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Initiation of Protein Synthesis by a Labeled Derivative of the *Lactobacillus casei* DN-114 001 Strain during Transit from the Stomach to the Cecum in Mice Harboring Human Microbiota

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Although studies on the survival of bacteria in the digestive tract have been reported in the literature, little data are available on the physiological adaptation of probiotics to the digestive environment. In previous work, a transcriptional fusion system (i.e., luciferase genes under the control of a deregulated promoter) was used to demonstrate that a derivative of the *Lactobacillus casei* DN-114 001 strain, ingested in a fermented milk and thus exhibiting initially a very weak metabolic activity, synthesized proteins de novo after its transit in the digestive tract of mice harboring human microbiota (known as human-microbiota-associated mice). With the same genetic system and animal model, we here investigate for the first time the ability of *L. casei* to reinitiate synthesis in the different digestive tract compartments. In this study, most ingested *L. casei* cells transited from the stomach to the duodenum-jejunum within 1 h postingestion. No luciferase activity was observed in these digestive tract compartments after the first hour. At later times, the bulk of bacteria had transited to the ileum and the cecum. Luciferase synthesis was detected between 1.5 and 2.0 h postingestion at the ileal level and from 1.5 h to at least 6.0 h postingestion in the cecum, where the activity remained at a maximum level. These results demonstrate that ingested *L. casei* (derivative of the DN-114 001 strain) administered via a fermented milk has already reinitiated protein synthesis when it reaches the ileal and cecal compartments.

It is well accepted that probiotic bacteria should remain alive during intestinal transit to exert beneficial effects on the host (10, 12), although one should not exclude the possibility that cell fragments or components may also exert physiological benefits especially related to potential immunomodulation (22, 23). The digestive tract (DT) is known to display active defense against exogenous bacteria (2). Since most probiotic strains are originally selected for their fermentation properties, regardless of their survival rate or metabolic activity in the DT, their intestinal fate is worth investigating. *Lactobacillus casei* is considered a beneficial bacterium. Studies have suggested that *L. casei* DN-114 001 reduces acute diarrhea in healthy children (19, 20), modulates ex vivo the production of proinflammatory cytokines in Crohn's disease (4) and enhances the immune system during its transit in the DT (18, 21). Little work has been carried out on *L. casei* recovery in DT content and, furthermore, on its pharmacokinetics in the human gastrointestinal tract. Viable *L. casei* (strain Shirota) cells were detected at 10^7 CFU · g^{-1} of feces after a 3-day consumption of about 10^{10} bacteria per day (26). When a PCR-based detection system was used, *L. paracasei* (strain LHT 2579) cells were recovered at between 10^7 and 10^8 CFU · g^{-1} of feces (5). In addition to intestinal survival, an active metabolism for transiting bacteria is certainly an important feature which may act on parameters, leading to beneficial effects. The survival and metabolic activity of a derivative of *L. casei* strain DN-114 001 (*L. casei* DN-114 001^{lux}) has previously been investigated in feces in a model that used mice harboring human microbiota (known as human-microbiota-associated [HMA] mice) (17). A modified *L. casei* DN-114 001 strain carrying a transcriptional fusion between a partial *lacTEGF* operon promoter and the *luxAB* genes from *Photorhabdus luminescens* was constructed. This strain initiated new protein synthesis, as revealed by fecal sample analysis.

In the present work, we address the following question: in what compartment of the DT does *L. casei* DN-114 001^{lux} start to initiate protein synthesis during the intestinal transit? It is observed that metabolically inactive *L. casei* (DN-114 001^{lux}) cells restart luciferase activity 1.5 to 2 h after ingestion, when the bulk of bacteria reaches the ileum. These results reveal that the digestive environment is far from being hostile to the *L. casei* DN-114 001 derivative strain.

MATERIALS AND METHODS

Bacterial strains and media. *L. casei* DN-240 041 (*L. casei* DN-114 001^{lux}) is a derivative of the strain DN-114 001 (CNCM I-1518). It contains, integrated in the chromosome, a transcriptional fusion of *luxAB* genes from *P. luminescens* and the *lacT* promoter lacking the ribonucleic antiterminator sequences and a gene coding for erythromycin resistance (1). DN-114 001^{lux} was grown at 37°C in MRS medium (7) or glucose-milk (GM) medium (174 g of milk powder per liter of water [autoclaved for 15 min at 110°C], 5% glucose). When required, erythromycin or rifampin (Merck, Darmstadt, Germany) was added to achieve 5 $\mu g \cdot ml^{-1}$ or 100 $\mu g \cdot ml^{-1}$ final concentrations, respectively. Spores of thermoresistant *Bacillus stearothermophilus* were used as transit markers and were enumerated after activation at 60°C in G-spore medium according to the method described by Contrepois and Gouet (6).

