530	This work
DN-240 034	*L. casei* strain DN-114 001 containing the plasmid pDN13	This work
DN-240 041	DN-114 001, Em^r, *lacTp*::*luxAB* on pDN26 integrated in the chromosome	This work
Plasmids		
pBluescript	Amp^r, M13*ori* pBR322*ori*	Stratagene
pGEM-T	Amp^r, M13*ori* pBR322*ori*, linear T-overhang vector	Promega
pDN2	Amp^r, 423-bp fragment of *L. casei* containing *lacTp* cloned in pGEM-T	This work
pmj763	Cm^r, pSH71*ori*	21
pJIM2279	Em^r, derivative of pIL252 with *copF*::linker	32
pJIM2530	Em^r, derivative of pJIM2279 with *luxAB* genes from *V. harveyi*	32
pDN13	Em^r, derivative of pJIM2279 with *luxAB* genes from *P. luminescens*	This work
pVE6007	Cm^r, thermosensitive derivative of pGKV12	22
pDN20	Em^r, *ori* (pWV01 Δ*repA*) with *luxAB* genes from *P. luminescens*, integrative vector	This work
pDN26	Em^r, derivative of pDN20 carrying *lacTp*::*luxAB*	This work

Plasmid and strain constructions. In order to test different *lux* genes, a plasmid similar to pJIM2530 (32) but carrying *lux* genes from *P. luminescens* was constructed as follows. *luxAB* genes from *P. luminescens* were obtained by *Eco*RI and *Bam*HI digestion of the plasmid pmj763. The resulting fragment of 2.35 kb was cloned into pBluescript. This plasmid was digested with *Sac*I and ligated with pJIM2279 also cut by *Sac*I. This large hybrid plasmid was then digested with *Bcl*I and *BssH*II, and blunt ends were created with T4 DNA polymerase and religated in order to place *lux* genes under the control of the promoter of the plasmid resolvase as in plasmid pJIM2530.

A transcriptional fusion of the *L. casei* DN-114 001 *lacTEGF* operon promoter with the *lux* genes from *P. luminescens* was constructed. The sequences of the *lacTEGF* operons from the strains *L. casei* 64H (1) and ATCC 393 pLZ15⁻ (17), available in GenBank (accession no. U21391 and Z80834, respectively), were used to design primers OFF21 (5′-GGCATCGTCGATGAGCCAA-3′) and OFF25 (5′-GCCTAATTAAATAGTCACAATCCAC-3′). The PCR fragment of 423 bp obtained with the primers OFF21 and OFF25 and containing the *lac* promoter (*lacTp*) lacks the stem-and-loop structure involved in the antiterminator (LacT)-dependent induction in the presence of lactose. The promoter region was cloned into pGEM-T, generating plasmid pDN2. pDN2 was digested with *Sal*I and ligated with pDN20, an integrative vector carrying the *luxAB* genes from *P. luminescens*, also digested with *Sal*I. The resulting plasmid was cut with *Pvu*II, leading to the transcriptional fusion of the constitutive lactose promoter, *lacTp*, and the *luxAB* genes. This plasmid, called pDN26, was used to introduce the construction into the *L. casei* DN-114 001 strain. DN-114 001 was first transformed with pVE6007, a thermosensitive plasmid (22) used as helper plasmid to provide the replicase for pDN26 replication. The strain was then transformed with pDN26 which could replicate in the presence of pVE6007 at a permissive temperature (30°C). A shift to a restrictive temperature (42°C) inhibiting plasmid pVE6007 replication allowed the insertion of pDN26 into the chromosome by homologous recombination. Integrants were analyzed by PCR with primers OFF43 (5′-GGTCATTAAAACCGGTCGCGTG-3′), located upstream of the *lacTp*, and OFF44 (5′-CCCAGATTAACCAATCGC-3′), located in the *luxA* gene, to check for correct insertion into the chromosome.

Growth curves and luminescence measurements. GM medium was inoculated at a 1/10 dilution with exponentially growing *L. casei* culture (optical density at 600 nm of around 0.3 in a Jenway 6400 spectrophotometer [Felsted, United Kingdom]). Cultures were grown at 37°C and samples were collected at regular intervals for measurement of luminescence and enumeration of bacteria. The luciferase activity was measured immediately after the addition of 5 μl of decanal (Sigma, Detroit, Mich.) to 1 ml of a broth culture. Light emission was measured in a luminometer (LB9501; Berthold, Germany). The values obtained were expressed as micro-relative light units (μRLU) per CFU or RLU per optical density unit at 600 nm.

