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Research article

Evaluation of conditions for the intestinal probiotic-mediated biotransformation of Sennosides into Physcion in *Cassia occidentalis* extracts

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Abstract: Anthraquinone compounds (AQCs) are functional constituents of various medicinal and edible plants belonging to families such as Fabaceae, Polygonaceae, and Liliaceae. They include sennosides, emodin, rhein, aloe-emodin, chrysophanol, and physcion, which show physiological activities, such as bowel movement promotion, hepatoprotection, anti-inflammatory effects, and antioxidation, as well as antimicrobial properties. The seeds of *Cassia occidentalis* L. (Fabaceae), an annual herb widely distributed in tropical and subtropical regions, are abundant in anthraquinone compounds, of which sennosides account for approximately 80% of the total anthrone-related constituents, and have traditionally been used as stimulant laxatives. However, sennosides are inactive forms and do not exert physiological effects until they reach the colon, where they are biotransformed

by intestinal microbiota into downstream metabolites with biological activity. The major bioactive sennoside metabolites are rhein anthrone and rhein, which can be detected in the mouse colon within 2–3 h after oral administration of sennosides, confirming the crucial role of gut microbiota, particularly the intestinal probiotics *Bifidobacterium* and *Lactobacillus*, in sennoside conversion. This study aimed to investigate whether commercially available common gut probiotics possess the ability to hydrolyze sennosides in *C. occidentalis* extracts, to determine the suitable conditions for such hydrolysis, and to further evaluate the efficiency of sennoside conversion into physcion and rhein. Of the 20 commonly used intestinal probiotic strains tested in this study, *Lactobacillus delbrueckii* subsp. *lactis* (BCRC 14069) and *Lactobacillus helveticus* (BCRC 14092) were the most efficient at sennoside hydrolysis. Under suitable conversion conditions, the hydrolysis rate reached over 95%, reducing the residual sennoside content to 6–7 mg/g while significantly enhancing the production efficiency of the downstream metabolites, physcion and rhein. Furthermore, even when administered at relatively high doses according to different application needs, the hydrolyzed products remained compliant with both Taiwan Food and Drug Administration (TFDA) (12 mg/day) and European Food Safety Authority (EFSA) (10 mg/day) regulatory limits. These findings provide scientific support for microbial biotransformation and the potential use of anthraquinone compounds, and a way to mitigate the adverse effects (such as intestinal dependence and electrolyte imbalance) of traditional sennoside-based products. This work facilitated the development of *C. occidentalis* or sennoside-based products for use in functional foods and nutraceuticals and promotes their modernization in herbal applications.

Keywords: *Cassia occidentalis* L.; sennosides; biotransformation; physcion; intestinal probiotics; Anthraquinone compounds

1. Introduction

Anthraquinone compounds (AQCs), including sennosides, emodin, chrysophanol, aloe-emodin, physcion, and rhein, are among the most widely distributed quinone substances in nature [1,2]. They include sennosides, emodin, rhein, aloe-emodin, chrysophanol, and physcion, which show physiological activities, such as bowel movement promotion, hepatoprotection, anti-inflammatory effects [3], antioxidation, reactive oxygen species (ROS) regulation [1,4], mitochondrial protection, and regulation of various signaling pathways [5]. Due to these physiological activities, AQCs are major bioactive materials used for developing pharmaceutical preparations and functional health foods [6]. However, most plant anthraquinones occur as glycosidic forms, which have relatively low bioavailability and reduced physiological activity, and, therefore, require metabolic conversion in order to fully exert their health-promoting effects [5]. Sennosides themselves do not possess direct physiological activity and require metabolic conversion by intestinal microorganisms to show their main effects [7]. In animal experiments, orally administered sennosides are largely unabsorbed by the stomach and small intestine and must be hydrolyzed in the large intestine by enzymes, such as β-glucosidase, or transformed into active metabolites, such as rhein anthrone and rhein, by intestinal probiotics [8]. These metabolites stimulate the mucosa, promote water and electrolyte secretion, inhibit water reabsorption, and enhance intestinal peristalsis in order to produce a laxative effect [9]. However, long-term or high-dose use of sennosides may cause adverse effects such as melanosis coli, electrolyte imbalance, and functional dependence of the intestine [10]. Therefore, the Taiwan Food and Drug

Administration (TFDA) stipulates that the daily intake of sennosides must not exceed 12 mg [11], and amounts exceeding this must be declared as medicinal products; the European Union's European Food Safety Authority (EFSA) sets an even stricter limit of <10 mg/day [12,13].

Rhein is the anthroquinone with the highest pharmacological value, being widely present in medicinal plants such as rhubarb (*Rheum palmatum*), senna, aloe, and *Polygonum cuspidatum* [14]. It is also an important terminal product of sennoside metabolism in the intestine, with an oral bioavailability of approximately 35%–55%. Many studies have demonstrated that rhein and other anthraquinones possess diverse biological activities, including bowel movement promotion, hepatoprotection, renoprotection (such as ameliorating renal fibrosis in AKI and CKD) [2,15], and anti-inflammatory [14,16], antioxidant [17], antitumor [18,19], and antibacterial [15,16] effects. *Cassia occidentalis* (Fabaceae), known as Shi Jue Ming (Goat's Horn Bean) in Chinese, is an annual herb that has traditionally been used to treat fever, malaria, liver diseases, skin infections, and disorders of the digestive system. Its seeds are rich in anthraquinones, including emodin, rhein, aloe-emodin, chrysophanol, physcion, and sennosides [13]. This compound composition is the main source of the functional efficacy of *C. occidentalis*. Compared to other natural laxatives such as rhubarb, *Aloe vera* contains a lower level of anthraquinones and is safer for long-term use, but is less effective. Rhubarb (Rheum spp.) is a classic source of anthraquinones with broad pharmacological effects but carries a higher risk of hepatotoxicity compared to *C. occidentalis* [17]. The sennoside content of *C. occidentalis* (approximately 2.5%–4.0%) is higher than that of aloe (<1%) but lower than that of rhubarb (4%–6%), which makes it a more effective treatment for constipation while having a lower risk of hepatotoxicity [20].

Because of sennosides' highly polar nature and low membrane permeability, their intrinsic biological activity is relatively weak. Only after undergoing biotransformation-mediated deglycosylation does the resulting product show increased lipid solubility and enhanced cellular permeation, and thus stronger bioactivity [5]. Therefore, biotransformation techniques, such as glycosidic hydrolysis, side-chain redox reactions, hydroxylation, and methylation, are commonly applied for structural tailoring [17]. Microbial metabolism plays a major role in the transformation of anthraquinone glycosides, and genera such as *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, and *Clostridium* have all been reported to participate in the conversion of sennosides into downstream metabolites [21]. The prevailing scientific consensus has been that the conversion of sennosides primarily relies on bacterial β-glucosidase-mediated hydrolysis [7]. For instance, Akao et al. demonstrated that *Bifidobacterium* strains that produce sennoside-hydrolyzing β-glucosidase can effectively catalyze the deglycosylation of sennosides, achieving in vitro conversion rates exceeding 80% [8]. Human fecal culture experiments showed that microbial communities enriched in *Bifidobacterium* have more pronounced laxative effects [22]. Furthermore, in in vivo studies of mice, oral administration of hydrolytic *Bifidobacterium* (*Bifidobacterium animalis* subsp. *lactis* LKM512) induced rhein anthrone production within the intestinal lumen, significantly promoting gut motility [23].

However, recent studies have revealed that the metabolic activation of sennosides is more complex than previously understood, involving not only deglycosylation (hydrolysis) but also reductive cleavage of the C10–C10' bond. Liu et al. (2025) recently demonstrated that nitroreductases (NTRs), rather than β-glucosidases, are the key enzymes responsible for this reductive cleavage step, and further identified NTR homologs in several bacterial strains such as *Bifidobacterium pseudocatenulatum* and *Streptococcus thermophilus* [21,24]. Their quantitative enzyme analysis revealed that the catalytic efficiency of the StNfrA enzyme from *S. thermophilus* is substantially higher than that of the BpNfrA

enzyme from *B. pseudocatenulatum* [24], indicating major strain-dependent differences in metabolic activation efficiency. These findings challenge the long-standing single-parameter model in which sennoside conversion efficiency is evaluated solely on the basis of β-glucosidase activity [24].

In addition, sennosides are highly polar molecules whose biological activity is limited by their poor ability to penetrate cell membranes [21]. Even when certain bacterial strains (such as *Escherichia coli*) possess NTRs, the absence of an effective transmembrane transporter prevents sennosides from entering the cell for metabolism, which prevents them from exerting their biological effects [24]. This emphasizes that the transmembrane transport mechanism is a prerequisite that determines the overall metabolic efficiency of a given strain [24]. Although reductive enzymes have been reported in *Lactobacillus* species, such as the NADH-dependent enoate reductase LacER in *Lactobacillus casei* [25] and the NTR PnbA in *L. plantarum* [26], whether these enzymes are homologous to the specific NTRs required for sennoside activation, and whether the overall metabolic capacity of *Lactobacillus* strains surpasses that of the traditionally reported *Bifidobacterium* strains, remains unclear [27].

