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Dual-Color Bioluminescence Imaging for Simultaneous Monitoring of the Intestinal Persistence of *Lactobacillus plantarum* and *Lactococcus lactis* in Living Mice

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Lactic acid bacteria are found in the gastrointestinal tract of mammals and have received tremendous attention due to their health-promoting properties. We report the development of two dual-color luciferase-producing *Lactobacillus* (*Lb.*) *plantarum* and *Lactococcus* (*Lc.*) *lactis* strains for noninvasive simultaneous tracking in the mouse gastrointestinal tract. We previously described the functional expression of the red luciferase mutant (CBRluc) from *Pyrophorus plagiophthalamus* in *Lb. plantarum* NCIMB8826 and *Lc. lactis* MG1363 (C. Daniel, S. Poiret, V. Dennin, D. Boutillier, and B. Pot, Appl Environ Microbiol 79:1086–1094, 2013, http://dx.doi.org/10.1128/AEM.03221-12). In this study, we determined that CBRluc is a better-performing luciferase for *in vivo* localization of both lactic acid bacteria after oral administration than the green click beetle luciferase mutant construct developed in this study. We further established the possibility to simultaneously detect red- and green-emitting lactic acid bacteria by dual-wavelength bioluminescence imaging in combination with spectral unmixing. The difference in spectra of light emission by the red and green click beetle luciferase mutants and dual bioluminescence detection allowed *in vitro* and *in vivo* quantification of the red and green emitted signals; thus, it allowed us to monitor the dynamics and fate of the two bacterial populations simultaneously. Persistence and viability of both strains simultaneously administered to mice in different ratios was studied *in vivo* in anesthetized mice and *ex vivo* in mouse feces. The application of dual-luciferase-labeled bacteria has considerable potential to simultaneously study the interactions and potential competitions of different targeted bacteria and their hosts.

Lactococci and lactobacilli are lactic acid bacteria (LAB) that have been used for thousands of years for the production and preservation of fermented food, such as milk, vegetables, and meat. Some specific strains are commercialized as probiotics and claimed to have health-promoting properties (1). The gastrointestinal tract (GIT) is the most important field of activity of LAB, although distal effects outside the gut have been described (2). Thus, it is important to understand the interactions of the administered bacteria with their host GIT system. LAB are able to survive and adapt to the GIT conditions, as shown by comparative and functional genomic characterization of various human isolates, essentially lactobacilli (for a review, see references 2 and 3). Some LAB, when present in the GIT of mice or humans, express a number of common characteristics that may relate to their intestinal niche adaptation (3, 4). However, direct *in vivo* tracking of these actions in terms of both spatial and temporal evolution would allow a better understanding of the survival and metabolic activities of these LAB in the gut.

In the past decade, bioluminescence imaging (BLI) has become essential for *in vivo* noninvasive monitoring of biological processes (4). The technique relies on the detection of photons emitted from cells or tissues in a living organism. Bioluminescence is a biological process that requires an enzyme known as luciferase, an enzyme-specific substrate (e.g., luciferin), oxygen, magnesium, and/or ATP, depending on the luciferase. Luciferases encompass a wide range of enzymes that catalyze light-producing chemical reactions in living organisms. The most used luciferase for *in vivo* bioluminescence imaging is represented by the *Photinus pyralis* (firefly) luciferase (Fluc) and the click beetle luciferase from *Pyrophorus plagiophthalamus* (CBluc). Both use D-luciferin as the substrate, depend on ATP, Mg, and O_2, and result in the production of green light. Red click beetle (CBRluc) and firefly luciferase variants with higher emission wavelengths also have been developed and greatly enhance the penetration and sensitivity of BLI in deep tissues (4). We previously showed that CBRluc produced in *Lactobacillus* (*Lb.*) *plantarum* NCIMB8826, one of most studied strains in LAB research, and *Lactococcus* (*Lc.*) *lactis* MG1363, a dairy starter derivative, can be successfully detected in the GIT of anesthetized mice by noninvasive BLI after oral administration (5).

