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Research Article

Proteomic Characterization of Human Gut Habitual *Bacteroides intestinalis* against Common Intestinal Bile Acid Stress

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Bacteroides intestinalis (*B. intestinalis*) is an abundant gastrointestinal commensal bacterium and is able to produce secondary bile acids (BAs) among other important metabolic functions. However, deoxycholic acid (DCA) is known to suppress *Bacteroides*, suggesting differential molecular impact of different BA species on *Bacteroides*. Among major human gastrointestinal BA components, we first demonstrated that DCA and chenodeoxycholic acid (CDCA) and their taurine-conjugated species at 1 mM showed significantly higher inhibitory effects on the growth of *B. intestinalis* than cholic acid (CA) and lithocholic acid (LCA) and their taurine-conjugated species. Then, high-throughput proteomic strategy was used to show that both TCDCA and TDCA caused more proteome-wide modulation than TCA and TLCA. In response to incremental BA toxicity, the main functional changes of *B. intestinalis* include enhanced protein synthesis, DNA integrity maintenance, and suppressed central metabolic activities. Importantly, key energy and BA metabolism enzymes of *B. intestinalis* were inhibited by TCDCA and TDCA. These findings provide a basis for future studies to explore how *Bacteroides* respond to bile stress and how BA composition modulate gut microbiome homeostasis.

1. Introduction

Bile acids (BAs) are steroid acids found predominantly in vertebrates. There are four major BAs, i.e., cholic acid (CA), lithocholic acid (LCA), deoxycholic acid (DCA), and chenodeoxycholic acid (CDCA), in human gastrointestinal (GI) lumen [1, 2]. These BAs are conjugated with taurine or glycine in the liver and secreted to the small intestine where they play important roles in digestion by acting as detergents. Many indigenous GI microorganisms can enzymatically deconjugate BAs and further bioconvert them to secondary BAs before being absorbed back to the host via GI mucosa. Although it has been 40 years since the discovery of enterohepatic cycling metabolism of BAs, the list of secondary BA metabolites and BA-converting gut microbial species is still growing, and our understanding about such a large family of BA components is still incomplete.

The differential impact of BA species on a multitude aspects of host metabolism has been widely explored [3–5]. On the other hand, BAs also exhibit bactericidal activities and therefore are key modulators of gut microbial homeostasis. Unfortunately, our understanding of influence by individual BA on major gut communal bacteria is still incomplete.

One of the most common residents of GI lumen is *Bacteroides*. Together with *Firmicutes*, they make up more than 90% of the total gut indigenous microbiota [6]. The *Bacteroides* is conserved evolutionally among mammalian species, implicating a beneficial symbiotic relationship that was not thoroughly investigated so far. The metabolic influence of *Bacteroides* on host has been shown in recent studies elaborating their connection to obesity and metabolic syndromes [7–10]. *Bacteroides intestinalis* (hereafter *B. intestinalis*) is a highly abundant member of this family that shows ability to ferment dietary fibers and produce short-chain fatty acids

(SCFAs) which are key nutrients for colonic epithelium cells [11–14]. Like many other GI bacteria, *B. intestinalis* also produces polycationic amines including spermidine, spermine, and putrescine which play a variety of critical biological roles including maintenance of mucosal homeostasis [15, 16] and maintenance of DNA and protein stability in host cells [17] and thus was thought to render anticarcinogenic and anti-inflammatory properties [18–20].

It is worthy to note that *Bacteroides*, the vast majority of them express bile salt hydrolase (BSH) and 7a-hydroxysteroid dehydrogenase (7a-HSDH), are key contributors to the hydrolysis of conjugated BAs and generation of secondary BAs [3, 21–23]. Interestingly, unlike other GI bacteria that convert cholic acid CA and CDCA into DCA and LCA, which are known carcinogens in the human GI tract [24], *Bacteroides* instead produce 7-oxo derivatives of DCA and LCA which can be later converted to ursodeoxycholic acid, 7-ketolithocholic acid, or 7-epi-cholic acid, thus contributing to the GI health [3, 21, 23]. On the other hand, *Bacteroides* is influenced by a multitude of host and microenvironmental factors, including the intestinal BA composition. The previous report showed that *Bacteroides* can be inhibited by DCA [25] produced by other major BA bioconvertors such as *Clostridium* and *Eubacterium* [26].

Given that the composition of intestinal BAs varies individually and each BA species has distinct chemical properties and biological activities, it is eligible to propose that differential BA formula contributes to both constitutional and functional diversities of gut microbiota. To understand such complex relationship, it is important to explore the molecular response of gut bacteria against BA insults. To our best knowledge, it is till recently that the differential impact of BA components on gut microbial species starts to draw attention [4]. In addition, little is known regarding the molecular effect of BAs on *Bacteroides* species. Here in this study, we employed the high-throughput proteomic technology to explore the differential impact of two major primary BA species, cholic acid (CA) and chenodeoxycholic acid (CDCA), and their microbiota-transformed species, deoxycholic acid (DCA) and lithocholic acid (LCA), on one of the major gut habitual member, *B. intestinalis*. Our investigation showed that both CDCA, TCDCA, DCA, and TDCA inhibit the growth of *B. intestinalis* substantially. Each BA species exerts their influence on the *B. intestinalis* proteome in a unique pattern; no protein was found up- or downregulated across all BA-treated samples as compared to controls, while TCDCA and TDCA offer the most significant impacts on multiple functional groups.

