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Daisuke Sasaki

daisuke@port.kobe-u.ac.jp

Kobe University <https://orcid.org/0000-0003-3781-1352>

Tomoya Shintani

Kobe University

Yasushi Matsuki

Strategic Planning Office, Kobe University

Akihiko Kondo

Kobe University <https://orcid.org/0000-0003-1527-5288>

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Ecological stratification of the human gut microbiome resolves metabolic heterogeneity via precision substrate optimization

Daisuke Sasaki ^{a,b,*}, Tomoya Shintani ^a, Yasushi Matsuki ^c, Akihiko Kondo ^{a,b}

^a Graduate School of Science, Technology and Innovation, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe city, Hyogo, 657-8501, Japan

^b Bacchus Bio innovation Co., Ltd., 6-3-7 Minatojima Minami-machi, Chuo-ku, Kobe city, Hyogo 650-0047, Japan

^c Strategic Planning Office, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe city, Hyogo, 657-8501, Japan

* Corresponding author:

Daisuke Sasaki

Telephone: +81-78-803-6192, Fax: +81-78-803-6192

E-mail: daisuke@port.kobe-u.ac.jp

Abstract

Microbiome-targeted dietary interventions are frequently impeded by profound inter-individual metabolic heterogeneity. Traditional in vitro models fail to capture this diversity due to trade-offs between physiological fidelity and throughput. To address this limitation, we aimed to establish a high-throughput, physiologically relevant in vitro screening platform, and thus present the Systematic-Kobe University Human Intestinal Microbiota Model (S-KUHIMM). Screening 177 functional ingredients across 52 human donors revealed distinct metabolic landscapes governed by deterministic ecological rules. First, targeted propionate production is determined by the dynamic expansion capacity of *Bacteroides* spp., rather than their static baseline abundance. Second, butyrate synthesis is constrained by functional decoupling, where unchecked expansion of primary degraders (*Bifidobacterium* spp.) exceeds the intrinsic buffering capacity, triggering rapid acidification that inactivates downstream butyrate producers. By matching host-specific buffering limits with mechanistically distinct substrates, we developed a precision optimization strategy bypassing these bottlenecks. The S-KUHIMM serves as a translational platform for mechanism-driven precision nutrition.

Introduction

The human gut microbiota forms a complex ecosystem governing critical host functions, from metabolic homeostasis to immune modulation^{1,2}. These physiological benefits are principally mediated by microbial fermentation of indigestible carbohydrates into short-chain fatty acids (SCFAs), predominantly acetate, propionate, and butyrate, the colonic ecosystem's primary metabolic currency. Extensive evidence demonstrates that these three core SCFAs collectively orchestrate vital host functions, including epithelial energy homeostasis, targeted systemic endocrine signaling via FFAR2/3, and mucosal immune regulation via regulatory T cell induction³⁻⁵. Consequently, quantifying the dynamic flux of these major SCFAs provides a primary and highly robust functional readout for evaluating microbiome-targeted dietary interventions.

Inter-individual variability frequently impedes the clinical effectiveness of these precision nutrition strategies in that identical dietary substrates may benefit one individual (responder) while failing another (non-responder)^{6,7}. Although large-scale metagenomic profiling and machine learning have advanced precision nutrition research, these approaches face a fundamental predictive limitation because static baseline taxonomy indicates genetic potential but often fails to predict actual metabolic flux under environmental stress. This heterogeneity occurs because specific metabolic outputs (e.g., targeted propionate production) are driven predominantly through direct primary fermentation pathways, whereas butyrogenesis depends on intricate cross-feeding networks in which specialized secondary fermenters must efficiently consume primary intermediates (e.g., acetate and lactate)⁸. Elucidating the mechanisms by which diverse microbial architectures coordinate these highly interdependent metabolic relays remains challenging. Small-scale in vivo human trials lack necessary mechanistic resolution, whereas traditional murine models are fundamentally limited by significant species-specific discrepancies in microbiome composition and gastrointestinal anatomy⁹.

In vitro fermentation models offer a controlled alternative. Comprehensive in vitro studies have demonstrated that inter-individual microbiota variation primarily drives distinct fermentation profiles¹⁰. Dynamic simulators mimic gut physicochemical dynamics with high fidelity but lack throughput¹¹⁻¹³. Conversely, high-throughput microplate systems can screen vast compound libraries¹⁴, but miniaturization often compromises ecological stability, leading to dysbiotic shifts, such as *Escherichia* spp. blooming and strict anaerobe loss¹⁵⁻¹⁷, thereby diminishing predictive value.

Furthermore, although recent high-throughput platforms successfully utilize single-strain isolates or synthetic microbial communities¹⁸, they inherently fail to capture the complex physicochemical and thermodynamic constraints of native gut microbiomes. Thus, a critical trade-off remains between physiological relevance and throughput.

To address this critical trade-off and decode the deterministic mechanisms underlying metabolic heterogeneity, in this study, we aimed to establish a high-throughput, physiologically relevant in vitro screening platform. Building upon our previously validated batch fermentation technology^{19,20}, we developed the Systematic Kobe University Human Intestinal Microbiota Model (S-KUHIMM). To assess this model, we tested the hypothesis that dynamic functional tracking could overcome the predictive limitations of static taxonomic profiling. Our findings revealed silent heterogeneity and functional decoupling. Ultimately, by mapping physical and thermodynamic constraints, we present a hierarchical optimization strategy to bypass individualized metabolic bottlenecks, establishing a mechanism-driven predictive roadmap for precision nutrition.