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TABLE 1. In vitro luciferase regeneration profiles from 4-day cultures[a]

Time (h)	Culture in medium without antibiotic		Culture in medium with rifampin	
	L. casei (log CFU · ml^{-1})	Luciferase (log μRLU · CFU^{-1})	*L. casei* (log CFU · ml^{-1})	Luciferase (log μRLU · CFU^{-1})
0.00	8.4 ± 0.1	ND[b]	8.4 ± 0.1	ND
0.25	8.4 ± 0.2	ND	8.2 ± 0.1	ND
0.50	8.3 ± 0.2	ND	8.4 ± 0.1	ND
1.50	8.4 ± 0.1	0.6 ± 0.2	8.4 ± 0.1	ND
3.00	8.3 ± 0.2	1.9 ± 0.4	8.3 ± 0.1	ND

[a] Regenerations were assessed by diluting (1/10) 4-day *L. casei* DN-114 001^{lux} cultures in fresh GM medium. The growth of *L. casei* DN-114 001^{lux} and luciferase activity were monitored until 3 h after the dilution. Regeneration experiments were performed in the absence of antibiotic or with the addition of rifampin.
[b] ND, no detectable luciferase activity.

Animal model. C3H/He mice were reared in sterile Trexler-type isolators (La Cahlène, Velizy, 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. HMA mice were obtained as previously described (17).

Preparation of the inocula. GM medium (10 ml) was inoculated at 1/1,000 with a preculture of *L. casei* DN-114 001^{lux} at an optical density at 600 nm between 2.0 and 3.0. The culture was maintained at 37°C for 4 days. The inoculation to HMA mice was performed using homogenous inocula in the different experiments. For each inoculum, luciferase activity was checked as well as the homogeneity of the regeneration profile of luciferase activity after a 10-fold dilution (Table 1). The regeneration of luciferase activity from 4-day-old cultures was monitored as previously described (17). Briefly, cultures were diluted (1/10) in prewarmed medium containing erythromycin and were incubated at 37°C. Samples were collected from 15 min to 3 h after dilution for enumeration and luminescence measurements. Regeneration experiments were carried out in the absence of antibiotic or with the addition of rifampin to the medium (17).

Inoculation of mice and digestive sample collection. For each experiment, at least three HMA mice (6 to 8 weeks old) were used. Each received 0.5 ml of a *L. casei* DN-114 001^{lux} GM culture (approximately 10^9 CFU · ml^{-1} of inoculum) mixed with *B. stearothermophilus* spores (approximately 10^8 CFU · ml^{-1} of inoculum). To facilitate the comparison between spore and *L. casei* data, spore concentrations were multiplied by a 10-fold factor, as indicated on the graphs. Orogastric inoculation made direct injection of a known volume of bacterial suspension possible. The animals were slaughtered at different moments after inoculation for analysis of the digestive contents.

Apparent survival of *L. casei* DN 114 001^{lux} cells compared to that of spore markers in the DT. The total contents of the stomach, small intestine (divided into two equal parts, SI1 and SI2, corresponding to duodenum-jejunum and ileum, respectively), and cecum were collected immediately after slaughtering and weighed. The average weight of the sampled digestive contents was 0.15 ± 0.02 g, 0.15 ± 0.01 g, 0.18 ± 0.02 g, or 0.23 ± 0.01 g for the stomach, the SI1, the SI2, or the cecum, respectively. Samples were then diluted (1/10) in liquid casein-yeast extract medium and separated into two sets. The first set was used to measure the luciferase activity immediately after dilution. Enumerations of *L. casei* DN-114 001^{lux} and *B. stearothermophilus* cells were made with the second set: samples were serially diluted in sterile saline water (0.9%), and 0.1 ml of each dilution was evenly spread on plates of freshly prepared medium. *L. casei* DN-114 001^{lux} survival was determined on selective MRS agar plates containing erythromycin, since this derivative is erythromycin resistant. The plates were incubated in anaerobic jars for 2 days at 37°C in an Anaerocult A system (Merck). Colonies grown under these conditions were checked for luciferase activity to confirm that they were actually *L. casei* DN-114 001^{lux} colonies. Spores were enumerated on agar plates aerobically incubated at 60°C, a temperature at which intestinal bacteria were unable to grow (6).

