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Author(s)	Gowda, Divyavani; Li, Yonghan; Gowda, Siddabasave Gowda B.; Ohno, Marumi; Chiba, Hitoshi; Hui, Shu-Ping
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1 **Determination of short-chain fatty acids by *N, N*-dimethylethylenediamine**
2 **derivatization combined with liquid-chromatography/mass spectrometry**
3 **and their implication in influenza virus infection**

4
5 Divyavani Gowda ^{1#}, Yonghan Li ^{2#}, Siddabasave Gowda B. Gowda ^{1,3}, Marumi Ohno ⁴,
6 Hitoshi Chiba ⁵, and Shu-Ping Hui^{1*}

7 1. Faculty of Health Sciences, Hokkaido University, Kita-12 Nishi-5, Kita-Ku,
8 Sapporo 060-0812, Japan

9 2. Graduate school of Health Science, Hokkaido University, Kita-12, Nishi-5, Kita-ku,
10 Sapporo 060-0812, Japan

11 3. Graduate School of Global Food Resources, Hokkaido University, Kita-12 Nishi-
12 5, Kita-Ku, Sapporo 060-0812, Japan

13 4. International Institute for Zoonosis Control, Hokkaido University, Kita 20, Nishi 10,
14 Kita-ku, Sapporo, 001-0020, Japan

15 5. Department of Nutrition, Sapporo University of Health Sciences, Nakanuma Nishi-
16 4-3-1-15, Higashi-Ku, Sapporo 007-0894, Japan

17
18 # Equally contributing author.

19 ***Correspondence:**

20 Prof. Shu-Ping Hui

21 Faculty of Health Sciences, Hokkaido University, Kita-12 Nishi-5, Kita-Ku, Sapporo 060-
22 0812, Japan ORCID: 0000-0001-9973-6461.

23 e-mail address: keino@hs.hokudai.ac.jp

24
25 **Keywords:** SCFAs, DMED derivatization, Mass spectrometry, Liquid chromatography,
26 colon and cecum contents, influenza infection,

27 **Abstract**

1 Short-chain fatty acids (SCFAs) are the end products of the fermentation of complex
2 carbohydrates by the gut microbiota. Although SCFAs are recognized as important
3 markers to elucidate the link between gut health and disease, it has been difficult to
4 analyze SCFAs with mass spectrometry technologies due to their poor ionization
5 efficiency and high volatility. Here, we present a novel and sensitive method for the
6 quantification of SCFAs, including C2–C6 SCFAs and their hydroxy derivatives, by liquid
7 chromatography/tandem mass spectrometry (LC-MS/MS) upon *N,N*-
8 dimethylethylenediamine (DMED)-derivatization with a run time of 10 min. Moreover, the
9 quantification method of DMED-derivatized SCFAs in intestinal contents using isotope-
10 labeled internal standards was also established. The method validation was performed
11 by analyzing spiked intestinal samples; the limits of detection and quantification of
12 SCFAs with this method were found to be 0.5 and 5 fmol, respectively; the recovery was
13 greater than 80% and good linearity (0.9932 to 0.9979) of calibration curves was
14 obtained over the range from 0.005 to 5000 pmol/μL; the intra-day and inter-day
15 precision were achieved in the range of 1–5%. Furthermore, the validated method was
16 applied to analyze SCFAs in the cecum and colon contents of mice infected with the
17 influenza virus. The results showed that the concentration of most of the SCFAs tested
18 here decreased significantly in a time-dependent manner after the infection, suggesting
19 a possibility that SCFAs in intestinal samples could be used as severe disease markers.
20 Overall, we here successfully developed a simple, fast, and sensitive method for SCFA
21 analysis by LC-MS/MS combined with DMED-derivatization. The method for the
22 quantification of SCFAs will be a useful tool for both basic research and clinical studies.

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28 **1. Introduction**

1 Gut microbial metabolites such as short-chain fatty acids (SCFAs) are produced by
2 the fermentation of dietary fiber and have important functions in the modification of
3 pathological and physiological processes [1]. SCFAs are highly volatile fatty acids that
4 consist of one to six carbons, with acetic, propionic, and butyric acids accounting for 90-
5 95% of the total [2]. SCFAs produced in the gut reach the circulation via the portal vein
6 and significantly alter the host energy metabolism not only in the gut but also in other
7 tissues [3]. In addition to energy metabolism, both *in vitro* and *in vivo* studies have
8 demonstrated the anti-inflammatory and anti-tumor properties of the SCFAs [4]. For
9 example, SCFAs inhibit lipopolysaccharide- and cytokine-induced inflammatory
10 responses via activation of G-protein coupled receptors (GPR41 and GPR43) and
11 inhibition of histone deacetylases [5]. SCFAs regulate innate immune cells, regulatory T
12 cells, and an increase in their biosynthesis are linked to preventing the progression of
13 type 2 diabetes and nonalcoholic fatty liver disease [6, 7]. Therefore, the analysis of
14 SCFAs is important for understanding their roles in complex biological systems.

15 Despite the importance of SCFAs in human health and disease, there is a limited
16 number of sensitive analytical methods are reported. SCFAs are often derivatized and
17 analyzed by conventional techniques such as gas chromatography-mass spectrometry
18 (GC-MS) [8, 9] and high-performance liquid chromatography-ultraviolet detection
19 (HPLC-UV) [10], nuclear magnetic resonance (NMR) [11], capillary electrophoresis [12],
20 and stable isotope tracer technique [13]. However, these techniques have problems such
21 as low sensitivity, the requirement of a large amount of sample, and time-consuming. On
22 the other hand, liquid chromatography-mass spectrometry (LC-MS) has considerable
23 advantages in terms of its high sensitivity, minimal sample requirement, and rapid
24 performance. However, direct analysis of SCFAs by LC-MS is difficult because of their
25 low mass range in mass spectra, insufficient ionization in electrospray ionization, poor
26 chromatographic separation, and the need for harsh experimental conditions such as a
27 highly acidic mobile phase. Hence, SCFAs are often quantitated by LC-MS by
28 derivatizing with various reagents such as 3-nitrophenylhydrazine [14, 15], O-

1 benzylhydroxylamine [16], ¹²C or ¹³C labeled aniline [17], and 4-acetamido-7-mercapto-
2 2,1,3-benzoxadiazole[18]. However, these methods are time-consuming or require drastic
3 conditions such as high temperature to derivatize SCFAs, which could result in
4 evaporation and degradation of SCFAs during the procedures. Recently, *N, N*-
5 dimethylethylenediamine (DMED) reagent is widely applied for the derivatization of fatty
6 acids and their analogs at the carboxylic acid moiety [19–21], which results in the
7 enhancement of detection sensitivity by several folds. In the present study, we aimed to
8 develop a simple and sensitive analytical method for the quantification of SCFAs and
9 their hydroxy derivatives (HSCFAs) in intestinal contents of mice using DMED as a
10 derivatizing agent under mild conditions. The derivatization reaction was optimized, and
11 the analytical validation was performed using standard solutions and a fecal matrix. The
12 method was successfully applied to determine the concentrations of SCFAs and HSCFAs
13 in the colon and cecum contents of mice, which further provide the lipid profile in a severe
14 influenza mouse model.

