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Akkermansia muciniphila upregulates the expression of γ-aminobutyric acid in response to deoxycholate exposure

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Abstract

Akkermansia muciniphila is a beneficial gut bacterium because of its improving metabolic effect. However, it is not fully understood how *A. muciniphila* interacts with host substances to inhabit the human gut. To examine the effect of deoxycholate (DCA) produced by the combination of host and gut bacteria, which enhances the growth of *A. muciniphila*, on the metabolic changes of *A. muciniphila* using transcriptome and proteome analyses. Transcriptome analysis showed that carbohydrate metabolism, including glycosyl hydrolase activity and glycosyl bond activity, was significantly upregulated. Notably, transcriptome and proteome analyses demonstrated that the γ-aminobutyric acid (GABA) production pathway, which is related to acid or osmotic stress responses, was upregulated in the presence of DCA. Our results demonstrated that carbohydrate metabolism and GABA production were altered in response to DCA. Therefore, DCA may be a key intestinal substance for the physiological regulation and persistence of *A. muciniphila* in the gut. This study provides valuable insights into understanding the interaction between host and gut bacterium to persist in the gut.

Impact Statement

This is the first study demonstrating changes in gene and protein expression in *Akkermansia muciniphila* driven by deoxycholate (DCA), a major circulating secondary bile acid. Our study revealed that changes in stress response–related pathways, such as GABA production and carbohydrate metabolism, were caused by DCA. Moreover, this study revealed the effect of DCA on the metabolism of *A. muciniphila* and proposed DCA as a potential regulator of *A. muciniphila* persistence in the gut.

Keywords: bile; gut bacteria; γ-aminobutyric acid; omics; *Akkermansia muciniphila*

Introduction

Bacteria inhabit various parts of the body, and gut microbiota is strongly related to host health via the gut (de Vos et al. 2022). Gut flora is considered a forgotten organ because a wide variety of specific gut bacteria and microbiota are strongly associated with human health and diseases, such as inflammation, obesity, and arteriosclerosis (Ley et al. 2006, Ansaldo et al. 2019). Elucidating the mechanism by which gut bacteria inhabit and survive in the gut may have health-related benefits.

The composition of gut microbiota is influenced by dietary and host factors. Bile acids are one of the most important factors linking host health and gut microbiota (Chen et al. 2020). Primary bile acids, produced and secreted by the liver, promote fat absorption and antibacterial activity associated with the regulation of gut microbiota. Moreover, primary bile acids are deconjugated by bile salt hydrolases and converted into secondary bile acids such as deoxycholate (DCA) by 7α-dehydroxylation of gut bacteria (Wahlström et al. 2016). A recent analysis of the correlation between bile acids and human age showed that the level of isoallolithocholic acid, which has strong antibacterial activity and is produced by gut bacteria, is higher than that in centenarians (Sato et al. 2021). Although DCA suppresses the pathogenicity of *Clostridioides difficile* (Saenz et al. 2023), a high-fat diet inducing DCA production is associated with carcinogenesis in the colon (Bernstein et al. 2011). The gut bacteria interacting with DCA are considered keystone species for human health.

Our previous report demonstrated that DCA may improve the growth of *Akkermansia muciniphila* (Hagi et al. 2020). *Akkermansia muciniphila* is one of the most dominant and beneficial gut bacteria on the mucus layer and is reported to possess anti-obesity and anti-diabetic activities (Plovier et al. 2017, Depommier et al. 2019). A recent study in C57BL/6 J mice showed a positive relationship between the *A. muciniphila* population and DCA (Zhang et al. 2024). Another study demonstrated that 3-succinylated cholic acid, a previously unidentified compound produced by *Bacteroidetes uniformis*, could promote the growth of *A. muciniphila* (Nie et al. 2024). These studies strongly indicate the interaction between *A. muciniphila* and DCA, and deciphering this interaction will contribute to promoting human health.