Results

Establishment and scalable miniaturization of the S-KUHIMM

We optimized basal culture conditions to develop a high-throughput in vitro model recapitulating human gut microbial ecosystems (Fig. 1a). The Gifu anaerobic medium (GAM)-based model (KUHIMM; 2nd generation)^{19,20} yielded the highest total bacterial load (mean 10.9 Log₁₀ copies/mL) and Pearson correlation coefficients with the original fecal inoculum compared to models based on other media (Fig. 1b), serving as the base platform. To prevent progressive depletion of functionally important taxa and model the mucosal environment, we screened additives including dietary fibers, bile acids, and mucin. Supplementation with 0.8% (w/v) mucin yielded the highest structural retention and alpha diversity (Shannon index) at 48 h (Fig. 1c). Titrating mucin concentration revealed that 0.8% mucin significantly enhanced total SCFA production ($P = 0.031$ vs. 0.0% mucin; Supplementary Fig. 1a) and stabilized the community, whereas excessive loading (4.0%) caused a marked decline in diversity, likely driven by rapid substrate fermentation disrupting ecological balance (Supplementary Fig. 1b). Thus, 0.8% mucin was used to simulate in vitro metabolic maturation.

Evaluating time-course stability revealed that increasing inoculum volume (200 to 500 μ L) transiently improved 24 h stability, but progressive community drift occurred by 48 h. Integrating 0.8% mucin with 500 μ L inoculum (S-KUHIMM; 3rd

generation) successfully prevented this time-dependent collapse, maintaining significantly higher centered log-ratio (CLR)–Pearson correlations than conventional models (false discovery rate (FDR)–corrected $q < 0.05$; Fig. 1d). This mucin-primed S-KUHIMM platform effectively suppressed in vitro dysbiotic expansion of *Escherichia* spp. while promoting the maintenance of critical butyrate producers such as *Faecalibacterium* spp. throughout 24 and 48 h culture periods (Supplementary Fig. 1c). Maintaining high fidelity up to 72 h supports dynamic models such as the Kobe University Library of Functional Food Ingredients (KULFFI) system, where test xenobiotics or functional foods added at 24 h are subjected to an additional 48 h of fermentation. Although *Prevotella* spp. abundance progressively declined by 72 h (Fig. 1e), this baseline depletion provides a broad dynamic range for detecting targeted proliferation upon specific substrate introduction.

To enable massive parallelization, we miniaturized the system 100-fold from a 100 mL jar fermenter into a 1 mL 96-well deep-well format. When tested under dynamic stress conditions, using a donor microbiota with a natural temporal shift, the 96-well format accurately recapitulated the ecological trajectory of the macroscopic 100 mL jar over 96 h of cultivation (Fig. 1f). At 72 h, the 96-well system preserved complex community structures (Shannon index > 5.0) and high bacterial biomass. Owing to exceptional technical reproducibility, minor fluctuations in total bacterial load and culture pH between formats were significant ($P = 0.0259$ and 0.0152 , respectively; Mann–Whitney U test) and remained biologically conserved within a tightly constrained range (Fig. 1g). Total SCFA production was indistinguishable between formats ($P = 0.3939$; Fig. 1g), demonstrating the scalable 1 mL S-KUHIMM platform achieves high functional and ecological fidelity comparable to macroscopic models, unlocking massive parallel screening.

High-throughput S-KUHIMM screening decodes the metabolic landscape

Using the miniaturized S-KUHIMM platform, we screened the KULFFI library across fecal samples from 52 healthy donors to evaluate metabolic outputs and microbiome dynamics (Fig. 2a). The KULFFI library encompasses 177 functional ingredients, ranging from foods to over-the-counter drugs, selected for their potential to modulate gut microbiota independent of predicted in vivo colonic reachability. Under unsupplemented control conditions (CUL), the system demonstrated analytical stability. The coefficient of variation for total organic acids was 11.5% across 52 donors (Fig. 2b). Coefficients of variation for individual SCFAs were similarly low despite intrinsic biological variation (acetate: 16.8%, propionate: 23.9%, and butyrate: 30.4%, consistent

with its role as a secondary metabolite). Although transitioning to the in vitro environment caused a significant contraction in absolute alpha diversity, the cultured ecosystem retained a complex community structure (median Shannon index: 5.22 vs. 5.58, for CUL and native feces, respectively; $P = 1.5 \times 10^{-10}$; two-sided Wilcoxon signed-rank test) (Supplementary Fig. 2a). Taxonomic analysis confirmed that core phyla and families were broadly conserved (Supplementary Fig. 2b). Although in vitro cultivation inevitably introduced specific environmental pressures, such as relative expansion of *Escherichia* spp. (median 12.80% vs. 0.40% in CUL and native feces, respectively), this localized outgrowth did not obscure each donor's unique baseline microbiome signature. Thus, the S-KUHIMM provides a functional screening environment where individualized metabolic variations can be reliably evaluated despite in vitro adaptive shifts.

We calculated genus-level CLR–Pearson correlation coefficients between in vitro cultured ecosystems and their respective native inoculum feces (Fig. 2c). The CUL group maintained a strong structural correlation with the native inoculum (median $r = 0.646$). Supplementation with diverse functional ingredients, including complex polysaccharides (resistant maltodextrin (RMD); median $r = 0.645$) and highly accessible substrates (inulin, median $r = 0.648$; xylitol, median $r = 0.618$), did not induce severe structural deviation or collapse. All evaluated conditions preserved stable structural fidelity relative to native feces. Coupled with low technical noise in SCFA production (Fig. 2b), the S-KUHIMM platform precisely captures specific metabolic impacts while preserving the core individualized microbial architecture of each donor.

Dynamic *Bacteroides* spp. expansion as the obligate predictor of propionate drive

Focusing on the propionate production pathway, we strategically selected RMD, RS, and D-mannose. Although RMD²¹ and RS²² have established human clinical evidence, D-mannose demonstrates propionate-driving capacity primarily in vitro²³. Ecologically, these substrates engage a functional axis wherein *Bacteroides* spp. act as a keystone primary degrader, driving propionate production directly via the succinate pathway or indirectly through complex cross-feeding²⁴. Global heatmap visualization of propionate production across the KULFFI library confirmed that the S-KUHIMM successfully reproduced and stratified highly individualized production profiles specific to these substrates (Fig. 3a). RMD was identified as a statistically significant global driver of propionate (Log2FC = 0.128, FDR-corrected $q < 0.001$), as visualized in a volcano plot (Fig. 3b). Conversely, RS and D-mannose showed no significant global enhancement ($q = 0.536$ and 0.638 , respectively). However, relying solely on relative metrics such as

Log2FC mathematically amplifies stochastic noise in low-baseline donors and compresses substantial metabolic outputs in high-baseline donors (Supplementary Fig. 3a, b). As luminal SCFAs exist at high baseline concentrations and undergo rapid mucosal clearance in vivo, physiological relevance must be evaluated by absolute physical yield to avoid mathematical artifacts.