It has been reported that dual-color BLI can be applied *in vitro* and *in vivo* using appropriate emission filters for the separation of bioluminescent signals and mathematical corrections for their deconvolution using spectral unmixing algorithms (6–8). Dual-

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
Strains		
Lactococcus lactis subsp. *cremoris* MG1363	Plasmid free	12
Lactobacillus plantarum NCIMB8826	Originally isolated from human saliva	NCIMB
Escherichia coli MC1061	*araD139* Δ(*ara-leu*)*7696 lacX74 galV galK hsr-hsm rpsL*	Invitrogen
Plasmids		
pNZ8148	Cm^r, *L. lactis* pSH71 replicon	MoBiTech
pMEC256	pNZ8148 carrying CBRluc cDNA optimized for *L. plantarum* codon fused to the *L. plantarum* P*ldh* promoter (lactate dehydrogenase)	5
pMEC257	pNZ8148 carrying CBRluc cDNA optimized for *L. lactis* codon fused to the *L. lactis* P*usp45*	5
pMEC271	pNZ8148 carrying CBGluc cDNA optimized for *L. plantarum* codon fused to P*ldh*	This study
pMEC269	pNZ8148 carrying CBGluc cDNA optimized for *L. lactis* codon fused to P*usp45*	This study

[a] Cm^r, resistance to chloramphenicol.

color bioluminescence has been described with red and green luciferases expressed by mammalian cells that can be separated *in vivo* (9). Very recently, Chang et al. showed that Fluc and CBRluc, which emit distinct light wavelengths, allow simultaneous imaging of two mycobacterial strains during infection (10).

As LAB are studied and given to humans and animals mostly in mixtures with multiple strains (11), we describe the development and application of two dual-color luciferase-expressing *Lb. plantarum* and *Lc. lactis* isolates for noninvasive simultaneous monitoring in the GIT of small laboratory animals. We demonstrate that CBRluc is a better-performing luciferase *in vivo* than the green click beetle luciferase (CBGluc) mutant, which also is described in this study. We also explore the possibility of simultaneously detecting red- and green-emitting LAB by dual-wavelength bioluminescence imaging in combination with spectral unmixing.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids are listed in Table 1. The *Lb. plantarum* codon-optimized *cbgluc* gene under the control of P*ldh* and the *Lc. lactis* codon-optimized *cbgluc* gene under the control of P*usp45* (the two DNA fragments were synthetized by Eurogentec [Belgium]) were cloned into pNZ8148 as BglII-XbaI fragments. The two resulting constructs subsequently were introduced into *Lc. lactis* MG1363 and *Lb. plantarum* NCIMB8826 by electrotransformation as described elsewhere (12) and named *Lc. lactis*-CBGluc (Ll-CBGluc) and *Lb. plantarum*-CBGluc (Lp-CBGluc), respectively. *Lc. lactis* MG1363 and *Lb. plantarum* NCIMB8826 containing the empty vector pNZ8148 (named Ll-pNZ8148 and Lp-pNZ8148, respectively), described previously (5), served as controls in all of the *in vitro* and *in vivo* experiments. Lp-CBRluc and Ll-CBRluc were described previously (5). Strain stability was tested as described previously (13).

Escherichia coli was cultured in Luria-Bertani broth at 37°C. *Lc. lactis* was grown at 30°C in M17 medium (Difco, Becton Dickinson, Sparks, MD) supplemented with 0.5% glucose. *Lb. plantarum* was grown at 37°C in MRS medium (Difco, Becton Dickinson). Chloramphenicol (Sigma-Aldrich, St. Quentin Fallavier, France) was added to culture media for bacterial selection, when necessary, at a final concentration of 20 μg/ml for *E. coli* and 10 μg/ml for LAB strains.

