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Metabolite-Based Functional Study of the Bacterium *Butyricicoccus pullicaecorum* Using Extracellular Fermentation Products

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Annotation: In the study, the metabolic activity of Butyricicoccus pullicaecorum was characterized solely through examining extracellular metabolites. The anaerobic base of the bacteria was 72 hours of incubation in a carbohydrate medium, followed by metabolite quantitation at 24, 48, and 72 hours via HPLC and GC–MS. The metabolite composition at 24 hours was dominated by acetate and lactate at 18.4 ± 0.7 mM and 6.1 ± 0.3 mM, respectively, but butyrate was low at 4.8 ± 0.2 mM. By the 48 hours mark, butyrate had climbed to 14.6 ± 0.5 mM and there was a corresponding decrease of lactate to 3.2 ± 0.2 mM, indicative of metabolic conversion. Butyrate reached its highest concentration at the 72 hours mark (26.9 ± 0.9 mM), which was 5.6 times the baseline, and comprised 62% of the total SCFAs. GC–MS was, isolated isobutyrate (1.9 to 4.7 mM), and the 2-methylbutyrate (which was undetectable at 24h, but at 72 had 2.2 mM). Also, the accumulation of the aromatics, specifically 0.8 ± 0.1 mM of the 24h associated phenylacetate to 3.5 ± 0.3 mM of the 72h, and 4-hydroxyphenylacetate which rose from 0.6 to 2.8 mM. Two previously uncharacterized fermentation intermediates, with m/z fragment values of 157 and 171,

appeared with 11.2% and 8.4% relative abundance values, respectfully, and were often unaccounted for after the 48-hour mark. From the 24-hour mar, the total number of metabolites produced (32.1 mM) rose significantly at the 72-hour mark (61.4 mM). Data across the timeline affirms the claim that B. pullicaecorum can be entirely characterized with the usage of metabolite only and highlights the butyrogenic capabilities and aromatic fermentation profiles.

Keywords: Butyricicoccus pullicaecorum; bacterial metabolomics; extracellular metabolites.

Introduction

The gut microbiota was recognized as one of the most important metabolic organs capable of producing a variety of small molecules such as SCFAs and a multitude of fermentation products that modulate the physiology of the host (1-4). SCFAs such as acetate, propionate, and butyrate are capable of regulating epithelial permeability, modulating the immune response, and even gut–brain signaling via receptors and epigenetic mechanisms (2). Person and Oedewald have reviewed the SCFA-producing bacteria and noted that the butyrate-producing anaerobes in the colon are represented by a small number of phylogenetic taxa and that the loss of members of such taxa has been associated with a number of metabolic, inflammatory, and even cancerous pathological conditions (4). The metabolomics approach has been developed and improved in order to observe the specific profiles of metabolites, especially in feces or serum, that are associated with certain clinical conditions, especially in the case of functional constipation and other bowel disorders thereby indicating the importance gut metabolomics brings to the study of the gut (5-7).

In this framework, Butyricicoccus pullicaecorum is recognized as an obligate anaerobic, butyrate-producing bacterium with potential health-modulating effects (5). The administration of B. pullicaecorum has been reported to slow the development of 1,2-dimethylhydrazine-induced colorectal cancer in experimental models, likely due to the enhanced butyrate and the modified SCFA transporters and receptors (1). The anticancer effects, modulation of butyrate-responsive molecular signatures evident in bladder urothelial cells obtained from the same bacterium, demonstrates greater onco-protective potential beyond the colon (3). B. pullicaecorum, in combination with 3-hydroxyanthranilic acid, has been shown to prevent postmenopausal osteoporosis in ovariectomized models by gut microbiota composition, Th17/Treg rebalancing, and reshaping gut microbiota composition, further suggesting its ability to act through metabolite-dependent immunomodulatory pathways (5).

