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Article

Lighting Up Gut Dysbiosis: Red-Shifted Whole-Cell Bioluminescence Enables Functional Sensing of Bile Salt Hydrolase Activity in Human Feces

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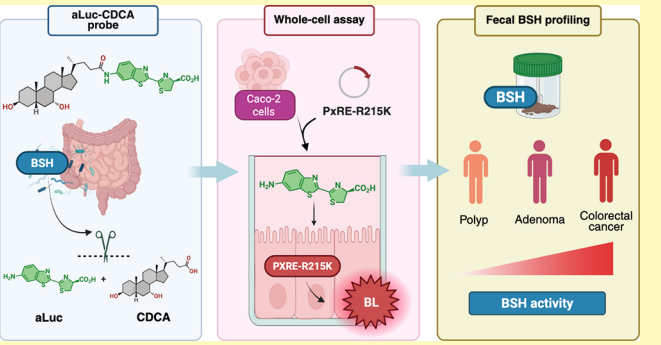
ABSTRACT: Bile salt hydrolase (BSH) activity is an emerging functional biomarker of gut microbiota dysbiosis and altered bile acid (BA) metabolism, but its direct quantification in feces remains challenging because stool components strongly attenuate optical signals. Here, we report a red-shifted whole-cell bioluminescent (BL) bioassay for sensitive and accurate quantitative BSH sensing in human feces. The platform couples amino-luciferin chenodeoxycholic acid (aLuc-CDCA), a BSH-cleavable caged luciferin probe, with Caco-2 cells expressing the engineered far-red-emitting *Phrixothrix hirtus* luciferase PxRE-R215K (λmax ≈ 640 nm). In cell-free assays, purified choloylglycine hydrolase (CH) generated a linear BL response with a limit of detection (LOD) of 0.010 ± 0.002 U/mL; however, analysis of fecal extracts was severely affected by matrix interference. Intracellular luciferase expression mitigated these effects, and PxRE-R215K outperformed the *Amydetes vivianii* green-emitting AmyLuc reporter in diluted stool suspensions (1 mg/mL), providing LOD and limit of quantification (LOQ) values of 0.02 ± 0.01 and 0.06 ± 0.04 U/mL, respectively. Spike-and-recovery experiments confirmed higher accuracy for PxRE-R215K than AmyLuc (83.3−93.4% vs 66.7−87.5%) and improved interassay precision (CV: 14% vs 29.9%). In clinical samples, the PxRE-R215K assay detected significantly higher fecal BSH activity in colorectal cancer (CRC) than in hyperplastic polyp or adenoma groups. UHPLC-MS/MS BA profiling of matched serum samples corroborated the functional readout, revealing CRC-associated increases in total BA levels, secondary-to-primary BA ratio, and unconjugated secondary BA fractions. This red-shifted whole-cell platform offers a simple and scalable strategy for functional monitoring of microbiota-associated BA metabolism in fecal samples with minimal handling.



KEYWORDS: *bile salt hydrolase, gut dysbiosis, bioluminescence, whole-cell assay, bile acid metabolism, colorectal cancer*

The human gut microbiota is a highly complex and dynamic community that plays a crucial role in host physiology, contributing to nutrient metabolism, immune system modulation, and maintenance of intestinal homeostasis.[1] Alterations in gut microbial composition and metabolic function, commonly referred to as dysbiosis, have increasingly been associated with gastrointestinal and metabolic disorders, including inflammatory bowel disease (IBD), metabolic dysfunction, and colorectal cancer (CRC).[2,3] Growing evidence indicates that dysbiosis is characterized not only by compositional changes but also by profound alterations in microbial metabolic activities that directly affect host physiology.[2,4,5] Among the metabolic pathways linking the gut microbiota to the host, bile acid (BA) metabolism represents a major functional interface.[2,6]

BAs are cholesterol-derived molecules synthesized in the liver that play key roles in lipid digestion and in metabolic and immune signaling.[7] After *de novo* synthesis from cholesterol by host hepatocytes, primary BAs, such as cholic acid (CA) and chenodeoxycholic acid (CDCA), are conjugated with glycine or taurine to form conjugated BAs. BAs are then released into the small intestine, where they facilitate lipid digestion and are largely reabsorbed in the terminal ileum as part of the enterohepatic circulation.[6] The fraction that escapes reabsorption reaches the colon, where primary BAs undergo further microbial biotransformations and are converted into secondary BAs, such as deoxycholic acid (DCA) and lithocholic acid (LCA).[7]

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BAs act as signaling molecules that modulate host metabolic pathways through nuclear and membrane receptors, including farnesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 (TGR5), thereby influencing glucose and lipid homeostasis, inflammation, and intestinal physiology.[8,9] Dysregulation of these pathways has been associated with chronic inflammation, metabolic imbalance, and carcinogenesis.

The initial and rate-limiting step in microbial BAs metabolism is catalyzed by bile salt hydrolases (BSHs; EC 3.5.1.24), a highly conserved family of microbial enzymes widely distributed across the major gut microbiota phyla.[1,10] BSHs hydrolyze conjugated BAs, producing free BAs and amino acids that subsequently serve as substrates for downstream microbial transformations.[11,12] Through this activity, BSHs act as key regulators of BA pool composition and, consequently, of microbiota-host metabolic crosstalk.[5] Elevated BSH activity has been associated with significant shifts in BA profiles, including the accumulation of cytotoxic secondary BAs such as DCA, which have been implicated in epithelial damage, oxidative stress, chronic inflammation, and CRC progression.[13] Moreover, BSH-driven changes in BA composition can further reshape microbial communities by selectively enriching bile-resistant, potentially proinflammatory bacterial taxa, thereby reinforcing dysbiosis-associated pathological states.[1] At the same time, modulation of BSH activity has also been linked to beneficial effects, including reduced cholesterol levels, improved intestinal homeostasis, and protection against inflammatory disorders, highlighting the context-dependent role of BSH-mediated BA metabolism in host health and disease.[14] Consequently, BSH activity is increasingly emerging as a functional biomarker of gut microbiota dysregulation in gastrointestinal and metabolic diseases.[13,15]

Despite its biological relevance, quantifying BSH activity directly in complex biological matrices remains analytically challenging. The intrinsic heterogeneity of fecal samples, together with the dynamic and spatially heterogeneous nature of the gut microbiota, substantially limits the applicability of current analytical approaches.[16,17] Existing methods mainly rely on indirect measurements, including metagenomic sequencing,[18] chromatographic BA profiling,[19] or *in vitro* assays performed on isolated bacterial strains.[20] While informative, these approaches often fail to provide a direct functional assessment of enzymatic activity within intact biological samples. In addition, they generally require extensive sample processing, sophisticated instrumentation, and time-consuming workflows, limiting their applicability to rapid, scalable, or minimally invasive analyses.