2. Materials and Methods

2.1. B. intestinalis Culture Conditions and BA Stimuli. Frozen stocks of *B. intestinalis* (DSM17393, DSMZ, Germany) were reactivated in modified DSMZ Medium 104 (per liter contained trypticase peptone 5 g, peptone 5 g, yeast extract 10 g, beef extract 5 g, glucose 5 g, K_2HPO_4 2 g, Tween 80 1 mL, cysteine-HCl 0.5 g, ATCC trace mineral supplement 10 mL, haemin 5 mg, vitamin K1 1 μL, and pH 7.2) and incubated overnight at 37°C in an anaerobic cabinet (Electrotek Anaerobic Workstation, Shipley BD18 4EW, United Kingdom) filled with 10% H_2, 10% CO_2, and 80% N_2. For growth with BA, the medium was supplemented with each of the following BA species at physiologically relevant concentration (1 mM) [27], CA, CDCA, DCA, and LCA, or their taurine-conjugated form: taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), and taurolithocholic acid (TLCA). All chemicals were acquired from Sigma (MO, USA) unless specified.

2.2. Growth Curve and Colony Counts. Fresh medium was inoculated with log phase culture grown from a single colony by 1 : 100 dilution, and growth curve under anaerobic conditions was determined by recording the optical density of cultures at 600 nm (OD_{600}) over 80 hrs. Mid-log-phase (~0.3 of OD_{600}, unless mentioned otherwise) cells were diluted 100-fold, spread onto plates (medium with 1.5% agar), and incubated overnight, and colonies were counted. For cultures with BAs, colony counts were recorded after 3 days.

2.3. BA Stimulus and Sample Preparation for Proteomic Study. For proteomic comparison regarding different BA stimuli, *B. intestinalis* grew in BA-free medium into log phase ($OD_{600} = 0.3$), which were then evenly divided and incubated with 1 mM either TCA, TCDCA, TDCA, or TLCA for 2-hour stimulation. Bacterium cultures were then centrifuged at 3000 × g for 15 min at 4°C and washed by 10 mL precooled PBS twice. The overall proteomic workflow is summarized in Figure 1. Briefly, the pellets were then heated in boiling water for 10 min and immediately incubated in -80°C for 1 h before protein extraction by 1 mL 7 M urea, 2% SDS, and 15 mM dithiothreitol. The cell lysate was sonicated for 5 min in ice bath. Protein concentration was determined by RC-DC kit (Bio-Rad). Before tryptic digestion, proteins (200 μg) in lysis buffer were applied to a 10 kDa MWCO spin filter (Millipore) for ultrafiltration and washed by 200 μL 8 M urea. Protein was then alkylated in the dark with 100 μL 30 mM iodoacetamide for 45 min, followed by wash steps using 200 μL 8 M urea once and 200 μL 50 mM triethylammonium bicarbonate (TEAB) twice. Protein was then digested with 4 μg trypsin (Promega, WI) in 200 μL 50 mM TEAB buffer for 16 hrs at 37°C. Postdigestion peptides were collected by ultrafiltration. All ultrafiltration steps were carried out at 12, 000 × g for 15 min.

2.4. iTRAQ Multiplexing and Peptide Fractionation. Peptides (50 μg) were labeled according to the manufacturer's 8-plex iTRAQ protocol (Sigma). Two separate isobaric 8-plex iTRAQ reagent sets were used to accommodate 3 replicates for TCA-, TCDCA-, TDCA-, and TLCA-treated samples plus 3 normal controls (N) using the design summarized in Figure 1. Briefly, N1, TCA1, TCDCA1, TDCA1, TLCA1, N3, and TCA3 were tagged by 113, 114, 115, 116, 117, 118, and 119 labels in the iTRAQ run A, while N2, TCA2, TCDCA2, TDCA2, TLCA2, TCDCA3, and TLCA3 were tagged by 113, 114, 115, 116, 117, 118, and 119 labels in the iTRAQ run B. TDCA3 tagged by 121 label was used in both runs as run-to-run reference.

The iTRAQ-multiplexed peptides were first separated on a 1260 HPLC System (Agilent) equipped with an Acquity