As described previously, bile acids, including DCA, possess antibacterial activity. Stress resistance systems against bile are required for bacterial persistence in the gut. Bile resistance systems, such as outer membrane proteins and transporters, play key roles in protecting gut bacteria from the antimicrobial activity of bile (Watson et al. 2008, Doranga and Conway 2023). Our previous study demonstrated that the bile resistance system of *A. muciniphila* includes squalene biosynthesis and two transporters (ABC- or RND-type) and is crucial for its survival in the gut (Hagi et al. 2020).

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Recent study showed the ability to produce γ-aminobutyric acid (GABA) in *A. muciniphila* (Konstanti et al. 2024). GABA, produced by gut bacteria is a potential metabolites linked to gut-brain axis (Braga et al. 2024). The regulation of GABA production by *Bacteroides* could influence mental health (Strandwitz et al. 2019). *Akkermansia muciniphila* can also influence gut-brain axis (Xu et al. 2023). It is important for human health to understand how GABA production by gut bacteria, including *A. muciniphila* is regulated in the gut. Investigation of the interaction between host-derived substances such as bile acids and GABA production may contribute to decipher the regulation of GABA production in the human gut.

In this study, we used transcriptome and proteome analyses to investigate the types of metabolism associated with growth and stress response of *A. muciniphila* in the presence of DCA. Transcriptome and proteome analyses showed that DCA significantly altered carbohydrate metabolism and GABA production. Moreover, *A. muciniphila* produced higher levels of GABA in the presence of DCA. Our results provide significant insights into the metabolic changes of *A. muciniphila* driven by DCA.

Materials and methods

Growth conditions

Akkermansia muciniphila MucT (DSM 22959) was anaerobically grown at 37°C in 10 ml of modified basal medium (mBM) as described previously (Hagi et al. 2020). For the medium supplemented with bile acid, 100 mmol l^{-1} sodium DCA (SIGMA, St. Louis, MO, USA) was sterilized using a 0.22-μm membrane filter (mdi Membrane Technologies; Harrisburgh, PA, USA) and added to the culture medium at a final concentration of 1 mol ml^{-1}. The concentration of DCA is physiologically relevant to *in vivo* bile acid level (Saito and Sagae 2023, Wahlström et al. 2024). Then, 200 μl of 10-fold diluted fully grown preculture of *A. muciniphila* in mBM was inoculated into 10 ml of mBM supplemented with a carbohydrate mixture. After incubation at 37°C, the optical density at 600 nm (OD600) was measured at different time points. Statistical analysis was performed using the Welch or paired *t*-test.

RNA extraction, transcriptome and real-time quantitative reverse-transcriptional PCR (qRT-PCR) analysis

RNA extraction was performed as previously described (Hagi et al. 2020). Briefly, 2 μl of a fully grown preculture of *A. muciniphila* in mBM was inoculated into 10 ml of mBM supplemented with or without DCA. After incubation at 37°C, 400 μl of cells in the late exponential phase (final OD600 = ~3.0 for control group and ~4.0 for DCA treatment group) was added to 800 μl of RNAprotect Bacteria Reagent (Qiagen GmbH, Hilden, Germany). RNA extraction was performed using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instruction. RNA sequencing (RNA-seq; 2 G raw data per sample) was performed by Novogen (Cambridge Science Park, Cambridge, UK) using the HiSeq platform with 150-bp paired-end reads. After removing low-quality reads and those containing adapters, genome mapping was performed using Bowtie2 v2.3.4.3. The RNA-seq reads were assembled according to the reference genome of *A. muciniphila* (GCF_000020225.1) using Rockhopper (McClure et al. 2013). Differential expression analysis was performed using DESeq2, with biological replicates for each condition, and adjusted *P*-value (padj) was calculated. Differences obtained at the padj < 0.05 level ($n = 3$) were considered significant. A volcano plot was constructed using VolcaNoseR (https://goedhart.shinyapps.io/VolcaNoseR/). Based on Gene Ontology (GO) analysis, upregulated genes were annotated to biological processes, molecular functions, or cellular components in a directed acyclic graph structure using clusterProfiler. According to fragments per kilobase of exon per million fragments mapped (FPKM), highly expressed genes were defined as having > 500 FPKM, as reported by Chao et al. (2017). The transcriptome data has been deposited in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA1233894.