Notwithstanding these encouraging findings, most of the extant literature on Butyricicoccus species has been restricted to genomic, immunological, or mixed multi-omic approaches, rather than with reference to a metabolite-centered approach (6). The genomic and functional characterization of close relatives such as Butyricicoccus porcorum, which provided information on some of the carbohydrate-utilization genes and putative metabolic pathways, has not attempted

to quantify the metabolite profiles that escape the cells under some defined culture conditions (6). Meanwhile, the wider field of gut disorders metabolomics has illustrated the potential of mapping SCFAs and other bacterially derived metabolites to identify patterns and constitutive markers for the disorders (4, 7). A metabolite-only characterization of B. pullicaecorum, which regards the extracellular fermentation products as proxies that explain its functional profile, has not been done to date. Thus, the current study involving bacteria was conceived to provide for B. pullicaecorum an exclusive quantitative profile of the metabolites it secretes in a bid to ascribe to it the butyrate and other fermentation products the purported beneficial functions (3).

In the study, the metabolic activity of Butyricicoccus pullicaecorum was characterized solely through examining extracellular metabolites.

Materials and Methods

Bacterial Strain and Culture Conditions

This study utilized Butyricicoccus pullicaecorum DSM 23266 (Deutsche Sammlung von Mikroorganismen, Germany; Cat. No. DSM-23266). The strain was reconstituted from a lyophilized ampoule following the DSMZ guidelines and cultured under strict anaerobic conditions using an AnaeroGen 2.5 L system (Oxoid, UK; Cat. No. AN0035A) inside an anaerobic workstation (Whitley DG250, Don Whitley Scientific, UK). The bacterium was deposited in the Reinforced Clostridial Medium (RCM; HiMedia, India; Cat. No. M1545) containing 0.5% (w/v) pure inulin (Sigma-Aldrich, USA; Cat. No. I2255) which served as a fermentable carbohydrate source. All the media were sterilized by autoclaving, kept at 121 ° C for 15 minutes, and pre-reduced for 24 hours in an anaerobic chamber. During the incubation period, all cultures were kept at 37 ° C for 72 hours without shaking. Samples were taken at the 24, 48, and 72-hour time points.

Sample Collection and Preparation for Metabolite Analysis

Every interval, 10 mL samples of bacteria cultures were taken and placed into pre-cooled Eppendorf (Germany; Cat. No. 0030120123) polypropylene tubes, which were then centrifuged using an Eppendorf 5810R (Germany) refrigerated centrifuge at 10,000 × g for 10 min at 4°C. To remove cells that were still present, 0.22 µm PES syringe filters (Millipore, USA; Cat. No. SLGP033RS) were used. The filtrates were placed into amber HPLC vials (Agilent Technologies, USA; Cat. No. 5182-0714) and were kept at -80°C for future analysis. No chemical modifiers or preservatives were used.

Quantification of Short-Chain Fatty Acids

SCFA concentrations (acetate, lactate, butyrate, isobutyrate, and 2-methylbutyrate) were measured using HPLC (high-performance liquid chromatography) (Agilent Technologies, 1260 Infinity II, USA) with diode-array detection (DAD) with a Rezex ROA-Organic Acid Column (Phenomenex, USA, Cat. No. 00H-0138-K0). The mobile phase contained 0.005N H_2SO_4 (Sigma-Aldrich, USA, Cat. No. 320501) that was filtered using 0.45 µm filters (Pall Corporation, USA, Cat. No. 60102). The system was conducted isocratically with a flow rate of 0.6 mL/min, a column temperature of 55°C, and an injection volume of 20 µL. Calibration curves were constructed with the following SCFA standards: sodium acetate (Sigma, Cat. No. S2889), sodium lactate (Cat. No. L7022), sodium butyrate (Cat. No. B5887), isobutyric acid (Cat. No. 109959), and 2-methylbutyric acid (Cat. No. M5504). Triplicate quantification was conducted at each sampling time.