To address these limitations, several activity-based sensing strategies have recently been developed for BSH detection.[21−23] Fluorogenic probes enabled continuous monitoring of BSH activity in bacterial lysates and fecal microbiome samples; however, fluorescence-based approaches remain intrinsically susceptible to matrix autofluorescence, optical interference, and signal quenching in complex fecal environments, often requiring sample preprocessing and relatively high probe concentrations for reliable detection.[21] Recently, bioluminescent (BL) BSH probes enabled highly sensitive functional measurements in fecal samples and *in vivo* models, demonstrating the potential of BL sensing for monitoring microbiota-associated enzymatic activity.[22] Caged aminoluciferin-bile acid probes allow selective BSH-dependent release of luciferin, enabling quantitative detection of BSH activity in intact stool samples and even the gastrointestinal tract of human subjects with minimal sample manipulation.[22] Miniaturized BL sensing platforms based on immobilized luciferase and air-segmented continuous-flow microfluidics have also demonstrated the feasibility of sensitive BSH detection using the synthetic probe amino-luciferin chenodeoxycholic acid (aLuc-CDCA), highlighting the potential for compact and portable biosensing applications in biological fluids and fecal samples.[24] However, accurate signal detection in complex fecal matrices remains challenging because stool components can strongly attenuate light transmission and interfere with optical readouts. Shorter-wavelength BL emissions are more strongly affected by optical scattering; endogenous chromophore absorption, especially porphyrin derivatives; and matrix-dependent signal attenuation in highly heterogeneous biological samples.[22,25,26] These limitations highlight the need for BL platforms with improved emission compatibility for fecal environments and enhanced signal robustness under clinically relevant conditions.

The present study extends the established BL BSH-activatable luciferin/whole-cell sensing concept by demonstrating that reporter emission wavelength selection can improve quantitative performance in complex fecal matrices.

We implemented the sensing platform using Caco-2 cells expressing the previously engineered Brazilian far-red-emitting *Phrixotrix* railroad worm luciferase, PxRE-R215K.[27,28] Compared with the *Amydetes vivianii* green-emitting luciferase, AmyLuc,[29−31] the red-shifted optical emission of PxRE-R215K exhibited reduced matrix-associated attenuation and improved BL signal in fecal samples, thereby enabling sensitive functional profiling of microbiota-associated BSH activity. As a proof of concept, the assay was tested across colorectal disease stages and compared with matched fecal and serum BA profiles measured by HPLC-MS.

■ EXPERIMENTAL SECTION

Chemical Reagents

Dulbecco's modified Eagle medium (DMEM), phenol red-free DMEM, OPTI-MEM-reduced serum medium, fetal bovine serum (FBS), One Shot TOP10 chemically competent cells, and Lipofectamine 3000 transfection kit were purchased from Thermo Fisher Scientific (Waltham, MA, USA). LB Broth, agar A, and ampicillin sodium salt were obtained from Bio Basic (Markham, ON, Canada). Phosphate-buffered saline (PBS) tabs (137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer solution, pH 7.4), dimethyl sulfoxide (DMSO), Tris-EDTA (TE), trypsin-EDTA, 100X antibiotic solution (10,000 U/mL penicillin and 10 mg/mL streptomycin), L-glutamine, cholylglycine hydrolase (CH) from *Clostridium perfringens*, 6′-amino-D-luciferin, D-luciferin, DL-dithiothreitol (DTT), adenosine 5′-triphosphate (ATP) disodium salt, magnesium sulfate ($MgSO_4$), and Tris−HCl were purchased from Sigma-Aldrich (St. Louis, MO, USA). X-ShiningTM luciferase (*Photuris pennsylvanica* luciferase, 10 mg/mL aqueous solution with glycerol) was purchased from Biosynth (Staad, Canton of St. Gallen, Switzerland). The Wizard Plus Maxipreps DNA purification system was purchased from Promega (Madison, WI, USA). 6-Aminobenzo[d]thiazole-2-carbonitrile was purchased from Fluorochem (Cork, Ireland). D-cysteine, CDCA, and all other reagents were purchased from Merck Life Science (Milan, Italy).

Analytical standards of BAs, comprising primary, secondary, conjugated, and oxo-bile acid species, along with isotopically labeled internal standards (IS), were either purchased from Sigma-Aldrich (St. Louis, MO, USA), Steraloids (Newport, CA, USA), or synthesized in-house as previously described.[32] LC-MS grade solvents, including methanol (MeOH), acetonitrile (ACN), and isopropanol, as well as reagents

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such as formic acid, acetic acid, and ammonium hydroxide, were obtained from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland). Ultrapure water was generated using a Milli-Q purification system from Millipore (Molsheim, France). Plasmids harboring *Phrixotrix hirtus* red-emitting luciferase (pCMV-RE-R215K) and *Amydetes vivianii* luciferase (pCMV-AmyLuc) were previously developed[29,30,33] and provided by one of the authors (VRV).

Human Subjects and Sample Collection

The ALMA0118 study was approved by the Local Ethics Committee of the Emilia-Romagna region (protocol no. 601/2018/Sper/AOUBo) and conducted in accordance with the Declaration of Helsinki and its subsequent amendments. This exploratory single-center study was performed at Sant'Orsola Malpighi Hospital (Bologna, Italy). A total of 54 male participants were enrolled and stratified into three groups: hyperplastic polyps (n = 7; mean age 63.3 ± 8.6 years), low- to high-grade dysplastic adenomas (n = 27; mean age 61.3 ± 9.7 years), and CRC (n = 20; mean age 68.3 ± 0.5 years). Written informed consent was obtained from all participants prior to enrollment. Fecal and blood samples were collected for subsequent analyses, yielding 39 fecal samples and 53 serum samples (not all enrolled patients were available to provide a fecal sample, and one patient withdrew from the study). All human-derived biological samples were handled in accordance with institutional biosafety regulations under biosafety level 2 (BSL-2) conditions.