Transitioning to the absolute concentration change (Δ mM) via waterfall plots unmasked substantial inter-individual heterogeneity (Fig. 3c–e). Absolute quantification for RMD confirmed a consistent cohort-wide increase in net propionate production (Δ mean = 4.86 mM). For RS, although the global mean was slightly negative (Δ mean = -0.84 mM), 26 of 52 donors exhibited net positive propionate production. D-mannose similarly yielded a negative global mean (Δ mean = -1.33 mM), yet 25 donors exhibited positive responses. Positive propionate output from responders was mathematically offset by stalled metabolic flux of non-responders, accounting for the lack of significance in global Log2FC analysis.

Quantifying the absolute abundance of *Bacteroides* spp. revealed that RMD induced significant, population-wide expansion ($q = 2.23 \times 10^{-5}$). RS and D-mannose did not induce uniform expansion ($q = 0.736$ and $q = 0.736$, respectively; Fig. 3f), but localized *Bacteroides* spp. proliferation occurred within distinct responder subsets. Static microbiome profiling failed to predict this individual variance because the initial absolute *Bacteroides* spp. abundance in original feces did not correlate with final net propionate yield for RS ($r = 0.113$, $q = 0.838$) or D-mannose ($r = 0.029$, $q = 0.838$). Instead, personalized propionate drive was strongly associated with dynamic expansion capacity (Δ Log10 *Bacteroides* spp.), highlighting the fundamental inadequacy of relying solely on static baseline profiling. We identified strong positive Pearson's correlations between dynamic spp. growth and absolute propionate production for RMD ($r = 0.607$, $q = 2.71 \times 10^{-6}$), RS ($r = 0.563$, $q = 1.40 \times 10^{-5}$), and D-mannose ($r = 0.732$, $q = 2.16 \times 10^{-9}$) (Fig. 3g–i). The reliability of these linear trends was rigorously confirmed via non-parametric Spearman's rank correlation to ensure that they are not mathematical artifacts driven by extreme outliers. This obligate relationship was consistently corroborated via 16S rRNA gene sequencing (Supplementary Fig. 3c–e). By directly capturing dynamic proliferation capacity, the S-KUHIMM could identify precisely which hosts possess actual ecological competence to respond to specific functional substrates.

Functional decoupling in cross-feeding networks: breakdown of butyrate drive

To investigate the butyrate drive governed by multi-species cross-feeding interactions, we mapped global acetate and butyrate production across the 177-ingredient library (Fig. 4a, b). Substantial increases in butyrate production were prominently detected for sugar alcohols, specifically xylitol and erythritol^{25,26}. When baseline ecological competence was assessed using inulin, a prebiotic that stimulates *Bifidobacterium* spp. and subsequent butyrate cross-feeding^{9,27}, significant acetate depletion occurred (FDR-corrected $q = 1.22 \times 10^{-5}$, Fig. 4c). However, global butyrate production remained entirely non-significant ($q = 0.769$, Fig. 4d), which was an unexpected metabolic response. This global stall contradicted quantitative polymerase chain reaction (qPCR) analysis, which demonstrated inulin successfully induced highly significant, cohort-wide proliferation of the cross-feeding consortium, driving marked absolute expansions of *Bifidobacterium* spp., *Faecalibacterium* spp., *Blautia* spp., *Butyrivicoccus* spp., and *Anaerostipes caccae* (all FDR-corrected $q < 0.01$, Fig. 4f). Comprehensive 16S rRNA profiling corroborated these profound structural shifts (Supplementary Data 1). This discrepancy, where bacteria physically expand but expected metabolic output stalls, represents fundamental functional decoupling.

The absolute concentration changes (Δ mM) revealed hidden individual heterogeneity. For inulin, the cohort was markedly stratified with 25 donors exhibiting net positive butyrate production (responders) and 27 donors experiencing severe metabolic stalling, neutralizing the global mean (Δ mean = -0.17 mM, Fig. 4e). Correlation analyses between dynamic bacterial expansion (Δ Log10) and SCFA flux (Δ mM) showed that the primary degrader did not accurately predict its own metabolic product; thus, dynamic *Bifidobacterium* spp. expansion did not correlate with acetate flux ($r = -0.148$, $q = 0.294$, Fig. 4g), whereas *Faecalibacterium* spp. positively correlated only modestly with net butyrate yield ($r = 0.313$, $q = 0.036$, Fig. 4h). Efficient cross-feeding predicts a negative correlation between Δ acetate and Δ butyrate; however, inulin exhibited a significant positive correlation ($r = 0.382$, FDR-corrected $q = 0.015$, Fig. 4i), indicating a severely stalled and decoupled state characterized by concurrent reductions in acetate and butyrate production. These decoupled relationships were corroborated by evaluating compositional shifts via 16S rRNA gene sequencing (Supplementary Fig. 4a–d). Individualized metabolic yield depends on bacterial expansion and the host ecosystem maintaining a delicate stoichiometric balance throughout the primary-to-secondary metabolic relay.

Divergent cross-feeding architectures and niche-specific butyrate drivers

Comparing cross-feeding dynamics of inulin against RS, xylitol, and erythritol, we evaluated net absolute butyrate production (ΔmM) (Fig. 5a). Inulin and RS failed to induce significant global butyrate increases (FDR-corrected $q = 0.820$ for both). Conversely, sugar alcohols elicited highly significant and consistent cohort-wide butyrogenic responses (xylitol $\Delta\text{mean} = 23.54 \text{ mM}$, $q = 1.06 \times 10^{-8}$; erythritol $\Delta\text{mean} = 34.42 \text{ mM}$, $q = 2.98 \times 10^{-9}$), substantially exceeding those of conventional polysaccharide prebiotics. Upon evaluating correlations between $\Delta\text{acetate}$ and $\Delta\text{butyrate}$ as a stoichiometric indicator of cross-feeding efficiency (Fig. 5b–d), RS ($r = 0.048$, $q = 0.733$) showed a similarly ineffective trend to inulin. Highly butyrogenic sugar alcohols exhibited strong negative correlations (xylitol: $r = -0.722$, $q = 6.21 \times 10^{-9}$; erythritol: $r = -0.675$, $q = 8.11 \times 10^{-8}$). This inverse dynamic illustrates optimal cross-feeding where accumulated acetate is continually and efficiently consumed to fuel effective butyrate conversion.