15 **2. Materials and Method**

16 **2.1 Materials**

17 Triethylamine (TEA), *N, N*-Dimethylethylenediamine (DMED), 3-hydroxybutyric acid
18 (3HC4), and acetic acid-d4 (C2-d4), were obtained from Sigma-Aldrich (St. Louis, MO,
19 USA). 2-chloro-1-methyl pyridinium iodide (CMPI), propionic acid (C3), butyric acid (C4),
20 valeric acid (C5), and hexanoic acid (C6) were purchased from Tokyo Chemical Industry
21 Co. LTD., (Tokyo, Japan). Acetic acid (C2) and 1M ammonium acetate solution were
22 obtained from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). LC-MS grade
23 methanol and acetonitrile were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).
24 3-hydroxypropionic acid (3HC3), 4-hydroxy pentanoic acid (4HC5), 3-hydroxyhexanoic
25 acid (3HC6), and pentanoic acid-d9 (C5-d9) were obtained from Cayman Chemical (Ann
26 Arbor, MI, USA). All the three reaction reagents (TEA, DMED, and CMPI) were dissolved
27 in the LC-MS grade acetonitrile at a concentration of 2 mM and stored in a –28°C freezer.

28 **2.2 Animal samples**

1 As reported previously [22], influenza virus A/Puerto Rico/8/34 (H1N1; PR8) was
2 obtained from the National Institute of Infectious Diseases, Japan. The virus was
3 propagated in 10-day-old embryonated chicken eggs at 35°C for 48 h, and aliquots of
4 collected allantoic fluids were stored at -80°C. Male C57BL/6 mice were purchased from
5 Hokudo (Sapporo, Japan) and were kept at a BSL-2 laboratory (temperature 22°C ± 2°C,
6 relative humidity 50% ± 10%) at the International Institute for Zoonosis Control, Hokkaido
7 University. The mice were fed with a standard CE-2 chow diet (CLEA, Sapporo, Japan)
8 with water *ad libitum*. Experiments were performed on 11–12 week-old mice. PR8 virus
9 at 500 plaque-forming units (PFU) in 50 µL of PBS or PBS only (control) were intranasally
10 inoculated into the mice under inhalation anaesthesia with isoflurane. On 1, 3, or 6 days
11 post-infection (dpi), the mice were euthanized, and their colon and cecum contents were
12 collected into a 1.5-mL Eppendorf tube (n=5 mice in each group). Each cecum and colon
13 contents were weighed and homogenized (30 sec × 2 cycles) in 10 volumes of
14 acetonitrile with 5–6 ceramic beads (1.4 mm, Cat. No. 15-340-159, Fisherbrand,
15 Pittsburgh, PA, USA), using a BeadMill 4 homogenizer (Fisherbrand). The homogenates
16 were stored at -80°C until further analysis.

17 **2.3 Extraction of SCFAs from intestinal contents**

18 For the extraction of SCFAs, 50 µL of sample homogenates (5 mg) in acetonitrile were
19 transferred into an Eppendorf tube (Hamburg, Germany) containing 50 µL of acetonitrile,
20 and 100 µL of 20 µM acetic acid-d4 [internal standard (IS)] in acetonitrile and 100 µL of
21 20 µM pentanoic acid-d9 (IS) in acetonitrile were added. The prepared mixture was then
22 vortexed vigorously for 3 min at 3,500 rpm at room temperature (RT) and centrifuged
23 (15,000 rpm, 10 min, 4°C), and the supernatant was transferred to a new tube as an
24 SCFA extract.

25 **2.4 Derivatization of SCFAs with DMED**

26 To optimize reaction conditions, the DMED-derivatization of authentic SCFAs (5 µM) in
27 the presence of TEA and CMPI was performed under different conditions: 20 µL of TEA,
28 10 µL of CMPI, and 20 µL of DMED (stock concentrations 0.5–4 mM each) were added

1 to 100 μ L of the standard and 200 μ L of the IS mixture and the mixtures were vortexed
2 for 15–60 min at 3,500 rpm at RT–60°C. After centrifugation (15,000 rpm, 10 min, 4°C),
3 200 μ L of the supernatant was transferred to an LC vial for LC-MS analysis as described
4 below. Based on the obtained peak areas, the optimal DMED-derivatization condition
5 was determined as follows; 2 mM for stock concentrations of DEMD, TEA, and CMPI,
6 RT for reaction temperature, and 30 min for reaction duration.

7 For the DMED-derivatization in the biological samples, 20 μ L of TEA (2 mM), 10 μ L of
8 CMPI (2 mM), 20 μ L of DMED (2 mM), and 200 μ L of the IS mixture were added to 100
9 μ L of the SCFA extract containing 5 mg of intestine contents as prepared above. The
10 reaction mixtures were vortexed for 30 min at 3,500 rpm at RT and centrifuged (15,000
11 rpm, 10 min, 4°C). The DMED-derivatized sample centrifugate (200 μ L) was transferred
12 to the LC vials.