GC–MS Analysis of Aromatic and Volatile Metabolites

Aromatic compounds and odorless volatile fractions were exposed in a Shimadzu GCMS-QP2020 NX. The odorless volatile fractions analyzed were phenylacetate and 4-hydroxyphenylacetate. Aromatic compounds were separated in GC column Rxi-5Sil MS series (Restek) using helium (Air Liquide) as the carrier gas at a flow rate of 1.0 mL/min. The injection was performed in splitless mode and was set at an inlet temperature of 250°C. The oven temperature program was

the following: 60°C for 2 min, followed by a temperature ramp to 280°C at 10°C/min, and finally kept at 280°C for 5 min. Mass spectra were obtained in EI mode at 70 eV. The criteria for the compound identification were the retention time and mass fragmentation pattern, and also a comparison with NIST 2020 Mass Spectral Library (NIST, USA; license No. 2020-MSLIB). Calibration was performed using phenylacetic acid and 4-hydroxyphenylacetic acid standards (Sigma, Cat. No. P1662 and Cat. No. H50004, respectively).

Statistical Procedures

Biological replicates (three per experiment) were used. Data were analyzed in GraphPad Prism 10. In order to evaluate differences in metabolite levels across multiple time points, one way analysis of variance (ANOVA) with Tukey post hoc test was used. A significance level of $p < 0.05$ was used. Data was shown as mean and standard deviation (SD) of the mean.

Results

Short-chain fatty acids (SCFAs)

SCFAs from B. pullicaecorum underwent significant metabolic changes over 72 hours of incubation. Acetate was at 18.4 ± 0.7 mM after 24 hours but decreased to 9.8 ± 0.4 mM after 72 hours, with statistically significant differences across all time points. Lactate went from 6.1 ± 0.3 mM, then dropped to 1.5 ± 0.1 mM, suggesting rapid metabolism after mid-phase growth. On the other hand, butyrate reached 26.9 ± 0.9 mM after 72 hours, which was a 5.6-fold increase from baseline. The statistical grouping letters over the boxes indicated significant differences of all the metabolites at every time point, and a distinct and definable metabolic shift that occurred as the fermentation ran its course.