To evaluate transcriptomic result related to GABA synthesis, qRT-PCR was performed as described before (Hagi et al. 2015). Genes associated with GABA production were selected from previous report (Konstanti et al. 2024). Reverse transcription PCR was performed using a PrimeScript R II 1st strand cDNA Synthesis Kit (Takara, Otsu, Japan) according to the manufacturer's instructions. cDNA was synthesized from 0.5 μg of total RNA using a random primer. The resulting cDNA solutions were diluted with 60 μl of sterile distilled water and used as templates for real-time PCR. PCR primers were designed using Primer BLAST design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). To specifically amplify glutamate decarboxylase (gad: AMUC_RS02075), amino acid permease (gadC: AMUC_RS00210), and glutaminase (glsA: AMUC_RS00215), we used the primer set gadF 5′- CCGCTATTGAGATGCGTTGC-3′/gadR 3′- TGTGCCAGCAGATTTGGACA-5′ (amplicon length:216 bp), glsAF 5′- GGAGTCAATCCCGTCAGCAA-3′/glsAR 5′- CCCCTTGACGGTATTCCCTG-3′ (amplicon length: 231 bp), and gadCF 5′-GTGCAATCCGCCTACCAGAT-3′/gadCR 5′-ACATGGTACTGCTGCCTACG-3′ (amplicon length: 250 bp). 16S rRNA gene transcript level was also monitored as reference gene for normalization; AM1 5′- CAGCACGTGAAGGTGGGGAC-3′/AM2 5′-CCTTGCGGTTGGCTTCAGAT-3′ (amplicon length: 327 bp) (Collado et al. 2007). Real-time quantitative PCR was performed using THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) and the Thermal Cycler CFX Duet Real-Time System (Bio-Rad, Hercules, CA, USA). The thermal cycling conditions used were as follows: 95°C for 10 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. Relative normalized expression was calculated using CFX Maestro Ver.2.3.

Proteome analysis

After the full growth of *A. muciniphila* (following incubation for 48 hours), the cells were collected via centrifugation at 17 800 *g* and 4°C. Protein extraction and liquid chromatography–mass spectrometry (LC–MS) analysis were performed by Proteobiologics Co. Ltd. (Osaka, Japan), as previously described (Takimoto et al. 2023). Briefly, protein extraction, digestion, and purification were performed using a phase-transfer protocol (Masuda et al. 2008). Purified peptides derived from digested proteins were analyzed via LC–MS/MS using the UltiMate 3000 Nano LC system (Thermo Scientific, Bremen, Germany) and an HTC-PAL autosampler