Evaluating dynamic expansion (ΔLog10) of key secondary fermenters showed that each substrate selectively enriched different taxa (Fig. 5e), which was confirmed via comprehensive 16S rRNA profiling (Supplementary Data 2–4). However, dynamic expansion of *Butyricoccus* spp. on RS, and *A. caccae* on erythritol, was not significantly correlated with net butyrate yield ($r = 0.023$, $q = 0.925$ and $r = 0.016$, $q = 0.925$; Fig. 5f, g). Comprehensive screening across absolute (qPCR) and relative (16S rRNA) datasets for taxa exhibiting strong correlations ($r > 0.4$) with butyrate production unmasked a highly niche-specific division of labor. Smooth butyrogenic output driven by xylitol strongly and uniquely correlated with dynamic expansion of minor *Anaerostipes* spp. identified through 16S sequencing ($r = 0.613$, $q = 9.08 \times 10^{-6}$, Fig. 5h). The pronounced butyrate response induced by erythritol was strongly associated with the specific proliferation of *Butyricoccus* spp. determined by qPCR ($r = 0.467$, $q = 0.003$, Fig. 5i). Initial basal abundances of these niche-specific taxa showed no predictive value for final metabolic yield, suggesting distinct functional substrates bypass global dominant networks to activate specific, low-abundance niche drivers, achieving smooth acetate-to-butyrate conversion.

Universal pH-driven metabolic stall and ecological stratification

By mapping median *Bifidobacterium* spp. expansion (ΔLog10) against net butyrate yield (ΔmM) across the KULFFI library (Fig. 6a), the systemic discrepancy between primary degradation and secondary fermentation became clear. Conventional prebiotics such as inulin and RS strongly stimulated primary bifidogenic expansion (median $\Delta\text{Log10} = 0.24$ and 0.25 , respectively), but median butyrate yields remained effectively

blunted (-0.44 and -0.58 mM, respectively). The highly butyrogenic sugar alcohols, xylitol and erythritol, effectively bypassed this bifidogenic bottleneck to drive substantial butyrate production (25.78 and 38.04 mM, respectively). Evaluating dynamic bacterial expansion, culture pH, and butyrate yield showed that severely acidified ecosystems fail to synthesize butyrate, independent of *Bifidobacterium* spp. proliferation (Fig. 6b). Extending the analysis to the 177-ingredient library identified a universal pH threshold (Fig. 6c, d). Substantial drops in luminal pH profoundly impaired whole-ecosystem butyrogenic capacity, broadly repressing secondary fermentation pathways across diverse functional substrates.

Stratifying the 52 healthy donors into three ecotypes based on the tertile distribution of their absolute pH drop during inulin fermentation, severe ($\Delta\text{pH} \leq -0.80$, $n = 17$), moderate ($-0.80 < \Delta\text{pH} < -0.44$, $n = 17$), and mild ($\Delta\text{pH} \geq -0.44$, $n = 18$) (Fig. 6e), unmasked a highly significant positive correlation between ΔpH and net butyrate yield ($r = 0.577$, FDR-corrected $q = 7.48 \times 10^{-6}$; Fig. 6f). This strongly suggests that maintaining a physiological pH range serves as a critical ecological constraint for successful cross-feeding. Microbial and metabolic dynamics indicated that the severe ecotype exhibited profound suppression in butyrate synthesis (median -5.17 mM) compared to that of the smoothly operating mild ecotype (median $+4.38$ mM; FDR-corrected $q = 6.14 \times 10^{-4}$) (Fig. 6g–j). This metabolic stall was not caused by absent bacterial growth because the severe ecotype exhibited a significantly higher absolute expansion of *Bifidobacterium* spp. than the mild ecotype (median $\Delta\text{Log10} = 0.36$ vs. 0.20 , FDR-corrected $q = 0.035$). *Faecalibacterium* spp. successfully proliferated across all groups to a similar degree ($q = 0.704$). Functional decoupling is primarily an acid-driven metabolic stall. When highly accessible substrates selectively over-stimulate primary degraders, triggering rapid organic acid accumulation, pH drops below the physiological tolerance threshold of secondary fermenters if their ecosystem lacks sufficient buffering capacity, effectively terminating the metabolic relay despite physical expansion of the cross-feeding consortium.

Mathematical determination of ecological tipping point and personalized metabolic restoration

Segmented regression analysis mapping $\Delta\text{butyrate}$ against ΔpH during inulin fermentation precisely identified the inulin-specific ecological tipping point (ETP) at $\Delta\text{pH} = -0.81$ (Fig. 7a). While non-parametric bootstrap resampling ($n = 1,000$) indicated a wide 95% confidence interval reflecting substantial inter-individual biological variation, integrating metabolic trajectories from 5 distinct bifidogenic

substrates (n = 260 data points) yielded a highly concordant global ETP of $\Delta\text{pH} = -0.83$ (Supplementary Fig. 5a). The convergence of these values defines a critical tipping zone where ecosystem buffering capacity is fundamentally exhausted, independent of age-dependent baseline variations across the cohort (6–78 years). We established a conservative global ecological stability margin at $\Delta\text{pH} = -0.80$ to stably sustain functional cross-feeding. Implementing a sequential stratification strategy strictly limited to 13 bifidogenic substrates revealed responder rates that reached an absolute plateau at 46.2% (24 of 52 donors) (Supplementary Fig. 5b, c). This mathematically demonstrates the strict capacity limit of bifidogenic cross-feeding; once an individual's ETP is exceeded, repeated cycling through mechanistically similar substrates is metabolically ineffective, highlighting the need to redirect metabolic pathways using distinct niche drivers.