Calibration Curve of Purified Choloylglycine Hydrolase (CH) by aLuc-CDCA Cleavage via a Cell-Free BL System

Deconjugation of the synthetic substrate aLuc-CDCA was evaluated *in vitro* using purified CH from *C. perfringens*. CH solutions (0.06−0.30 U/mL) were prepared in PBS supplemented with 1 mM DTT (PBS-DTT). Briefly, 50 μL of CH solution was incubated with 50 μL of aLuc-CDCA (50 μM in PBS) at 37 °C for 30, 60, and 120 min. Following incubation, the BL reaction was triggered by adding 60 μL of luciferase reaction mixture containing X-Shining luciferase (0.5 mg/mL in 0.1 M Tris−HCl, pH 7.5, containing 80 mM $MgSO_4$ and 40 mM ATP). BL reactions were performed in black flat-bottom 96-well microplates. BL emission was monitored for 90 min using a Varioskan Lux multimode plate reader (Thermo Fisher Scientific, Waltham, MA, USA) maintained at 37 °C. Curves were generated by plotting BL intensity vs CH concentration and fitting the data with linear regression. The limits of detection (LOD) and quantification (LOQ) of the assay were determined as the concentrations of aLuc-CDCA that yielded BL signals equal to the blank plus 3 times and 10 times its standard deviation, respectively.

Preparation of Human Fecal Samples

Human fecal samples were suspended in PBS supplemented with 1 mM DTT (PBS-DTT) to a final concentration of 1 mg/mL. DTT was added to maintain the catalytic cysteine residues of fecal BSH enzymes in a reduced state, thereby preserving enzymatic activity during sample preparation and incubation.[34] Fecal suspensions were incubated for 60 min under continuous rotation, vortexed, and centrifuged at 13,000 rpm for 15 min. Following centrifugation, the clarified supernatants were collected and used for all subsequent cell-free and whole-cell BL experiments.

Quantification of Fecal BSH Activity in a Cell-Free System

Human fecal supernatant was prepared as described above and diluted in serial steps (range 0.01−1 mg/mL). For BL measurements, 100 μL of each fecal dilution was incubated with aLuc-CDCA (final concentration: 50 μM in PBS) at 37 °C for 60 min. The BL reaction was triggered by adding 60 μL of luciferase reaction mixture containing X-Shining luciferase (0.5 mg/mL in 0.1 M Tris−HCl buffer, pH 7.5, supplemented with 80 mM $MgSO_4$ and 40 mM ATP), as described before. In parallel, CH standard solutions (0.06−0.30 U/mL in PBS-DTT) were analyzed under identical conditions to generate calibration curves (as previously described) for quantifying BSH activity in fecal samples. BL emission was monitored using a Varioskan Lux multimode plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Cell Culture

Human colorectal adenocarcinoma cells (Caco-2) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Caco-2 cells were grown in DMEM high glucose, supplemented with 10% heat-inactivated FBS, 2.5 mM L-glutamine, and penicillin/streptomycin, and maintained at 37 °C in an atmosphere of 5% CO_2. Cells were routinely passaged at 70−80% confluence.

Brazilian Engineered Beetle Luciferases

The engineered cDNAs for the Brazilian *P. hirtus* red-emitting mutant R215K (PxRE-R215K) and *A. vivanii* firefly luciferases inside the pCold vector (Takara) were previously developed[27,31] and provided by the Federal University of São Carlos (Sorocaba, Brazil). The respective codon optimized luciferase DNAs in mammalian plasmid vectors, pCMV-Amy and pCMV-PxRE-R215K, were also previously developed.[28−30] Plasmid DNAs were purified using the Wizard Plus Maxiprep DNA Purification System (Promega).

Transfection of Caco-2 Cells

Caco-2 cells were transfected with each plasmid encoding either the green-emitting AmyLuc or the red-emitting PxRE-R215K luciferase using the Lipofectamine 3000 transfection reagent according to the manufacturer's instructions. Briefly, cells were seeded at a density of 10×10^4 cells/well in clear-bottomed, black-walled 96-well tissue-culture-treated plates and cultured in phenol red-free DMEM supplemented with 10% FBS, 2.5 mM L-glutamine, and penicillin/streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h prior to transfection, reaching approximately 70−100% confluence.

BL Emission Spectra

BL emission spectra were recorded using a Varioskan Lux multimode plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Spectral scans were acquired at 37 °C using bandpass filters ranging from 400 to 700 nm for AmyLuc and from 600 to 800 nm for PxRE-R215K, with an integration time of 1000 ms per wavelength. Emission spectra were measured in Caco-2 cells transfected with either AmyLuc or PxRE-R215K following the addition of 6′-amino-D-luciferin (final concentration: 0.1 mM). To evaluate the effect of the fecal matrix on the BL emission profiles, the same measurements were repeated in the presence of six representative fecal supernatants (1 mg/mL)

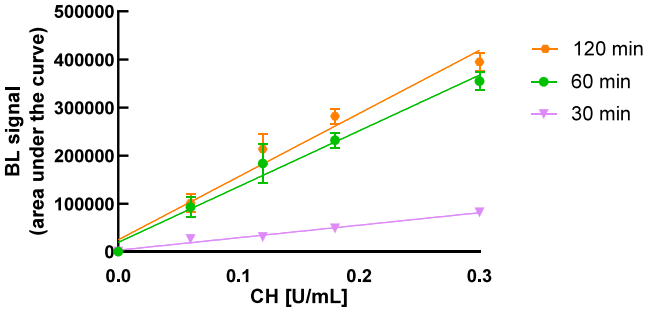

Figure 1. Calibration curves of purified CH (range 0.06−0.30 U/mL) obtained by analyzing BL signals after 30, 60, and 120 min upon *Photuris pennsylvanica* firefly luciferase addition. Each point represents the mean ± SD of three independent measurements.

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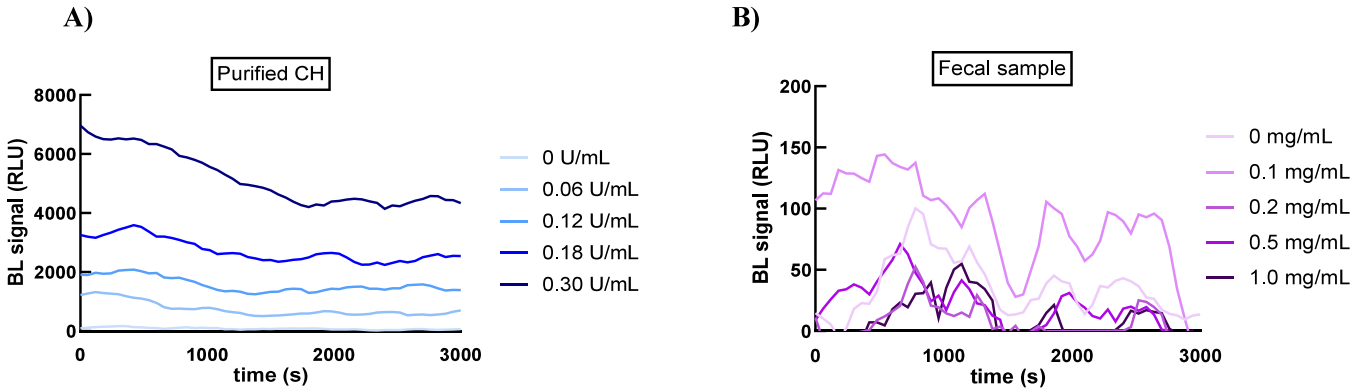

Figure 2. (A) BL kinetic profiles obtained in the presence of different CH concentrations after 60 min of exposure (range 0.06−0.30 U/mL) in PBS-DTT. (B) BL kinetic profiles obtained in the presence of different concentrations of a diluted human fecal sample (range 0.1−1.0 mg/mL).