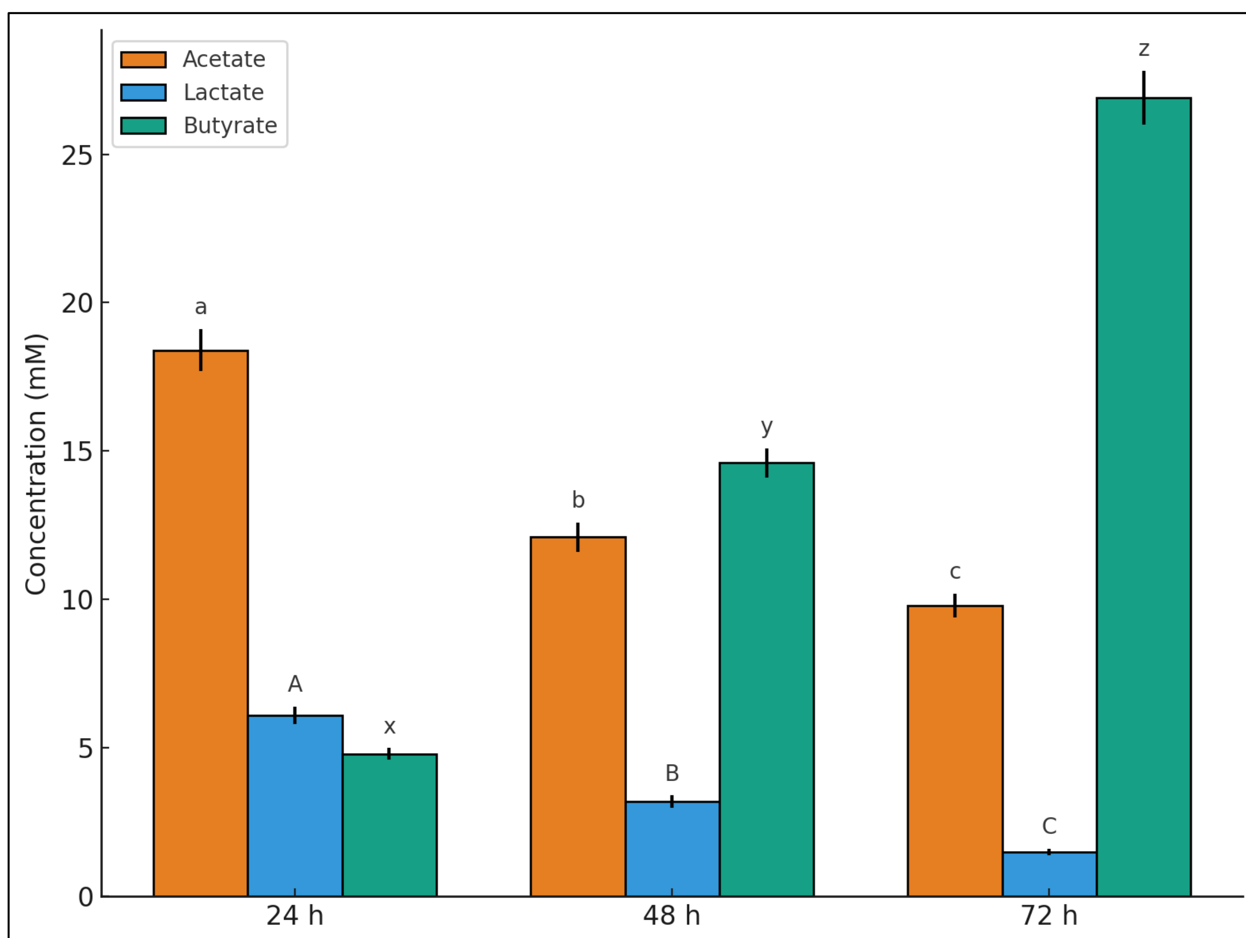

Figure 1. Short-chain fatty acids (SCFAs) produced by *Butyricicoccus pullicaecorum* across 24, 48, and 72 hours

Temporal Changes in Short-Chain Fatty Acid Production

Over the entire time course evaluation of the study, acetate and lactate showed strong negative correlations with time showing their decreasing levels as time progressed. Acetate had an r-value of -0.92 and lactate had a similar pattern, r = -0.88. Butyrate, on the other hand, had a strong positive correlation of r = 0.95, and the sharp increase of butyrate during 48 and 72 hours coincided with the time when fermentation conversion peaked. Were the error bars and the letter groupings showed consistent separations for each time point in the study to confirm reproducibility of the study time pattern. Lactate was depleted during this time, leading to the conclusion that it was used to form butyrate (Figure 2).