Tracking individualized metabolic trajectories across diverse functional substrates, stratified by baseline inulin-induced ecotypes, illustrated the severe limitations of conventional prebiotics (Fig. 7b). Although mild ecotypes successfully maintained consistent butyrate production, moderate ecotypes exhibited substrate-dependent vulnerability (e.g., stalling with RS), and severe ecotypes were consistently forced past the ETP into a profound metabolic stall. Ecological tracking across the KULFFI library revealed severe acidifiers consistently experienced profound metabolic stalls across a broad spectrum of highly accessible bifidogenic substrates (e.g., galacto-oligosaccharides, fructo-oligosaccharides, lactulose) (Supplementary Data 5). Although these substrates possess excellent primary fermentation capacity, this demonstrates that functional decoupling manifests intrinsic ecological vulnerability and limited buffering capacity in severe ecotypes. An ecological-functional quadrant framework, integrating the ETP stability margin ($\Delta\text{pH} \geq -0.80$) and functional efficacy threshold ($\Delta\text{butyrate} \geq 5.0 \text{ mM}$), highlighted stark disparities (Fig. 7c). Inulin clustered a substantial portion beyond the ETP limit, whereas RS largely failed to cross the efficacy threshold. By bypassing the rapid primary fermentation bottleneck, the niche-specific drivers xylitol and erythritol avoided the ETP boundary, driving highly efficacious butyrate production stably within buffering limits.

Implementing a stepwise combinatorial stratification framework, host ecosystems failing to achieve efficacy with inulin were sequentially allocated to alternative substrates (RS → xylitol → erythritol) bypassing the global dominant networks (Fig. 7d). Relying solely on gold-standard inulin achieved stable functional efficacy in only 28.8% (15 of 52). Sequential step-up to RS compensated an additional 9.6% (n = 5). Deploying xylitol achieved successful metabolic restoration in 48.1% (n =

25), predominantly among severe or moderate baseline ecotypes inherently susceptible to acid-driven stall. Erythritol effectively covered an additional 7.7% (n = 4). Dynamically matching unique ecological architecture and buffering limits to precise functional substrate kinetics achieved optimal metabolic modulation in 94.2% (49 of 52) of the highly heterogeneous human cohort.

Discussion

Although recent clinical and large-scale metagenomic studies have unequivocally demonstrated that microbiome responses to dietary interventions are profoundly personalized^{28,29}, deciphering this heterogeneity exclusively through in vivo trials remains unfeasible in terms of cost and throughput. Moreover, while advanced metagenomics provides comprehensive genetic blueprints, relying solely on static baseline taxonomic profiling faces fundamental predictive limitations under environmental stress³⁰. By breaking through these barriers, the S-KUHIMM platform facilitates a shift from static metagenomic observation to dynamic functional tracking. Demonstrating exceptional analytical reliability and physiological fidelity (Figs. 1 and 2), our mucin-priming protocol provides essential ecological scaffolding that maintains critical strict anaerobes, such as butyrate producers, and stabilizes intricate cross-feeding networks often lost in conventional microplate systems. By creating a reliable, functional digital twin of the donor's gut ecosystem, this platform provides a rigorous analytical foundation for decoding complex diet-microbiome interactions and distinguishing true biological non-responders from in vitro technical artifacts.

A pivotal challenge in nutritional science is addressing this silent heterogeneity. By establishing predictive validity, the S-KUHIMM accurately reproduced distinct in vivo approaches to RMD, clinically established as a key global driver enriching *Bacteroides* spp. and propionate²¹, and RS, which exhibits a highly personalized metabolic route distinctly dividing cohorts^{22,31} (Fig. 3). Building upon this, the metabolic trajectory of D-mannose serves as a prime example decoding silent heterogeneity. Although recent murine models demonstrated D-mannose specifically enriches *Bacteroides* spp. and improves host metabolism²³, its capacity to similarly drive *Bacteroides* spp. and propionate in the highly diverse human gut remained unclear. Although population-average responses were negligible, high-throughput screening successfully stratified the cohort into distinct functional ecotypes. Crucially, across these propionate-driving substrates, personalized metabolic potential was strictly not dependent on initial taxonomic abundance of *Bacteroides* spp. in native feces but

rather on its dynamic proliferative capacity (ΔLog10). The physical presence of a degrader species possessing genetic potential does not guarantee functional realization within a complex community under environmental stress. Consequently, although conventional models relying strictly on static initial compositions face fundamental predictive limits⁷, the dynamic predictive capability of the S-KUHIMM offers a powerful screening filter bridging fundamental animal models with precision human nutrition.

In the human gut, colonic butyrogenesis relies on these complex cross-feeding networks⁸. In our baseline evaluations, intermediate metabolites did not exhibit pronounced accumulation as lactate and succinate remained below 1.0 mM or completely undetectable in most subjects (50 and 43 of 52 donors, respectively), precisely mirroring the healthy in vivo physiological state in which rapid turnover prevents thermodynamic stalling. Although bifidogenic substrates actively drive this pathway, human clinical trials have consistently reported highly variable butyrogenic capacity across individuals despite marked *Bifidobacterium* spp. expansion²⁷. Comprehensive screening showed that inconsistent butyrogenic effects of inulin are mechanistic consequences of ETPs. Expanding the analysis to include a subset of 13 well-established bifidogenic prebiotics showed that these substrates, despite there being differing degrees of polymerization, universally induced primary degrader expansion yet predictably stalled downstream butyrate synthesis in severe responders (Supplementary Fig. 5). When acid production exceeds the ecosystem's buffering and cross-feeding capacity, it induces a self-limiting feedback loop. Successful cross-feeding to butyrate is determined by the magnitude of environmental acidification (ΔpH), selectively constraining pH-sensitive butyrate producers such as *Faecalibacterium* spp. (Figs. 4 and 6). Crucially, untargeted screening confirmed that although a few low-abundance taxa correlated with specific functional trajectories (FDR-corrected $q < 0.05$, Supplementary Data 1–4), their low absolute abundances preclude them from driving massive stoichiometric shifts. Instead, these minor responders serve as secondary ecological indicators, supporting the conclusion that macroscopic outputs are predominantly driven by primary keystone guilds (*Bacteroides* and *Bifidobacterium*–*Faecalibacterium* axes).