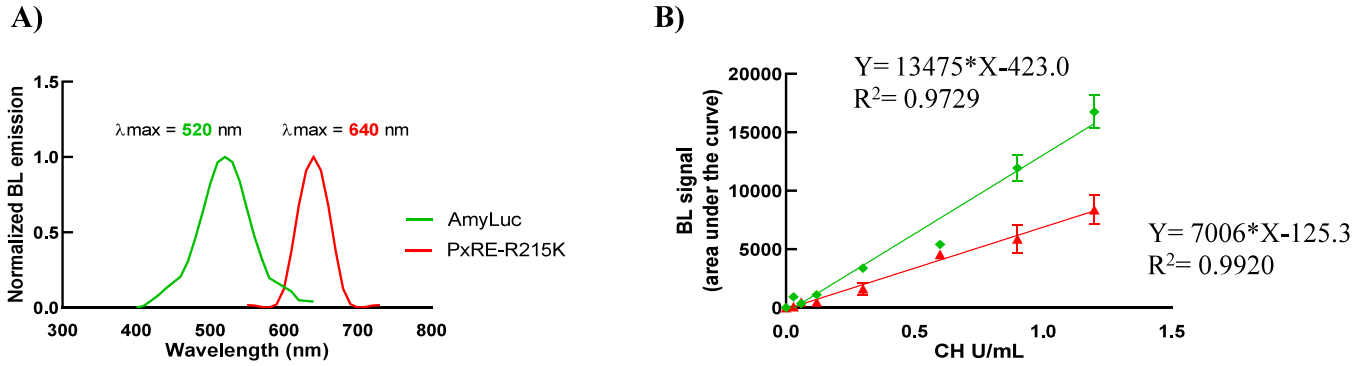

Figure 3. (A) Normalized BL emission spectrum of AmyLuc and PxRE-R215K plasmids in transfected Caco-2 cells. Spectra were normalized to the maximum emission intensity. (B) BL signal curves of Caco-2 cells transfected with AmyLuc or PxRE-R215K, treated with purified CH (range 0.03−1.2 U/mL) in PBS-DTT solution, and then exposed for 60 min to the aLuc-CDCA probe.

prepared as previously described. The backgrounds were automatically subtracted from each spectrum.

Whole Cell-Based BL Bioassay Assessment

For BL measurements, CH solutions (range 0.03−1.2 U/mL in PBS-DTT) were preincubated with aLuc-CDCA (50 μM in PBS) at 37 °C for 60 min and subsequently added to transfected cells. 6′-Amino-D-luciferin and D-luciferin (0.1 mM in PBS) were used as positive controls to confirm successful luciferase expression following transfection. BL emission was monitored for 90 min using a Varioskan Lux multimode plate reader (Thermo Fisher Scientific, Waltham, MA, USA). BL imaging experiments were also performed on transfected Caco-2 cells following the addition of D-luciferin (0.1 mM) to better characterize the whole-cell reporter system and confirm the intracellular origin of the detected BL signal, as described in the Supporting Information. To identify the optimal fecal concentration for subsequent analyses, fecal supernatants from three independent donors were prepared at different concentrations (range 0.25−1.0 mg/mL) and analyzed using both AmyLuc- and PxRE-R215K-transfected Caco-2 cells under identical experimental conditions. 100 μL of fecal supernatant for each concentration was incubated with 50 μL of aLuc-CDCA (50 μM in PBS) at 37 °C for 60 min. Following probe deconjugation, reaction mixtures were added to transfected Caco-2 cells for BL detection. Based on these preliminary optimization experiments, a fecal concentration of 1.0 mg/mL was selected for all subsequent studies. The assay accuracy was evaluated using spike-and-recovery experiments performed on diluted fecal samples (final concentration 1 mg/mL) from three independent donors. Samples were spiked with purified CH (range 0.12−1.20 U/mL) and analyzed using both the AmyLuc- and PxRE-R215K-based whole-cell BL assays. BL signals were interpolated against a calibration curve generated with purified CH under identical assay conditions and expressed as CH-equivalent activity values. Recovery (%) was calculated according to the following equation:

$$\text{Recovery (\%)} = [(\text{measured activity in spiked samples} - \text{endogenous activity})/\text{added CH activity}] \times 100$$

On the same three donors, interassay precision was assessed in fecal samples spiked with purified CH at an intermediate activity of 0.30 U/mL. These spiked fecal samples were analyzed in three independent experiments performed on different days under identical assay conditions. Precision was expressed as the coefficient of variation (CV%), calculated as CV (%) = (SD/mean) × 100, where SD is the standard deviation and mean is the average CH-equivalent activity measured across independent experiments. To further investigate the mechanisms underlying reporter performance, additional characterization experiments were conducted to assess the optical behavior of six representative fecal supernatants (1 mg/mL). Absorbance spectra (400−700 nm) were acquired using a Varioskan Lux plate reader, and wavelength-dependent light transmission was calculated from absorbance values (Supporting Information). Signal-retention experiments were performed to compare AmyLuc and PxRE-R215K BL signal generated in PBS-DTT and fecal supernatants following addition of

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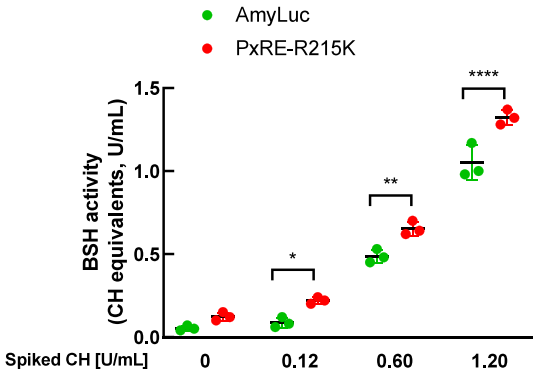

Figure 4. Spike-recovery analysis performed in fecal samples from three donors. Samples were supplemented with increasing concentrations of purified CH (range 0.12−1.20 U/mL) and analyzed using Caco-2 cells expressing either AmyLuc or PxRE-R215K luciferase. Data are reported as mean ± SD. Statistical significance between reporter systems at each CH concentration revealed significant differences among the compared groups (*$p < 0.05$; **$p < 0.001$; ****$p < 0.0001$).