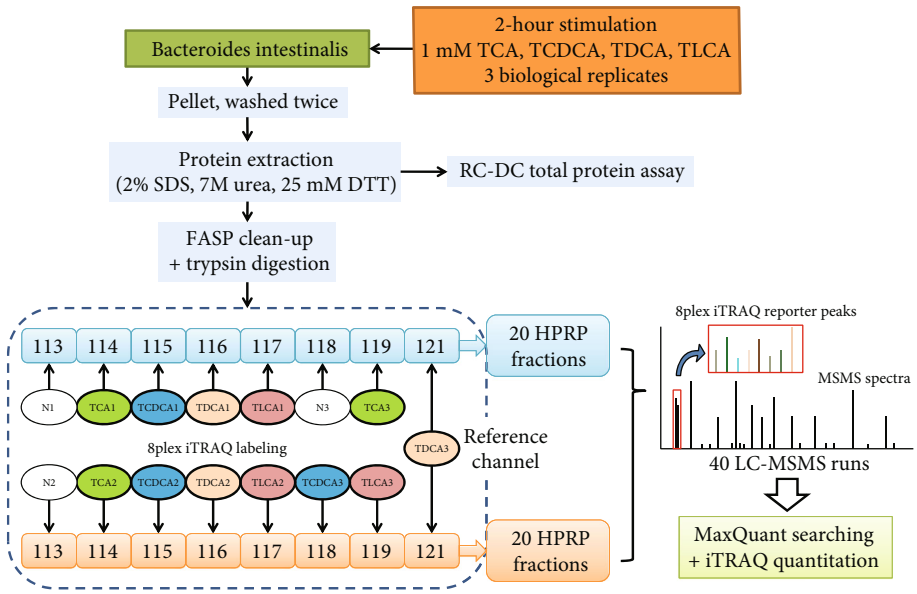
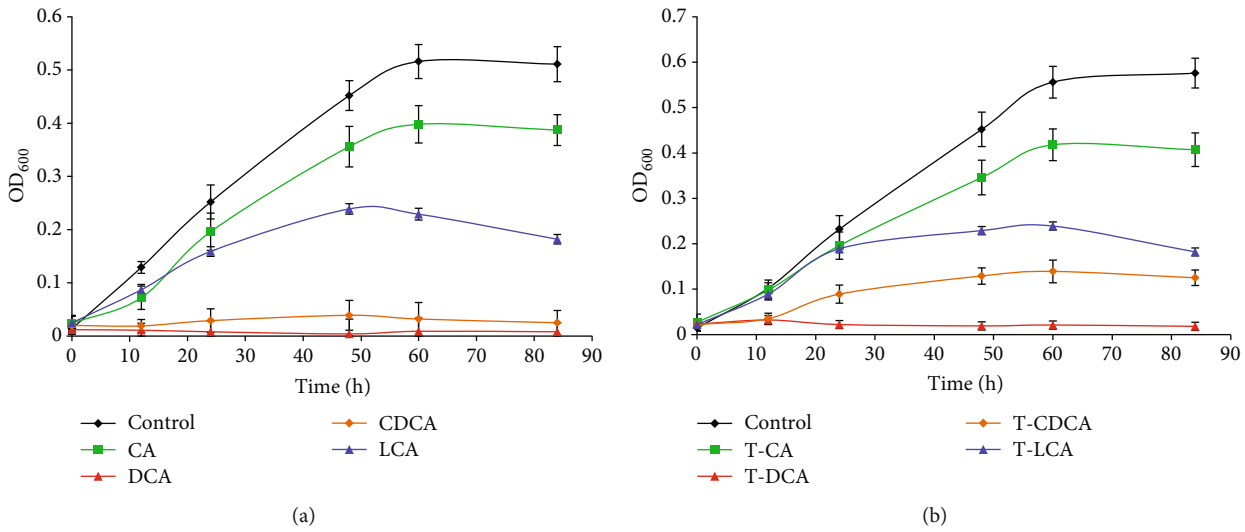


Figure 1: Overall design of two parallel 8plex iTRAQ shotgun LC-MSMS experiments to quantify proteome changes over 15 *B. intestinalis* samples challenged with different BA species.



Medium supplement	Control	CA	CDCA	DCA	LCA	TCA	TCDCA	TDCA	TLCA
Colony counts	187	105	1	0	40	120	20	0	45

(c)

Figure 2: The effect of 1 mM of free BAs (a) or taurine-conjugated BAs (b) on *in vitro* growth of *B. intestinalis*. Colony counts of each agar plates supplemented with different BA were shown (c).

Peptide BEH C18 column (1.7 μm, 130 Å, and 2.1 mm × 150 mm, Waters) kept at 45°C. Mobile phase A contains 0.1% NH_4OH and B contains 0.1% NH_4OH in ACN. The 45 min solvent gradient at a flow rate of 0.2 mL/min was set as follows: 5% B within 2 min, 5–18% B in 25 min, 18–32% B in 13 min, and 32–95% B in 1 min, maintained at 95% B for 4 min. Eluted peptides were monitored by UV at 280 nm. Forty fractions were collected every 1 min from 3 to 42 min, were combined into 20 fractions via a concatenated fashion, and then were dried by SpeedVac. Dried peptides were resuspended in 10 μL 0.1% FA in 2% ACN for nanoLC-MS/MS analysis.

2.5. Mass Spectrometric Acquisition. Peptides (~500 ng) were loaded and enriched on a Symmetry C18 nanoACQUITY Trap Column (100 Å, 5 μm, and 180 μm × 20 mm) on a nanoACQUITY UPLC system (Waters, Milford, MA).