HFA mice. Germfree C3He/J mice were reared in sterile Texler-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 sterilized water. To obtain HFA mice, a breeding stock of donors was prepared: one healthy human volunteer donated fecal material used as inoculums over 3 intervals of 3 weeks in germfree animals. Germfree animals for the experiments received 0.5 ml of a 1/10 fecal dilution from donor mice associated with human flora three times in a 3-week interval.

Experimental design. One hundred milliliters of GM medium containing erythromycin was inoculated with an overnight culture of *L. casei* DN-240 041 and maintained at 37°C for 5 days. Inoculum size was chosen such that the culture was saturated after 1 day and little initial luciferase activity was detected. The viability of bacteria and the regeneration of the luciferase activity (see below) were studied daily in culture and in the DT of HFA mice.

Regeneration of luciferase activity. (i) In culture. Culture samples were diluted (1/10) in prewarmed medium containing erythromycin and incubated at 37°C. The experiment was carried out with and without addition of chloramphenicol or rifampin to the medium. Samples were collected at different times. Enumerations and luminescence measurements were performed until 12 h after dilution.

(ii) In DT. Mice received 0.5 ml of a mix of 4 ml of *L. casei* culture and 1 ml of *B. subtilis* spore solution (10^8 CFU of each per ml) at time zero by oral administration. Feces were collected individually 6 h later. Enumerations and luminescence measurements were performed with feces diluted to 1/10 in saline buffer.

Fecal elimination of *L. casei* in HFA mice. Each mouse received, by oral administration, 0.5 ml of a mix containing *L. casei* culture and spores as described above. Fecal samples were collected from different animals every 2 h after inoculation until 24 h. Enumerations of *L. casei* and spores were immediately performed as described above.

RESULTS

Genetic constructions and luciferase expression in GM broth. (i) Construction of *L. casei* with luciferase activity. Several vectors have been previously developed with the *lux* genes isolated from *V. harveyi* and used successfully in *L. lactis* (5, 6, 13). One of these, pJIM2530, was introduced into *L. casei* DN-114 001. The resulting strain, DN-240 013, exhibited very poor luciferase activity (3,500 RLU per optical density unit) in contrast to the level of luciferase activity obtained with the same plasmid introduced in *L. lactis* IL-1403, strain JIM4930 (500,000 RLU per optical density unit). *lux* genes isolated from another microorganism, *P. luminescens*, were tested in *L. casei*. For this purpose, plasmid pDN13 was constructed in such a

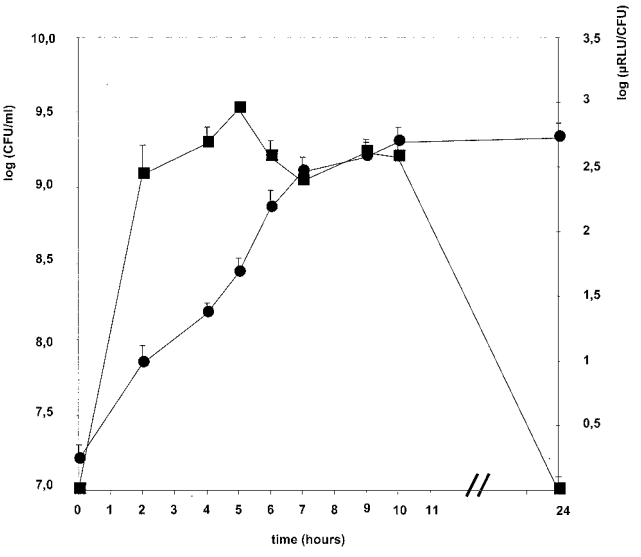

FIG. 1. Growth and luciferase expression of *L. casei* DN-240 041 GM broth. Cultures of *L. casei* DN-240 041 were grown at 37°C and samples were removed at timed intervals for bacterial enumeration (circles) and luminescence measurement (squares). The luciferase activity was measured immediately after the addition of 5 μl of decanal to 1 ml of culture.