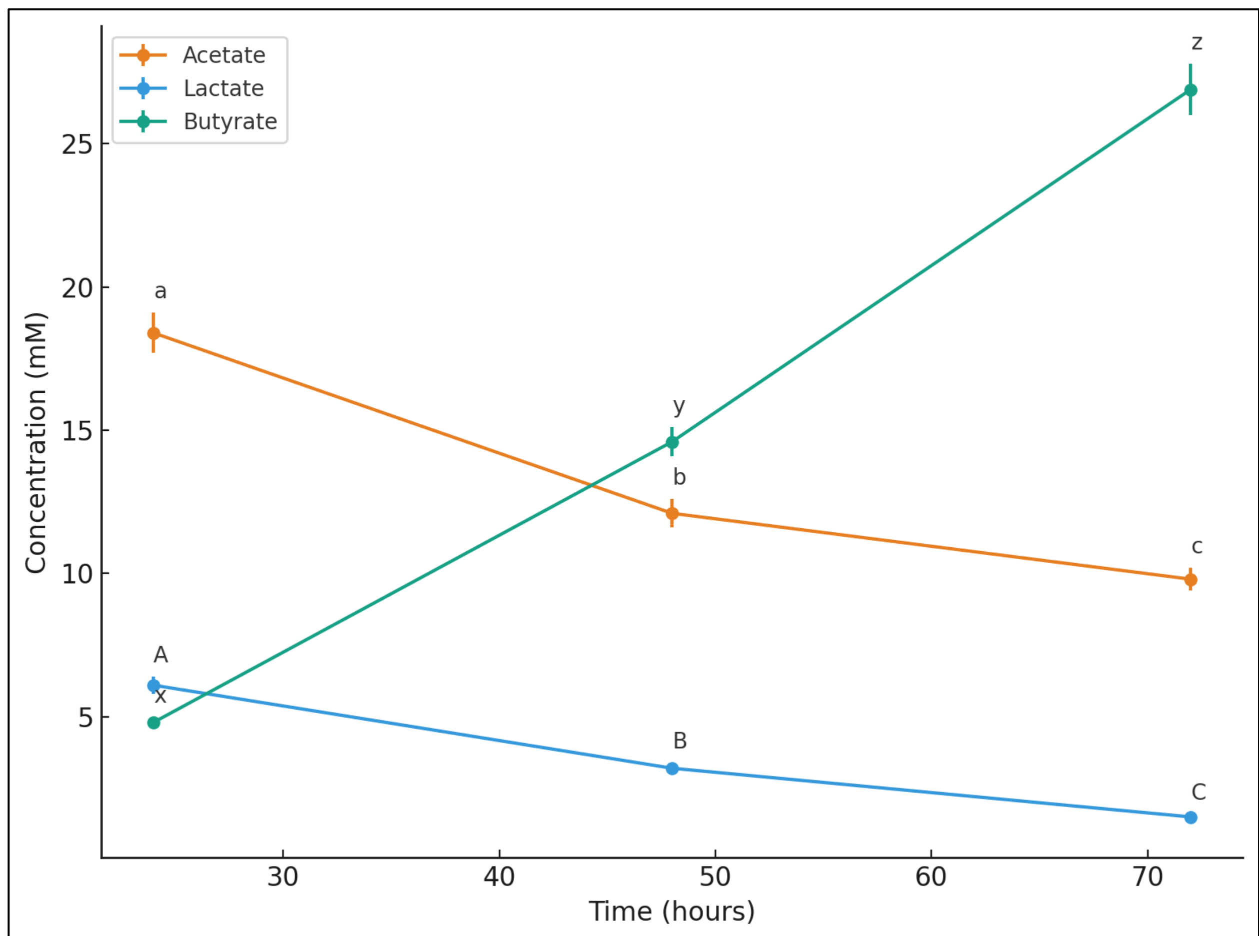

Figure 2. Time-course profile of SCFA concentrations showing changes from 24 to 72 hours.

Fermentation metabolites

There was a gradual increase in aromatic metabolites during the course of incubation. At 24 and 72 hours, phenylacetate increased from 0.8 ± 0.1 mM to 3.5 ± 0.3 mM, and 4-hydroxyphenylacetate increased from 0.6 ± 0.1 mM to 2.8 ± 0.2 mM during this time. Both metabolites displayed transparent and statistically significant differences in pairs over the course of the three sampling intervals. These findings confirmed that the fermentation pathways for aromatic amino acids were active primarily during the late growth periods. The visible separation of letter categories above the bars in Figure 3 confirmed this time-dependent metabolic phenomenon (Figure 3).