6′-amino-D-luciferin (0.1 mM). The pH of PBS-DTT, fecal supernatants, and post-transfection culture supernatants was also measured to exclude pH as a potential confounding factor; all samples exhibited comparable pH values (7.09−7.34).

Application of the Red-Shifted Whole-Cell-Based BL Bioassay to Measure Fecal BSH Activity in Clinical Samples

Frozen human fecal samples collected from subjects enrolled in the clinical study ($n = 39$; 6 hyperplastic polyps, 22 adenomas, and 11 CRC samples) were processed as previously described and analyzed using Caco-2 cells expressing the red-emitting PxRE-R215K luciferase. Briefly, 100 µL of fecal supernatant (1 mg/mL) was incubated with 50 µL of aLuc-CDCA (50 µM in PBS) at 37 °C for 60 min. Following probe deconjugation, reaction mixtures were added to transfected Caco-2 cells for BL detection. In parallel, CH standard solutions (0.03−1.2 U/mL in PBS-DTT) incubated with aLuc-CDCA under identical experimental conditions were used to generate calibration curves for BSH activity quantification. 6′-Amino-D-luciferin and D-luciferin (0.1 mM in PBS) were included as positive controls to confirm successful luciferase expression following transfection. BL emission was recorded for 90 min using a Varioskan Lux multimode plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

UHPLC Mass Spectrometry Analysis of BAs in Human Serum and Fecal Samples

Serum sample preparation was performed according to a previously published method[35] with slight modifications. Briefly, 100 µL of serum was spiked with 10 µL of IS solution, diluted with 0.1 N NaOH, and incubated at 64 °C for 30 min prior to solid-phase extraction (SPE) extraction.

Fecal samples were prepared using an established procedure.[36] Briefly, 300 mg of wet, homogenized samples was extracted with isopropanol and homogenized using a high-speed homogenizer. The samples were then stirred for 2 h, sonicated for 20 min, and centrifuged twice at 13,000 rpm. The resulting supernatant was diluted 1:10 (v/v) with a solution of 40% isopropanol and 15 mM ammonium acetate (pH 8.0), transferred to autosampler vials, and analyzed using UHPLC-ESI-MS/MS.

Liquid chromatography analyses were carried out using a UHPLC 1290 Infinity II (Agilent Technologies, Inc., Milan, Italy) system coupled to a 6495 LC/TQ triple quadrupole mass spectrometer (Agilent Technologies, Inc., Milan, Italy) operating in multiple reaction monitoring (MRM) mode.

For serum analysis, the method enabled the comprehensive profiling of 18 analytes, including free and conjugated primary and secondary BAs, as well as selected sulfated species. Chromatographic separation was achieved using an ACQUITY Premier CSH Phenyl-Hexyl column (150 × 2.1 mm, 1.7 µm; Waters), maintained at 40 °C. The mobile phases were water containing 15 mM ammonium acetate (pH 8.0) and ACN:MeOH (75:25, v/v), delivered at a flow rate of 0.25 mL/min under gradient elution conditions. The total run time was 27 min. Method development and validation have been previously described.[35]

Fecal analysis targeted 27 BA-related analytes, including primary and secondary BA and their downstream metabolites in order to capture the main BA metabolic pathways. The separation was performed using an ACQUITY UPLC BEH C18 column (150 × 2.0 mm, 1.7 µm; Waters), which was maintained at 40 °C. The mobile phases were water containing 15 mM ammonium acetate (pH 8.0) and methanol, with a flow rate of 0.3 mL/min. Gradient elution provided a total run time of 30 min with an injection volume of 2 µL. MRM transitions for all analytes have been reported previously.[3]

Statistical Analysis

GraphPad Prism v6.0 (GraphPad Software, La Jolla, CA, USA) was used for data analysis and graphical representation. Calibration curves were generated by linear regression analysis of BL intensity vs CH concentration. Differences between AmyLuc and PxRE-R215K reporter systems across increasing spiked CH concentrations were analyzed by two-way ANOVA followed by Šídák's multiple-comparisons test. Differences in fecal BSH activity among clinical groups were evaluated using the Kruskal-Wallis test followed by Dunn's multiple comparisons test. A p value < 0.05 was considered statistically significant. Unless otherwise specified, experiments were performed using at least three technical replicates and repeated independently at least twice. Data are presented as mean ± standard deviation (SD). Calibration curves and fecal sample analyses were reproduced in independent experimental batches to assess assay reproducibility.

■ RESULTS AND DISCUSSION

Design Principle of the BSH Activity Sensing

The design strategy is based on the conjugation of the D-amino luciferin scaffold to the primary BA, CDCA, via an amide bond that is cleaved by BSH enzymes.

Since the addition of this group to the luciferin scaffold results in a caged molecule that can no longer be recognized as a substrate by the luciferase, subsequent photon emission is then dependent on the cleavage of the "caged" amino-luciferin. Previous studies have demonstrated that the amidated BA bond of aLuc-CDCA is stable in biological fluids and is selectively hydrolyzed by BSH, supporting the probe's specificity for microbiota-associated BSH activity.[24]

Firstly, we evaluated aLuc-CDCA deconjugation in a cell-free system using purified CH from *C. perfringens*, a representative member of the BSH enzyme family with well-characterized structural and biochemical properties.[18,22,37] The "caged" amino luciferin hydrolysis was previously compared with the catalytic effect on physiological glycine-conjugated CDCA (GCDCA), while proline conjugated to CDCA (PCDCA) was used as a negative control since it was stable toward CH activity by using HPLC-MS,[24] demonstrating the selective recognition and hydrolysis of the aLuc-CDCA by BSH enzymes.