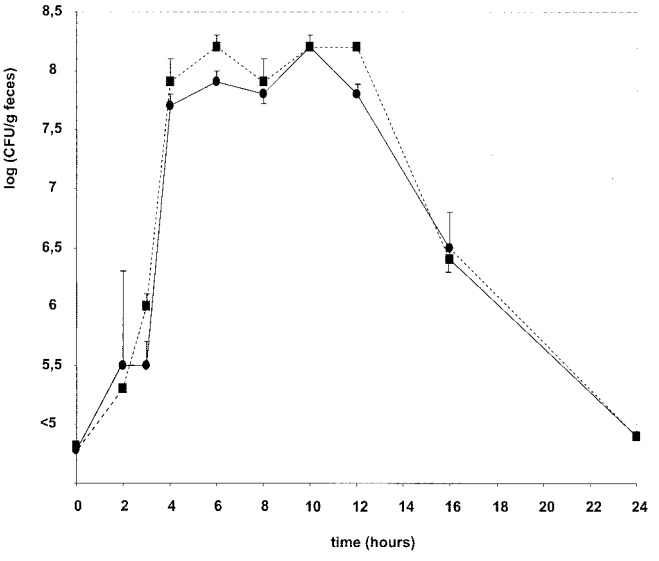

FIG. 2. Fecal elimination kinetics of *L. casei* DN-240 041. Mice received 0.5 ml of a mix containing *L. casei* culture (about 10^8 CFU/ml) and *B. subtilis* spores (same quantity) (transit markers) by oral administration. Fecal samples were collected every 2 h after inoculation until 24 h. Bacteria (circles) and spores (squares) were analyzed in triplicate; fecal samples from each mouse were plated on MRS agar with erythromycin and on G-spore medium to enumerate *L. casei* DN-240 041 and *B. subtilis*, respectively.

way that the *lux* genes from *P. luminescens* were in the same genetic context (vector, promoter) as the *lux* genes from *V. harveyi* in pJIM2530. Plasmid pDN13 was introduced in *L. casei* strain DN-114 001 and luciferase activity was measured for the resulting strain, DN-240 034. This strain exhibited a 90-fold increase in luciferase activity compared to strain DN-240 013. *lux* genes from *P. luminescens* were therefore used to construct a *lacTp*::*luxAB* transcriptional fusion.

This fusion was integrated in the DN-114 001 chromosome by Campbell-like recombination using a two-step procedure previously described for *L. lactis* (15) involving the use of a thermosensitive helper plasmid. The resulting strain, used in this study, was named DN-240 041.

(ii) Growth and luciferase expression in GM broth. Growth of strain DN-240 041 in GM broth was assessed by enumeration of CFU (Fig. 1). Strain DN-240 041 exhibited a generation time of about 1 h. Moreover, growth of modified strain DN-240 041 was similar to the growth of its parental strain, DN-114 001 (data not shown). When the strain DN-240 041 was grown in GM broth, luciferase expression was high during the exponential phase and remained high until the culture reached a density of 10^9 CFU/ml (6 h). Then luciferase activity gradually decreased and no luciferase activity was detected in the late stationary phase. No luciferase activity was observed after 1 day of incubation at 37°C.

Influence of an environmental shift from GM to the DT. (i) *L. casei* survival during intestinal transit in HFA mice. An experiment was carried out to determine the kinetics of *L. casei* fecal excretion after a single oral inoculation into HFA mice. Thermoresistant *B. subtilis* spores were added to a fresh *L. casei* culture before inoculation and served as an inert intestinal transit marker (Fig. 2). The number of *L. casei* bacteria and spores in the feces reached a plateau of 10^8 CFU/g between 4 and 12 h after inoculation. Then the levels of both bacterial species decreased to reach 10^6 CFU/g after 14 h and levels below 10^5 CFU/g 1 day after inoculation. The ratio between bacteria and spores remained constant during the elimination period. In the following experiments, fecal samples were collected 6 h after inoculation.

The effect of the age of the initial culture in GM broth on the survival during intestinal transit was analyzed. The viability of *L. casei* remained constant during the 5-day incubation period in GM broth at 37°C. The same viability of bacteria was found in fecal samples collected 6 h after inoculation (Fig. 3).

(ii) Luciferase activity after transit. Experiments were designed to determine whether *L. casei* shows metabolic activity during transit. Measurement of luciferase activity during the DT transit is the way chosen to estimate if *L. casei* is able to synthesize protein during transit in the DT.