13 **2.5 LC-MS analysis**

14 Targeted analysis of SCFAs and HSCFAs was performed on a Prominence UFLC
15 (Shimadzu, Kyoto, Japan) connected to TSQ Quantum Mass Spectrometer System
16 (Thermo Fisher Scientific, San Jose, CA, USA) operated in the positive-ion mode.
17 Chromatographic separation was achieved using a Hypersil gold C8 column (50 \times 2.1
18 mm, 5 μ m; Thermo Fisher Scientific) maintained at 40°C and mobile phases A: 20 mM
19 ammonium acetate, B: methanol and acetonitrile (1:1) with a flow rate of 0.4 mL/min.
20 Elution gradient as follows: 0–2 min (90% B), 3–5 min (50% B), 6–7 min (20% B). 7.5–
21 10 min (90% B). The sample injection volume was set to 5 μ L. The LC-MS/MS was
22 performed using Heated Electrospray Ionization (H-ESI) probe with the following
23 optimized parameters: The DEMD derivatized analytes were directly infused into the
24 triple quadrupole mass spectrometer and MS parameters were optimized for each
25 compound. The schematic representation of DMED derivatization of SCFAs and their
26 ionization pattern are shown in **Figure 1A**. The precursor ion, product ion, tube lens, and
27 collision energies for each of the analytes are described in **Table. 1**. The MS/MS spectra
28 of each of the SCFAs derivatized with DMED are shown in **Figure 1B**. The loss of 45 Da

1 was commonly observed for all the analytes in their MS/MS spectra. The spray voltage
2 was set to 3500 V, the capillary temperature was 250°C, sheath gas and auxiliary gas
3 were set to 40 and 25 arbitrary units, and the H-ESI vaporizer temperature was set to
4 150°C. Extracted ion chromatograms and MS spectra were obtained using Xcalibur 2.2
5 (Thermo Fisher Scientific). The peak area of the analytes was obtained using Xcalibur
6 2.2 (Thermo Fisher Scientific) software, and their absolute concentrations were
7 calculated from the standard calibration curve.

8 **2.6 Method validation**

9 The method validation was performed following the guidelines of previous reports [23,
10 24]. Stock solutions of each SCFA and HSCFA standard (100 mM) were prepared in
11 acetonitrile solution. The standard stock solutions were mixed and made into a
12 homogenous stock solution containing 1 mM each in acetonitrile. The IS solutions (C2-
13 d4 and C5-d4) were also prepared in acetonitrile at a concentration of 20 µM and kept
14 at -80°C. Three sets of samples containing SCFA and HSCFA standards at three
15 different concentrations: low (25 pmol each), medium (50 pmol each), and high (125
16 pmol each) were prepared for precision, recovery, and matrix effect validation.

17 Set A was prepared by spiking the analytes and IS mix solution in acetonitrile. Set B
18 was prepared by spiking the analytes and IS mix solution into the feces samples after
19 extraction (i.e., post-spiked samples). Set C was prepared by spiking the analytes and
20 IS mix solution into the feces samples before extraction (i.e., pre-spiked samples). Five
21 replicates were prepared for each set of samples. Recovery was calculated as the area
22 ratio of the post- to pre-spiked samples (i.e., $\text{recovery (\%)} = \text{set B}/\text{set C} \times 100$). The
23 matrix effect was calculated as the area ratio of the post-spiked samples to the
24 acetonitrile samples (i.e., $\text{matrix effect (\%)} = \text{set B}/\text{set A} \times 100$). Intra- and inter-day
25 precisions were calculated as the relative standard deviation (RSD) of the spiked analyte
26 and IS peak areas of the feces samples (i.e., $\text{RSD (\%)} = \text{standard deviation}/\text{mean} \times 100$).
27 The chemical stability of the analytes was determined from the pre-spiked samples. The
28 stability was calculated by comparing the spiked analyte and IS peak areas of freshly

1 extracted feces samples with those of samples after 1-week storage at 4 °C. Analyte
2 stabilities were found to be >80%.

3 **2.7 Statistics**

4 Statistical analyses were conducted using the GraphPad Prism 8.1 software (GraphPad
5 Software, San Diego, CA, USA). Student's t-tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,
6 ns: not significant) were used to detect statistically significant differences between the
7 groups.

9 **3. Results and Discussion**

10 Qualitative and quantitative measurements of SCFAs are important to understand
11 their roles in regulating host energy metabolism and immune responses in both
12 physiological and pathological processes. Until now, many methods have been used to
13 analyze SCFAs in the biological samples [8–18]. However, these techniques have
14 problems such as low sensitivity or drastic conditions such as derivatization at high
15 temperatures. Since SCFAs are highly volatile compared to long-chain fatty acids, a
16 novel analyzing method with a very mild condition needs to be established for accurate
17 analyses of SCFAs. DMED, a low molecular weight reagent, is known to react with a
18 carboxylic acid moiety under mild conditions to form amide which enables efficient mass
19 ionization of fatty acids. Past studies by Feng Y-Q *et al.* demonstrated several
20 applications of the DMED reagent for the quantitative analyses of various bioactive
21 molecules [19, 21, 25, 26]. In this study, SCFAs and their hydroxy derivatives were
22 derivatized with DMED under various conditions in terms of reaction temperature (RT to
23 60°C) and concentrations of DMED, CMPI, and TEA (0.5 to 4 mM) to find the optimal
24 condition to obtain DMED-SCFAs with maximum yield (**Figure 2**).

25 We first investigated the impact of reaction temperature from RT to 60°C on the
26 derivatization of SCFAs and found that at the ambient temperature, the SCFAs are
27 derivatized with DMED efficiently. Optimal concentrations of DMED, CMPI, and TEA for
28 the derivatization were examined in the range of 0.5–4 mM of each reagent, and it was

1 found that the reaction with all agents at 2 mM resulted in better intensity and peaks in
2 the LC-MS. On the other hand, when the concentration of the derivatizing agents
3 exceeded 2 mM, the peak of the analyte became unstable, and the signal intensity
4 detected by the LC-MS system decreased. After optimization for reaction temperature
5 and concentrations of reagents, we also checked the derivatization time from 15 to 60
6 min and determined 30 min for derivatization time based on obtained intensities of
7 DMED-SCFAs in LC-MS. Therefore, the derivatization of SCFAs and their hydroxy
8 derivatives with DMED, CMPI, and TEA at concentrations of 2 mM each for 30 min at RT
9 was selected as the optimized condition for further experiments.

10 After the optimization of DEMD derivatization conditions and single reaction
11 monitoring channels for each SCFA and HSCFA, the analytical validation was performed
12 using mouse feces as a matrix. For chromatographic separations we have tested Shim-
13 Pac Scepter C18 (100 × 2.1 mm, 5 μm; Shimadzu), Atlantis T3 C18 (2.1×150 mm, 3 μm,
14 Waters, Milford, MA, USA), and Hypersil gold C8 column (50 × 2.1 mm, 5 μm; Thermo
15 Fisher Scientific) columns and found the best peak shapes in the later one. Gradient
16 including isopropanol, methanol, acetonitrile, and milli-Q were used, but significantly
17 improved peak shapes were detected with 20 mM ammonium acetate and methanol:
18 acetonitrile (1:1). However, the study limits the peak resolution of SCFAs and very few
19 molecular species of great importance are targeted. The linearity for each standard was
20 examined for a concentration range of 0.005 to 5000 pmol/μL. Linear regression
21 correlation coefficients were greater than 0.99. The calibration curves were obtained
22 from the area ratio of analytes to the IS vs concentration, and the calibration curves of
23 C2 (IS C2-d4) and C5 (IS C5-d9) were used for the calculation of concentrations for
24 analytes with the C2–C3 and C4–C6 carbon chain, respectively. The limit of detection
25 (LOD) in an S/N above 3; the limit of quantitation (LOQ) in an S/N above 10 were
26 determined. The LOD and LOQ for SCFAs are 0.5 and 5 fmol, respectively. These results
27 suggest the method is comparatively highly sensitive to the methods previously reported
28 (LOD/LOQ: 0.1 to 5 pg/ 0.1 -10 pg and 1 to 7 pg/ 3-19 pg respectively) [9, 14]. However,