***In vitro* bioluminescence measurement.** The bioluminescence of each recombinant luciferase was quantified as described previously (5). Briefly, recombinant bacteria were grown overnight, harvested by centrifugation, and washed with phosphate-buffered saline (PBS). Fifty microliters of each culture, adjusted to approximately 2.5×10^{11} CFU/ml, was distributed in black microplates (Nunc, Thermo Fisher, NY, USA) and imaged after addition of 50 μl of the Bright-Glo luciferase (Promega, Madison, WI, USA). Luminescence was measured at room temperature on the IVIS Lumina XR imaging system (PerkinElmer, Alameda, CA, USA) using the Living Image software (PerkinElmer). Each individual well was manually selected as a region of interest (ROI). Strains were compared on the basis of the total flux of emitted light, expressed as photons per second (p/s) measured per 1.2×10^{10} CFU of each bacterial culture. *Lc. lactis* MG1363 and *Lb. plantarum* NCIMB8826 containing the empty vector pNZ8148 were used to measure the background luminescence.

Preparation of bacteria and administration to mice. Bacterial strains were grown overnight, harvested by centrifugation, and washed with PBS. Mice received 5×10^{10} CFU in 200 μl gavage buffer (0.2 M $NaHCO_3$ buffer containing 1% glucose, pH 8). Eight-week-old female BALB/c mice were purchased from Charles River (St. Germain sur l'Arbresle, France). Experiments were performed in an accredited establishment (no. A59107; Institut Pasteur de Lille) according to European guidelines (number 86/609/CEE), and animal protocols were approved by the local ethics committee.

***In vivo* persistence of LAB in the GIT of mice.** Groups of five mice received a unique, daily dose of 5×10^{10} CFU of live recombinant strains (Lp-CBRluc, Ll-CBRluc, Lp-CBGluc, or Ll-CBGluc) or different doses of the two different recombinant strains (one expressing CBRluc and the other CBGluc) administered for four consecutive days by oral gavage. Control mice received Lp-pNZ8148 and Ll-pNZ8148 in the different experiments. Fecal samples were individually collected at different time points and mechanically homogenized in PBS. No chloramphenicol-resistant bacteria were detected in noninoculated mice. Mice were anesthetized with 2% isoflurane and sacrificed by cervical dislocation, and mouse digestive tracts were immediately excised before *ex vivo* bioluminescence imaging. As described previously, the intestines were injected with air to enhance the bioluminescent signal (5). Experiments were repeated twice.

***In vivo* bioluminescence imaging.** Bioluminescence imaging was performed using a multimodal IVIS Lumina XR imaging system (PerkinElmer) as described previously (5). Mice were anesthetized with 2% isoflurane, and D-luciferin potassium salt (PerkinElmer) was administered to animals intragastrically 15 min before the oral administration of the bacteria. Two hours later, mice were placed into the specimen chamber of the IVIS imaging system, where a controlled flow of 1.5% isoflurane was administered through a nose cone via a gas anesthesia system (Tem Sega, Lormont, France). A grayscale reference photograph under low illumination was taken as an overlay prior to detection of emitted photons over 1 s to 5 min, depending on signal intensity and using Living Image software (PerkinElmer). A pseudocolor image representing detected light intensity was generated using Living Image software and superimposed over the grayscale reference photograph. For each individual mouse, the ROI corresponding to the mouse digestive tract was selected manually.