When *L. casei* was cultured in GM medium, 12 h after inoculation the luciferase activities were high in pure culture and in HFA mouse feces (Fig. 3). When the culture was older than 1 day, however, the luciferase activity was not detectable in the inoculum. The acidity is not the main reason for the drop of luciferase activity since a shift to neutral pH did not enhance the activity except for in the 2-day culture (data not shown). After passage through the DT, luciferase activities were enhanced 30- to 100-fold (Fig. 3). The reinitiation of luciferase activities in the DT occurred while no bacterial growth was observed.

(iii) Restart of growth and luciferase activity of *L. casei* in culture. Experiments in pure culture were carried out to determine if reinitiation of luciferase activity is linked to new protein synthesis. Stationary overnight cultures in GM medium reinitiated growth and showed luciferase activity very quickly after dilution (1/10) in fresh medium (data not shown). For

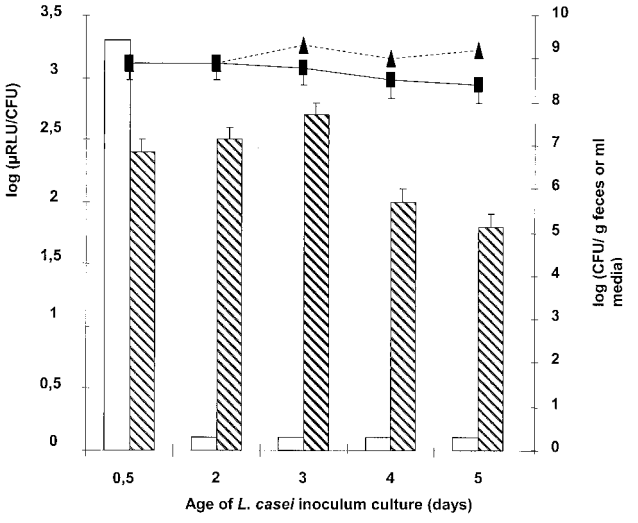

FIG. 3. Regeneration of luciferase activity in vivo from GM culture. Mice received 0.5 ml of a mix of *L. casei* (10^8 to 10^9 CFU/ml) culture and spores (same quantity) of *B. subtilis* (transit markers) daily at 10 a.m. by oral administration. Feces were collected 6 h later. Enumerations (E) and luminescence (L) measurements were performed with inoculum (E, triangles; L, empty columns) and feces (E, squares; L, hatched columns) diluted to 1/10.

older cultures the reinitiation of growth was delayed, although the number of viable cells directly after dilution was the same as at the beginning of the stationary phase. Figure 4 illustrates this with *L. casei* strain DN-240 041 cultivated for 3 days in GM medium: cultures were diluted in prewarmed medium and kept at 37°C. The luciferase activity increased very rapidly, whereas multiplication was delayed for 4 h. Under the same conditions

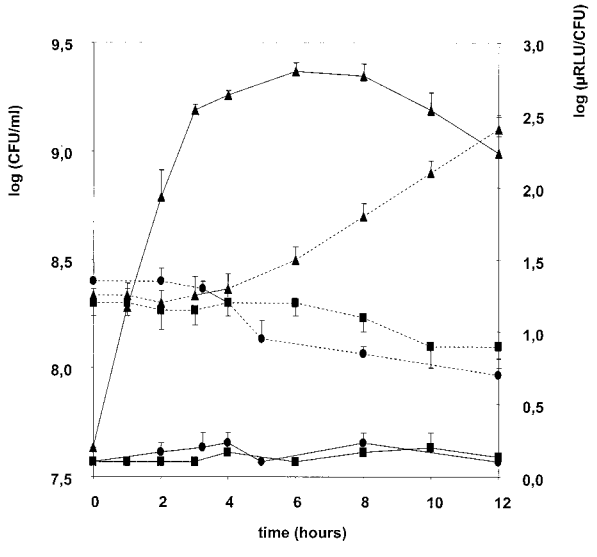

FIG. 4. Regeneration of luciferase activity in pure culture of *L. casei*. Three-day-old culture samples were diluted (1/10) in prewarmed GM medium containing erythromycin (triangles) and incubated at 37°C. Further experiments were carried out with a supplement of chloramphenicol (squares) or rifampin (circles) in the medium. Enumerations (dotted lines) and luminescence (solid lines) measurements were performed until 12 h after dilution.

during the first 4-h period, chloramphenicol or rifampin addition blocked luciferase activity but did not affect *L. casei* viability.