This acidification-driven stall mirrors physiological dynamics of the proximal colon. Early radiotelemetry studies demonstrated that rapid fermentation of accessible substrates significantly lowers proximal colonic pH, reaching 5.0^{32,33}, physiologically constraining acid-sensitive guilds^{34,35}. The deliberate omission of strong artificial buffering agents in the S-KUHIMM prevents artificial distortion of highly pH-sensitive

competitive exclusion and cross-feeding networks, acting as a high-resolution ecological stress test. This amplified intrinsic acidifying potential enabled us to mathematically define a universal ETP ($\Delta\text{pH} \approx -0.80$) where cross-feeding networks deterministically collapse (Fig. 7a). This functional transition perfectly aligns with physiological constraints: a ΔpH drop of -0.80 from baseline forces the microenvironment below pH 6.0, an absolute boundary that is thermodynamically predicted to restrict the enzymatic flux (e.g., butyryl-CoA:acetate CoA-transferase) of acid-sensitive butyrate producers such as *Faecalibacterium* spp. Functional classification did not align with traditional enterotypes (*Bacteroides*- or *Prevotella*-dominant)³⁶, indicating the limitation of static taxonomic profiling (Supplementary Fig. 6a, b). Dynamic functional responses often uncouple from baseline compositions³⁷. Functional ecotypes defined based on ecosystem thermodynamic balance are superior predictors. The gut ecosystem is governed by multifactorial constraints. Rapid primary fermentation can lead to accumulation of H₂ and intermediate metabolites, altering thermodynamic redox balance. If an individual lacks sufficient hydrogen-consuming guilds (e.g., methanogens or acetogens), then the elevated partial pressure of H₂ can thermodynamically inhibit upstream fermentation pathways independently of pH³⁸. Furthermore, initial substrate competition at the strain level, particularly for complex polysaccharides such as inulin, may influence the efficiency of downstream cross-feeding networks³⁹. Local accumulation of specific organic acids, such as lactate, can also exert direct inhibitory effects⁴⁰. The ETP identified likely represents a composite threshold where pH acts as the most visible macroscopic indicator of a broader physicochemical and thermodynamic collapse.

To contextualize ETPs, we expanded the analysis to the 177-ingredient library (Fig. 6a, d; Supplementary Data 5). Establishing strict functional thresholds grounded in physiological kinetic requirements, including the rapid mucosal clearance of SCFAs⁴¹, and demanding a substantial 25% elevation above the classical human colonic baseline (acetate:propionate:butyrate $\approx$ 60:20:20 mM)⁴², required absolute increases of ≥ 15.0 mM for acetate, and ≥ 5.0 mM for propionate and butyrate to achieve substantial epigenetic modulation (e.g., regulatory T cell induction via histone deacetylase inhibition)^{3,4} and targeted systemic endocrine responses via FFAR2/3^{5,43}. Over half of the library (100 substrates) fell below this population-level efficacy threshold, acting instead as highly personalized drivers of silent heterogeneity. Thirty-four substrates spanning diverse structural classes fell into the same acid-driven functional decoupling category as inulin (Supplementary Data 5). Phenotypic diversity observed within identical chemical sub-classes demonstrates simple structural profiling is fundamentally

insufficient; substrate fate is governed by dynamic fermentation profiles relative to intrinsic buffering capacity. Our comprehensive evaluation identified a distinct dual-predictor model governing metabolic prediction. For simple substrates such as D-mannose, absolute increment in biomass (Δ abundance) directly drives metabolic production. Conversely, for complex polysaccharides, relative expansion magnitude (fold change) emerges as a highly sensitive predictor, mechanistically corroborated by the requirement of specific polysaccharide utilization loci to dynamically acquire niche dominance when digesting complex glycans^{44,45}.

Dynamic screening compels re-evaluating functional ingredients capable of bypassing ecological bottlenecks. For these target carbohydrates, evaluated at a physiologically conservative concentration (0.3% w/v), corresponding to a theoretical colonic delivery of approximately 4.5–6.0 g based on 1.5–2.0 L of chyme entering the colon daily⁴⁶, and well below the clinical tolerance threshold (18–20 g/day) that triggers osmotic diarrhea⁴⁷, xylitol emerges as a potent metabolic synchronizer^{25,48}. Xylitol possesses exceptional potency to recruit lactate-utilizing butyrate producers (e.g., *Anaerostipes* spp.), thereby promoting butyrogenesis with high reliability. This strong unique correlation ($r = 0.613$) provides mechanistic evidence of functional redundancy. By activating an alternative metabolic bypass avoiding the highly fragile *Bifidobacterium*–*Faecalibacterium* cross-feeding axis, these acid-tolerant guilds efficiently convert accumulated primary metabolites, physically buffering the environment to prevent rapid pH collapse. Erythritol presents a completely independent bypass. Historically considered non-fermentable and biologically inert⁴⁹, it holds exceptional potency specifically for severe ecotype donors resistant to traditional prebiotics (Fig. 7). This metabolic restoration is driven by profound specific expansion of potent butyrate-producing taxa, notably *Eubacterium* spp. (Log2FC = 5.33) and *Anaerobutyricum* spp. (Log2FC = 4.54) (Supplementary Data 4), which aligns with recent findings on their erythritol-utilizing capacity²⁶. Evading primary degraders, erythritol acts as a specific metabolic trigger selectively enriching latent butyrogenic guilds. Although almost entirely absorbed in the human small intestine in vivo⁵⁰, the S-KUHIMM unmask its true metabolic potential independent of host absorption. This provides a precise functional rationale for engineering future colon-targeted drug delivery systems⁵¹, which could safely maintain effective localized concentrations without triggering rapid spikes in luminal osmolality⁵², transforming erythritol into a precision tool for restoring butyrogenesis.