Calibration curves at different incubation times (30, 60, and 120 min) showed a linear correlation between BL emission intensity and increased CH activity (range 0.06−0.30 U/mL) (Figure 1), confirming that probe cleavage is directly

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Table 1. Recovery of Purified CH Spiked into Three Human Fecal Samples from Three Independent Subjects in the Red- and Green-Emitting Whole Cell-Based Assay

spiked CH [U/mL]	recovered CH-equivalent activity [U/mL] in stool samples (PXRE-R215K)	recovery % (PXRE-R215K)	recovered CH-equivalent activity [U/mL] in stool samples (AmyLuc)	recovery % (AmyLuc)
0.12	0.10 ± 0.02	83.3 ± 12.4	0.08 ± 0.03	66.7 ± 12.8
0.60	0.54 ± 0.05	90.2 ± 7.2	0.48 ± 0.04	80.4 ± 8.4
1.2	1.12 ± 0.08	93.4 ± 8.3	1.05 ± 0.11	87.5 ± 7.5

transduced into BL signal generation. This response is consistent with previous fluorescence- and colorimetric-based methods developed for BSH activity measurements,[21,38] while the luciferin-based design benefits from the intrinsically low signal-to-noise ratio typically associated with BL reporters.[22,39]

Among the incubation times tested, 60 min yielded a stronger, more consistent BL signal than 30 min, with no significant improvement at 120 min. Under these conditions, the assay achieved an LOD of 0.010 ± 0.002 U/mL and an LOQ of 0.040 ± 0.001 U/mL. The lack of signal enhancement at longer incubation times suggests that extended exposure does not improve assay sensitivity and could contribute to increased non-specific background, as previously observed in luciferase-based systems.[40] Therefore, a 60 min incubation was considered the best compromise between analytical sensitivity and experimental throughput and was adopted for further experiments.

Detection of BSH Activity in Fecal Matrices *In Vitro* in a Cell-Free System

To test whether the aLuc-CDCA probe could be applied in physiologically relevant samples, BSH was next investigated in human fecal samples. Consistent with the results previously shown in Figure 1 using purified CH, the probe produced robust concentration-dependent BL responses after 60 min of exposure (CH range 0.06−0.30 U/mL) (Figure 2A). However, we found that light output markedly decreased when the luciferase was exposed to fecal material (dilution range: 0.1−1 mg/mL) (Figure 2B), rendering these measurements unsuitable for this purpose. Accordingly, although the engineered X-Shining luciferase was initially selected because of its enhanced stability and suitability for caged luciferin-based assays, it did not overcome the strong fecal matrix interference that impairs either luciferase catalytic activity or photon emission. Similar inhibitory effects have been previously described in BL assays performed in complex biological samples[22,41] where BAs, heme derivative compounds, or reactive oxygen species can interfere with luciferase stability or light transmission.[42] In addition, fecal samples contain proteases, redox-active molecules, and microbial metabolites that may further affect luciferase activity or interact with the amino-luciferin substrate, thereby decreasing signal intensity.[21,43,44] These findings indicate that although the aLuc-CDCA probe performs efficiently in purified systems, its direct application in fecal samples is significantly limited by matrix effects. This represents a critical challenge, since stool samples are among the most accessible and clinically relevant materials for monitoring gut microbial enzymatic activity. Similar limitations have been reported for other luminescence- and enzyme-based stool assays.[22,41,45] The pronounced inhibitory effect observed in fecal samples, therefore, highlighted the need for an alternative assay configuration capable of preserving signal generation under physiologically relevant conditions.

In Vitro Detection of BSH Activity in Human Fecal Samples Using a Whole Cell-Based BL Assay: Comparison of Green- and Red-Emitting Brazilian Luciferases

To overcome the limitations of the previously described BL assay, we extend the established BSH-activatable luciferin/whole-cell sensing concept[22] by demonstrating that the selection of the luciferase-based reporter displaying different emission wavelengths can improve quantitative performance in complex fecal matrices.

Caco-2 cells were selected for their human intestinal epithelial origin and robustness under chemically variable conditions.[46] Moreover, the intracellular expression of luciferases in mammalian cells has been shown to substantially improve the assay performance in complex biological matrices compared with purified enzyme-based systems.[22,47,48]

In particular, two previously engineered Brazilian luciferases were evaluated since they can efficiently emit light with 6'-Amino-D-Luciferin:[49] the green-emitting luciferase from *Amydetes vivianii* (AmyLuc)[31] and the red-emitting mutant PxRE-R215K derived from *Phrixothrix hirtus*,[27] the only beetle luciferase known to naturally emit red light with improved properties.[27,49,50] Because red-shifted emission is less affected by absorption and scattering in biological matrices, PxRE-R215K was compared with the brighter green-emitting AmyLuc to identify the construct that provides the best sensitivity and robustness for BSH detection in a whole-cell format.[26,27,49,51,52] This comparison was particularly relevant for fecal applications, since shorter-wavelength green emissions are more susceptible to optical scattering and endogenous absorption, potentially limiting quantitative BL detection in minimally processed samples. Emission spectra recorded from transfected Caco-2 cells in the presence of 6′-amino-D-luciferin (0.1 mM final concentration) showed a maximum peak at

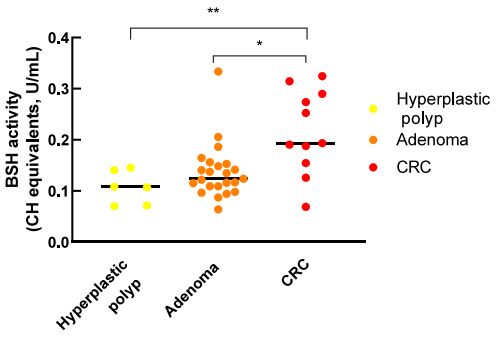

Figure 5. Quantitative assessment of fecal BSH activity in clinical samples from subjects with hyperplastic polyps ($n = 6$), adenomas ($n = 22$), and CRC ($n = 11$), using PxRE-R215K-transfected Caco2 cells. Each dot represents individual sample values, while horizontal lines indicate the median for each group. Statistical analysis revealed significant differences among the three groups ($^*p < 0.05$; $^{**}p < 0.01$).