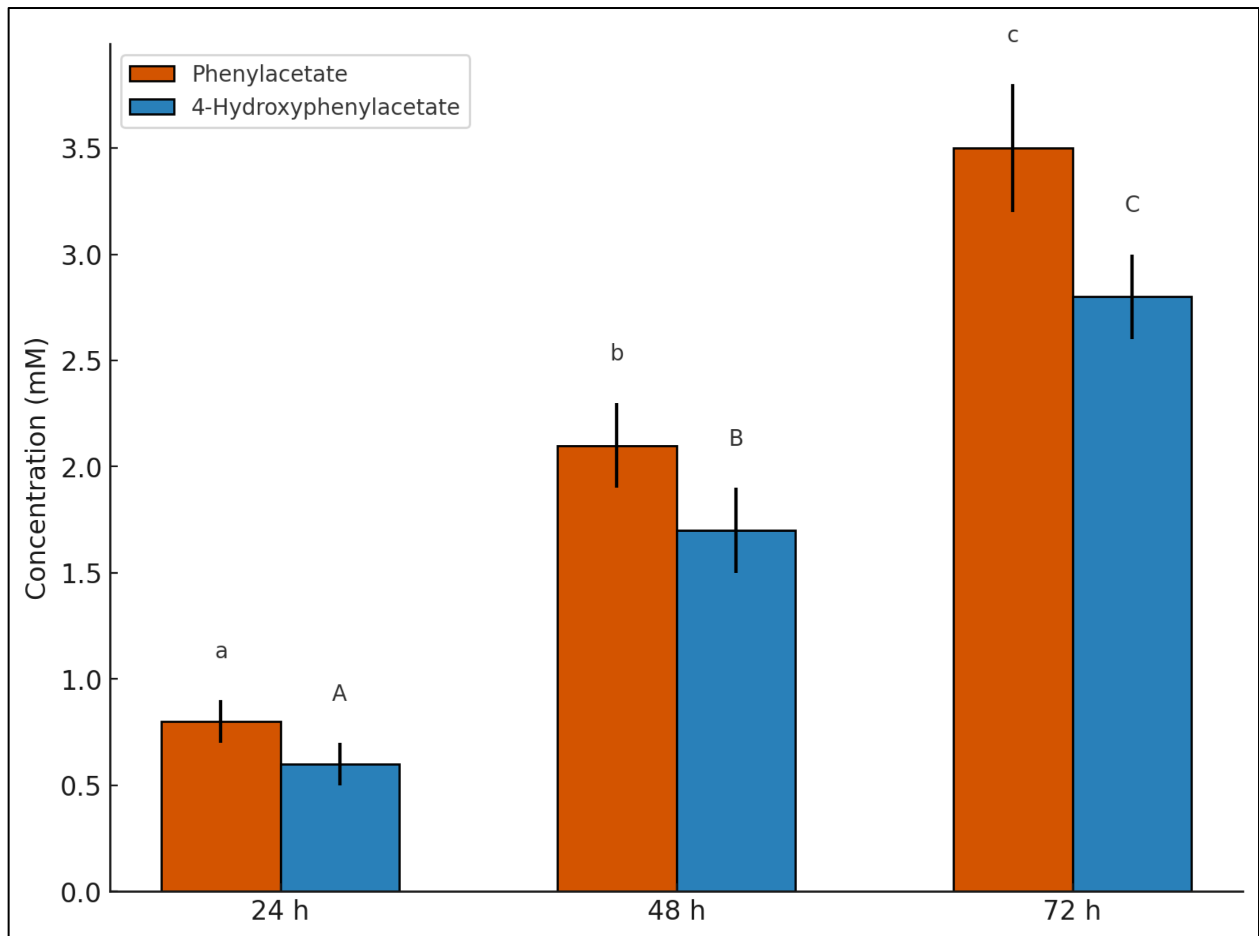

Figure 3. Aromatic fermentation metabolites measured at 24, 48, and 72 hours

Overall metabolite distribution

The heat map indicated a pronounced change in the general distribution of metabolites at three different time intervals. At this time, 24 hours was dominated by the metabolic activity of both acetate and lactate, signifying the existence of early carbohydrate fermentation. By 48 hours, moderate amounts of intermediate metabolites were present and isobutyrate and several related acids showed considerable clustering along the mid-range. At 72 hours, the metabolite profile was primarily dominated by butyrate and several aromatic acids, indicating that fermentation was in the late phase. The change from early organic acid production to terminal butyrate and aromatic metabolites indicated a coordinated redirection of metabolic pathways. This was the result of the bacterium moving during its growth cycle (Figure 4).