Integrating these dynamic and physicochemical findings, a mechanistic working model contrasts metabolic trajectories of diverse substrates (Fig. 8), providing

a functional blueprint for a stepwise precision nutrition stratification framework (Fig. 7d). Dynamically matching intrinsic buffering limits to precise substrate-specific metabolic shifts ensures nearly every individual can benefit from targeted microbiome modulation. We acknowledge some limitations. First, the closed in vitro system lacks host-derived absorption. However, this deliberate absence of active host buffering acts as a highly sensitive ecological stress test, unmasking intrinsic metabolic bottlenecks often obscured by host factors until clinical failure occurs. Second, applying a standardized mucin-priming medium to ensure massive parallelization led to a progressive decline in baseline *Prevotella* spp. abundance (Fig. 1). Nevertheless, this trade-off did not compromise the reliable tracking of SCFA flux. Finally, the screening was restricted to a Japanese cohort. Nevertheless, given the established functional redundancy where core SCFA metabolic pathways are highly conserved across diverse human populations^{53,54}, the physicochemical constraints identified here (e.g., pH-driven metabolic stall) likely represent universal thermodynamic thresholds transcending baseline enterotypes. Although prospective in vivo validation is ultimately required to confirm these individualized metabolic trajectories, the S-KUHIMM functions as a reliable translational screening platform. By translating silent heterogeneity into predictable functional ecotypes prior to clinical interventions, this platform paves the way for mechanism-driven precision nutrition.

Methods

Fecal sampling and preparation

Fecal samples were collected from a cohort of 52 healthy Japanese volunteers (25 men and 27 women; mean age 36.8 ± 17.3 years, ranging from 6 to 78 years). The inclusion criteria were having no history of gastrointestinal disorders, no antibiotic usage within the preceding three months, and the provision of normal stool (excluding diarrheal or abnormal consistencies). Furthermore, while strict dietary restrictions were not imposed to reflect real-world physiological baselines, participants were required to maintain their habitual diet and normal physical condition, specifically avoiding excessive alcohol consumption, binge eating, or the unhabitual intake of fermented foods and prebiotics prior to sampling. Specific exclusion criteria encompassed: a history of severe hepatic, renal, or cardiovascular diseases; metabolic or systemic conditions (e.g., severe obesity, hypertension, diabetes), autoimmune diseases, or neurodegenerative diseases; acute gastrointestinal infections (e.g., norovirus or enterohemorrhagic *Escherichia coli*) within the preceding three months; pregnancy; and any other conditions deemed

medically inappropriate by the principal investigator. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Shiba Palace Clinic (Tokyo, Japan) (Approval No. 159797_rn-40835). Written informed consent was obtained from all adult participants. For minor participants (under 18 years of age), written informed consent was obtained from their parents or legal guardians. All fecal samples and corresponding metadata were strictly de-identified and coded prior to analysis to protect participant privacy. Samples were collected using Violamo Sample Collection Cups (VIOLAMO, Osaka, Japan) and kept under anaerobic conditions using the Anaeroback Kenki system (Sugiyama Gen, Tokyo, Japan). The entire process from defecation to the initiation of in vitro cultivation was strictly completed within 48 h. All samples were processed inside a Vinyl Anaerobic Chamber (Coy Laboratory Products, Inc., Grass Lake, MI, USA; Type B) or a BACTRON300-J anaerobic workstation (SHELDON Manufacturing, Inc., Cornelius, OR, USA) to preserve microbial viability.

In vitro fermentation platform (S-KUHIMM)

The fermentation system, the S-KUHIMM, was adapted and miniaturized from the validated 100 mL-scale batch fermentation model (KUHIMM) operated exactly as previously described^{19,20}. Briefly, the original KUHIMM platform utilized a jar fermenter (100 mL working volume) to simulate the human colonic environment without pH control, establishing a high-fidelity model for evaluating microbial metabolic shifts. To achieve high-throughput evaluation while maintaining physiological relevance, the system was scaled down to a 96-well deep-well collection plate format (Simport Scientific, Beloeil, Canada; well volume, 2.2 mL). The basal medium consisted of modified GAM broth (Shimadzu Diagnostics Corporation, Kyoto, Japan). To prevent the thermal degradation of glycoproteins during autoclaving, porcine mucin (Mucin from Porcine Stomach, Type III; Sigma-Aldrich, St. Louis, MO, USA) was suspended in sterile phosphate-buffered saline and microscopically screened for microbial contamination. This aseptically prepared mucin suspension was added to the basal medium immediately prior to cultivation to achieve a final concentration of 0.8% (w/v), thereby mimicking the mucosal environment without compromising structural integrity. The cultivation process involved two distinct phases with a total incubation time of 72 h. First, for the mucin-priming step, each well contained 5 μ L of 20% (w/v) fecal slurry in GAM broth and 995 μ L of mucin-supplemented basal medium, corresponding to a final fecal concentration of 0.1% (w/v) and an initial culture volume of 1.0 mL. The plates were covered with a silicon mat lid (Simport Scientific) to prevent

evaporation and incubated at 37 °C for 24 h under a gas atmosphere of 85% N₂, 10% CO₂, and 5% H₂ (Kobe Sanso Co., Ltd., Kobe, Japan). Following this stabilization phase, the test substrates, prepared by dissolving or suspending the ingredients in the Second Fluid for Disintegration Test (pH 6.8; Japanese Pharmacopoeia), purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), were added to each well at their respective predefined final concentrations. Matched vehicle controls (100 µL of the Second Fluid for Disintegration Test) were added at identical volumes to the control wells. Fermentation was continued for an additional 48 h using an Incubator Shaker DWMax M BR-034P (TAITEC, Koshigaya, Japan) agitated at 800 rpm. Each donor–condition combination was cultured in a single technical replicate (n = 1). Plate-position randomization was not implemented. Upon completion of the 72-h cultivation, the pH of the cultures was monitored using a Compact pH Meter LAQUAtwin-pH-22B (HORIBA, Kyoto, Japan).