Assay for luciferase production by *L. casei* DN-114 001^{lux}. Luciferase activities were measured immediately after the addition of 5 μl of decyl-aldehyde (Sigma, Detroit, Mich.) to 1 ml of 10-fold-diluted digestive content or broth culture. Light emission was measured with a luminometer (LB9501; Berthold, Bad Wildbad, Germany). The obtained values were expressed thereafter as micro-relative light units (μRLU) per CFU. The detection limit of the luminometer for luciferase activity measurement was fixed at 500 RLU. On this basis and in accordance with the *lacT* promoter features (17), the minimal amount of cells that could be used for luciferase measurement was 10^7 CFU.

Calculation of *L. casei* DN-114 001^{lux} apparent survival. The percentage of survival of *L. casei* in the different DT compartments was determined with respect to the spore counts by the following calculation: [(count of *L. casei* DN-114 001^{lux} bacteria in a DT compartment/count of spores in the same compartment)/(count of *L. casei* DN-114 001^{lux} bacteria in the inoculum/count of spores in the inoculum)] as previously described (8).

RESULTS

A 4-day GM culture was used to inoculate HMA mice; in such a culture, *L. casei* DN-114 001^{lux} is in the late stationary phase and no longer has luciferase activity (17). The apparent survival of bacteria compared to that of spore markers and luciferase activity were then estimated in the different parts of the DT.

Transit of spore markers. At 15 min after mice were inoculated, *B. stearothermophilus* spores were recovered mainly from the stomach (9.6×10^7 CFU · g^{-1} of digestive content) (Fig. 1) and to a lesser extent from the SI1 (2.2×10^7 CFU · g^{-1} of digestive content) (Fig. 2), the SI2 (1.3×10^7 CFU · g^{-1} of digestive content) (Fig. 3), and the cecum (2.5×10^2 CFU · g^{-1} of digestive content) (Fig. 4). Spore concentrations remained stable at these levels for 1 h in the stomach and for 2 h in the SI1 before decreasing progressively to undetectable levels in the next 4 h. In contrast, spore concentrations increased in the SI2 and the cecum during 1 and 1.5 h, respectively. Then, the spore content declined gradually in the SI2 to 9.8×10^2 CFU · g^{-1} of digestive content at 6 h postinoculation, while the spore content of the cecum reached a plateau at 10^7 to 10^8 CFU · g^{-1} of digestive content and remained stable until the end of the experiment.

Apparent survival of transiting *L. casei* DN-114 001^{lux} bacteria compared to that of spore markers. (i) Stomach. At 15 min after inoculation, the majority of the inoculated bacteria and spores were detected in the stomach (Fig. 1). During the first hour, the apparent survival of *L. casei* DN-114 001^{lux} bacteria compared to that of spore markers was at least 90% in this compartment. Thereafter, the results of enumeration of the spores and the lactic acid bacteria differed markedly. The level of *L. casei* DN-114 001^{lux} declined rapidly to 10^2 to 10^3 CFU · g^{-1} of digestive contents, while spore numbers decreased slowly.

(ii) Duodenum-jejunum (SI1). Within 15 min of inoculation, *L. casei* DN-114 001^{lux} was already detectable in the SI1 at 2×10^8 CFU · g^{-1} of digestive content (Fig. 2). The apparent survival of *L. casei* DN-114 001^{lux} bacteria compared to that of spore markers dropped gradually after inoculation.