In vitro and *in vivo* spectral unmixing of emission wavelengths. To

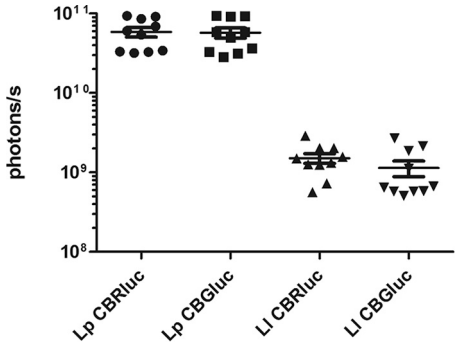

FIG 1 Quantification of bioluminescent signals in cultures of Lp-CBRluc, Lp-CBGluc, Ll-CBRluc, and Ll-CBGluc. The values displayed correspond to the means from 10 independent cultures. Results are given in photons per second for 1.2×10^{10} CFU of each strain. Error bars indicate standard deviations. Lp, *Lb. plantarum*; Ll, *Lc. lactis*. Differences between groups were assessed using the Kruskal-Wallis nonparametric test, and no statistical difference between Lp CBRluc and Lp CBGluc or between Ll CBRluc and Ll CBGluc, respectively, was found. There was a statistical difference ($P < 0.001$) between the two *Lb. plantarum* and the two *Lc. lactis* strains. For clarity purposes, the statistics are not shown.

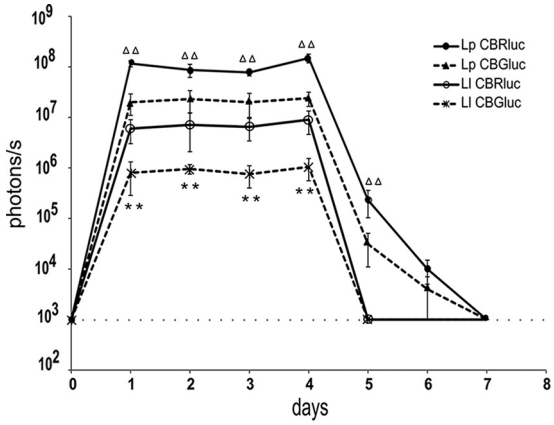

FIG 2 Persistence of *Lc. lactis* and *Lb. plantarum*, both producing CBR and CBG, in mice after oral administration. Plots represent the bioluminescence from day 1 to day 7 from each set of five mice, with standard deviations. The background level for the bioluminescent signal is displayed as a dashed line. Differences between groups were assessed using the Kruskal-Wallis test, and those found to be statistically significant are indicated with triangles for comparison between Lp-CBRluc and Lp-CBGluc (two triangles, $P < 0.005$) and asterisks for comparison between Ll-CBRluc and Ll-CBGluc ($P < 0.005$).

separate the red bioluminescent signal (CBRluc with an emission peak at 620 nm) from the green signal (CBGluc with an emission peak at 540 nm) in mixed cultures of *Lc. lactis* and *Lb. plantarum*, either (i) *in vitro* in microplates, (ii) *in vivo* in mice after coadministration of both strains by oral gavage, (iii) *ex vivo* on dissected GITs, or (iv) directly in feces, images were taken using a set of 20-nm step emission filters (500 series high-spectral-resolution emission filters; PerkinElmer) from 500 nm to 620 nm. Living Image software was used for generating spectrally unmixed images and quantification of each signal.

RESULTS

In vitro comparison of the production of CBGluc and CBRluc in *Lc. lactis* and *Lb. plantarum*. The bioluminescent signals produced by the different recombinant *Lc. lactis* and *Lb. plantarum* isolates were quantified and compared *in vitro* on bacterial cultures adjusted to 2.5×10^{11} CFU/ml (Fig. 1). Results show that maximum bioluminescence is obtained with Lp-CBRluc and Lp-CBGluc with mean values of 5.1×10^{10} and 4.1×10^{10} p/s (per 1.2×10^{10} CFU, equivalent to 50 μl of bacterial culture). Approximately 36-fold lower bioluminescent signals were obtained with *Lc. lactis* strains producing CBGluc and CBRluc. No statistical difference was found between the production of CBGluc and CBRluc for the respective strains. Plasmids 271 and 269 in *Lc. lactis* and *Lb. plantarum*, respectively, were remarkably stable, with 100% bioluminescent colonies even after approximately 100 generations (10 subcultures) in nonselective medium.

As observed earlier for CBRluc-producing strains (5), there was an excellent correlation between CFU counts and *in vitro* bioluminescent signals from CBGluc-expressing strains (data not shown). The bioluminescence system allowed the detection of bacterial amounts as low as 5×10^4 CFU/ml for Lp-CBGluc (similar to Lp-CBRluc [5]) and 5×10^5 CFU/ml for Ll-CBGluc (similar to Ll-CBGluc [5]).