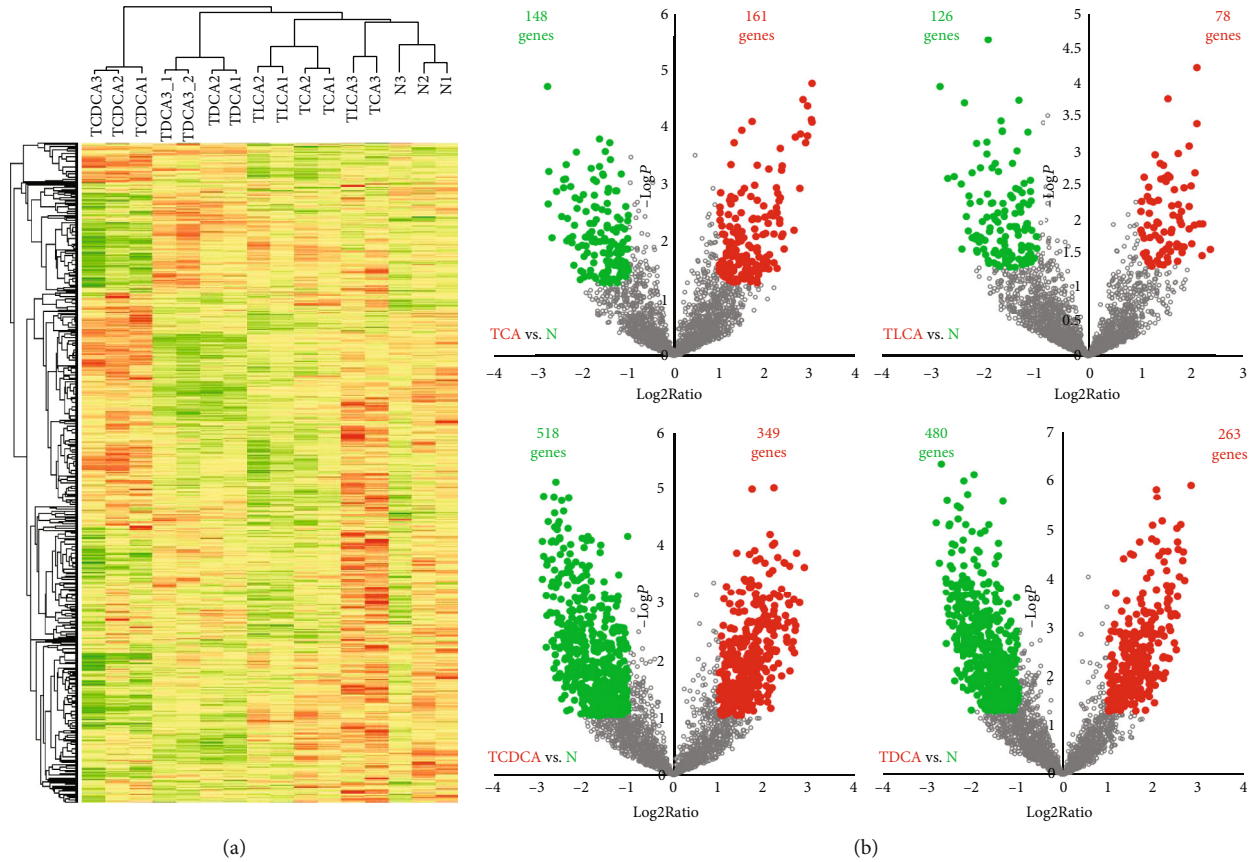


FIGURE 3: Differentially expressed proteins (DEPs) selected by proteomics. Expression level of DEPs across samples was transformed into Z-score by rows and summarized in heatmap with K-means-based clustering analysis performed both sample- and gene-wise (a). Shades of red or green represent elevation or decrease, respectively, of a protein expression level relative to the median levels in each row. Selection of DEPs based on differences (log2Ratio) and level of significance (−log P of t-test) in BA vs. N comparisons (b). Red and green dots indicate up- and downregulated DEPs.

Peptide separation was performed by a BEH C18 nanoACQUITY Column (130 Å, 1.7 μm, and 75 μm × 250 mm) with a 120 min gradient with flow rate set at 200 nL/min. Mobile phase A contains 0.1% FA in water and B contains 0.1% FA in ACN.

Eluted peptides were analyzed by Quadrupole-Orbitrap mass spectrometry (Q Exactive, Thermo). The nanospray source was charged at 1.8 kV. The MS was operated in data-dependent acquisition mode scheduling a MS^1 full survey scan at the 70,000 FWHM resolution (at m/z 200 Th) with automatic gain control (AGC) set to 3e6, followed by 20 MS^2 scans of precursor ions fragmented by higher-energy collision dissociation with normalized collision energy set to 27%. All MS^2 spectra were acquired at 17,500 FWHM resolution with AGC set to 2e5. Dynamic exclusion was set to 30 sec.

2.6. Proteomic and iTRAQ Data Analysis. Proteomic data were processed according to the previous method [28]. The acquired mass spectrometry data were searched first against the *B. intestinalis* DSM 17393 UniProt database (ID: UP000004596) supplemented with common contamination sequences using MaxQuant (version 1.6.0.13). The search was performed under the MS2 report ion quantitation (iTRAQ 8plex) mode. Trypsin was set as protease with up to two missed cleavages. The main database search was performed with mass tolerance of 7 ppm. Oxidation on methionine and carbamidomethylation on cysteine were set as variable and fixed modifications, respectively. The search result was filtered by 1% FDR at protein.

For quantitative analysis, only protein IDs with at least two peptide hits and commonly identified in both iTRAQ runs were used. The iTRAQ report ion intensities of each protein were first normalized against the reference ion intensity of 121 label in both runs A and B to correct run-to-run variations. For each sample, relative protein intensities were normalized against the median value to correct label-to-label variations. Differentially expressed proteins (DEPs) were generated based on log2 fold change (FC) ≥ 1 or $\leq$-1, and Student's t-test was $p < 0.05$.