This study aimed to investigate intestinal probiotic species by screening for highly efficient sennoside-converting strains and identifying suitable biotransformation conditions in order to systematically verify (1) whether intestinal probiotic strains can exhibit faster (short-time) and more efficient (higher conversion rate) hydrolysis of sennosides in vitro than the traditional *Bifidobacterium* strains, thereby providing a novel technical scheme for industrial pre-biotransformation; and (2) whether suitable conversion conditions can markedly reduce the residual sennoside content to within the safety limits stipulated by TFDA/EFSA. This investigation not only verifies the application potential of these highly efficient strains but also provides an experimental basis, through enzymatic activity and reaction-rate perspectives, for elucidating the unique cooperative mechanism in *Lactobacillus*-mediated sennoside metabolism (e.g., β-glucosidase together with NTR systems or a superior transport mechanism), thereby demonstrating the substantial potential of *C. occidentalis* products rich in sennosides for further development.

2. Materials and methods

This study used an aqueous extract of *C. occidentalis* rich in sennosides. The experimental process first involved identifying and quantifying the sennoside content of the extract. Then, the process was split into two major steps: the first step involved screening commonly marketed gut probiotic strains to evaluate their efficiency in hydrolyzing sennosides, and the second step examined the suitable conditions for hydrolysis in these selected strains, as well as the efficiency of conversion of sennosides into physcion and rhein.

2.1. Materials

2.1.1. Plant extract

The *C. occidentalis* seed aqueous extract used in this study was provided by the laboratory of Professor Yuh-Shuen Chen of HungKuang University (Shalu District, Taichung, Taiwan). The extract was prepared by extracting dried seeds with distilled water at a ratio of 1:10 (w/v) at 80 °C for 2 h, filtering, and lyophilizing. The extract was verified to contain approximately 100 mg/g of total sennosides (equivalent to 10%), as determined by high-performance liquid chromatography (HPLC)

analysis, as described in Section 2.5. In the biotransformation experiments, the extract was dissolved in distilled water to prepare 10%, 20%, and 25% (w/v) working solutions.

2.1.2. Microbial strains

Twenty microbial strains were used in this study. Lactic acid bacteria (LAB) comprised 16 strains: *Lactobacillus delbrueckii* subsp. bulgaricus (BCRC 12256), *Lactobacillus acidophilus* (BCRC 10695), *Lactobacillus johnsonii* (BCRC 17010), *Lactobacillus fermentum* (BCRC 14019), *Lactobacillus reuteri* (BCRC 17011), *Lactobacillus casei* (BCRC 10697), *Lactobacillus delbrueckii* subsp. *lactis* (BCRC 14069), *Lactobacillus helveticus* (BCRC 14092), *Lacticaseibacillus paracasei* (BCRC 17415), *Lactobacillus gasseri* (BCRC 14680), *Lactobacillus plantarum* (BCRC 10069), *Lactobacillus rhamnosus* (BCRC 16000), *Lactococcus lactis* subsp. *lactis* (BCRC 12327), *Lactobacillus taiwanensis* (BCRC 17755), *Lactobacillus agilis* (BCRC 14655), and *Streptococcus thermophilus* (BCRC 13869). *Bifidobacterium* strains comprised three strains: *Bifidobacterium adolescentis* (BCRC 14602), *Bifidobacterium longum* (BCRC 14634), and *Bifidobacterium infantis* (BCRC 14602). An additional strain included the yeast *Kluyveromyces marxianus* (BCRC 21067). All strains were provided by the same laboratory. Note that *Bifidobacterium* spp. and *K. marxianus* are not classified as LAB sensu stricto but were included as comparators given their known roles in food fermentation and anthraquinone biotransformation.

2.1.3. Chemicals and instruments

Sennoside A and sennoside B standards (purity ≥98%), rhein (purity ≥98%), and physcion (purity ≥98%) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). β-glucosidase from *Aspergillus niger* (specific activity: 50 U/mg; Enzymes.bio, Taichung, Taiwan) was used; 1500 U and 3000 U doses were prepared by dissolving the enzyme in 50 mM sodium phosphate buffer (pH 6.0) immediately before use. MRS broth (de Man, Rogosa and Sharpe; Difco, Becton Dickinson, Sparks, MD, USA), potato dextrose broth (PDB; Difco), sodium bicarbonate, phosphoric acid (Choneye Pure Chemicals, Taipei, Taiwan), acetonitrile (HPLC grade; J.T. Baker, Phillipsburg, NJ, USA), and methanol (HPLC grade; Honeywell Burdick & Jackson, Muskegon, MI, USA) were used as received. Gallic acid and rutin standards were purchased from Sigma-Aldrich. Instruments used were an HPLC system (LC-2050C; Shimadzu, Kyoto, Japan) equipped with a photodiode array (PDA) detector, an anaerobic workstation (Whitley A35; Don Whitley Scientific, Bingley, UK), a high-speed refrigerated centrifuge (5430R; Eppendorf AG, Hamburg, Germany), a thermostatic shaking incubator (ZQZY-88BN; Zhicheng Analytical Instruments, Shanghai, China), an ultrasonic extraction apparatus (KQ-500DE; Kunshan Ultrasonic Instrument Co., Kunshan, China), a pH meter (FE28; Mettler Toledo, Columbus, OH, USA), and a UV-Vis spectrophotometer (Spectroquant Pharo 300; Merck KGaA, Darmstadt, Germany).

2.2. Activation of probiotic strains

LAB strains were activated by streaking a loopful of each frozen stock onto MRS agar (pH 6.2) and incubating anaerobically at 37 °C for 48 h in the anaerobic workstation (5% CO_2, 10% H_2, 85% N_2). Single colonies were then transferred to 10 mL of MRS broth and subcultured twice under the same conditions

to ensure full metabolic activity before use. *K. marxianus* was cultivated on PDB (pH 5.6) at 30 °C under aerobic conditions.

2.3. β-glucosidase-assisted hydrolysis

For enzyme-assisted hydrolysis, the *C. occidentalis* extract was dissolved in distilled water to give a final concentration of 20% (w/v, 100 mL total volume) in 250-mL Erlenmeyer flasks. β-glucosidase was added at either 1500 or 3000 U per 100 mL of reaction volume. The reaction was carried out at 37 °C with shaking at 150 rpm for 2 h. The hydrolysis was terminated by heating at 80 °C for 10 min, and the samples were stored at -20 °C until HPLC analysis.

2.4. Screening of gut probiotic strains

2.4.1. First-stage screening of probiotic strains

Activated LAB strains (2%, v/v inoculum; approximately 10^8 CFU/mL) were inoculated into 10 mL of 10% (w/v) *C. occidentalis* aqueous extract, which served as the sole carbon source, in addition to the growth medium. The cultures were incubated anaerobically at 37 °C for 24 h with shaking at 100 rpm in the anaerobic workstation. After cultivation, cultures were heat-inactivated at 80 °C for 10 min and stored at -20 °C until analysis. *K. marxianus* was screened under aerobic conditions at 30 °C for 24 h. All experiments were performed in triplicate (n = 3).

2.4.2. Second-stage screening of probiotic strains

Based on the first-stage screening results, eight strains with superior hydrolytic activity were selected for second-stage screening. These strains were cultivated in 10%, 20%, and 25% (w/v) *C. occidentalis* extract for 24 h at 37 °C under anaerobic conditions (n = 3) to evaluate the effect of substrate concentration on hydrolysis efficiency and to identify strains maintaining high conversion rates at higher substrate concentrations.

2.5. Investigation of suitable biotransformation conditions

Two strains selected from the first-stage screening were used to investigate suitable biotransformation conditions using a one-factor-at-a-time approach. Three parameters were investigated sequentially:

(1) Substrate concentration: 10%, 20%, and 25% (w/v) *C. occidentalis* extract were tested with a fixed inoculum (2%, v/v) at 37 °C for 24 h, anaerobically.

(2) Hydrolysis time: At the suitable substrate concentration (20%), the effect of hydrolysis duration was examined at time points of 2, 4, 8, 24, and 48 h.

(3) β-glucosidase addition: After bacterial hydrolysis for 2 h or 4 h, β-glucosidase (1500 U) was added and incubated for an additional 2 h to assess whether supplemental enzyme hydrolysis could further reduce residual sennosides.

After confirming the suitable conditions, subsequent analytical experiments (Sections 2.7–2.10) were performed under these conditions. All experiments were performed in triplicate (n = 3).

2.6. Determination of sennoside content

Sennoside content was determined following the method of Ghassemi-Dehkordi et al. (2014) [28]. Briefly, 1 g of the homogenized, heat-inactivated sample was extracted with 20 mL of 70% methanol by ultrasonic agitation for 30 min, then centrifuged at 3000 × g for 5 min. The extraction was repeated twice, and the combined supernatants were brought to 50 mL with distilled water before filtration (0.45 μm membrane filter). HPLC analysis was performed on an LC-2050C system (Shimadzu) equipped with a Mightysil RP-18 GP250 column (250 mm × 4.6 mm, 5 μm; Kanto Chemical Co., Inc., Tokyo, Japan), using a mobile phase of acetonitrile–water–phosphoric acid (200:800:1, v/v/v) at a flow rate of 0.6 mL/min; column temperature, 40 °C; injection volume, 10 μL; and detection at 380 nm. Sennoside A and B contents were quantified using external standard curves ($R^2 > 0.999$).