(iii) Ileum (SI2). The ratio between spores and *L. casei* DN-114 001^{lux} bacteria remained stable in this part of the DT. Apparent survival of *L. casei* DN-114 001^{lux} bacteria compared

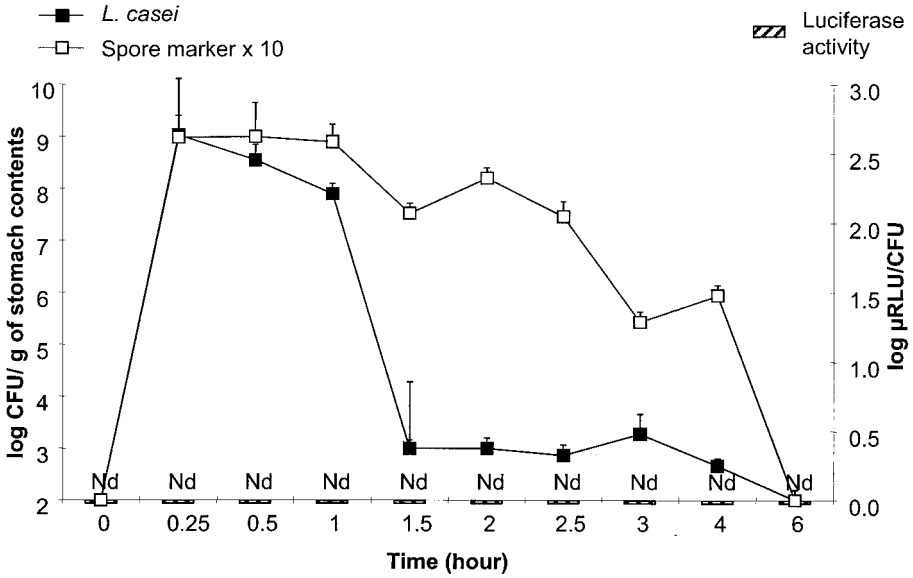

FIG. 1. Recovery of *L. casei* DN-114 001^{lux} bacteria (filled squares) and *B. stearothermophilus* spores (nonfilled squares) in the stomach. HMA mice were inoculated with 0.5 ml of a 4-day *L. casei* DN-114 001^{lux} GM culture mixed with *B. stearothermophilus*. The animals were slaughtered at different moments after inoculation. Enumeration of *L. casei* DN-114 001^{lux} bacteria and spores as well as luciferase activity measurement was performed on the DT contents. Column bars, luciferase activity; Nd, no detectable luciferase activity.

to that of spore markers was high (85 to 90%) in the DT environment once it had reached the ileum (Fig. 3). *L. casei* counts in the ileal samples progressively reached 10^8 CFU · g^{-1} of digestive content at 1 h after inoculation and remained stable for 2 h. *L. casei* DN-114 001^{lux} concentrations dropped steadily thereafter, following the same flux as the spores. The maximum concentration of *L. casei* DN-114 001^{lux} measured in the luminal content of the ileum was 5×10^8 CFU · g^{-1} of digestive content at 1 h.

(iv) Cecum. The flux levels of the lactic acid bacteria and transit marker were roughly parallel in this compartment (Fig. 4). The levels of *L. casei* DN-114 001^{lux} and spores rose steadily from 15 min to 1 h after inoculation. The apparent survival of *L. casei* DN-114 001^{lux} bacteria compared to that of spore markers remained at about 95% during the entire transit period.

Physiology of transiting bacteria. The use of a transcriptional fusion system with luciferase as a biosensor renders