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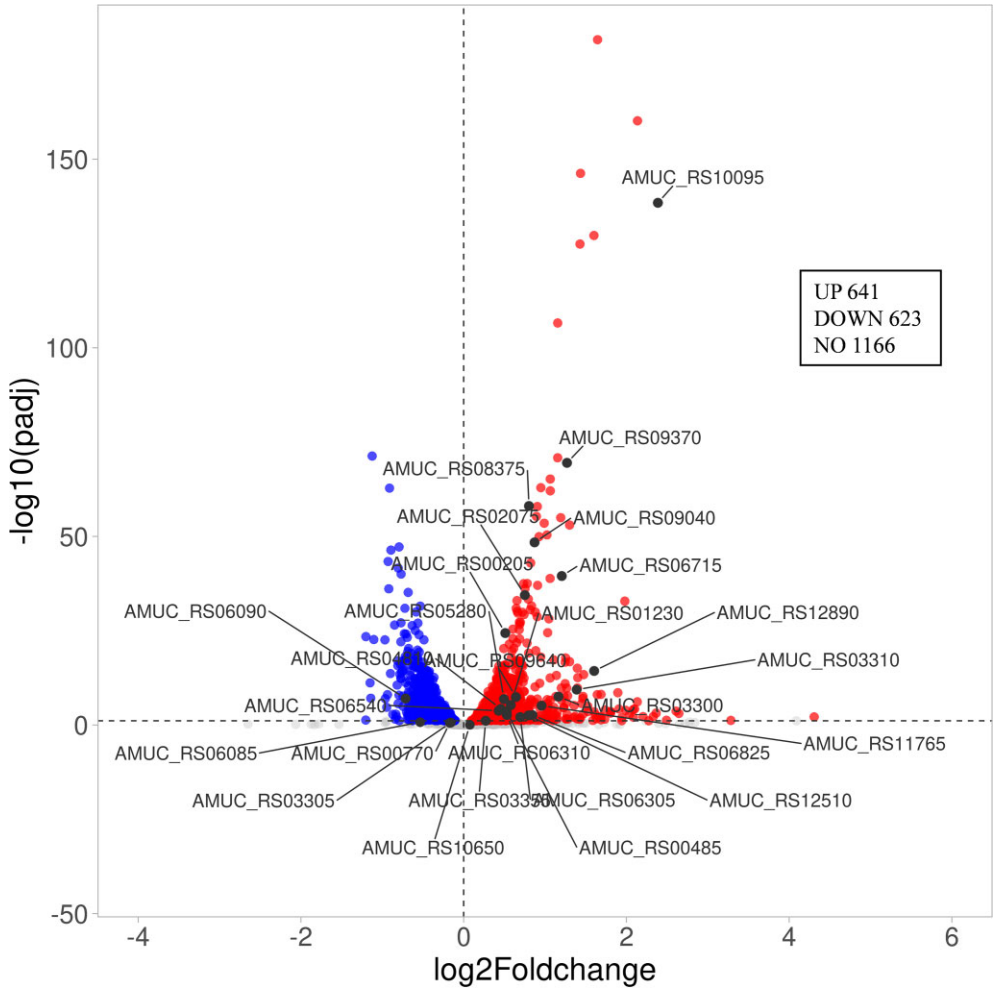

Figure 1. Volcano plot: differential expression of genes in the presence of deoxycholate (DCA). Differences obtained at the adjusted *P*-value (padj) < 0.05 level ($n = 3$) were considered significant. The volcano plot was generated using VolcaNoseR. Black plots with locus tag means the upregulated proteins with locus tag in the presence of DCA from proteome analysis (Supplementary Table S1).

(CTC Analytics, Zwingen, Switzerland) equipped with a trap column (0.075 × 20 mm; Acclaim PepMap RSLC Nano-Trap Column, Thermo Fisher Scientific). Subsequently, the peptides were ionized using nanoelectrospray ionization in the positive ion mode and a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific). The Q-Exactive instrument was operated in the data-independent acquisition mode (DIA). Raw MS data were processed using DIA-NN software (Ver. 1.8). Database searching included all entries from the *A. muciniphila* (UniProt Taxon ID: 239935) UniProt database and contaminant database (Tyanova et al. 2016). Statistical analysis via two-tailed Welch's *t*-test was conducted using Perseus ver. 1.6.14.0 based on the label-free quantification intensities of the two groups (DCA treatment and control groups).

GABA production

GABA concentrations in the bacterial supernatants were analyzed using an amino acid analyzer (L-8900; Hitachi Ltd., Tokyo, Japan), as previously described (Hagi et al. 2019). Statistical analysis of the GABA concentration data was performed using Welch's *t*-test.

Statistical analysis

The results are presented as the mean ± standard deviation (SD) from three independent experiments. Statistical analysis for transcriptome was carried out using DESeq2. An adjusted *P*-value (padj) < 0.05 level was considered significant. For proteome and GABA production analysis, Welch's *t*-test was performed to determine the statistical significance. A *P*-value < 0.05 was considered statistically significant.