2.7. Determination of physcion and rhein

Physcion and rhein were determined following the method of Lemli and Lemmens (1980) with modifications [29]. Extraction was identical to Section 2.6 but with a 70% methanol solvent system. HPLC analysis was performed using the same column and instrument, with the following gradient mobile phase [0.1% phosphoric acid (A) and acetonitrile (B)]: 0–40 min, 87% A/13% B; 40–50 min, 84% A/16% B; 50–60 min, 62% A/38% B; 60–65 min, 47% A/53% B; and 65–75 min, 40% A/60% B. Flow rate: 1.0 mL/min; column temperature: 30 °C; injection volume: 10 μL; and detection at 254 nm. Physcion and rhein contents were quantified using external standard curves ($R^2 > 0.999$).

2.8. Antioxidant analysis (DPPH radical-scavenging assay)

Free radical-scavenging activity was evaluated using the DPPH method according to Shimada et al. (1992) [30]. Methanol extracts at different concentrations (0.1–20 mg/mL, 4 mL) were mixed with 1 mL of freshly prepared 10 mM DPPH in methanol, vortexed, and incubated in the dark for 30 min. Absorbance was measured at 517 nm (Spectroquant Pharo 300; Merck KGaA, Darmstadt, Germany). The EC_{50} (mg/mL), defined as the concentration required to scavenge 50% of DPPH radicals, was calculated by linear regression. Scavenging effect (%) = [1 − (A517 sample / A517 control)] × 100. All measurements were performed in triplicate (n = 3).

2.9. Analysis of antioxidant components

2.9.1. Determination of total phenolic content (TPC)

TPC was determined according to [31]. Briefly, 100 mg of the methanol extract was dissolved in acidified methanol–water (60:40, v/v, containing 0.3% HCl). Then, 400 μL of the sample solution was mixed with Folin–Ciocalteu reagent (200 μL) and allowed to stand for 3 min, after which 40 μL of 10% Na_2CO_3 was added. The mixture was vortexed every 10 min for 1 h, then centrifuged at 13,000 rpm for 5 min at 4 °C. Absorbance of the supernatant was measured at 735 nm. Results were calculated from a gallic acid standard curve and expressed as mg of gallic acid equivalent (GAE) per g of extract. All measurements were performed in triplicate (n = 3).

2.9.2. Determination of total flavonoid content (TFC)

TFC was determined following [31,32]. The extract (200 mg) was dissolved in 4 mL of 70% ethanol. Then, 0.5 mL of the sample solution was mixed with 0.1 mL of 5% $NaNO_2$ and allowed to stand for 6 min. Next, 0.1 mL of 10% $AlCl_3$ was added (6 min), followed by 1 mL of 5% NaOH and 0.8 mL of RO water (15 min). Absorbance was measured at 510 nm. Results were calculated from a rutin standard curve and expressed as mg of rutin equivalent (RE) per g of extract. All measurements were performed in triplicate (n = 3).

2.10. Statistical analysis

All experiments were conducted in triplicate (n = 3), and results are expressed as mean ± standard deviation (SD). Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test, with $P < 0.05$ considered statistically significant.

3. Results and discussion

3.1. Analysis of sennosides in Cassia occidentalis *water extract*

High-performance liquid chromatography was performed using a PDA detector at 380 nm to determine the sennoside content in the *C. occidentalis* water extract. Based on a comparison with retention times of standard compounds, sennoside B was eluted at 6.7 min and sennoside A at 11.84 min. The 10% *C. occidentalis* extract showed corresponding chromatographic peaks at 6.774 and 11.843 min, confirming the presence of both sennoside A and B in the extract (Figures 1 and 2). Quantification using the standard curve indicated that the extract contained approximately 100 mg/g of total sennosides, which is equivalent to approximately 10% of the content. Typically, sennoside levels in seeds range from 2% to 4%, which is lower compared to rhubarb (4%–6%), but the resulting laxative effect is milder, as shown in Yang et al., 1996 [33]. In this study, the extract used was verified to contain approximately 10% sennosides and was subsequently adopted for further experiments.

The main objective of this study was to use lactic acid bacteria to facilitate the degradation of sennosides, thereby reducing their content in the final product. This process began with the screening of numerous intestinal probiotic strains to identify several strains with superior sennoside degradation capabilities. Then, the preferred transforming strains with high potential for producing downstream metabolites, such as physcion and rhein, were selected. Finally, the best conversion conditions were determined through the analysis of sennoside degradation capacity and the production of physcion and rhein.

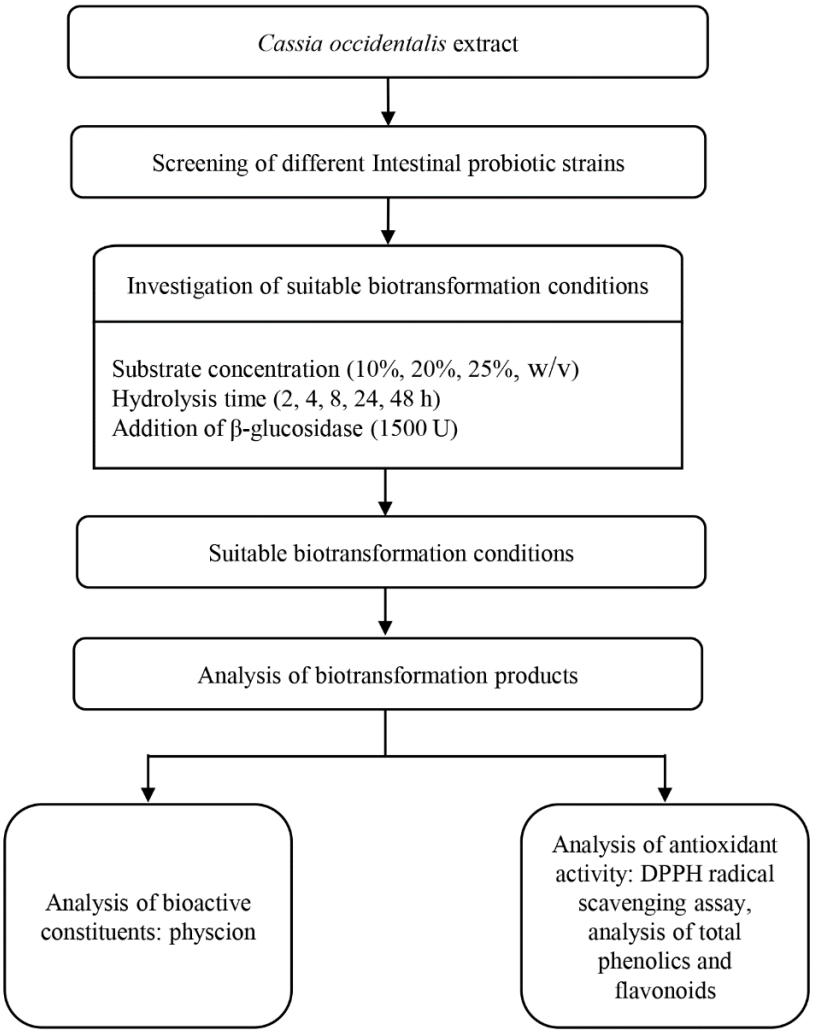

Figure 1. Biotransformation of sennosides in *Cassia occidentalis* extracts by intestinal probiotic strains. Investigated factors and their levels for suitable biotransformation conditions included substrate concentration (10%, 20%, and 25%, w/v), hydrolysis time (2, 4, 8, 24, and 48 h), and the addition of β-glucosidase (1500 U).

3.2. First-stage screening of probiotic strains

The gut microbiota functions as a critical metabolic organ that significantly modulates the bioactivity of dietary and medicinal compounds through complex biotransformation pathways [7,15,21,25]. Probiotics, particularly those belonging to the *Lactobacillus* and *Bifidobacterium* genera, possess a diverse array of enzymes—such as β-glucosidases and NTRs—that catalyze the conversion of inactive plant glycosides into functional aglycones. For instance, sennosides are biotransformed into rhein anthrone and rhein, which exhibit potent laxative, anti-inflammatory, and antioxidant properties [7,21]. Recent research has identified specific reductive enzymes within *Lactobacillus* species, such as NADH-dependent enoate reductases (e.g., LacER) and NTR homologs (e.g., PnbA), which may explain their high efficiency in metabolizing complex polyphenols and enhancing the therapeutic value of herbal extracts like *Cassia occidentalis* [21,25]. Also, sennosides themselves do not possess direct

physiological activity, and their effects rely mainly on metabolic conversion by intestinal microorganisms into downstream metabolites to enhance their absorption rate and exert physiological functions, such as promotion of bowel movements [7]. Different gut probiotic strains may produce different effects. According to Akao et al. (1994), *Bifidobacterium* sp. SEN produces β-glucosidase capable of effectively hydrolyzing sennosides, achieving a conversion rate of over 80% [29]. In fecal culture experiments, groups of intestinal microbiota rich in *Bifidobacterium* were found to show more pronounced laxative effects [31].