1 less sensitive compared to the methods using isotopic labeling coupled LC-MS (LOD:
2 0.005-0.4 pg and LOQ: 0.015-0.8 pg) and O-benzylhydroxylamine derivatization (LOQ:
3 0.5-1 fmol) analysis of SCFAs [16, 27]. There is a possibility that the use of isotopically
4 labeled DMED reagent could improve the sensitivity by several folds.

5 The recovery, matrix effect, and intra- and inter-day reproducibility were evaluated
6 at three different concentrations of each SCFA and HSCFA, and the results are
7 summarized in **Table 2**. The recovery for most of the standards was greater than 80%.
8 The matrix effect determination showed ion suppression (<100%) for most of the species,
9 except for C6, 4HC5, and 3HC6 with ion enhancement (>100%). The extracted ion
10 chromatograms of authentic standards and those detected in samples are shown in
11 **Figure 3**. The elution was completed within a period of 2.5 min. The validated method
12 was then applied for the quantitative analysis of SCFAs and HSCFAs in intestinal
13 samples from mice.

14 Although the microbiota-derived SCFAs affect host biological processes in the gut,
15 recent studies have indicated diverse roles of SCFAs in other tissues in regulating
16 inflammatory responses which are associated with the progression of type 2 diabetes
17 and nonalcoholic fatty liver disease [6, 7]. Furthermore, viral infections in respiratory
18 tissues have been demonstrated to disturb lipid metabolism in gastrointestinal tracts.
19 Particularly, past studies have demonstrated that the influenza virus infection leads to
20 the reduction of SCFAs in intestinal tissues, which results in the impairment of the gut
21 barrier properties to increase susceptibility to bacterial infections [28, 29]. These findings
22 clearly suggest a potential link between host health and SCFAs derived from gut-
23 microbiota. Actually, many studies have reported that SCFAs protect against viral
24 infections by influencing directly the growth of pathogens or indirectly via the host
25 immune system as summarized in a review article [30].

26 To understand the kinetic changes in SCFA levels in the intestine during the time-
27 course of severe influenza, in this study, we quantitatively analyzed concentrations of
28 SCFAs in intestinal contents of mice infected with the PR8 virus at 500 PFU as already

1 reported [22]. At 1, 3, or 6 dpi, mice were sacrificed, and their colon and cecum contents
2 were collected. The results of analyses of SCFA detected in the cecum and colon
3 contents are shown in **Figures 4** and **5**, respectively. In the cecum contents, the
4 concentrations of C2 and C3 fatty acids were not changed significantly after infection,
5 however, C4, C6, and 3HC3 fatty acids were significantly reduced at 3 and 6 dpi in mice
6 infected with the PR8 virus. The C5 SCFA and 3HC4 showed a slight but significant
7 increase at 1 and/or 3 dpi, but not 6dpi. In colon contents, at 1 dpi, no significant changes
8 were observed between the control and PR8 groups. However, at 3 dpi most of the
9 measured SCFAs (C2, C3, C4, C5, 3HC4, and 3HC6) were decreased significantly in
10 PR8 virus-infected mice. At 6 dpi C2, C4, and 3HC6 were significantly decreased in the
11 PR8 group compared to controls whereas the 3HC4 concentration was elevated. These
12 changes suggest the virus infection in the respiratory tissue has a suppressive effect on
13 the lipid metabolism by gut microorganisms producing SCFAs. Moreover, these results
14 are similar to those in previous studies representing the reduction of cecal concentrations
15 of SCFAs (C2, acetate; C3, propionate; and C4, butyrate) after infection with an influenza
16 virus, although acetate and propionate did not decrease in this study [28, 29]. This
17 discrepancy may have been attributed to differences in experimental conditions, such as
18 virus strains, infection titers, and the timing of sample collection. On the other hand, both
19 our results and theirs highlighted the reduction of butyrate in the cecum of mice after day
20 3 of infection with influenza viruses. Therefore, decreased butyrate could be used as a
21 biomarker for severe influenza as already suggested by a previous study representing a
22 reverse correlation between the abundance of butyrate-producing microbiota in the gut
23 and respiratory viral infection risk in humans [31]. In addition, given the capability of
24 butyrate of promoting differentiation of regulatory T cells [32], a potential role of butyrate
25 in the pathogenesis of or protection against influenza also should be noted. The
26 decreased SCFAs in the colon content could be attributed to the increased
27 transformation into lipids such as short-chain fatty acid esters of hydroxy fatty acids
28 (SFAHFAs) since SFAHFA levels are significantly elevated in the colon contents after

1 influenza virus infection [33]. Interestingly, 3HC4 increased both in the cecum and colon,
2 which may contribute to the increase of 3HC4 in the circulation, leading to ketoacidosis.
3 However, the biological significance of and the mechanisms behind the influenza-
4 associated changes observed here need to be further investigated. Further, this study
5 limits the information on the interference of isomeric SCFAs, especially when analyzing
6 colon and cecum contents.

7 We here established the quantitative analyzing method of SCFAs using LC/MS,
8 following the derivatization of SCFAs with DMED under a mild condition so that SCFAs
9 can be efficiently ionized in the positive mode. Method validation was performed with
10 mouse feces as a matrix, and the method was confirmed to have good linearity, LOD,
11 LOQ, measuring range, and repeatability. Further, this established method was applied
12 to analyze the kinetic SCFA profiles in colon and cecum contents of mice during severe
13 influenza. Significant decreases in concentrations of SCFAs in the cecum and colon
14 contents were observed in infected mice, suggesting a possibility that the reduction of
15 SCFAs, particularly butyrate, can be used as a biomarker of respiratory viral infections.
16 In conclusion, the SCFA analyzing method developed in this study is simple, rapid, and
17 sensitive, and will be a powerful tool for measuring the concentrations of SCFAs in
18 biological samples, whose function has been increasingly attracting attention concerning
19 human health and disease.