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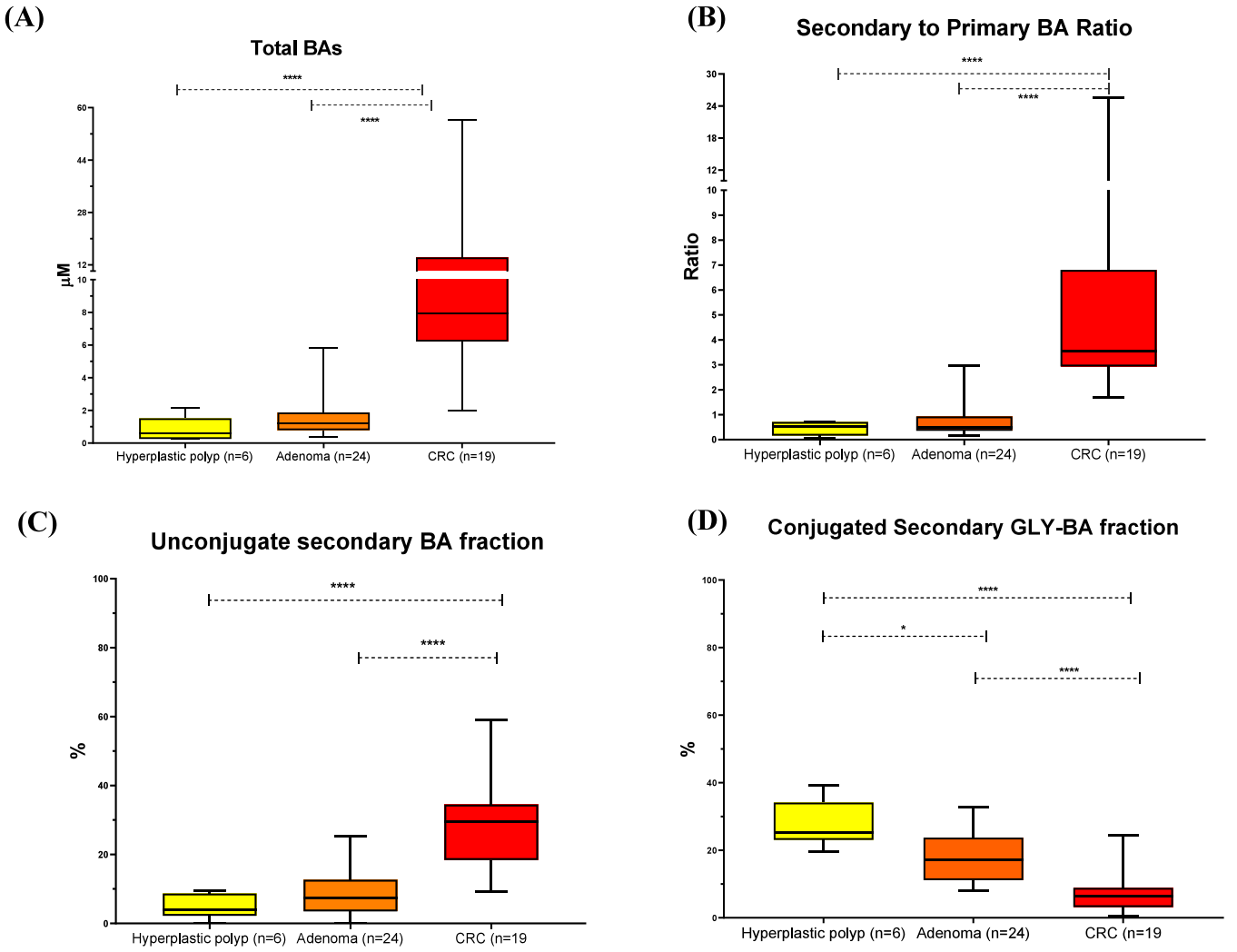

Figure 6. Box-and-whisker plots showing (A) total BA concentrations (μM), (B) secondary-to-primary BA ratio (unitless), (C) unconjugated secondary BA fraction (%), and (D) conjugated secondary glycine BA fraction (%). Statistical significance is indicated as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****)

approximately 520 nm for AmyLuc, consistent with green emission, whereas PxRE-R215K displayed a distinct maximum around 640 nm in the red/far-red region (Figure 3A). These emission profiles were consistent with previously reported spectra for *A. vivianii* and *P. hirtus* luciferases.[28] Spectral variations have been reported in cell-based luciferase systems and are generally attributed to differences in the intracellular microenvironment, substrate, and assay conditions, as well as different photodetection equipment, for which the photosensitivity spectral response has not been corrected.[53] To optimize the bioassay procedure, purified CH (range 0.03−1.2 U/mL) was incubated with the aLuc-CDCA probe at 37 °C for 60 min to allow enzymatic hydrolysis. The resulting reaction mixtures were then added to Caco-2 cells previously transfected with either AmyLuc or PxRE-R215K plasmids. For both constructs, BL intensity increased linearly with CH activity (Figure 3B). The calculated LOD and LOQ values were 0.03 ± 0.02 and 0.08 ± 0.03 U/mL for AmyLuc, and 0.02 ± 0.01 and 0.06 ± 0.04 U/mL for PxRE-R215K, respectively. Although PxRE-R215K produced a lower overall BL signal than AmyLuc, consistent with previous reports,[49] both constructs provided robust quantitative detection of enzymatic activity.

Analytical Performance of the Whole Cell-Based BL Bioassays

To identify the optimal dilution for fecal samples, different dilutions (range 0.1−1.0 mg/mL) were tested using both whole-cell reporter systems (Figure S1). AmyLuc generated higher absolute BL intensities, particularly at 1.0 mg/mL, whereas PxRE-R215K exhibited a more gradual concentration-dependent response with reduced signal fluctuations across the tested dilutions. Based on these results, 1.0 mg/mL was selected as the working fecal concentration for all subsequent experiments.

Spike-and-recovery experiments were performed in 1 mg/mL fecal samples from three independent subjects. BL signals were interpolated against a calibration curve generated with purified CH under identical assay conditions as described above, yielding fecal endogenous BSH activities of 0.12 ± 0.02 U/mL and 0.05 ± 0.01 U/mL with the PxRE-R215K and AmyLuc reporters, respectively. Recovery values, therefore,

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reflected the ability of the assay to accurately quantify added CH activity in the presence of fecal matrix components.