Table 1. Sennoside and hydrolysis rate after biotransformation of *C. occidentalis* extract (10% sennosides) by intestinal probiotics.

Strains	Sennoside B (mg/g)	Degree of hydrolysis (%)	Sennoside A (mg/g)	Degree of hydrolysis (%)
Control	100 ± 6.87	0 ± 0.00	100 ± 9.61	0 ± 0.00
L. delbrueckii subsp. bulgaricus (BCRC 12256)	34.06 ± 3.32a	64.60 ± 3.85d	37.06 ± 2.02d	62.30 ± 2.07d
L. acidophilus (BCRC 10695)	38.41 ± 3.33a	59.60 ± 1.93e	52.54 ± 3.14c	46.37 ± 2.11f
B. adolescentis (BCRC 14602)	46.97 ± 3.75a	49.80 ± 3.01e	69.68 ± 3.64b	28.72 ± 1.33g
B. longum (BCRC 14634)	31.07 ± 1.80a	68.03 ± 2.62c	44.65 ± 2.96d	54.49 ± 3.62e
L. johnsonii (BCRC 17010)	21.52 ± 1.24c	78.99 ± 2.63b	21.70 ± 1.51f	78.12 ± 4.83b
L. fermentum (BCRC 14019)	15.83 ± 0.84d	85.51 ± 6.62a	29.63 ± 1.88e	69.96 ± 5.20c
L. reuteri (BCRC 17011)	34.03 ± 3.18a	64.63 ± 5.06d	46.11 ± 4.22d	52.98 ± 2.84e
L. casei (BCRC 10697)	32.67 ± 2.62a	66.19 ± 4.66d	54.27 ± 3.68c	44.59 ± 1.60f
L. delbrueckii subsp. *lactis* (BCRC 14069)	18.11 ± 0.92d	82.89 ± 2.89a	23.65 ± 1.82f	76.11 ± 5.18b
L. helveticus (BCRC 14092)	20.48 ± 2.02d	80.18 ± 5.15a	27.83 ± 1.59e	71.81 ± 4.17c
L. paracasei (BCRC 17415)	35.24 ± 3.23a	63.25 ± 3.29d	66.94 ± 6.03b	31.54 ± 2.16g
L. gasseri (BCRC 14680)	28.59 ± 1.73b	70.87 ± 2.56c	42.74 ± 2.30d	56.45 ± 3.09d
L. plantarum (BCRC 10069)	23.33 ± 1.38c	76.90 ± 4.21b	29.09 ± 2.89e	70.51 ± 3.96c
L. rhamnosus (BCRC 16000)	38.31 ± 2.27a	59.72 ± 1.89d	33.62 ± 2.98e	65.84 ± 3.38c
Lc. lactis subsp. lactis (BCRC 12327)	29.15 ± 1.90b	70.23 ± 5.30c	44.05 ± 2.64d	55.11 ± 1.72d
K. marxianus (BCRC 21067) [yeast]	24.10 ± 1.84c	76.03 ± 3.26b	42.59 ± 2.14d	56.61 ± 2.00d
L. taiwanensis (BCRC 17755)	16.00 ± 1.29d	85.31 ± 4.78a	78.12 ± 6.75a	20.03 ± 1.00g
L. agilis (BCRC 14655)	29.22 ± 1.66b	70.15 ± 4.02b	48.92 ± 4.33c	50.09 ± 2.78e
S. thermophilus (BCRC 13869)	14.38 ± 1.05d	87.18 ± 5.99a	30.43 ± 1.70e	69.13 ± 3.49c
B. infantis (BCRC 14602)	15.18 ± 1.35d	86.26 ± 6.64a	14.28 ± 1.33f	85.75 ± 5.81a

*Note: Each value is expressed as mean ± SD (n = 3). Means with different letters within a column are significantly different ($P < 0.05$)**. ND: Not detected.

In this study, 20 commonly used gut probiotic strains were first tested with a 10% *C. occidentalis* aqueous extract containing 100 mg/g of sennosides to evaluate their ability to hydrolyze sennoside A and sennoside B, with the goal of screening the strains exhibiting higher hydrolysis rates for subsequent

biotransformation experiments. The results indicated that the ability to hydrolyze sennoside B was generally satisfactory for all strains, with hydrolysis rates ranging from 49.80% to 87.18% (Table 1). *S. thermophilus* (BCRC 13869) showed the best hydrolytic effect, achieving a hydrolysis rate of 87.18% ± 5.99%, leaving a residual sennoside B content of only 14.38 ± 1.05 mg/g. *B. infantis* (BCRC 14602), *L. fermentum* (BCRC 14019), and *L. taiwanensis* (BCRC 17755) also exhibited excellent hydrolytic ability, with hydrolysis rates of 86.26%, 85.51%, and 85.31%, respectively. All screened strains showed lower efficiency in hydrolyzing sennoside A compared to sennoside B, with hydrolysis rates ranging from 20.03% to 85.75%. *B. infantis* (BCRC 14602) showed the highest hydrolysis rate, reaching 85.75% ± 5.81%, significantly surpassing other strains. *L. johnsonii* (BCRC 17010) and *L. delbrueckii* subsp. *lactis* (BCRC 14069) also performed well, with hydrolysis rates of 78.12% and 76.11%, respectively. The rates of metabolism of sennoside A and sennoside B by gut probiotic strains were significantly different, which may be related to the different bonding positions of the anthraquinone structures in their molecular configurations. Dreessen et al. (1981) demonstrated that sennoside A is a rhein–aloe emodin dianthrone glycoside, whereas sennoside B is a rhein–rhein dianthrone glycoside [32]. Such structural differences may be the reason affecting enzyme reaction efficiency.

B. infantis (BCRC 14602) and *S. thermophilus* (BCRC 13869) both had a strong ability to hydrolyze sennoside A and sennoside B. Therefore, this study selected these two strains for further screening, along with several other strains, including *L. delbrueckii* subsp. *lactis* (BCRC 14069), *L. helveticus* (BCRC 14092), *L. paracasei* (BCRC 17415), *L. gasseri* (BCRC 14680), *L. plantarum* (BCRC 10069), and *L. rhamnosus* (BCRC 16000), which presented higher capacity to hydrolyze both sennosides.

3.3. Second-stage screening of probiotic strains

Based on the first-stage results, eight strains with superior hydrolytic activity for both sennoside A and B were selected for second-stage screening: *L. delbrueckii* subsp. *lactis* (BCRC 14069), *L. helveticus* (BCRC 14092), *L. paracasei* (BCRC 17415), *L. gasseri* (BCRC 14680), *L. plantarum* (BCRC 10069), *L. rhamnosus* (BCRC 16000), *S. thermophilus* (BCRC 13869), and *B. infantis* (BCRC 14602).

These eight strains were cultivated in 10%, 20%, and 25% (w/v) *C. occidentalis* extract for 24 h at 37 °C anaerobically (n = 3) to identify those that maintained high sennoside biotransformation efficiency at higher substrate concentrations. Under 10% extract concentration, sennoside B degradation ranged from 59.72% (*L. rhamnosus*) to 87.18% (*S. thermophilus*), and sennoside A degradation ranged from 31.54% (*L. paracasei*) to 85.75% (*B. infantis*) (Figure 2(A),(B)). This pattern is consistent with findings by Akao et al. (1994), who reported that *Bifidobacterium* sp. SEN produces β-glucosidase capable of hydrolyzing sennosides at >80% efficiency [10].

As the extract concentration increased to 20%, the hydrolysis rates of most strains increased further, with sennoside B hydrolysis rates reaching 86.26%–95.46% (highest: *L. helveticus*) and sennoside A hydrolysis rates reaching 85.75%–98.12% (highest: *L. delbrueckii* subsp. *lactis*), consistent with an improved substrate-to-enzyme ratio in line with Michaelis–Menten kinetics. However, at 25%, the hydrolysis rate of sennoside A showed a downward trend for several strains, possibly due to substrate inhibition, osmotic stress, or reduced bacterial viability. Therefore, 20% was identified as the suitable substrate concentration, with *L. helveticus* (BCRC 14092) and *L. delbrueckii* subsp. *lactis* (BCRC 14069) showing the strongest overall hydrolytic activity.