21 **Conflicts of interest:**

22 The authors declare that they have no known competing financial interests or personal
23 relationships that could have appeared to influence the work reported in this paper.

24 **Ethical approval:**

25 All the animal protocols were approved by the Animal Care and Use Committee of
26 Hokkaido University (approval no. 17-0003).

28 **Author contribution:**

1 DG, SGBG: Conceptualization, Methodology, Supervision. YL, DG, SGBG: Formal
2 analysis, Visualization, Writing - original draft. MO, YL: Data curation, Writing - review &
3 editing. HC, S-PH: Supervision, Resources, Writing - review & editing.

4

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19 **TABLES**

20 **Table 1.** Optimized analytes MS setting parameters. CE: collision energy.

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Analyte	Q1	Q3	CE (V)	Tube lens (V)
Acetic acid (C2)	131.1	86.3	12	45
Propionic acid (C3)	145.1	100.2	13	48
Butyric acid (C4)	159.1	114.2	11	52
Valeric acid (C5)	173.2	128.2	12	53
Hexanoic acid (C6)	187.2	142.2	11	51
3-hydroxypropionic acid (3HC3)	161.1	116.2	11	47
3-hydroxybutyric acid (3HC4)	175.1	130.2	9	46
4-hydroxypentanoic acid (4HC5)	189.1	144.1	14	54
3-hydroxyhexanoic acid (3HC6)	203.2	158.1	9	50
d4-Acetic acid (C2-d4)	134.1	89.3	12	50
d9-Valeric Acid (C5-d9)	187.2	142.2	11	51

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1 **Table 2.** Validation of the method at low (25 pmol), medium (50 pmol), and high (125
 2 pmol) concentrations of spiked standards to feces matrix. The data are shown as mean
 3 \pm SD, n=5. RSD: Relative standard deviation.

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SCFAs	Recovery (%)			Matrix effect (%)			Intraday RSD (%)			Interday RSD (%)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
C2	90 \pm 3	97 \pm 2	90 \pm 4	84 \pm 8	71 \pm 8	81 \pm 5	4	3	3	5	2	4
C3	93 \pm 4	102 \pm 7	89 \pm 3	90 \pm 6	80 \pm 7	86 \pm 2	3	4	4	2	5	2
C4	96 \pm 2	94 \pm 2	88 \pm 1	89 \pm 3	90 \pm 12	97 \pm 7	2	1	2	2	4	2
C5	94 \pm 3	93 \pm 5	88 \pm 2	99 \pm 6	94 \pm 2	101 \pm 6	2	1	3	2	3	2
C6	93 \pm 4	95 \pm 5	87 \pm 3	105 \pm 10	100 \pm 2	108 \pm 8	3	2	2	2	4	2
3HC3	89 \pm 3	85 \pm 1	86 \pm 2	75 \pm 4	82 \pm 5	81 \pm 3	2	1	4	4	1	2
3HC4	91 \pm 6	87 \pm 3	87 \pm 3	90 \pm 6	92 \pm 3	94 \pm 2	6	2	4	2	2	2
4HC5	91 \pm 2	71 \pm 2	81 \pm 1	104 \pm 3	121 \pm 2	104 \pm 3	3	2	3	3	3	3
3HC6	91 \pm 3	87 \pm 3	87 \pm 1	102 \pm 5	105 \pm 4	104 \pm 5	3	2	3	2	3	1
C2-d4	89 \pm 4	101 \pm 7	87 \pm 3	78 \pm 5	70 \pm 6	79 \pm 2	3	2	4	4	2	3
C5-d9	94 \pm 2	94 \pm 4	88 \pm 2	96 \pm 5	87 \pm 3	99 \pm 8	2	1	2	1	4	3

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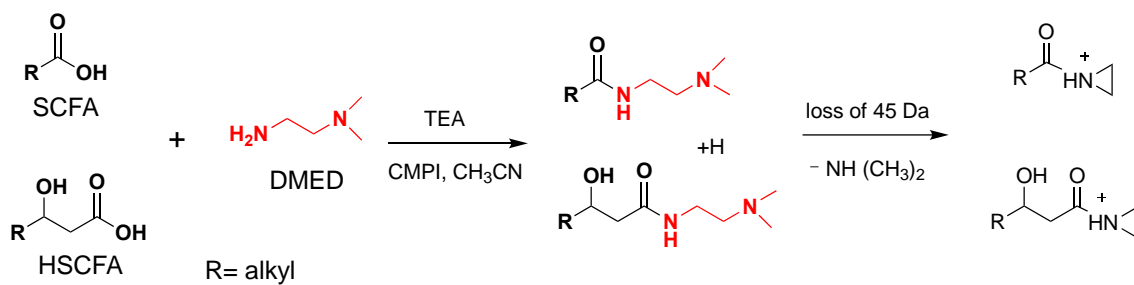
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1 **Figure 1. A.** DMED derivatization and ionization patterns of SCFAs and HSCFAs. **B.** The
 2 MS/MS spectra of DMED-derivatized SCFAs and HSCFAs. DMED: *N, N*-
 3 dimethylethylenediamine, TEA: triethylamine, CMPI: 2-chloro-1-methyl pyridinium iodide.