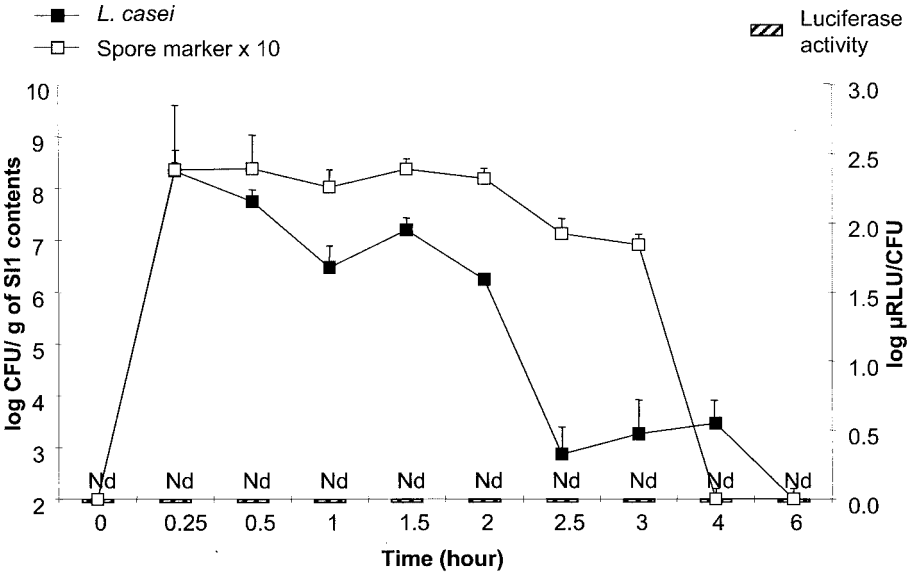

FIG. 2. Recovery of *L. casei* DN-114 001^{lux} bacteria (filled squares) and *B. stearothermophilus* spores (nonfilled squares) in the duodenum-jejunum (SI1). Inoculation of HMA mice and SI1 content analyses were performed as described for Fig. 1. Column bars, luciferase activity; Nd, no detectable luciferase activity.

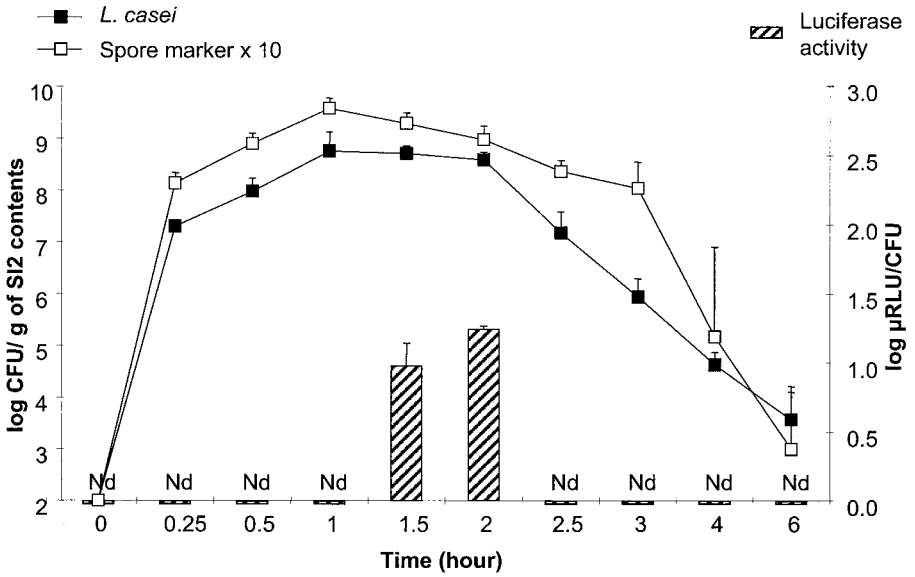

FIG. 3. Recovery of *L. casei* DN-114 001[lux] bacteria (filled squares) and *B. stearothermophilus* spores (nonfilled squares) in the ileum (SI2) after inoculation. Inoculation of HMA mice and SI2 content analyses were performed as described for Fig. 1. Column bars, luciferase activity; Nd, no detectable luciferase activity.

possible the estimation of the physiological state of *L. casei* DN-114 001[lux] in the different parts of the DT. In culture, luciferase activity restart was observed 1.5 to 2 h after a dilution of the inoculum in fresh medium (Table 1). The addition of rifampicin to the medium prevented the luciferase activity without affecting *L. casei* DN-114 001[lux] viability (Table 1).

No luciferase activity was detected in the stomach or the duodenum-jejunum compartments throughout the experiment (Fig. 1 and 2). In contrast, luciferase activity appeared significantly in the ileum 1.5 and 2 h after inoculation (Fig. 3). In the cecum compartment, luciferase activity at a mean value of log 1.5 µRLU · CFU^{-1} in all samples was detected from 1.5 to 6 h after dose ingestion.