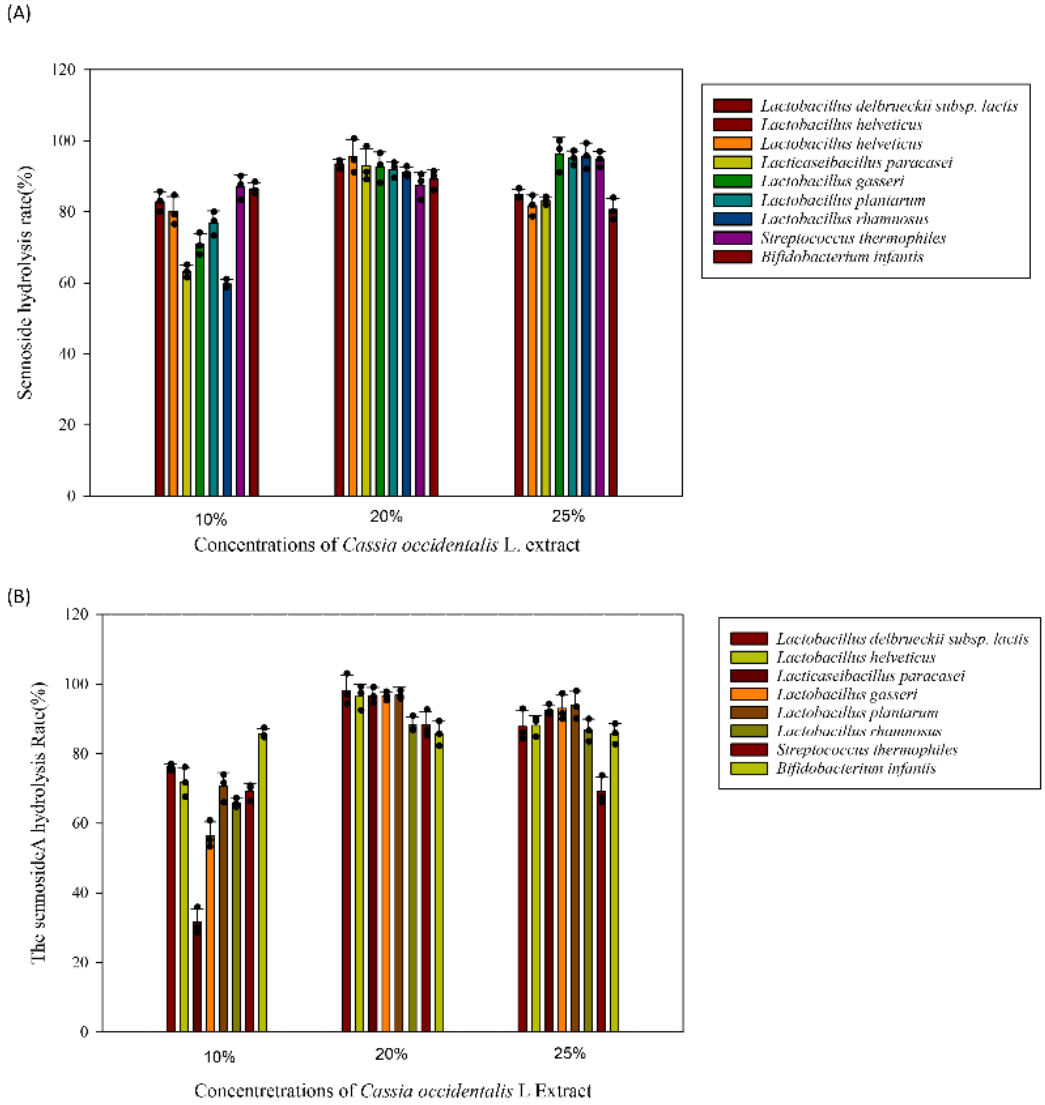

Figure 2. (A) Sennoside B hydrolysis rates (%) after biotransformation of *C. occidentalis* extract at different substrate concentrations (10%, 20%, 25%) by selected intestinal probiotic strains. Conditions: MRS broth, 37 °C, anaerobic (5% CO_2/10% H_2/85% N_2), 24 h, n = 3. (B) Sennoside A hydrolysis rates (%) after biotransformation under the same conditions as (A).

3.4. Effect of hydrolysis time

L. delbrueckii subsp. *lactis* (BCRC 14069) and *L. helveticus* (BCRC 14092) were cultivated in 20% *C. occidentalis* extract (100 mg/g sennosides) at 37 °C anaerobically for 2, 4, 8, 24, and 48 h (n = 3) to determine the suitable hydrolysis duration (Table 2).

With *L. delbrueckii* subsp. *lactis*, the residual sennoside B concentration dropped rapidly to 8.78 ± 0.34 mg/g after 2 h (hydrolysis rate: 98.36% ± 0.46%) and remained stable throughout the 4–48 h period (7.92–8.60 mg/g; 98.45%–98.57%), with no significant differences among time points. Sennoside A showed a similar pattern (residual: 5.67–7.45 mg/g; 95.86%–97.91%), confirming stable hydrolytic performance. In contrast, *L. helveticus* showed an unusual fluctuation in sennoside A

concentration: At 2 h, the residual was 18.99 mg/g (89.46%), which decreased to 3.70 mg/g at 4 h (97.12%), rebounded to 17.89 mg/g at 24 h (90.01%), and then decreased again to 2.26 mg/g at 48 h (97.39%). This fluctuation may reflect reductase activity (possibly sennoside A reductase homologs) producing intermediate sennidins that temporarily reconvert to sennoside A, as reported for *B. pseudocatenulatum* by Wang et al. (2024) [34]. Both strains showed faster and higher hydrolytic efficiency than *Bifidobacterium* strains reported in previous studies, which typically require >8 h to reach 90% conversion [10,35].

Table 2. Sennosides A and B and hydrolysis rates after different hydrolysis times (2–48 h) by *Lactobacillus delbrueckii* subsp. *Lactis* and *Lactobacillus helveticus.*

Hydrolysis time (h)	Strains	Sennoside B (mg/g)	Degree of hydrolysis (%)	Sennoside A (mg/g)	Degree of hydrolysis (%)
2	*L. delbrueckii*	7.68 ± 0.24a	97.86 ± 0.34a	6.83± 0.14b	96.81 ± 0.34b
4	subsp. *lactis*	7.27 ± 0.35a	98.06 ± 0.15a	5.75 ± 0.30b	97.35 ± 0.16b
8	(BCRC 14069)	7.50 ± 0.31a	97.95 ± 0.21a	6.51 ± 0.30b	96.96 ± 0.12b
24		7.25 ± 0.28a	98.07 ± 0.24a	6.04 ± 0.17b	97.20 ± 0.48b
48		7.27 ± 0.18a	98.06 ± 0.28a	5.97 ± 0.16b	97.23 ± 0.48b
2	*L. helveticus*	7.68 ± 0.19a	97.86 ± 0.41a	19.29 ± 0.35a	90.56 ± 0.42c
4	(BCRC 14092)	7.39 ± 0.16a	98.00 ± 0.18a	4.00 ± 0.15c	98.22 ± 0.22a
8		6.82 ± 0.31b	98.27 ± 0.30a	5.55 ± 0.22b	97.45 ± 0.13b
24		6.37 ± 0.24b	98.49 ± 0.33a	18.19 ± 0.44a	91.11 ± 0.37c
48		6.50 ± 0.27b	98.43 ± 0.11a	2.56 ± 0.13c	98.94 ± 0.27a

*Note: Each value is expressed as mean ± SD (n = 3). Means with different letters within a column are significantly different ($P < 0.05$)**. ND: Not detected.

3.5. *β-glucosidase hydrolysis test*

In this experiment, β-glucosidase at different enzyme activities (1500 and 3000 U) was used to hydrolyze the 20% *C. occidentalis* extract for 2 h to investigate its hydrolytic efficiency against sennoside A and sennoside B. In the 1500 U group, the residual amount of sennoside B was 22.73 mg/g, corresponding to a hydrolysis rate of 90.69%, while the residual amount of sennoside A was 10.53 mg/g, also corresponding to a hydrolysis rate of 90.69% (Figure 3). In the 3000 U group, the residual amount of sennoside B was 40.28 mg/g, with a hydrolysis rate of 82.33%, and the residual amount of sennoside A was 18.35 mg/g, corresponding to a hydrolysis rate of 86.59%.

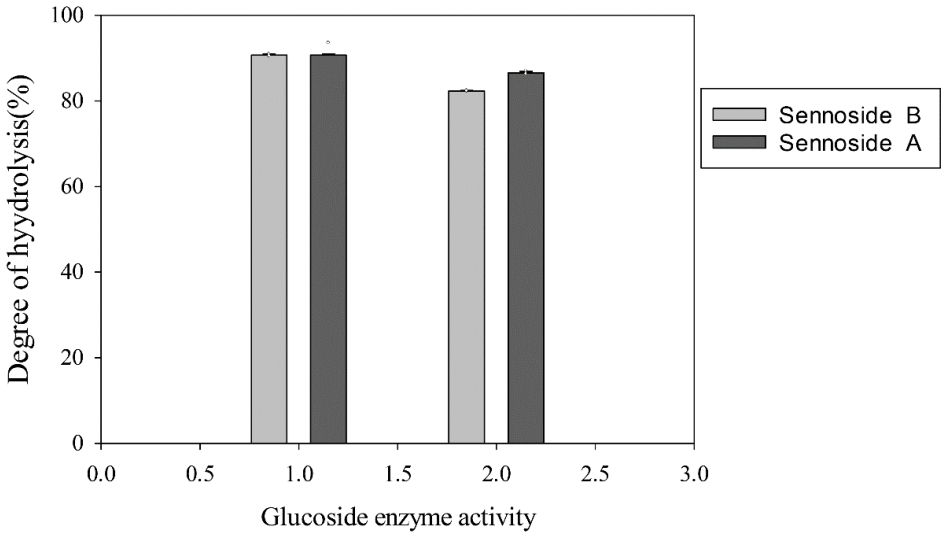

Figure 3. Sennoside A and B hydrolysis rates (%) at different β-glucosidase activities (1500 U vs. 3000 U). Substrate: 20% (w/v) *C. occidentalis* aqueous extract containing 100 mg/g sennosides. Conditions: 37 °C, pH 6.0 (50 mM sodium phosphate buffer), 150 rpm, 2 h, n = 3.