Comparison of the persistence of CBGluc- and CBRluc-producing *Lc. lactis* and *Lb. plantarum* in the digestive tract of mice after 4 daily oral administrations. As we had previously shown that differences in bacterial persistence in mice are more pronounced after multiple daily oral administrations (5), groups of mice were given a daily dose of 5×10^{10} CFU of each recombinant strain for four consecutive days. The bioluminescent signal was quantified each day by direct imaging in 5 anesthetized mice (Fig. 2). The same mice were used during the whole experiment. The signal was very strong for the four strains on day 1 and remained at a similar level until the last day of oral administration (day 4). Results show that from day 1 to day 4, Lp-CBRluc emitted the highest bioluminescent signal (mean value of 1.2×10^8 p/s), 6-fold higher than that of Lp-CBGluc, 14-fold higher than that of Ll-CBRluc, and 111-fold higher than that of Ll-CBGluc. Signals of both *Lc. lactis* strains decreased to background levels at day 5, whereas signals of both *Lb. plantarum* strains decreased to background levels only at day 7.

Simultaneous monitoring of red and green bioluminescent signals by dual-color *in vivo* imaging. In order to test the CBRluc/CBGluc pair of luciferases for *in vivo* applications, groups of mice were given an oral dose of Lp-CBRluc, Lp-CBGluc, or a mixture (50/50) of *Lb. plantarum* expressing CBRluc and expressing CBGluc for 4 days. The bioluminescent signal was measured each day in mice, and a series of images at different wavelengths, ranging from 500 to 620 nm, were acquired (Fig. 3A). Representative images of a spectral unmixing of the red and green signals are shown in Fig. 3. The respective red and green signals are separated and quantified after applying the spectral unmixing algorithm to the acquired image series. The resulting Unmix1 image displays the green signal from Lp-CBGluc, while the resulting Unmix2 image displays the red signal from Lp-CBRluc (Fig. 3B). The red signal was not detected in the green emission light image, and the green signal was not detected in the red emission light image, which confirmed the good spectral unmixing of both signals. Moreover, both signals were detected in mice administered a mixture of Lp-CBRluc and Lp-CBGluc.

Simultaneous monitoring of red and green bioluminescent signals after coadministration of *Lb. plantarum*-CBRluc and *Lc. lactis*-CBGluc by dual-color *in vivo* imaging. In order to study the persistence of *Lb. plantarum* and *Lc. lactis* in mice simultane-