DISCUSSION

The definition of probiotics (10, 12) states that bacteria in the product are in a living state when transiting through the gastrointestinal tract. As reported recently, the mechanisms of probiotic response might, however, involve cell fragments or components (22, 23). In a previous study (17), the analysis of mouse fecal content demonstrated that the probiotic bacteria

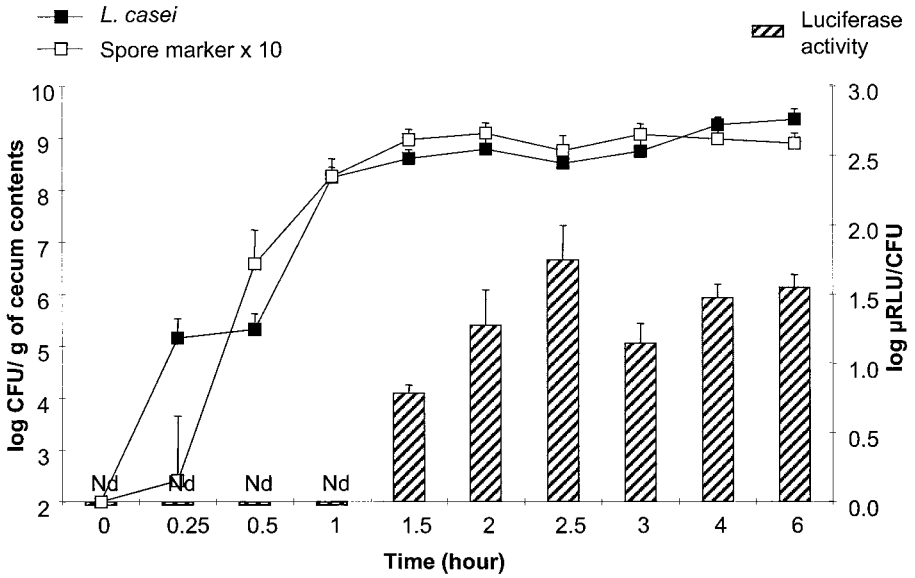

FIG. 4. Recovery of *L. casei* DN-114 001[lux] bacteria (filled squares) and *B. stearothermophilus* spores (nonfilled squares) in the cecum after inoculation. Inoculation of HMA mice and cecum content analyses were performed as described for Fig. 1. Column bars, luciferase activity; Nd, no detectable luciferase activity.

L. casei DN-114 001^{lux} synthesized protein de novo after its transit in the DT of HMA mice. In the present work, the main goal was to determine the transit time after which metabolically inactive bacteria reentered an active physiological state and thus restarted protein synthesis. For this purpose, the pharmacokinetics of *L. casei* DN-114 001^{lux} in the digestive content of HMA mice were defined and the bacterial physiological state in the different gastrointestinal compartments was monitored. A transcriptional fusion system composed of luciferase genes controlled by a deregulated promoter was used to look for evidence of protein synthesis (17). As previously reported (8), the use of *B. stearothermophilus* spores as a microbial transit marker made it possible to estimate the in vivo apparent survival rate of *L. casei* DN-114 001^{lux} bacteria compared to that of spore markers. By focusing on the fate of ingested *L. casei* DN-114 001^{lux} in the different parts of the DT, determinations of whether viable cells were in significant quantity and in a physiological state compatible with exerting a biological effect on the host were made.

Ingested microorganisms are exposed to adverse conditions starting as soon as they reach the stomach. Indeed, the pH may fall as low as 1.5 in this compartment (13). In the present experiments, the apparent survival rate of *L. casei* DN-114 001^{lux} bacteria compared to that of spore markers in the stomach depended on the time required for the bacteria to leave this compartment: the pool of cells transiting rapidly escaped the drastic conditions of the stomach environment, while cells remaining longer died. This observation is in agreement with results obtained for humans by Berrada et al. (3). Indeed, those authors report that two distinct *Bifidobacterium* strains were present at about 90% in the stomach 15 min after ingestion but that the level dropped to only 10% after 1.5 h (3). The gastric emptying rate is obviously an important feature for the survival of bacteria in the subsequent compartments of the DT.

The small intestine, and especially its proximal part (i.e., the duodenum-jejunum), contains hydrolytic enzymes and bile salts known to have lethal effects on microorganisms when they are cultivated in vitro (13). Thus, the passage through this compartment may also significantly affect the survival and the physiological state of ingested bacteria. Nevertheless, the percentage of survival of *L. casei* cells compared to that of *B. stearothermophilus* spores was at least 90% but no luciferase activity was detected in the stomach and in the SI1 throughout the experiment. Indeed, the in vitro experiments demonstrate that *L. casei* DN-114 001^{lux} originating from a 4-day culture needed a delay of at least 1.5 h to synthesize luciferase de novo. It is likely that, in vivo, ingested bacteria need at least this delay to reinitiate protein synthesis. However, at 2 h after ingestion, the concentrations of bacteria were the highest in the ileum and the cecum. Cell concentrations in the stomach and the SI1 were then too low to elicit a measurable luciferase activity. Thus, one cannot exclude the possibility that at 2 h after ingestion, the few bacteria remaining in the SI1 could have reinitiated the luciferase synthesis, as observed with bacteria mainly present in the ileum and in the cecum.