When the enzyme dosage was increased to 3000 U, the hydrolysis rates of both sennosides decreased by approximately 4%–8%, which may be due to the enzyme already reaching saturation, with the excessive enzyme dosage triggering nonspecific reactions or leading to the accumulation of inhibitory intermediate products, such as inactive fragments of degraded sennosides, or local pH changes that suppress enzyme activity. Hattori et al. (1988) reported that β-glucosidase is inhibited by glucose and shows suitable activity at pH 6.0, explaining why a rapid increase in glucose produced during hydrolysis at high enzymatic activity may lead to saturation effects [7,9].

In this study, when enzyme hydrolysis alone was applied, the hydrolytic efficiency was clearly inferior to that achieved by the selected probiotic strains; the use of *L. delbrueckii* subsp. *lactis* (BCRC 14069) for hydrolysis resulted in hydrolysis rates of over 96% (Table 2), considerably higher than the >90% hydrolysis rate achieved with 1500 U of enzyme, suggesting that further enhancement of hydrolytic efficiency might be achieved by combining bacterial culture with β-glucosidase supplementation.

3.6. Investigation of suitable biotransformation conditions: β-glucosidase addition

Recent advancements in microbial metabolism have redefined the mechanism of sennoside activation, shifting the focus from exclusive β-glucosidase-mediated hydrolysis to a more complex pathway involving NTRs [5,18,21,34]. Studies published in 2024 and 2025 emphasized that NTRs are the primary enzymes responsible for the reductive cleavage of sennosides into active anthrones, and the efficiency of this process is highly dependent on specific bacterial transmembrane transporters. Furthermore, clinical studies and human fecal culture experiments underscore that the therapeutic efficacy of sennoside-containing extracts, such as *C. occidentalis*, is intrinsically linked to the individual composition of the gut microbiota [18,21,34]. Clinical observations regarding the safety of anthraquinone-based laxatives highlight the risk of melanosis coli under long-term use, although recent

clinical reports indicate that such conditions are often reversible upon cessation of intake [5]. By achieving over 97% sennoside hydrolysis within a 2-h period, our biotransformation model using *L. delbrueckii* subsp. *lactis* (BCRC 14069) provides a robust technical scheme to meet clinical safety requirements and regulatory limits set by TFDA and EFSA [5,21,34].

In this experiment, the probiotic strain *L. delbrueckii* subsp. *lactis* (BCRC 14069) was used for hydrolysis at 2 and 4 h, after which β-glucosidase was additionally applied for 2 h of hydrolysis to investigate hydrolytic activity against sennoside A and sennoside B. In the 2-h hydrolysis group, the residual amount of sennoside B was 28.92 mg/g, corresponding to a hydrolysis rate of 87.74%, while the residual amount of sennoside A was 20.44 mg/g, with a hydrolysis rate of 89.99% (Table 3). In the 4-h hydrolysis group, the residual amount of sennoside B was 33.02 mg/g, corresponding to a hydrolysis rate of 85.79%, while the residual amount of sennoside A was 10.89 μg/mL, corresponding to a hydrolysis rate of 94.77%.

Table 3. Sennosides A and B and hydrolysis rate after different hydrolysis times (2–4 h) by *L. delbrueckii* subsp. *lactis* (BCRC 14069) and 1500 U β-glucosidase.

Hydrolysis time (h)	Strains	Sennoside B (mg/g)	Degree of hydrolysis (%)	Sennoside A (mg/g)	Degree of hydrolysis (%)
2	*L. delbrueckii* subsp. *lactis* (BCRC 14069)	28.92 ± 1.45	87.74 ± 1.75	20.44 ± 1.43	89.99 ± 1.80
4		33.02 ± 2.31	85.79 ± 2.15	10.89 ± 0.76	94.77 ± 1.90

*Note: Each value is expressed as mean ± SD (n = 3). Means with different letters within a column are significantly different ($P < 0.05$)**. ND: Not detected.

These results indicate that the hydrolysates after 4 h of bacterial hydrolysis showed significantly greater susceptibility to further hydrolysis by β-glucosidase than those obtained at 2 h. This phenomenon is consistent with the finding of Yang et al. (1996) that β-glucosidase is inhibited by glucose and exhibits suitable activity at pH 6.0 [33]. The 2-h hydrolysis group had already reached >97% hydrolysis of both sennoside A and sennoside B (Table 2), suggesting that the hydrolysis reaction was saturated, and glucose accumulation likely inhibited β-glucosidase activity, whereas the consumption or reduction of glucose over a longer incubation period might explain the significantly higher enzymatic hydrolysis observed in the 4-h group.

These results, together with Tables 2 and 3, show that *L. delbrueckii* subsp. *lactis* (BCRC 14069) alone achieved 97%–98% hydrolytic efficiency within 2 h, and additional β-glucosidase supplementation not only failed to further improve the hydrolysis rate but, in some cases, even resulted in a reduction in hydrolytic efficiency, likely due to reductase activity inherent to anthraquinone compounds and other potential competing biochemical reactions. Therefore, the selected probiotic strain alone was sufficient to hydrolyze >97% of sennoside A and sennoside B without requiring supplemental β-glucosidase. External enzymatic addition would not only increase production costs but also risk decreasing hydrolytic efficiency.

3.7. *Chromatographic profiles of hydrolysis products*

For the *C. occidentalis* extract, retention times for sennoside B and sennoside A were approximately 6.7 and 11.7 minutes, respectively, although slight variation might have resulted from concentration effects (Figure 4(A)). After β-glucosidase treatment for 2 h, both sennoside A and sennoside B in the *C. occidentalis* extract were markedly reduced (Figure 4(B)). The hydrolysis rate exceeded 90%, and at a retention time of approximately 17–18 min, a new and pronounced peak appeared, which corresponded to the transformed product (Figure 4). Because β-glucosidase partially hydrolyzes the glycosidic moiety of sennosides to yield more hydrophobic aglycone forms, this peak was presumed to be physcion or another intermediate metabolite.