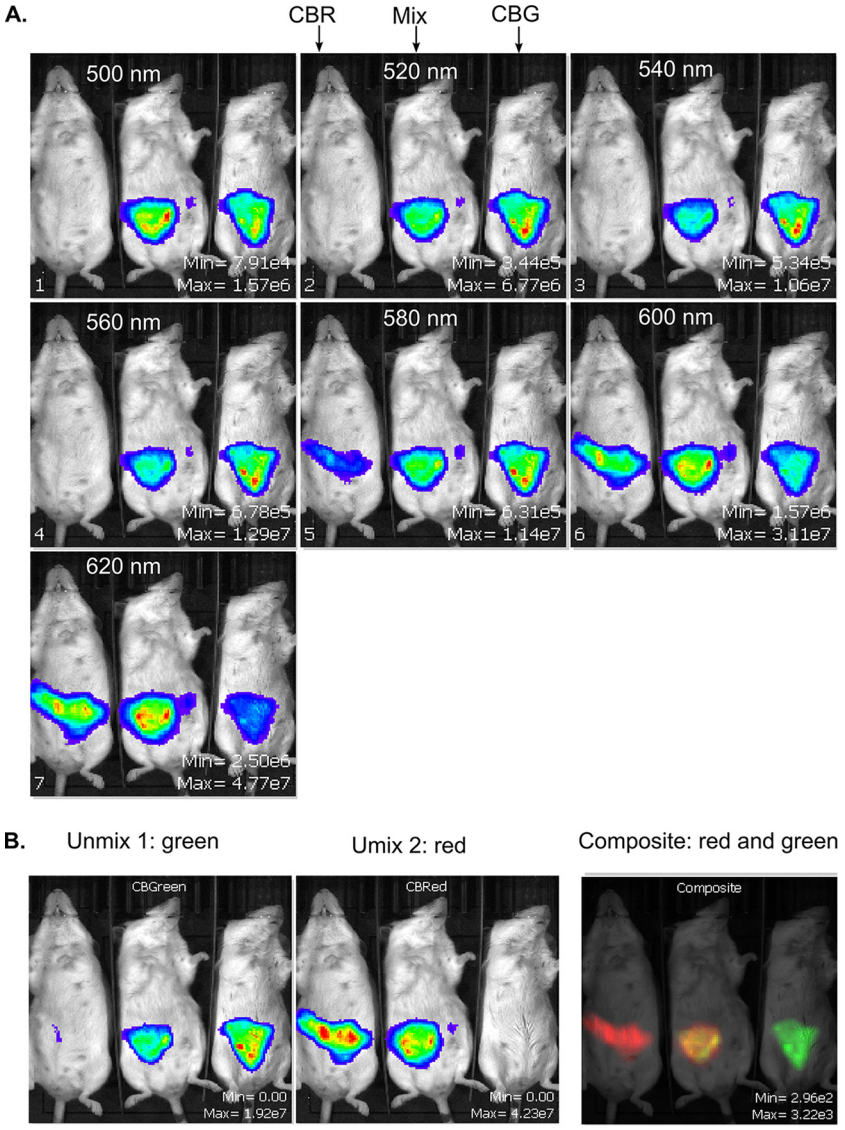

FIG 3 Representative images of spectral unmixing of red and green signals in mice after oral administration of bioluminescent bacteria. (A) Multispectral acquisition of red- and green-emitting *Lb. plantarum*. Groups of mice were fed with Lp-CBRluc (CBR), Lp-CBGluc (CBG), or a mixture of both strains (50/50; Mix). Images were taken using a set of 20-nm filter step emission filters ranging from 500 nm to 620 nm. (B) Resulting unmixed images used for quantification and recomposition of the two different luciferases in false colors (red for CBR, green for CBG, and yellow for both luciferases).

ously, groups of mice were given an oral daily dose of Lp-CBRluc, Ll-CBGluc, or different mixtures (20/80, 50/50, and 80/20) of Lp-CBRluc and Ll-CBGluc for 4 days. We found that red signals in mice administered a mixture of 50/50 or 80/20 of Lp-CBRluc–Ll-CBGluc are similar to red signals of mice administered 100% Lp-CBRluc (no statistical difference) from day 1 to day 6 (Fig. 4A). The same observation was made for green signals in mice administered only Ll-CBGluc (Fig. 4B). However, red signals in mice administered *Lb. plantarum* only are statistically higher than red signals in mice administered a mixture of 20% *Lb. plantarum* and 80% *Lc. lactis* from day 1 to day 6. Similarly, green signals in mice administered 100% *Lc. lactis* are statistically higher than green signals in mice administered a mixture of 20% *Lc. lactis* and 80% *Lb. plantarum*. The values of red signals from *Lb. plantarum* given alone (mean value of 1.4×10^8 p/s from day 1 to day 4) are statistically higher than green signals from *Lc. lactis* given alone ($P < 0.001$). Signals of *Lc. lactis* given alone or in mixtures decreased to background levels at day 5, whereas signals of *Lb. plantarum* given alone or in mixtures decreased to background levels only at day 7.

As observed previously (5), both strains separately administered were localized essentially in the cecum and colon from day 1 to day 4 (Fig. 4C). When both strains are administered simultaneously to mice (data not shown for the 20/80 and 80/20 mixtures), they both also localized in the cecum and colon. On days 5 and 6, *Lb. plantarum* was localized essentially in the cecum and colon of mice orally administered *Lb. plantarum* or in mixtures with *Lc. lactis*, while no green signal was detected.