As shown in Figure 4, the red-emitting PxRE-R215K cell-based system consistently generated stronger and increased BSH activity after the addition of purified CH enzyme (range 0.12−1.2 U/mL) than the green-emitting AmyLuc luciferase. This behavior is consistent with the improved optical transmission and reduced scattering typically associated with red-shifted BL in complex biological samples.[25,26,52] Recovery analysis further confirmed the superior analytical performance of the red-emitting reporter, presumably due to a better signal-to-noise ratio than the green-emitting system. Recovery values for the PxRE-R215K system ranged from 83.3% to 93.4%, compared with 66.7% to 87.5% for AmyLuc, indicating more accurate quantification of CH activity in the presence of fecal matrix components (Table 1). Also, the PxRE-R215K cell-based system showed improved reproducibility. Interassay precision was evaluated in fecal samples from the same three donors. The fecal samples were spiked with purified CH at an intermediate activity level (0.30 U/mL) and analyzed across three independent experiments conducted on different days. Under these conditions, PxRE-R215K exhibited an interassay coefficient of variation (CV) of 14%, whereas AmyLuc showed substantially higher variability (CV = 29.9%), further supporting the suitability of the red-emitting reporter for quantitative measurements in complex fecal matrices. Consistently, signal-retention experiment further demonstrated that the red-emitting PxRE-R215K reporter transmitted a significantly larger fraction of its emitted signal than AmyLuc in fecal matrices (Figure S2). PxRE-R215K was therefore selected for subsequent analyses. Absorbance spectra acquired from six representative fecal supernatants demonstrated consistently lower absorbance at 640 nm (Figure S3), and AmyLuc was more strongly attenuated than PxRE-R215K (Figure S5 and Table S1). These findings support improved transmission of the red-shifted signal through the fecal matrix. BL imaging also confirmed substrate-dependent emission from the transfected cell monolayer (Figure S4).

Evaluation of the Red-Shifted Whole Cell-Based BL Bioassay in Clinical Samples Including Hyperplastic Polyps, Adenomas, and Colorectal Cancer

As a proof of concept, BSH activity was quantified in all fecal samples collected from subjects with hyperplastic polyps, low- or high-grade dysplastic adenomas, and CRC using the PxRE-R215K reporter system. As shown in Figure 5, CRC patients displayed significantly higher BSH activity than subjects with hyperplastic polyps ($p < 0.01$) and adenomas ($p < 0.05$), whereas no significant difference was observed between hyperplastic polyps and adenomas. Importantly, the red-shifted whole-cell-based BL assay generated robust and reproducible luminescence signals across all clinical samples, further supporting its applicability as a non-invasive approach for rapid functional profiling of BSH activity in fecal samples.

EVALUATION OF SERUM BA PROFILES BY UHPLC-MS/MS

To validate the red-shifted whole cell-based BL assay for the detection of fecal BSH activity, BA profiles were analyzed in matched serum and fecal samples from the same cohort using UHPLC-MS/MS.

Serum BA analysis revealed significant alterations in CRC subjects compared with individuals with hyperplastic polyps and adenomas (Figures 6 and S6 and Table S2), supporting the increased fecal BSH activity detected by the red-shifted whole cell-based BL assay.

In contrast, fecal BA concentrations showed high interindividual variability and no statistically significant differences among the clinical groups (Figure S7 and Table S3). Interestingly, oxo-BA species did not differ significantly among the clinical groups, as reported in Supporting Information Table S2. These findings suggest that the most prominent BA metabolism alteration associated with CRC involves enhanced deconjugation and increased formation of secondary BAs, whereas downstream oxidative transformations appear to be less affected.

Collectively, the observed increase in fecal BSH activity, together with the alterations in circulating BA composition and the progressive increase in the secondary-to-primary BA ratio, supports the existence of disease-associated changes in microbial BA metabolism.

CONCLUSIONS

In this work, we optimized and analytically validated a red-shifted whole-cell BL configuration for detecting BSH activity in human fecal samples, designed to reduce matrix-associated interference and wavelength-dependent signal attenuation. Although validation in larger, well-characterized cohorts is required, these findings support the feasibility of the assay for monitoring fecal BSH activity in fecal samples. This assay could be further improved upon use of new variants of the far-red emitting luciferase with stronger signals and higher stability under development in one of our laboratories. Commercial translation, therefore, will require a stable PxRE-R215K reporter cell line supplied in a standardized, ready-to-use format, with validated post-thaw performance, reproducibility, storage conditions, and shelf life.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.6c02592.

Additional experimental details, including optimization of fecal sample dilution for the whole-cell BL assay (Figure S1), evaluation of BL signal retention in fecal matrices (Figure S2), BL emission spectra recorded in fecal supernatants (Figure S3), BL imaging of transfected Caco-2 cells (Figure S4), optical characterization of fecal supernatants (Figure S5 and Table S1), supporting experimental procedures, and serum and fecal bile acid quantification and profiling data (Tables S2 and S3; Figures S6 and S7) (DOCX)

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Author Contributions

A.P. conceived and designed the study, performed the experiments, analyzed the data, and wrote the manuscript. A.S. and G.G. contributed to experimental work and data collection. E.P. and R.C. performed UHPLC−MS analyses and contributed to data interpretation. V.R.B. contributed to luciferase purification, cell transfections, and assay establishment using Brazilian beetle luciferases. L.R. contributed to patient recruitment, clinical sample collection, and clinical data interpretation. B.C. and A.G. designed and synthesized the aLuc-CDCA probe. V.R.V. contributed by providing improved engineered Brazilian beetle luciferases, discussing experiments, and writing the manuscript. A.R. contributed to study supervision, data interpretation, and manuscript revision. C.C. conceived and supervised the study, contributed to data interpretation, secured funding, and revised the manuscript. All authors reviewed and approved the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

aLuc-CDCA	amino-luciferin chenodeoxycholic acid
AmyLuc	*Amydetes vivianii* firefly luciferase
ATP	adenosine 5′-triphosphate
BA	bile acid
BL	bioluminescence
BSH	bile salt hydrolase
CA	cholic acid
CDCA	chenodeoxycholic acid
CH	choloylglycine hydrolase
CRC	colorectal cancer
CV	coefficient of variation
DCA	deoxycholic acid
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
FBS	fetal bovine serum
FXR	farnesoid X receptor

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GCDCA	glycine-conjugated chenodeoxycholic acid
HPLC-MS	high-performance liquid chromatography−mass spectrometry
IBD	inflammatory bowel disease
IS	internal standard
LCA	lithocholic acid
LOD	limit of detection
LOQ	limit of quantification
MRM	multiple reaction monitoring
PBS	phosphate-buffered saline
PBS-DTT	phosphate-buffered saline supplemented with dithiothreitol
PCDCA	proline-conjugated chenodeoxycholic acid
PxRE-R215K	red-emitting luciferase mutant derived from *Phrixothrix hirtus*
SD	standard deviation
SPE	solid-phase extraction
TGR5	Takeda G protein-coupled receptor 5
UHPLC-ESI-MS/MS	ultra-high-performance liquid chromatography−electrospray ionization tandem mass spectrometry
UHPLC-MS/MS	ultra-high-performance liquid chromatography−tandem mass spectrometry

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