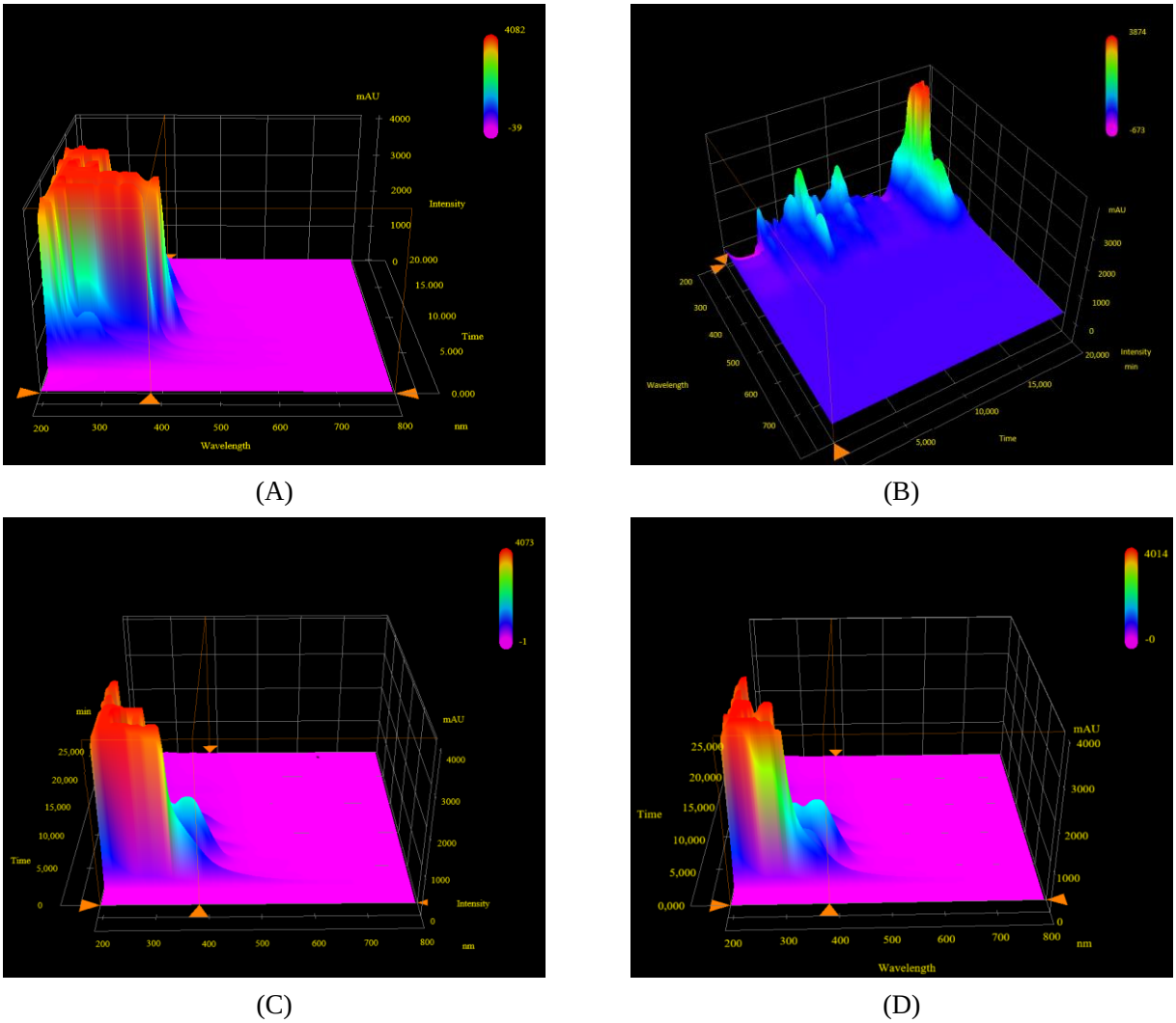

Figure 4. HPLC-PDA chromatogram of (A) the control (20% *C. occidentalis* extract), (B) extract treated with β-glucosidase for 2 h, (C) extract treated with *L. delbrueckii* subsp. *lactis* (BCRC 14069) for 2 h, and (D) extract treated with *L. delbrueckii* subsp. *lactis* (BCRC 14069) for 2 h and followed by β-glucosidase for 2 h. Detection was performed at 380 nm.

Nearly all sennoside peaks were hydrolyzed in the extract treated with *L. delbrueckii* subsp. *lactis* (BCRC 14069) for 2 h (Figure 4(C)); approximately 97%–98% of the sennosides were

hydrolyzed (Table 2). Since lactic acid bacteria can produce a variety of enzymes and co-metabolic reactions, multiple downstream metabolites may occur. (1) Reductase activity: Lactic acid bacteria possess NADH reductases that can reduce anthraquinone compounds into anthrones or anthranols, which may account for the increased abundance of peaks appearing earlier in the chromatographic profile. (2) Deglycosylation: Bacterial β-glucosidase hydrolyzes the glycosidic bonds to generate fully deglycosylated aglycones. (3) Side-chain modification: Various biochemical reactions, such as hydroxylation, methylation, or acetylation, may alter product polarity. Therefore, many downstream metabolites, including physcion and rhein, were likely generated, and nearly all sennosides were hydrolyzed, which is highly advantageous from a regulatory perspective for reducing residual sennosides. However, it was necessary to quantify downstream metabolites, such as physcion and rhein, to ensure that hydrolyzed products also possessed the desired functional bioactivity.

Panel (D) of Figure 3 shows the chromatogram after 2 h of hydrolysis by *L. delbrueckii* subsp. *lactis* (BCRC 14069) followed by 2 h of β-glucosidase treatment. β-glucosidase supplementation did not further increase the hydrolysis rate, and due to the presence of intermediate metabolites and reductase activity, the hydrolytic performance was not superior to that of *L. delbrueckii* subsp. *lactis* alone, while the hydrolysis rate was slightly reduced. This was likely due to glucose inhibition of β-glucosidase and its suitable activity around pH 6.0, and because nearly all sennosides had already been hydrolyzed after the initial 2-h treatment with *L. delbrueckii* subsp. *lactis* and product feedback inhibition, and even reconversion phenomena might have occurred.

The four chromatographic profiles in Figure 4 show that treatment condition (C) could serve as the most suitable approach for subsequent applications if the contents of the downstream metabolites physcion and rhein were confirmed, since this condition both minimizes sennoside residues and eliminates the need for exogenous β-glucosidase supplementation, thereby reducing cost while maintaining biotransformation efficiency.

3.8. Physcion content after biotransformation

Physcion content was determined in the hydrolysates obtained under suitable conditions (20% *C. occidentalis* extract, *L. delbrueckii* subsp. *lactis*, BCRC 14069, 37 °C, anaerobic) after 0 (control), 2, and 4 h of hydrolysis (Table 4). Compared with the control group (125.43 ± 6.27 mg/g), physcion content increased significantly after 2 h of hydrolysis to 156.39 ± 7.82 mg/g (a 24.7% increase, $P < 0.05$). After 4 h, physcion content decreased to 133.14 ± 4.44 mg/g, which was 6.1% higher than the control but 14.9% lower than the 2 h group. This decrease at 4 h may reflect secondary enzymatic reactions during prolonged fermentation, including oxidation of physcion to rhein or its reductive metabolization by LAB as an energy source.

The residual sennoside levels after 2 h of *L. delbrueckii* subsp. *lactis* hydrolysis (sennoside A: ~6.5 mg/g; sennoside B: ~8.8 mg/g; total: ~15.3 mg/g in the 20% extract concentrate) were substantially reduced compared to the control (200 mg/g total). When formulated at an application dose providing ≤12 mg of sennosides/day (TFDA limit) or <10 mg/day (EFSA limit), the biotransformed product complies with both regulatory requirements, while the increased physcion content (156.39 mg/g, + 24.7%) provides enhanced functional bioactivity compared to non-biotransformed extracts.

Table 4. Sennoside hydrolysis rates, physcion content after *L. delbrueckii* subsp. *lactis* (BCRC 14069) treatment of 20% *C. occidentalis* extract at 0, 2, and 4 h (37 °C, anaerobic, n = 3).

Hydrolysis time (h)	Strains	Sennoside B degree of hydrolysis (%)	Sennoside A degree of hydrolysis (%)	Physcion (mg/g)
Control		0%	0%	125.43 ± 6.27^{c}
2	*L. delbrueckii* subsp. *lactis* (BCRC 14069)	97.86 ± 0.34^{a}	96.81 ± 0.34^{b}	156.39 ± 7.82^{a}
4		98.06 ± 0.15^{a}	97.35 ± 0.16^{b}	133.14 ± 4.44^{b}

*Note: Each value is expressed as mean ± SD (n = 3). Means with different letters within a column are significantly different ($P < 0.05$)**. ND: Not detected.

This mechanism is consistent with findings from other studies showing that fermentation of sennosides using *Bifidobacterium* strains results in efficient conversion to physcion or related derivatives, thereby enhancing laxative activity. These results indicate that *L. delbrueckii* subsp. *lactis* (BCRC 14069), beyond its traditional role in dairy fermentation, can effectively catalyze the deglycosylation of plant polyphenols. Accordingly, the present findings may serve as a reference model for future biotransformation systems involving plant extracts rich in polysaccharides and anthraquinone derivatives.

The decrease in physcion content observed at 4 h suggests that, because LAB are metabolically active organisms, other enzymes may participate in secondary reactions during prolonged fermentation. Physcion may undergo further oxidation to rhein or other derivatives, be reduced, or even be metabolized as an energy source by the bacteria. Based on the analytical results of this study, a 2-h hydrolysis period using *L. delbrueckii* subsp. *lactis* (BCRC 14069) represents the suitable condition, achieving more than 97% sennoside hydrolysis, while increasing physcion production by more than 24%.

3.9. Antioxidant activity analysis: DPPH radical-scavenging capacity

Antioxidants often function by donating hydrogen atoms to reduce free radicals, thereby interrupting oxidative chain reactions. DPPH is a stable free radical system characterized by a strong absorbance at 517 nm. When DPPH is reduced by antioxidants through hydrogen donation, its absorbance decreases, and because the assay remains highly sensitive even at low concentrations, it is widely used to evaluate the hydrogen-donating ability of antioxidant compounds. In this study, the DPPH radical-scavenging capacity was used as an indicator of antioxidant activity (hydrogen-donating capacity). A 20% *C. occidentalis* extract served as the control group, while samples obtained after hydrolysis by *L. delbrueckii* subsp. *lactis* (BCRC 14069) for 2 and 4 h were analyzed. Antioxidant activity was quantified using the EC_{50} value, defined as the concentration required to scavenge 50% of DPPH radicals.

As shown in Table 5, the EC_{50} values of the control extract and the samples hydrolyzed for 2 and 4 h were 41.69 ± 0.37, 19.44 ± 0.23, and 20.00 ± 0.09 mg extract/mL, respectively. After 2 h of hydrolysis, the EC_{50} value decreased significantly to 19.44 mg/mL, indicating a substantial enhancement in DPPH-scavenging activity—a reduction of approximately 53% compared with the control. Extending the hydrolysis to 4 h resulted in an EC_{50} value of 20.00 mg/mL, which was not

significantly different from the 2 h group, suggesting that the antioxidant activity did not improve further beyond 2 h.