The distal part of the small intestine (i.e., the ileum) contains Peyer patches and Paneth cells (11) that constitute biological targets for transiting microorganisms. Marteau and Rambaud have suggested that probiotics have to reach the small intestine at a concentration of at least 10^6 CFU $\cdot$ g^{-1} of digestive content to induce immunostimulation (15). To get a better view of potential interactions between probiotic bacteria and host ileal structures, the presence of living and active bacterial cells at this level calls for further examination. With the HMA mouse model, the experiments show that at 1 h postinoculation, *L. casei* DN-114 001^{lux} reached 6×10^8 CFU $\cdot$ g^{-1} of digestive content in the SI2. In a recent clinical investigation, *L. casei* strain DN-114 001 was recovered at 10^6 CFU $\cdot$ ml^{-1} (2×10^8 CFU $\cdot$ h^{-1}) in human ileal fluid samples after 1 h (R. Oozeer, A. Leplingard, R. Michelin, A. Mogenet, I. Seksek, L. Diop, J. Doré, JL. Bresson and G. Corthier, Abstr. 9th Eur. Nutr. Conf., abstr. T.07, 2003). Regarding transit times to the ileum, the results obtained here with the HMA mouse model are consistent with those obtained with humans. In the present study, it was demonstrated that *L. casei* DN-114 001^{lux} synthesizes luciferase de novo in the SI2. Interestingly, the delay before synthesis that occurred in the DT was as short as that observed in vitro when metabolically inactive cells were inoculated in rich liquid medium. This finding suggests that *L. casei* DN-114 001 has a remarkable ability to adapt its physiology to the digestive environment.

Transport of ingested bacteria to the cecum occurred rapidly, achieving relative numbers as high as 10^9 CFU $\cdot$ g^{-1} of digestive content. Then, *L. casei* DN-114 001^{lux} numbers remained stable as a consequence of a slowing down of the transit in this compartment. A similar plateau has already been observed when *L. casei* DN-114 001^{lux} bacteria were enumerated in the feces of HMA mice (17). As in the ileum, luciferase activity was detected at as early as 1.5 h postinoculation. It then remained stable for the 6 h of the experiment. This observation can be related to previous results showing that luciferase activity can be recovered from feces at at least 12 h postinoculation (17).

Although human microbiota is known to exert bacteriostatic or lethal effects on transiting bacteria (9), we have here shown that *L. casei* DN-114 001^{lux} persisted at high levels in the cecum and, to a lesser extent, in the ileum. Moreover, protein synthesis took place in both compartments, demonstrating the active physiological state of the bacteria. Such a situation is favorable for potential interactions between the probiotic and the host digestive ecosystem.

The present results also reinforce the possibility for *L. casei* to be proposed as an alternative to *Lactococcus lactis* as an appropriate vector for the delivery of therapeutic proteins. Indeed, while *L. lactis* is advantageously used in protein delivery experiments because of its performance in genetic manipulations (16, 24, 26), it is recovered at low levels from the DT (14). In contrast, *L. casei* strains are found at high levels in the human ileum and persist in the feces (A. Leplingard, R. Oozeer, R. Michelin, A. Mogenet, I. Seksek, L. Diop, J. Doré, JL. Bresson and G. Corthier, Abstr. 9th Eur. Nutr. Conf.., abstr. T.08, 2003) (26). Here, it is demonstrated that *L. casei* DN-114 001^{lux} can produce proteins in specific parts of the DT of HMA mice. The high survival rate of bacteria such as *L. casei* or *L. plantarum* strains (25) is a valuable criterion for the choice of an in vivo delivery system of biologically active molecules such as cytokines or heterologous antigens (16, 24, 26).

In conclusion, this study investigated the physiological state of ingested bacteria in the different parts of the DT for the first time. The results demonstrate that *L. casei* DN-114 001^{lux}

survives DT transit and can synthesize proteins starting at the ileal level. These findings strengthen the credibility of this probiotic strain and claims concerning its health benefits (4, 18–21).

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