Result and discussion

Upregulated genes and proteins in the presence of DCA

In the presence of DCA, transcriptome analysis showed that 64 genes were upregulated and 623 were downregulated in *A. muciniphila* (Fig. 1). Black plots show the upregulated proteins with locus tag in the presence of DCA. Most of the upregulated proteins were the same results with transcriptome analysis (22 of 27 proteins, Supplementary Table S1). There may be a probability of a statistically significant outcome to be a false positive in proteome analysis because of Welch's *t*-test.

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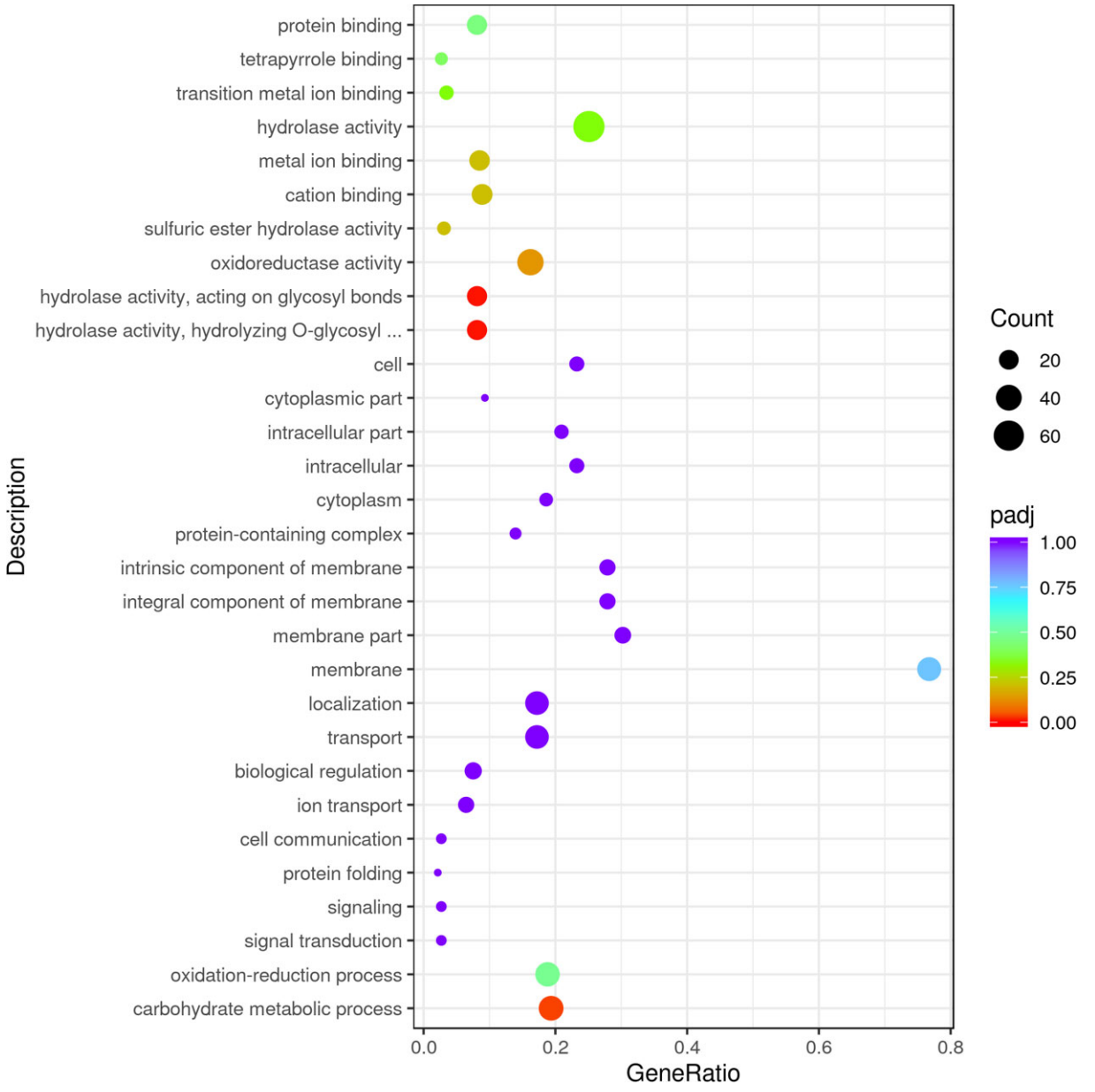