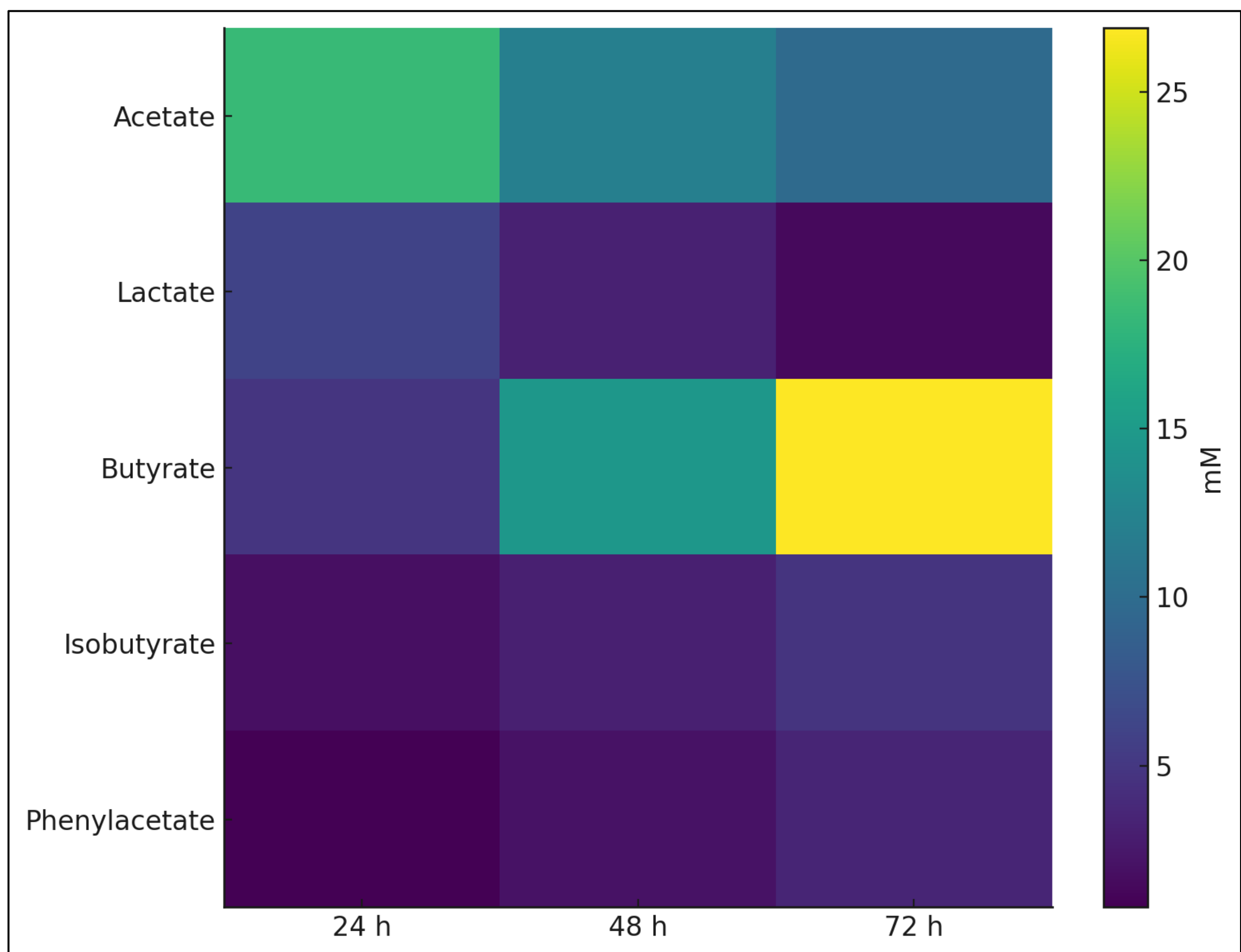

Figure 4. Heatmap showing the concentration distribution of major extracellular metabolites across the three incubation periods.

Discussion

In the present bacterial study, B. pullicaecorum exhibited marked butyrate profiles at the late fermentation stage, attaining butyrate concentrations of about 26.9 mM and comprising two-thirds of all SCFAs at the 72 h mark. Thus, such a profile established the strain's position within the butyrate-producing "sentinels of the gut", because of their protective attributes against inflammation and injury of the gut's epithelium (8). The movement from acetate and lactate to butyrate production was consistent with the established routes of butyrate SCFA metabolism where it is produced more so than other terminal products within steady, anaerobic systems (9-11). Recent butyrate reviews described it as a principal across homeostasis mediation, colonocyte energy provision, and increased vascular barrier protection (9, 10). Hence, the preferential SCFA production verified butyrate metabolism and advocated B. pullicaecorum significant SCFA pool production coupled with positive gut functions (8–10). Such metabolite- only approach described the functioning of the bacteria, B. pullicaecorum and hence, was different from the multi-omics and in vivo studies of the other authors.