2.7. Functional Enrichment Analysis. Clusters of DEPs were derived by K-means algorithm based on their similarities of expression profile across all samples. Clusters of interested *B. intestinalis* DEP were then annotated by KEGG GENES

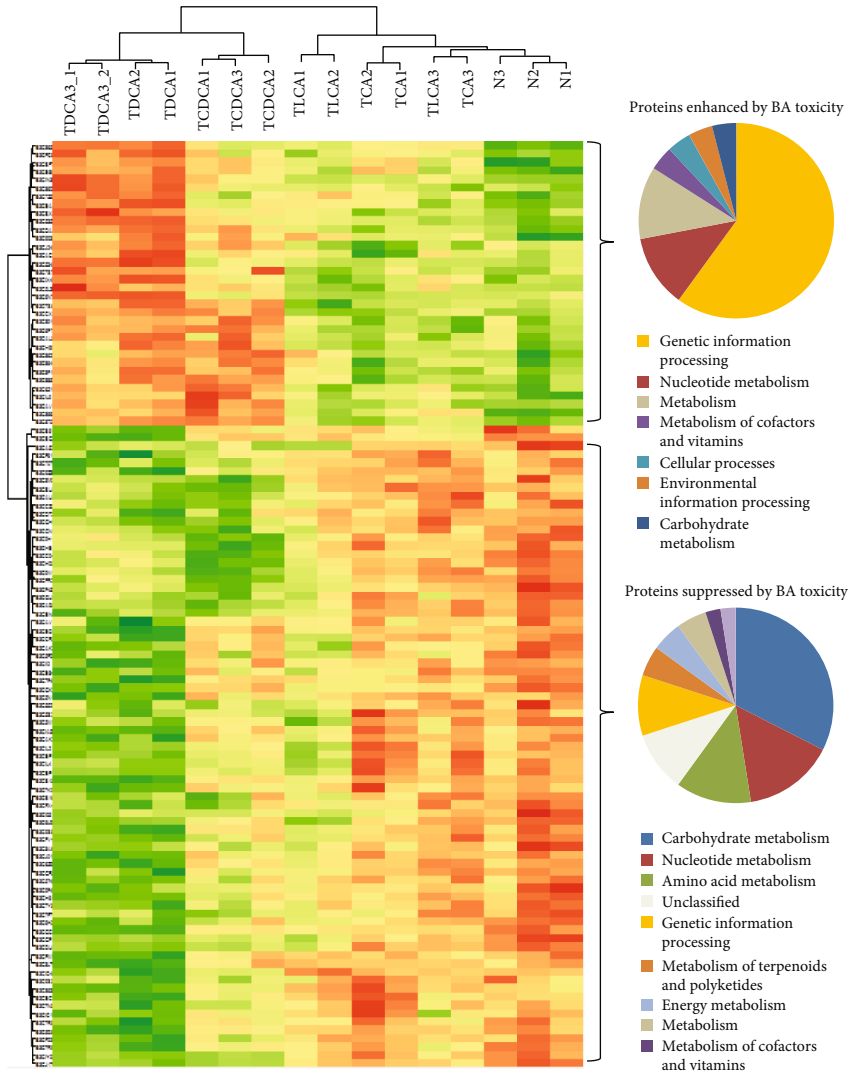


Figure 4: Identification of DEPs incrementally responds to increasing toxicity of different BA species (a). Functional summary of DEP clusters with expression enhanced or suppressed by BA toxicity (b).

based on sequence homology via BlastKOALA service [29] provided by KEGG (http://www.kegg.jp).

3. Results

3.1. Inhibitory Effect of Different Bile Acids on Growth of B. intestinalis. To investigate the effects of different BAs to *B. intestinalis*, the growth curves with medium supplemented with different BAs were determined by recording the OD_{600} value when supplemented with 1 mM different BA species. The growth curves indicated that all BA compounds inhibited *B. intestinalis* growth to a different degree (Figure 2(a)). Compared to the controls, DCA and CDCA totally block the growth of *B. intestinalis*, while the impacts of LCA and TLCA were also significant as OD_{600} values of *B. intestinalis* were almost half of that in each time point as compared to controls. In comparison, CA showed lowest inhibitory effect. Similar pattern of growth inhibition was observed in *B. intestinalis* incubated with T-BA, except TCDCA, a lower inhibition effect compared with CDCA. Notably, both DCA and TDCA at 1 mM can totally blocked the *B. intestinalis* growth. The colony count experiments supported the results of growth curve (Figures 2(b) and 2(c)).

3.2. Proteomic Characterization of B. intestinalis with Different Bile Acid Stimuli. A total of 28238 and 28693 unique peptide sequences were identified which mapped to 2567 and 2568 unique protein IDs from runs A and B, respectively. To ensure sound statistical analysis, 2378 unique proteins commonly identified from both runs A and B with at least 2 unique peptides were used for iTRAQ quantitation.

Clustering analysis of proteomic data suggested that there were apparent patterns of expression profile related to BA stimulus (Figure 3(a)). One major observation is that the TDCA and TCDCA inflicted more overall proteome perturbation than TCA and TLCA. In turn, there were more proteins differentially expressed in response to TDCA (263 up- and 480 downregulated) and TCDCA (349 up- and 518 downregulated)

Table 1: *B. intestinalis* DEP response to BA toxicity and differential expression between TDCA and TLCA.