Figure 2. Upregulated genes in the presence of deoxycholate (DCA): Gene Ontology (GO) annotations of genes to biological processes, molecular functions, and cellular components. Based on GO analysis, upregulated genes were annotated to biological processes, molecular functions, or cellular components in a directed acyclic graph structure using clusterProfiler.

GO annotation of genes to biological processes, molecular functions, and cellular functions using clusterProfiler is shown in Fig. 2. Of the 30 modules, 3 (carbohydrate metabolic process [GO:0 005 975], hydrolase activity [GO:0 004 553], and hydrolyzing O-glycosyl compounds and hydrolase activity, acting on glycosyl bonds [GO:0 016 798]) were identified with significant associations with carbohydrate metabolism. In *Ligilactobacillus salivarius*, carbohydrate metabolism was altered by bile acids (Wang et al. 2020). Another paper indicated that conjugated bile acid exposure could activate the growth and glycolysis of *Bifidobacterium* spp. (Tian et al. 2020). The enhanced growth of *A. muciniphila* caused by DCA is attributed to a change in carbohydrate metabolism, similar to *Bifidobacterium* spp. Figure 2 also showed the potential change in oxidation-reduction process [GO:0 055 114]. Previous study showed that aerobic stress enhanced the growth of *A. muciniphila* at the end of the exponential phase and that amino acid permease (AMUC_RS00210) may be one of the factors driving enhanced growth (Ouwerkerk et al. 2016). Changes in nutritional metabolism (carbohydrate metabolism and amino acid permease upregulation) in the presence of DCA might be a key aspect of *A. muciniphila* that helps facilitate adaptation in the gut.

To obtain a better understanding of the upregulated genes associated with the metabolism of *A. muciniphila* in transcriptome analysis, the top 25 genes that were highly expressed (FPKM $\geq$ 500) in the presence of DCA were determined (Supplementary Table S2). The expression levels of three chaperones (co-chaperone GroES: AMUC_RS07515, molecular chaperone DnaK: AMUC_RS07510, and chaperonin GroEL: AMUC_RS07520) were also higher in the DCA treatment group. Furthermore, genes encoding galactose mu-

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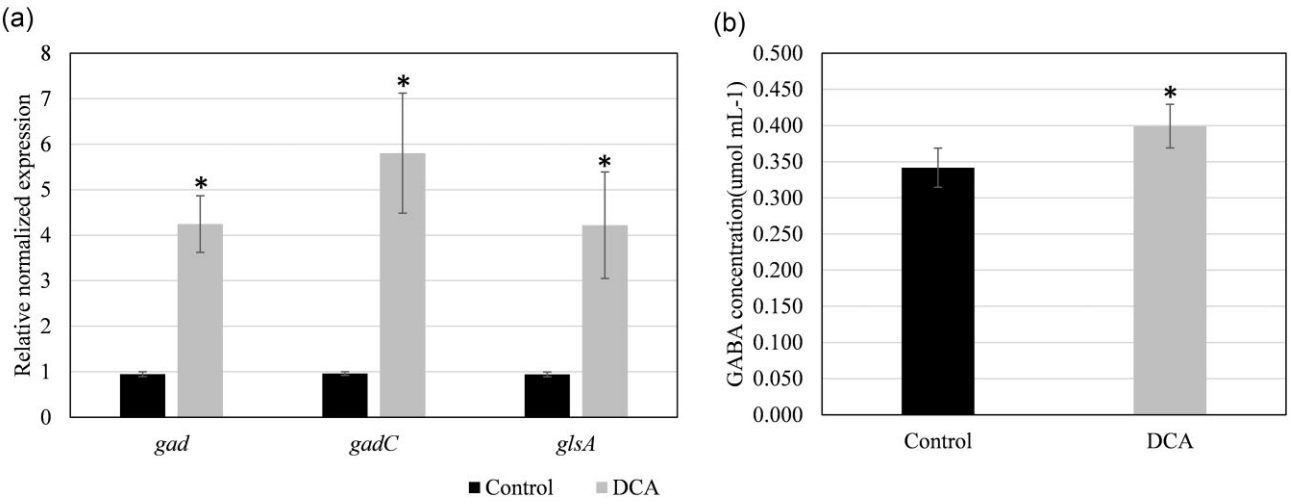