The persistence of both strains administered separately or in mixtures, as well as the respective red and green bioluminescent signals, also were examined in mouse feces after spectral unmixing (see Fig. S1 in the supplemental material). The *Lb. plantarum*

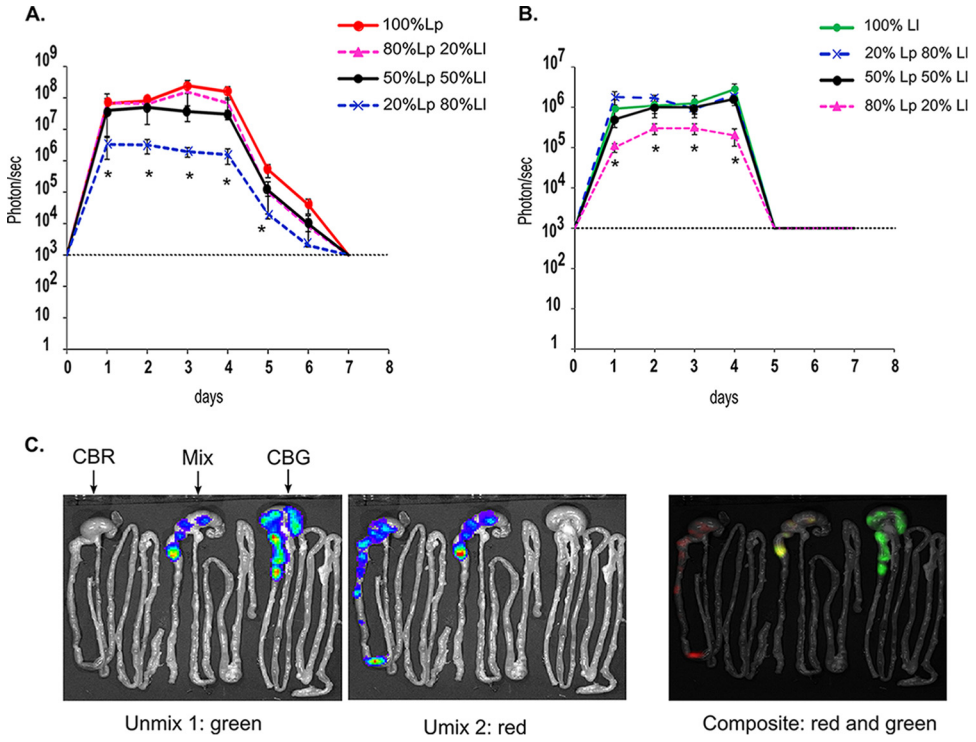

FIG 4 Spectral unmixing of red (A) and green (B) signals after oral administration of Lp-CBRluc, Ll-CBGluc, and a mixture of the two strains. Groups of mice were fed for four consecutive days with Lp-CBRluc, Ll-CBGluc, or a mixture of both strains (20/80, 50/50, and 80/20). Plots represent the bioluminescence of each set of five mice, with standard deviations. The background level for the bioluminescent signal is represented by a dashed line. Differences between groups were assessed using the Kruskal-Wallis test, and those found to be statistically significant ($P < 0.005$) are indicated with an asterisk for comparison between 100% Lp-CBRluc and 20% Lp-CBRluc–80% Ll-CBGluc and between 100% Ll-CBGluc and 80% Lp-CBRluc–20% Ll-CBGluc. (C) Two mice per group were sacrificed from day 4 to day 7, and a representative image of the digestive tract of one mouse per group is shown at day 4 in mice fed with Lp-CBRluc (CBR), a mixture of 50% Lp–50% Ll (Mix), and Ll-CBGluc (CBG).