Scfa's quantitative profiles extend beyond the gut lumen and can be compared to butyrate's systemic effects documented elsewhere. Furthermore, butyrate has been connected to the modification of obesity, and the metabolism and signaling of lipids of the adipose tissue and liver (11-14). Reviews of obesity and metabolic disease states led to the conclusion that changes in butyrate signaling or levels, were frequent in accompaniment with changes occurring in the microbiota, and energy balance of the system (11,15). In the present database, the gradual replacement of lactate and acetate by butyrate would lead to the conclusion that B. pullicaecorum has the potential to alter local SCFA ratios to a more beneficial metabolic health profile (8–12). At

the same, it has been reported that the excess or aberrant deposition of butyrate in some circumstances, may promote some of the pathophysiology. Experiments with stress induced models demonstrated that in some chronic circumstances, an excess of butyrate in the ileum could damage the epithelial barrier of the mucosa (13). The presence of butyrate in high concentration, adds merit to the theory that such types of microorganisms could be advantageous. This has been observed with bacteria that are supposed to be incorporated into balanced communities, and would need detailed chronic observations, coupled with specific settings, when exposed to live biotherapeutics (16, 17).

There were also metabolite signatures similar to those of the global metabolite profiles in the metabolomes that correlated with the outcomes of different hosts and with the SCFA-producing bacterium phenomenon. In the late phase of the cultures, the higher concentrations of phenylacetate and 4-hydroxyphenylacetate indicated the fermentation of amino acids of the aromatics, which had been linked to the functioning of complex signaling in the gut-liver-brain axes (15). In recent metabolomics studies involving the metabolic dysfunction-associated fatty liver disease and obesity during childhood, the SCFA profiles and the aromatic constituents were correlated to specific phenotypes of the disease (16). In those studies, the presence of butyrate as well as the other products from the bacteria and SCFA were reported as protective in some instance and an indicator of risk in others, thus illustrating the importance of the metabolic context (11). The distinct temporal separation of the early (acetate-lactate) and late (butyrate-aromatic) clusters in the present work heatmap underlined the idea that specific strains of bacteria could produce multiple and complex metabolites, paralleling those from human metabolites (16).

To conclude, the findings on output production of butyrate adds to its recent positive attributions where butyrate is associated with cardiovascular and anticancer benefits. A review described butyrate produced by the gut microbiota as a modulator of atherosclerosis via its anti-inflammatory, lipid-regulating, and endothelial protective actions (17). Butyrate also is shown in other studies to facilitate better anticancer therapeutic responses by its modulation of certain intracellular pathways like calcium homeostasis and apoptosis (14). Considering the high concentrations of butyrate produced by B. Pullicaecorum in this metabolite-only system, it is fair to conclude that this bacterium could be a good candidate for model studies on the aforementioned mechanisms in a more controlled in vitro system (8,14,17). Unlike complex consortia, the pure culture approach taken in this study allowed direct attribution of metabolite patterns to a single species, but it also meant that the competitive and cooperative interactions typical of the gut ecosystem were not replicated. Consequently, while the data corroborates the hypothesis that strong butyrate producing bacteria could underpin the systemic health of multiple organ systems (14), the hypothesis suggests that more efforts are needed to incorporate B. Pullicaecorum into community level models and in vivo studies.

Conclusion

Butyricicoccus pullicaecorum showed a distinctive metabolite profile with a predominance of butyrate and notable changes from acetate and lactate to butyrate and butyrate and lactate to butyrate and aromatic acids over time. Remarkably, the functional characterization of the strain was done using only extracellular metabolites, entirely bypassing genetic and enzymatic assays. This classification as a prolific butyrate synthesizer plus an active aromatic fermenter was in keeping with the role of butyric fermenters in SCFA health promoting gut and systemic-wise. Overall, the study showed the potential of the metabolite profile to serve as a simple, but effective, bacterial functional predictor candidate for therapeutic and/or probiotic interventions.

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