4 **A.**



6 **B.**

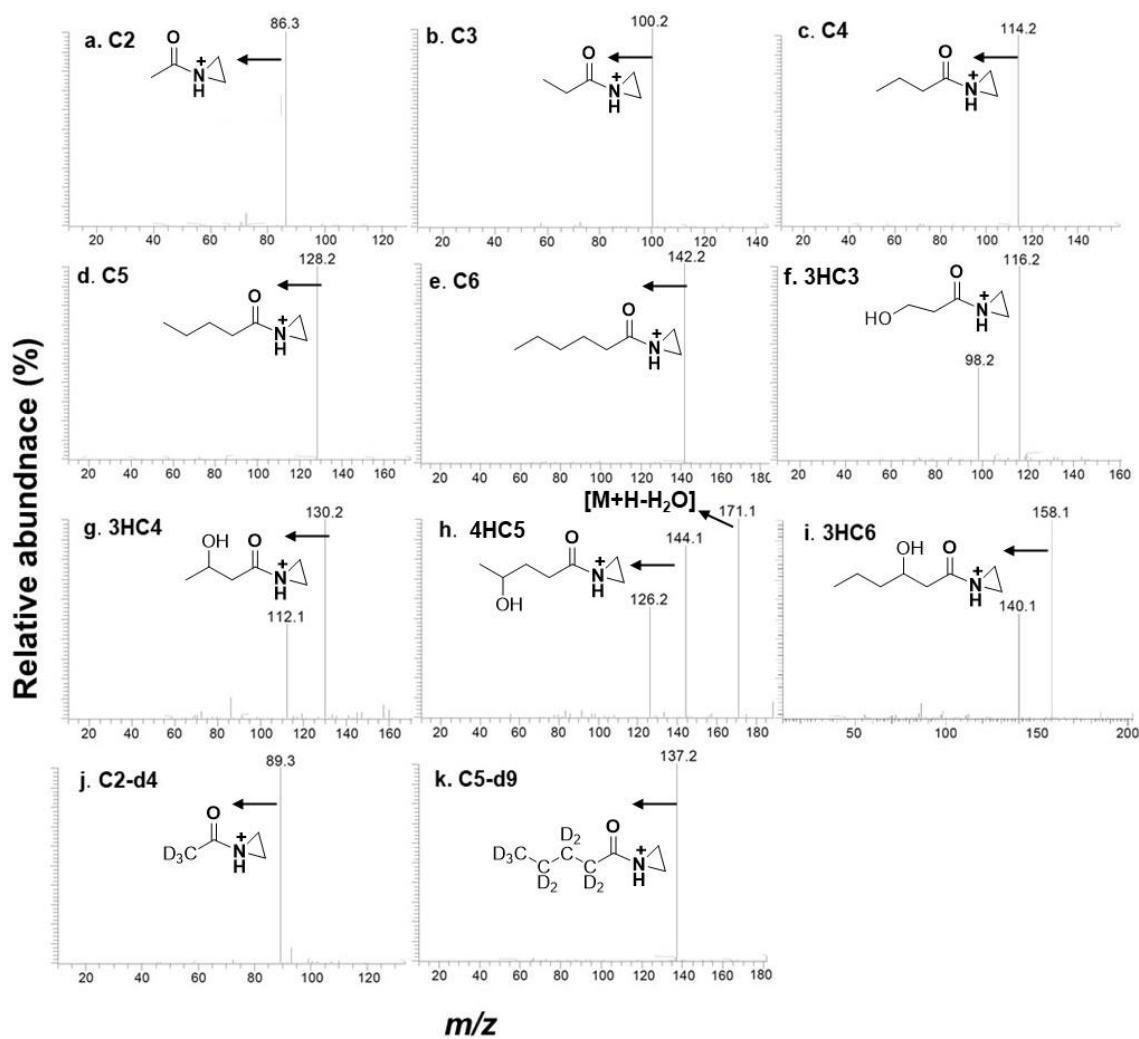