CBRluc administered alone and in mixtures persisted for 11 days after the last inoculation (day 4), whereas Ll-CBGluc given alone and in mixtures persisted for 4 days after the last inoculation. The bioluminescent signal of Lp-CBRluc given alone and in mixtures was detected 3 days after the last dose, while Ll-CBGluc was undetectable at day 5.

DISCUSSION

As LAB are studied and applied in humans and animals mostly in mixtures with multiple strains (11), there is a need to better understand the fate of the individual strains in the GIT as well as the possible *in vivo* interactions between the different strains of the mixture. Bioluminescence has been shown to be a useful approach to study the temporal and spatial evolution of live bacteria administered to mice (5). Dual-color luminescence has been described with red and green luciferases in other *in vivo* systems (8–10) but so far has never been used in Gram-positive LAB.

Here, we describe the development and application of a green-and-red variant of the click beetle luciferase, expressed by strains of *Lactobacillus plantarum* NCIMB8826 and *Lactococcus lactis* MG1363 for simultaneous detection of these strains in the GIT of mice. Both luciferases use the same D-luciferin substrate and can be imaged and discriminated simultaneously. We were able to separate the two signals spectrally, allowing the respective strains to be monitored and their respective bioluminescent signals to be quantified.

Our results show that *Lb. plantarum* expressing CBRluc and CBGluc produced the highest luminescent signal, 30 times brighter than the ones expressed by *Lc. lactis*. Similar production differences have been observed already for CBRluc in a previous study, even with strong optimized *Lc. lactis* promoters (5). *In vivo*, the bioluminescence of CBRluc was greater than the one from CBGluc in both strains, demonstrating that CBRluc is a more sensitive reporter than CBGluc for tracking bacteria in the GIT. Similar observations were made by Chang et al. with CBRluc and Fluc (with an emission spectrum similar to CBGluc's) in the lungs of mice infected with bioluminescent mycobacteria (10). Similar conclusions also have been made by Mezzanotte et al. on imaging the liver and prostate using a red-optimized Fluc with CBGluc (9). The lungs, the liver, the prostate, and the GIT are difficult sites to image optically due to the great tissue depth, and the use of red-emitting luciferases is more adapted to imaging in these tissues, as the longer wavelength enables better penetration through living tissues (4, 8–10).

However, even if the bioluminescence produced *in vivo* by CBGluc is lower than the one produced by CBRluc, CBGluc is still a good and reliable reporter for LAB in the GIT, as the kinetics of the bioluminescent signals from each respective strain expressing CBGluc and CBRluc were similar during all of the experiments. Both bioluminescent reporters, CBGluc and CBRluc, exhibiting different emission spectra, allowed for dual bioluminescence labeling, permitting us to study the persistence of *Lb. plantarum* and

Lc. lactis in the GIT of mice. We confirmed, based on bioluminescence, that *Lb. plantarum* persists better than *Lc. lactis* in the GIT of mice (5, 14). Signals from anesthetized mice and feces showed that the persistence of coadministered *Lb. plantarum* and *Lc. lactis* is similar to the persistence of each strain administered separately, illustrating that the strains are not competing with each other, even if they were colocalized in the cecum and colon as described previously (5). The survival and persistence of mixtures of LAB strains have been studied in mice and animals (15–17). However, to our knowledge, the comparison between the persistence of two coadministered LAB and the persistence of each strain administered separately has never been reported.

Our study supports the potential for multiple luciferases to be imaged simultaneously *in vivo* using spectral unmixing. Further studies are needed to combine both bioluminescence and fluorescence (18), allowing for highly sensitive optical imaging, ranging from single-cell analysis to *in vivo* whole-body bioluminescence imaging (19–21). The application of dual-luciferase labeled bacteria has significant potential to allow further study of the interactions of various LAB with their mammalian hosts. Our system may be used to perform competition assays between mutant and wild-type strains in the same animal, analyze immunological events in real time, e.g., by labeling a subset of immune cells (22) in conjunction with bioluminescent bacteria, or study the persistence of two LAB which have a different behavior *in vivo*, and improve our understanding of the effects of a mixture of LAB strains in the GIT.

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