ID	Protein name	Toxicity correlation	TDCA/TLCA ratio	Function
B3C6N7	Outer membrane efflux protein (tolC)	+	2.38	Efflux pump
B3C7S6	Arginine-tRNA ligase (ArgRS)	+	2.28	Protein synthesis
B3C7S7	DNA topoisomerase 1	+	2.12	Transcription
B3C860	Transcription termination/antitermination protein (NusG)	+	2.08	Transcription
B3CIX4	Zinc metalloprotease (rseP)	+	2.05	Protease
B3CIN3	Histidine-tRNA ligase (HisRS)	+	2.01	Protein synthesis
B3CAL5	Tetratricopeptide repeat protein	+	1.98	Unknown
B3CFC8	Peptidase, S41 family	+	1.95	Protein synthesis
B3C535	Trigger factor (tig)	+	1.93	Protein folding
B3CCX7	Antioxidant, AhpC/TSA family (PCB)	+	1.92	Antioxidant
B3CE86	Peptidyl-prolyl cis-trans isomerase D (ppiD)	+	1.77	Protein folding
B3C5L3	Hydrolase, NUDIX family	+	1.76	Nucleotide metabolism
B3CEA7	30S ribosomal protein S1	+	1.75	Protein synthesis
B3C6P7	50S ribosomal protein L19	+	1.64	Protein synthesis
B3C865	DNA-directed RNA polymerase subunit beta (rpoB)	+	1.58	Transcription
B3C7Y8	Phosphate butyryltransferase (ptb)	-	-1.56	SCFA production
B3C5T4	Methylmalonyl-CoA mutase (MUT)	-	-1.58	SCFA production
B3CJ01	Glucose-1-phosphate thymidylyltransferase	-	-1.60	Nucleotide metabolism
B3CGR4	Dihydroorotase (URA4)	-	-1.62	Nucleotide metabolism
B3CED1	Methionine aminopeptidase (MetAP, map)	-	-1.68	Protein synthesis
B3CB15	Pyridine nucleotide-disulfide oxidoreductase	-	-1.68	Energy metabolism
B3CGC4	Glucosamine-6-phosphate deaminase-like protein (nagB)	-	-1.70	Energy metabolism
B3CEC7	OmpA family protein	-	-1.77	Biofilm formation
B3CBG5	Hydroxylamine reductase (HCP) (prismane protein)	-	-1.80	Nitrate metabolism
B3CII2	Transcriptional regulator, effector binding domain protein (AraC)	-	-1.83	Transcription
B3C6Z9	Acetate kinase (acetokinase, ackA)	-	-1.85	SCFA production
B3CF22	4-Phosphoerythronate dehydrogenase (hprA)	-	-1.87	Energy metabolism
B3CH61	Uridylate kinase (UMPK, pyrH)	-	-1.88	Nucleotide metabolism
B3CGH3	Alpha-2-macroglobulin family protein	-	-1.89	Host colonization
B3C7F4	Peptidase dimerization domain protein (argE), acetylornithine deacetylase	-	-1.92	Amino acid/polyamine metabolism
B3C5N1	Polyprenyl synthetase geranylgeranyl diphosphate synthase (GGPS)	-	-1.94	Diterpene synthesis
B3C707	Cupin family protein	-	-1.99	Unknown
B3C9Z3	Fructokinase, PfkB family	-	-2.01	Energy metabolism
B3CFW4	Orotidine 5′-phosphate decarboxylase (OMPdecase, pyrF)	-	-2.03	Nucleotide metabolism
B3C5D8	DJ-1 family protein, protein deglycase (thiJ)	-	-2.06	Redox sensor
B3CF61	6-Phosphogluconolactonase (PGLS)	-	-2.09	Energy metabolism
B3CD72	Aspartate carbamoyltransferase regulatory chain (pyrI)	-	-2.09	Nucleotide metabolism
B3C863	50S ribosomal protein L10	-	-2.12	Protein synthesis
B3C8L7	Carbamoyl-phosphate synthase small chain (carA)	-	-2.17	Amino acid metabolism

Note: positively (+) and negatively (-) correlated with BA toxicity.

treatments in comparison to TCA (161 up- and 148 downregulated) and TLCA (78 up- and 126 downregulated) (Figure 3(b)). More importantly, both TDCA and TCDCA insults resulted in substantially more downregulated DEPs than upregulated ones, suggesting overall suppression roles of TDCA and TCDCA against multiple metabolic functions of B. intestinalis. The list of DEPs from all comparisons can be found in supplementary table 1.

3.3. Proteomic Change of B. intestinalis Related to Bile Acid Toxicity. The clustering analysis helped to identify a set of DEPs with incremental changes in response to different levels