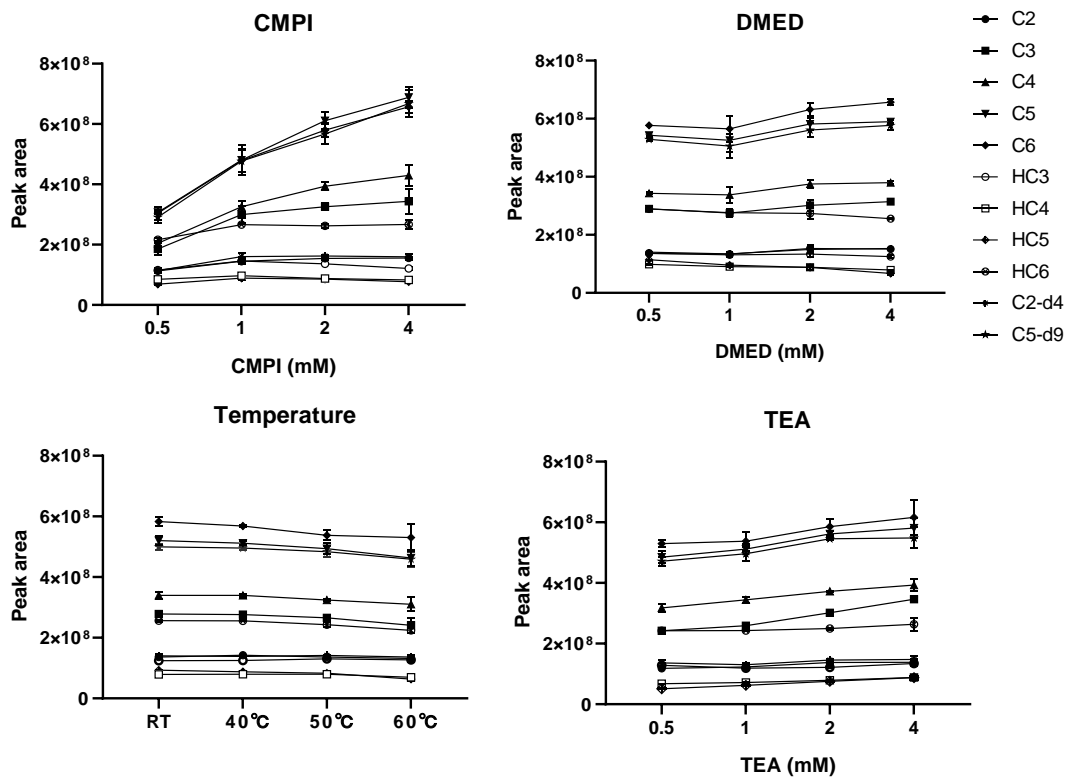
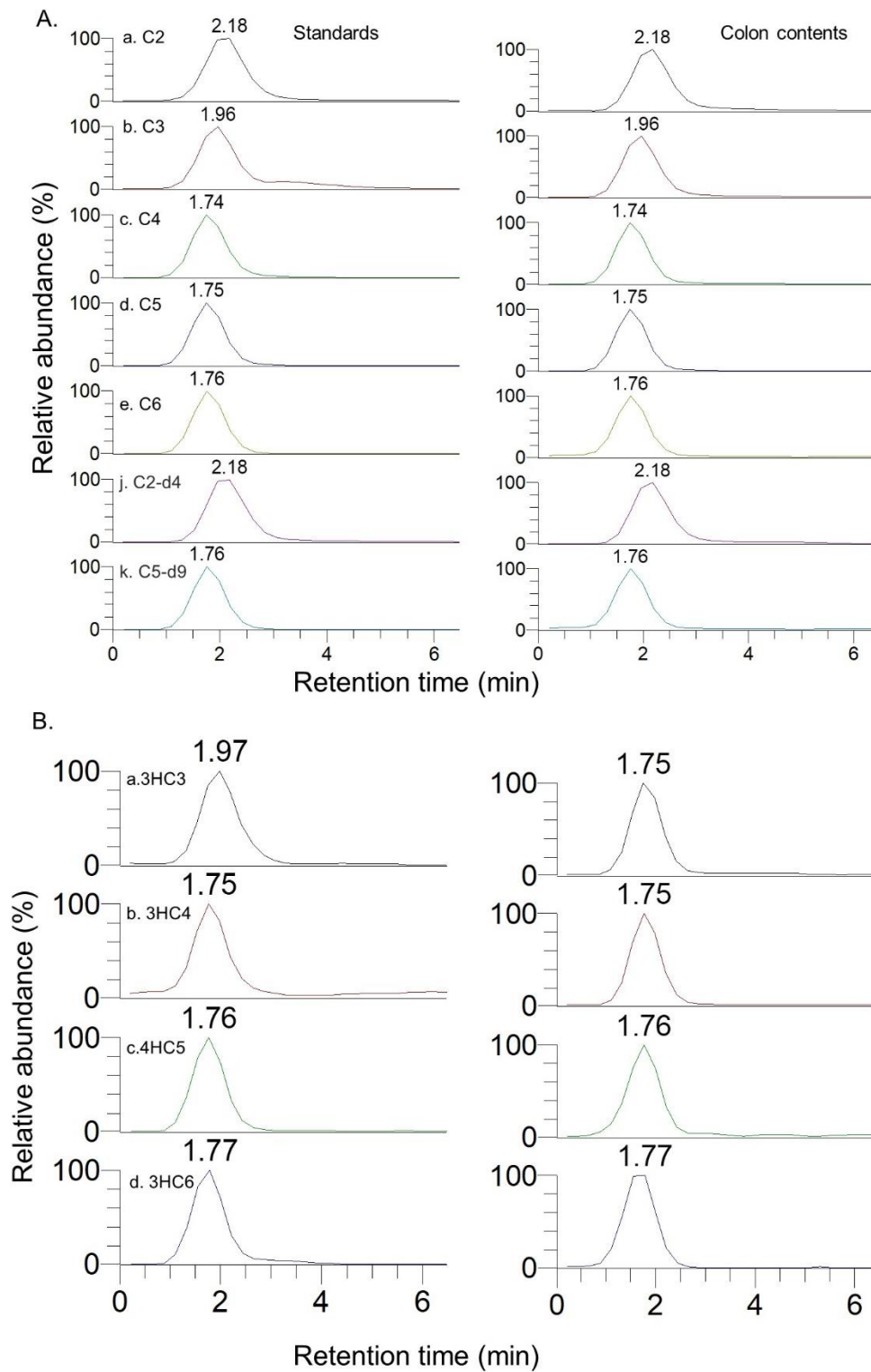