After the 4-h delay period, bacterial growth became visible in GM medium, and luciferase activities were similar to those observed in Fig. 1. Chloramphenicol or rifampin additions reduced viability after the 4-h delay.

DISCUSSION

A previous approach using a modified *L. lactis* strain (5, 6, 13) gave us the ability to evaluate how this lactic acid bacterium behaves during transit in HFA mice. No effect of *L. lactis* as a probiotic on human health was reported. In this work the approach was adapted to *L. casei*, which is known as a probiotic for humans. It represents the first successful attempt to express luciferase genes in *L. casei*. Luciferase from *P. luminescens* shows a higher activity in *L. casei* than luciferase from *V. harveyi*. This may be due to a higher stability of this enzyme at high temperature since the optimal temperature for the *P. luminescens* luciferase activity is 45°C compared to 30°C for the *V. harveyi* enzyme (19). In pure cultures, *L. casei* (DN-240 041) shared similar features with *L. lactis* (6) since luciferase activity was high in the exponential growth phase and reduced in the late stationary phase.

The HFA mouse model reflects the human situation concerning the antagonistic effect against lactic acid bacteria: *L. casei* did not multiply but survived well as demonstrated by the fact that the ratio between the bacteria and spores used as transit markers remained constant. This observation is consistent with what has been observed in humans where different lactic acid bacteria were progressively eliminated from the DT (16, 23, 37). We determined the transit time based on fecal excretion of *L. casei*: a maximum was observed between 4 and 12 h postinoculation. This period was not precisely estimated in humans since fecal emission is more rare than in mice. Bouhnik et al. (3) observed that the level of bifidobacterium decreases 1 day after product consumption. Pochart et al. (29) observed that the transit time was 2 h postingestion by using in vivo ileal perfusion. In this HFA model we chose a 6-h delay between inoculation and fecal sample collection for further experiments.

When exponential-phase cultures were used to inoculate mice, the luciferase activity per CFU measured in the inoculum was recovered in the feces. In contrast, when old stationary-phase cultures were used to inoculate mice, the luciferase activity per CFU observed in the corresponding fecal samples was high while it was not detectable in the inoculum. These data suggested that *L. casei* DN-240 041, which transited the DT without any detectable multiplication, underwent a physiological change resulting in the reinitiation of luciferase activity.

We have studied, in pure culture, the restart of luciferase activity with such aged *L. casei* cultures: during the first 4 h after dilution into fresh culture medium luciferase activity increased to a large extent while no multiplication could be observed. Such reinitiation of luciferase activity preceding *L. casei* multiplication was blocked by rifampin or chloramphenicol. These antibiotics are known to block transcription (24) and translation (30), respectively, and we therefore postulate that the reinitiation of luciferase activity corresponded to the synthesis of new proteins. The protein(s) could be LuxAB itself,

another protein(s) needed for the enzymatic reaction (36), or protein synthesis affecting the global physiology of the cell.

Based on our studies, performed in pure culture with chloramphenicol and rifampin, where reinitiation of luciferase activity was shown to be linked to the synthesis of a new protein(s), the reinitiation of luciferase activity observed after transit suggests that *L. casei* not only survives in the DT but also synthesizes new protein during its transit in the DT. The environmental shift from fermented milk to DT may induce protein synthesis in *L. casei* to adapt to the new conditions. This feature is observed without bacterial division. To our knowledge, this is the first study to suggest that lactic acid bacteria in transit in human flora synthesize proteins. The promoter used in the luciferase construct could influence this result. Other promoters may be more or less active as previously observed with the same luciferase reporter gene (10). This work opens a perspective for comparison of different *L. casei* promoters in HFA mice.

In previous work dealing with *L. lactis*, it was shown that bacteria adapted to diet stimulation (6), but the experiments were carried out in germfree mice. Here we investigated HFA mice. The presence of autochthonous microflora exerted an antagonistic effect on *L. casei* growth, but the bacterium was still able to adapt to the environment during transit. This feature is of importance for probiotic bacteria that are supposed to exert in the DT functions beneficial for human health. The next step will be to determine which functions are induced in the bacteria during digestive transit.

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