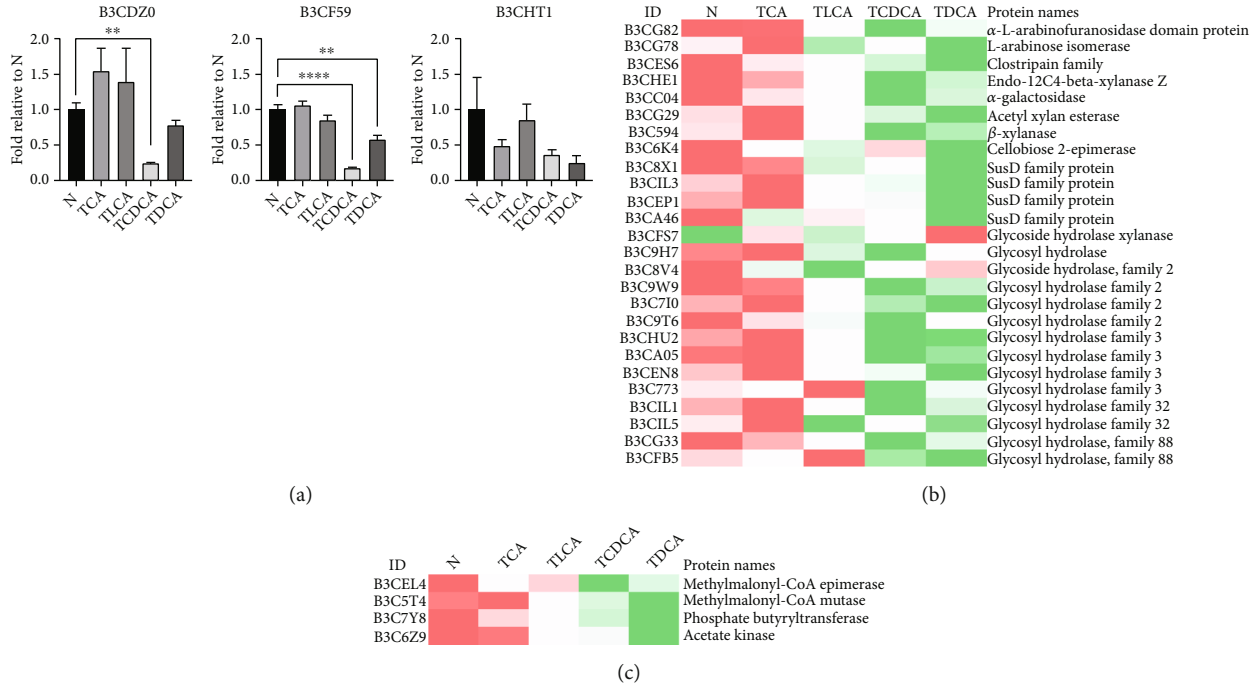


Figure 5: Impact of BAs on proteins related to BA metabolism (a), polysaccharides (b), and SCFA production (c).

of toxicities imposed by TCA, TLCA, TCDCA, and TDCA, respectively (Figure 4). The differential BA toxicity gradually upregulated 34 DEPs mainly related to genetic information processing and nucleotide metabolism and downregulated 77 DEPs mainly related to metabolism of carbohydrate, nucleotide, and amino acids according to BlastKOALA GO annotation analysis.

Since gut lumen BA pool is mainly comprised of secondary BA components (LCA and DCA), we then focused on the proteomic response against TDCA which is highly inhibitory for *B. intestinalis* and that against TLCA. Compared to TLCA treatment, TDCA upregulated 194 DEPs mainly related to genetic information processing and nucleotide metabolism and downregulated 243 DEPs mainly related to metabolism of carbohydrate, nucleotide, and amino acids according to BlastKOALA GO annotation analysis. This is generally in line with proteomic modulation by BA toxicity as we mentioned above. Table 1 summarizes key *B. intestinalis* DEPs in response to BA toxicity and also shows differential expression between TDCA and TLCA ($FC > 1.5$, $p < 0.05$). The list included multiple upregulated DEPs in transcription and translation and downregulated DEPs in energy metabolism.

3.4. Impact of BAs on Proteins Related to BA Metabolism. The expression of key *B. intestinalis* enzymes related to the bioconversion of BAs, the BSHs that deconjugate BAs and 7a-HSDH that produce secondary BAs, was investigated in this study (Figure 5(a)). The choloylglycine hydrolases (BSH, accession ID: B3CDZ0, B3CF59) showed little impact by TCA and TLCA stimuli, while TCDCA showed significant inhibitory effect for both choloylglycine hydrolases ($FC = -2.203$ for B3CDZ0 and $FC = -2.56$ for B3CF59) and TDCA also showed mild inhibitory effect for choloylglycine hydrolase B3CF59. Besides, TCA, TCDCA, and TDCA showed mild inhibitory effect without statistical significance on the expression level of 7-alpha-hydroxysteroid dehydrogenase (hdhA, accession ID: B3CHT1).

3.5. Proteins Related to Polysaccharide and SCFA Metabolisms Are Suppressed by BAs. Like other *Bacteroides* members, *B. intestinalis* converts dietary polysaccharide, including xylans, to SCFAs which is critical to the health of gut mucosa. Our data shows that the BA toxicity, particularly of TCDCA and TDCA, can significantly suppress the enzyme systems that utilize polysaccharides, including glycosyl hydrolases (B3C9H7, B3C9W9, B3C8V4, B3C7I0, B3C9T6, B3CHU2, B3CA05, B3CEN8, B3C773, B3CIL1, B3CIL5, B3CG33, and B3CFB5) and SusD proteins (starch utilization system, B3C8X1, B3CIL3, B3CEP1, and B3CA46), xylanases (B3CHE1 and B3C594), and acetyl xylan esterase (B3CG29) (Figure 5(b)). Several key enzymes related to monosaccharide or oligosaccharide metabolism were also inhibited, such as arabinose isomerase (B3CG78), galactosidase (B3CC04), and cellobiose 2-epimerase (B3C6K4). Additionally, enzymes related to SCFA production, such as methylmalonyl-CoA epimerase (B3CEL4) and methylmalonyl-CoA mutase (B3C5T4), phosphate butyryltransferase (B3C7Y8), and acetate kinase (B3C6Z9), were all similarly inhibited by TCDCA and TDCA (Figure 5(c)).