Figure 2. Optimization of DMED-derivatization conditions for SCFAs and HSCFAs at various concentrations of CMPI, DMED, TEA and different temperatures



1 **Figure 3.** Extracted ion chromatograms of SCFAs (A) and HSCFAs (B)- standards vs
2 Samples



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Figure 4. Concentrations of SCFAs and HSCFAs detected in the cecum contents of mice (n=5) infected with the influenza virus (PR8). Values are represented by box-and-whisker plots as follows: the central line in the box is the median, the bottom and top lines of the box are the first and third quartiles, respectively, and whiskers are the minimum to maximum values. Student's t-test, Control vs. PR8 at each time point, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. dpi, day-post-infection.

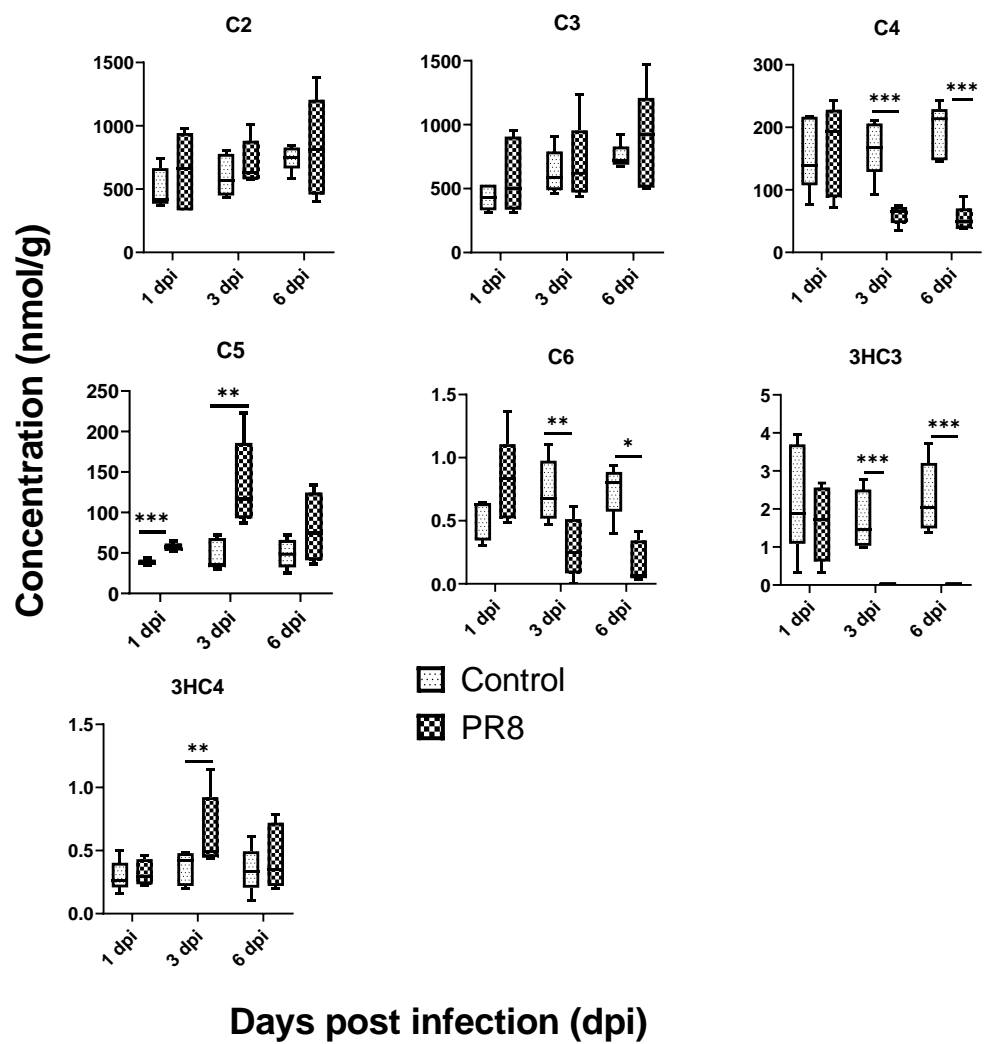
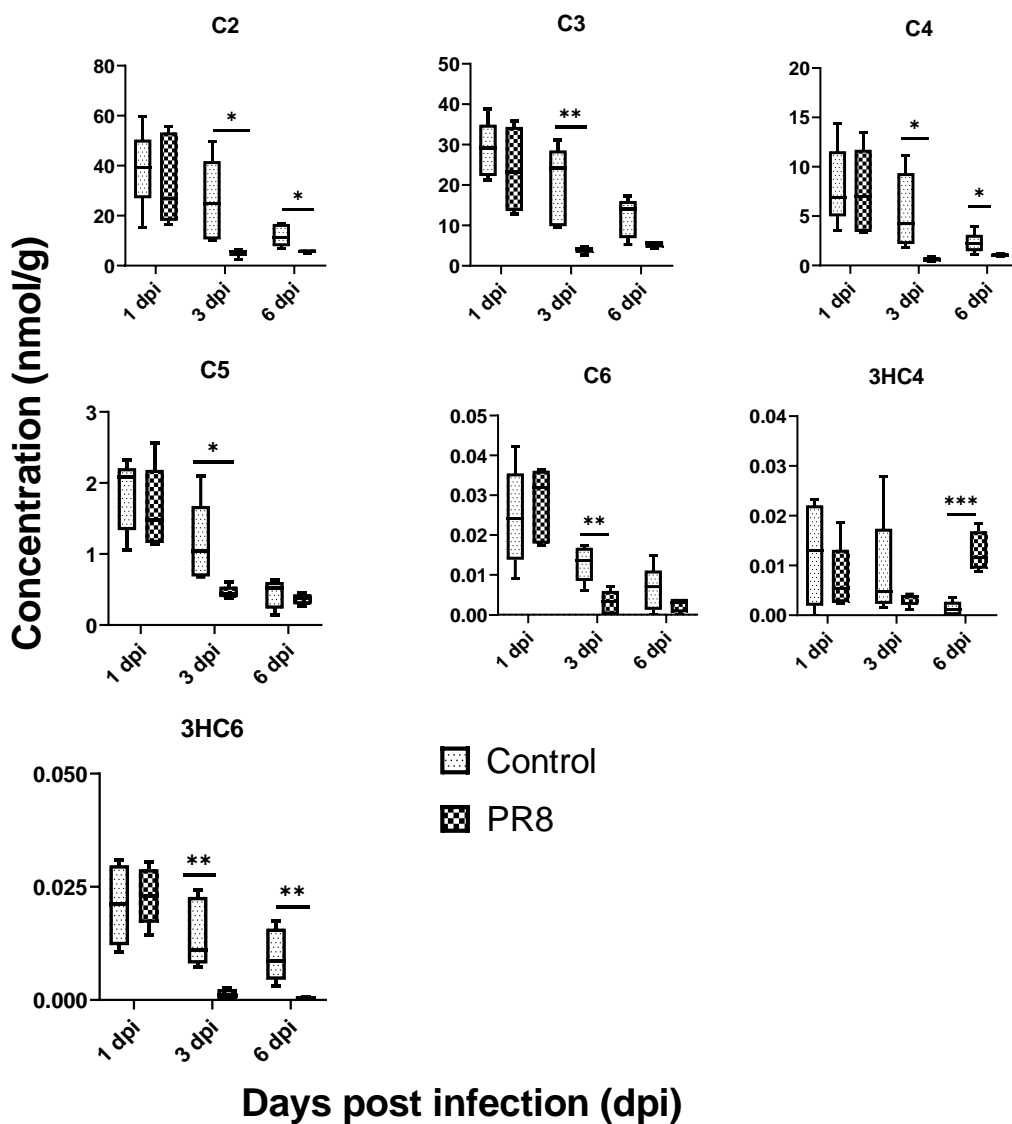
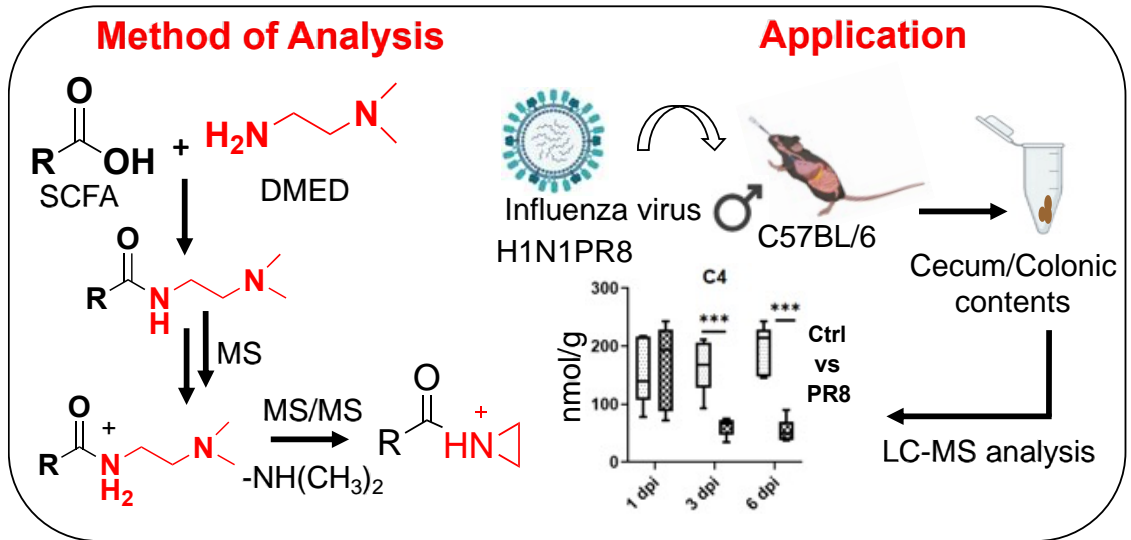


Figure 5. Concentrations of SCFAs and HSCFAs detected in the colon contents of mice (n=5) infected with the influenza virus (PR8). Values are represented by box-and-whisker plots as follows: the central line in the box is the median, the bottom and top lines of the box are the first and third quartiles, respectively, and whiskers are the minimum to maximum values. Students t-test, Control vs. PR8 at each time point, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. dpi, day-post-infection.



1

2 Graphical abstract



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