Table 5. Scavenging ability on DPPH radicals EC_{50} value of methanol extracts from *C. occidentalis* hydrolysis for 2–4 h by *L. delbrueckii* subsp. *lactis* (BCRC 14069).

Hydrolysis time (h)	Scavenging ability on DPPH radicals EC_{50} value (mg extract/mL)
Control	41.69 ± 0.37^{a}
2	19.44 ± 0.23^{b}
4	20.00 ± 0.09^{b}

*Note: Each value is expressed as mean ± SD (n = 3). Means with different letters within a column are significantly different ($P < 0.05$).

These results indicate that the decrease in EC_{50} may be attributed to the hydrolytic action of *L. delbrueckii* subsp. *lactis* on the glycosidic bonds of polymeric anthraquinone derivatives—such as sennosides—and abundant phenolic compounds present in *C. occidentalis*. Through the activity of β-glucosidase and other hydrolytic enzymes, these glycosides were cleaved to generate a greater quantity of small-molecule antioxidant constituents. Fermentation-mediated biotransformation by *L. delbrueckii* subsp. *lactis* (BCRC 14069) can release more aglycones while reducing lipid solubility, thereby facilitating reactions with free radicals such as DPPH and leading to a pronounced 53% reduction in EC_{50}.

The plateau in antioxidant enhancement between 2 and 4 h may result from the rapid hydrolysis within the first 2 h, causing a reduction in available substrates, as well as potential product inhibition or pH changes caused by lactic acid accumulation. This finding is consistent with Table 4 and other experimental results in this study, which indicate that sennoside hydrolysis exceeded 97% after 2 h. Collectively, these results demonstrate that 2 h is the suitable hydrolysis duration, not only for maximizing sennoside hydrolysis but also for enhancing antioxidant activity.

3.10. Antioxidant component analysis

Phenolic and flavonoid compounds possess strong antioxidant capacity because their structures enable them to chelate reactive oxygen species and metal ions; therefore, they are commonly used as indicators of antioxidant potential in food systems. In this experiment, total phenolic compounds (TPC) and total flavonoid content (TFC) were used as the basis for evaluating antioxidant components. A 20% *C. occidentalis* extract served as the control group, and samples hydrolyzed by *L. delbrueckii* subsp. *lactis* (BCRC 14069) for 2 and 4 h were analyzed to determine changes in TPC and TFC.

As shown in Figure 5, after 2 h of hydrolysis by *L. delbrueckii* subsp. *lactis* (BCRC 14069), the total phenolic content of the *C. occidentalis* extract increased significantly to 414.26 mg/g, representing an approximately 21% increase compared with the control group (343.14 mg/g). This indicates that during the hydrolysis process, the breakdown of macromolecular compounds may have generated or released additional phenolic constituents originally present in the extract. However, after 4 h of hydrolysis, the total phenolic content decreased to 405.03 mg/g. Although still higher than the control, it was slightly lower than the 2-h sample, suggesting that prolonged hydrolysis may have led

to further degradation or conversion of some phenolic compounds into other secondary metabolites, resulting in a decline in total phenolics.

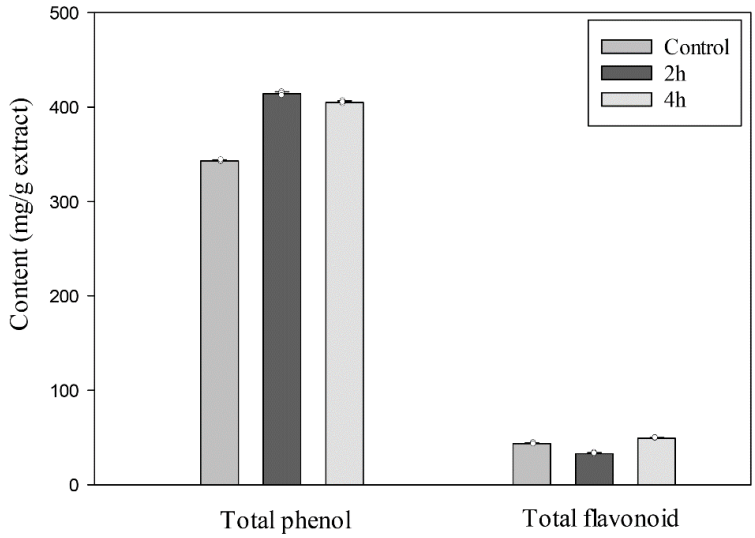

Figure 5. Changes in total phenolic content (TPC) and total flavonoid content (TFC) of 20% (w/v) *C. occidentalis* extract during hydrolysis by *L. delbrueckii* subsp. *lactis* (BCRC 14069) for 2 and 4 h. Data are presented as mean ± SD (n = 3).

In contrast to the TPC results, the trends in total flavonoid content differed. The 2-h hydrolyzed sample exhibited a flavonoid content of 33.07 mg/g, which was approximately 24.0% lower than the control group (43.49 mg/g). This may be attributed to insufficient production of flavonoids during the early hydrolysis stage and the possibility that flavonoids were degraded or transformed during microbial fermentation. However, after 4 h of hydrolysis, the flavonoid content increased markedly to 49.35 mg/g, representing an approximately 13.4% increase compared with the control and a 33% increase relative to the 2-h sample. This result may be due to extended fermentation by *L. delbrueckii* subsp. *lactis*, which facilitated the metabolic conversion of conjugated flavonoids (e.g., glycosides) into their free forms, thereby increasing total flavonoid content.

Overall, the results demonstrate that hydrolysis by *L. delbrueckii* subsp. *lactis* (BCRC 14069) significantly affects both the total phenolic and total flavonoid contents of *C. occidentalis* extract, but their response patterns differ. Total phenolics reached their highest level after 2 h of hydrolysis with a 21% increase, whereas total flavonoids increased most after 4 h, with a 13% enhancement. These differences may be related to the enzymatic system of *L. delbrueckii* subsp. *lactis* (BCRC 14069)—including esterases and glycosidases—which may act differently on distinct secondary metabolites.

Supplemental β-glucosidase did not improve, and in some cases reduced, the sennoside hydrolysis rate achieved by bacterial treatment alone, consistent with glucose-mediated product inhibition and the possibility that the rate-limiting step in sennoside metabolic activation resides in reductive enzymatic activities (e.g., NTR-type enzymes) rather than in β-glucosidase hydrolysis. Future research should investigate the molecular mechanisms of NTR-type reductases and transmembrane transport proteins in *L. delbrueckii* subsp. *lactis* to elucidate the mechanistic basis of its superior sennoside-converting capacity.

The present study has several limitations. All experiments were conducted in vitro using a simplified aqueous extract system, which may not fully reflect the complex colonic environment in

vivo. The microbial strains were not isolated from the human gut, and their behavior under in vivo gut conditions (pH gradients, competing microbiota, mucus layers, bile salts) may differ. Additionally, only physcion and rhein anthrone were quantified as downstream metabolites; a comprehensive metabolomic profiling of all biotransformation products, including sennidins and other anthraquinone derivatives, is warranted in future studies. Finally, safety and efficacy studies in animal models and clinical trials are required before these findings can be applied to the development of functional food products.

4. Conclusions

This study screened 20 microbial strains (16 LAB, 3 *Bifidobacterium* spp., and *K. marxianus*) for their ability to biotransform sennosides in *C. occidentalis* extracts. *L. delbrueckii* subsp. *lactis* (BCRC 14069) and *L. helveticus* (BCRC 14092) demonstrated superior in vitro biotransformation efficiency. Under suitable conditions (20% substrate concentration, 37 °C, anaerobic, 2 h), *L. delbrueckii* subsp. *lactis* achieved sennoside hydrolysis rates exceeding 97% within 2 h, substantially faster and more efficient than previously reported *Bifidobacterium* strains (~80% conversion rate, typically > 8 h). Physcion content increased by 24.7% (to 156.39 mg/g), and DPPH-scavenging EC_{50} decreased by ~56%, indicating significant enhancement of antioxidant activity. Residual sennoside levels were reduced to approximately 6–7 mg/g, ensuring compliance with both TFDA (≤12 mg/day) and EFSA (<10 mg/day) regulatory limits under realistic dosage scenarios.

Author contributions

Conceptualization: Yuh-Shuen Chen, Shuo-Wen Tsai; Methodology: Shuo-Wen Tsai, Sheng-Yuan Yang; Investigation: Sheng-Yuan Yang, Chung-Ju Chen, Chia-Sen Juo; Data curation: Sheng-Yuan Yang, Shih-Lun Liu, Chung-Ju Chen; Supervision: Shih-Lun Liu, Shuo-Wen Tsai; Validation: Yuh-Shuen Chen, Shih-Lun Liu, Chung-Ju Chen; Visualization: Sheng-Yuan Yang, Shih-Lun Liu, Chung-Ju Chen; Writing–original draft: Sheng-Yuan Yang, Shih-Lun Liu; Writing–review & editing: Yuh-Shuen Chen, Shih-Lun Liu. All authors have read and approved the final version of the manuscript for publication.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Conflict of interest

The authors declare no conflicts of interest.

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