Figure 3. Effects of DCA on GABA synthesis in *A. muciniphila*. (a) Effect of DCA on the gene expression level associated with GABA synthesis. (b) GABA concentrations in the cultures with or without DCA. Culture conditions with and without DCA are indicated by black and grey bars, respectively. Culture condition without DCA was used for control for relative gene expression levels determined by qRT-PCR. Target genes are follows: glutamate decarboxylase (*gad*: AMUC_RS02075), amino acid permease (*gadC*: AMUC_RS00210), and glutaminase (*glsA*: AMUC_RS00215). Results are averages of three independent experiments. Error bars indicate standard errors. $*P < 0.05$ (Welch's *t*-test).

tarotase and glycosyl hydrolases family 2, which were significantly associated with carbohydrate metabolism and hydrolase activity in GO annotations, were highly expressed in the presence of DCA. In addition, two genes associated with γ-aminobutyric acid (GABA) production (amino acid permease: AMUC_RS00210, glutamate decarboxylase: AMUC_RS02075:) were upregulated. The stress response chaperones encoding GroES, DnaK, and OsmC were upregulated in the presence of DCA. DCA can cause membrane damage because it is an ionic detergent that can solubilize membrane proteins. Membrane damage also induces DNA and protein damage as well as acid and oxidative stress (Begley et al. 2005). To ameliorate protein damage, DNA repair, and oxidative stress, chaperones described above might be upregulated, respectively.

Our previous study showed that ABC (AMUC_RS07345, AMUC_RS07350, MUC_RS07355, and AMUC_RS07360) and RND-type (AMUC_RS10890, AMUC_RS10895, and AMUC_RS10900) transporters were required for bile acid resistance (Hagi et al. 2020). The present study showed efflux transporter, RND family protein (AMUC_RS04810) was upregulated in the presence of DCA. Thus, efflux transporter-RND family as well as chaperones might play an important role in DCA stress resistance. DNA methyltransferase is also required for DCA stress response because DNA methyltransferase contributes to DNA repair control.

To validate transcriptome analysis and obtain a better understanding of the effect of DCA on the metabolic changes of *A. muciniphila*, proteomic analysis was performed. In total, 787 proteins were detected. Supplementary Table S3 shows the proteins that were significantly upregulated in the presence of DCA [log2 fold change of > 0.27 (>1.2-fold)]. Carbohydrates metabolism such as glycosyltransferases and hydrolases, oxoglutarate dehydrogenase (AMUC_RS09040), and glutamate decarboxylase (AMUC_RS02075) were upregulated in the presence of DCA, consistent with the results of transcriptome analysis (Supplementary Table S1).

Gene expression level of GABA synthesis and its production by *A. muciniphila* in the presence of DCA

The combination of transcriptome and proteome analyses showed that the GABA production pathway was significantly upregulated by DCA. To validate these results, qRT-PCR-targeted GABA synthesis (glutamate decarboxylase: AMUC_RS02075, amino acid permease: AMUC_RS00210) and glutaminase: AMUC_RS00215) was performed. All genes were highly upregulated (Fig. 3a). Amino acid analysis of the supernatants showed that the DCA treatment group had significantly higher levels of GABA than the control group (Fig. 3b).