4. Discussion

It is previously shown that BAs, as key determinant of gut microbiome, led to outgrowth of *Firmicutes*, *Clostridia*, and *Erysipelotrichia* species at the expense of *Bacteroidetes* [30]. This *in vitro* study confirmed the inhibitory effects of all

major BA components on *B. intestinalis*, which is a common *Bacteroidetes* member present in human GI. Both CDCA and DCA showed much stronger inhibitory effects on *B. intestinalis* growth than CA and LCA. Our observation fits with the previous study which showed that *Bacteroides* were strongly suppressed by DCA [25]. Acting as detergent, BAs solubilize bacterial membrane lipids and destabilize membrane proteins, a process that eventually leads to cell lysis [26]. It is known that CDCA and DCA, both containing 2 hydroxyl groups in their perhydrocyclopentanophenanthrene steroid ring, have unique hydrophobic and detergent properties [4, 31]. Therefore, we believe that the stronger bactericidal effects of dihydroxy-BAs could explain our observation that CDCA and DCA, whether in free or conjugated forms, cause significant inhibition to the growth of *B. intestinalis* at physiological relevant concentration. In agreement with growth curve data, the more profound proteomic responses to TCA and TLCA treatments were clustered together, while less shifted proteome profile of TCDCA and TDCA treatments was clustered together (Figure 3(a)).

Interestingly, the most highlighted protein upregulated in response to BA toxicity is outer membrane efflux tolC (Table 1). The AcrAB-TolC complex was studied intensively as a critical stress and multidrug resistance gene in Gram-negative bacteria that pumps out a variety of antibiotics, detergents, lipids, dyes, and quorum sensing molecules [5, 32–34]. Future researches are needed to clarify the role of tolC efflux protein related to BA metabolism and transportation and to explore the impact of BA components on bacteria multidrug resistance mechanism. As a part of stress response, *B. intestinalis* apparently also enhanced genetic information processing and protein synthesis upon BA insults. The upregulations of multiple ribosomal subunits and protein chaperones are probably due to the high demand of proteome reconfiguration and to maintain DNA integrity during BA challenges. On the other hand, central metabolisms of carbohydrate and amino acids were otherwise compromised. These data were in line with the previous proteomic studies that showed BA challenges lead to significant alterations of energy metabolism and gene transcription/translation in *Bifidobacterium longum*, *Lactobacillus reuteri*, *Lactobacillus johnsonii*, *Lactobacillus plantarum*, and *Lactobacillus salivarius* [35–39]. Related to important metabolic functions of *B. intestinalis*, we have revealed the negative impact of BA toxicity on enzyme system of polysaccharide utilization, including that of starch, xylans (i.e., noncellulosic polysaccharides), and ligo-/monosaccharides. Interestingly, another proteomic study on *Bacteroides fragilis* also documented the suppression effect of BA on the starch utilization system (Sus) [40], which is critical for polysaccharide metabolism in *Bacteroides* [41]. Moreover, we found that genes related to SCFA production, a key function of GI *Bacteroides*, were also suppressed by BAs.

As a part of general metabolic inhibition of *B. intestinalis* by BA toxicity, we also noticed that enzyme system related to BA conversion was also affected. The expression level of BSH, which enzymatically liberates the free BAs from conjugated BAs, was dampened by TCDCA or TDCA. In contrary, there were reports showing that BA challenge in *Lactobacillus johnsonii*, *Lactobacillus salivarius*, and *Lactobacillus plantarum* can actually upregulate BSH expression [36, 38, 39]. Such discrepancies in regulation of BSH genes by BAs are probably microbial species specific or BA species or concentration dependent. Additionally, the expression of hdhA, which converts primary BAs to secondary counterparts, was also slightly inhibited by BA, which further explain the strong bactericidal effects particularly of CDCA and DCA on *B. intestinalis*. It is worthy to point out that unlike major producer of DCA such as *Clostridium* and *Eubacterium* [26], *B. intestinalis* instead produce ursodeoxycholic acid, 7-ketolithocholic acid, or 7-epi-cholic acid, which provide benefits to the host [3, 21, 23]. The previous study has shown that the 7-ketolithocholic acid produced by *B. intestinalis* can be considered as a biomarker of GI health, and lower level of this secondary BA was documented as an indicator of microbiome dysbiosis in patients with liver cirrhosis [42].

As complex proteomic response of *B. intestinalis* by different BA species was revealed, this study may provide insights to the interindividual variability in intestinal microflora composition possibly caused by the different BA composition. However, because treatment with individual BAs did not fully reflect the BA pressure on the bacteria in actual GI environment, future investigations were needed to clarify *B. intestinalis* functional modulation with dynamic BA metabolism *in vivo*.

5. Conclusion

This work unraveled differential proteomic impact of BAs with incremental toxicity against *B. intestinalis*. We confirmed the greater growth inhibitory effects of both TCDCA and TDCA on *B. intestinalis* and thus caused more differentially expressed proteins than TCA and TLCA. We further discovered that the main functional changes of *B. intestinalis* involved with enhanced protein synthesis and DNA integrity maintenance and suppressed central metabolic activities in response to incremental BA toxicity. The key BA metabolic enzymes including hdhA and BSHs were inhibited by TCDCA and TDCA.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary Materials

Supplemental material for this article may be found at SupplTable1 (the list of DEPs from all comparisons in this study). *(Supplementary Materials)*

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