Our study demonstrated that the expression of glutamate decarboxylase leading to GABA production in *A. muciniphila* was upregulated in the presence of DCA. Glutamate decarboxylase is the main enzyme in the GABA shunt, which is known to be involved in stress response such as low-pH (Pennacchietti et al. 2018). *Akkermansia muciniphila* contains GABA-related proteins such as glutamate decarboxylase, glutaminase, and amino acid permease (Konstanti et al. 2024). Bile acids can decrease the intracellular pH by membrane damage leading to cell death in Lactobacilli and Bifidobacteria (Kurdi et al. 2006). The decreased intracellular pH may be one of the reasons for the increased GABA production because low intracellular pH induces GABA production in *A. muciniphila* by enhancing the expression of GABA production-related genes (Konstanti et al. 2024). However, in contrast to cell death effect on Lactobacilli and Bifidobacteria by DCA, DCA can improve the growth of *A. muciniphila*. Another report indicated that metabolites flux contributes to fit DCA stress and improve GABA production in *B. fragilis* P207 by regulating global transcriptional response, including stress responses, transporters, and carbon metabolism (Fiebig et al. 2024). The induction of GABA production by DCA indicates a specific physiology for some gut bacteria, including *A. muciniphila*. GABA is synthesized from glutamate (Glu). During Glu synthesis, the expression of glutamate synthase

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(ferredoxin; AMUC_RS06715) was upregulated in the presence of DCA (Supplementary Table S3). The increased expression of glutamate synthase may also contribute to higher GABA production. Further studies, including intracellular pH and metabolome analysis should be required for understanding the effect of DCA on GABA production. Our study showed the limited *in vitro* study using a single bacterial strain. The investigation of GABA production and therapeutic effects after DCA administration in animal would be a helpful for the clarification of the relationship between *A. muciniphila*, GABA, and DCA in the gut.

GABA is a neurotransmitter, and a metabolite induced by stress response. There are numerous GABA-producing bacteria in the gut, and GABA-producing *Bacteroides* is negatively correlated with depressive disorder (Strandwitz et al. 2019). A previous study showed that *A. muciniphila* may improve depressive-like symptoms by serotonin modulation (Guo et al. 2024). DCA might contribute to improve depressive-like disorder by promoting the growth of *A. muciniphila*, leading to higher GABA production as well as serotonin modulation. In addition, another study showed *Akkermansia* and *Parabacteroides* could synergistically increase hippocampal GABA/glutamate ratios leading to a prevention of seizures (Olson et al. 2018). *Parabacteroides* has an ability to convert primary bile acids into secondary bile acids (Wang et al. 2019), and here we demonstrated DCA regulates GABA production in *A. muciniphila*. Our study contributes to understand the bacterial interaction associated with GABA production via secondary bile acid in the human gut.

Conclusions

This study revealed the effect of DCA on gene/protein expression level in *A. muciniphila* by transcriptome and proteome analysis, and the change in a wide variety of metabolisms such as stress response and carbohydrates pathways was observed. Moreover, combination of transcriptome/transcriptome and metabolite analysis demonstrated that GABA synthesis is one of the metabolic changes in response to DCA. Our results suggested that DCA plays a role in the metabolic regulation, including GABA production of *A. muciniphila* in the human gut.

Author contributions

Tatsuro Hagi (Data curation [equal], Formal Analysis [equal], Funding acquisition [equal], Investigation [equal], Project administration [equal]), Masaru Nomura (Data curation [equal], Investigation [equal]), Clara Belzer (Supervision [equal], Writing – review & editing [equal])

Supplementary data

Supplementary data is available at *LAMBIO Journal* online.

Conflict of interest: None declared.

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Data availability

The transcriptome data in Table 1 has been deposited in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA1233894. The data underlying this article will be shared on reasonable request to the corresponding author.

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