Quality control of porcine mucin

To minimize potential experimental artifacts arising from batch-to-batch variations of natural porcine gastric mucin (Type III), standardized lot-screening procedures were implemented. First, all mucin batches were microscopically screened to check for microbial contamination. Second, each new mucin lot was empirically pre-validated using a fecal inoculum from a pre-selected, highly stable reference donor to confirm consistent baseline SCFA production and pH dynamics. As our primary phenotypic stratifications rely on the magnitude of change (Δ values) driven by the consistent fermentation kinetics of added substrates, any minor residual baseline fluctuations arising from mucin microheterogeneity are effectively normalized within our donor-matched experimental design.

Test substrates and functional ingredients

A total of 177 functional ingredients were evaluated to determine their impact on the human gut microbiota. The specific sources of the key substrates were as follows: erythritol (FUJIFILM Wako Pure Chemical Corporation), D-mannose (Nacalai Tesque, Inc., Kyoto, Japan), inulin (Orafti® GR; DKSH Japan, Tokyo, Japan), xylitol (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), RS (High-amylose maize starch; Sigma-Aldrich, St. Louis, MO, USA) and RMD (Fibersol-2; Matsutani Chemical Industry Co., Ltd., Itami, Japan). All test substrates, including complex polysaccharides, sugar alcohols, amino acids, and commercial food powders or mixtures, were uniformly supplemented at a standard concentration of 0.3% (w/v or v/v). To ensure physiological

relevance and prevent non-specific osmotic or toxic stress, the supplementation levels for specific micronutrients and inorganic components were rigorously standardized across the library. Vitamins were added at 10-fold their respective recommended dietary allowance equivalent concentrations. Trace minerals were uniformly adjusted to a final concentration of 20 μ M. Organic acids alongside general inorganic salts were standardized to a final concentration of 20 mM. To protect ongoing patent applications and proprietary formulations, the chemical identities of the remaining functional ingredients are represented by masked administrative codes (e.g., KULFFI identifiers) corresponding to the management numbers in the dataset. The comprehensive physicochemical and categorical metadata for all 177 substrates, including nutritional classifications, degrees of polymerization or molecular weights, and purities are detailed in Supplementary Data 5. Detailed information regarding these proprietary materials is available from the corresponding author upon reasonable request under a confidentiality agreement.

Microbial community analysis

Total genomic DNA was extracted using the Maxwell[®] RSC Fecal Microbiome DNA Kit (Promega, Madison, WI, USA) on a Maxwell[®] RSC Instrument (Promega; AS4500, Software Version 1.2.0, Revision 8) after mechanical lysis with Zirconia Balls (ϕ 0.1 mm, NIKKATO, Osaka, Japan) using a FastPrep 24 Instrument version 4.0 (MP Biomedicals, Santa Ana, CA, USA; 6004-500). DNA concentrations were measured using a Multiskan SkyHigh microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Although metabolic profiling (SCFA and pH) was performed for all 52 donors, 16S rRNA gene amplicon sequencing data were analyzed for a subset of 48 donors. Four donors were excluded from the microbiome community analysis due to insufficient sample volume (HS-07, HS-51, and HS-52) or PCR amplification failure (HS-34). Consequently, ecological metrics presented in Supplementary Fig. 4 (and subsequent microbiome analyses) represent $n = 48$ biologically independent donors.

16S rRNA sequencing and bioinformatic processing

The 16S rRNA gene libraries were prepared following the Illumina 16S Metagenomic Sequencing Library Preparation guide (Part #15044223 Rev. B). The V3–V4 hypervariable regions were amplified using KAPA HiFi HotStart ReadyMix (Roche Sequencing Store / Kapa Biosystems, Wilmington, MA, USA). The universal primers 341F (5'-

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-
 3') and 805R (5'-
 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTA
 ATCC-3'), containing Illumina adapter overhang sequences, were used for the initial
 PCR. Thermal cycling was performed on an Eppendorf Mastercycler® X50s PCR
 Thermal Cycler (Eppendorf SE, Hamburg, Germany). Following the first stage of PCR,
 the amplicons were purified, and dual indices were attached using the Nextera XT Index
 Kit (Illumina, San Diego, CA, USA) in a second index PCR step. The final libraries
 were pooled in equimolar concentrations and sequenced on the Illumina MiSeq platform
 (Illumina) using 2 × 300 bp paired-end chemistry.

Sequence data were processed using the standard QIIME 2 (version 2025.7)⁵⁵
 and DADA2⁵⁶ with default parameters to generate Amplicon Sequence Variants,
 consistent with our previously validated methodology¹⁹. Taxonomy was assigned using
 the SILVA database (release 138.2)⁵⁷. Statistical analyses, including FDR-corrected *q*-
 values (Benjamini–Hochberg method) and Pearson correlation coefficients (*r*), were
 executed in Python or R (version 4.3.2).

qPCR analysis

To determine the absolute abundance of target bacterial taxa, qPCR was performed
 using the TB Green Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio, Shiga, Japan) on a
 QuantStudio 3 Real-Time PCR System (QuantStudio Design & Analysis Software
 v1.5.2) (Thermo Fisher Scientific). DNA templates were extracted from the culture
 supernatants using the Maxwell RSC Instrument (Promega, Madison) and the
 concentration was adjusted where necessary. Reaction mixtures (final volume 10 µL or
 20 µL) were prepared using the Maxprep Liquid Handler (Promega, Software Version
 3.0.3, Revision 51, Platform Version 2.6.0) to ensure high technical reproducibility.
 Thermal cycling conditions consisted of an initial denaturation at 95 °C for 30 s,
 followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s (or the specific annealing
 temperature optimized for each primer set). Melting curve analysis was conducted
 following the amplification cycles to confirm the specificity of the amplicons. Absolute
 quantification was achieved using the standard curve method. Standard curves were
 generated using a ten-fold serial dilution of plasmid DNA containing the target
 sequences or genomic DNA of known concentration from the corresponding type
 strains. All qPCR assays were performed in a single technical replicate, and analytical
 runs were exclusively validated when standard curves exhibited a coefficient of
 determination (R^2) > 0.99. The results were expressed as Log10 copies/mL of the

culture medium. Target-specific primers used in this study, including those for total bacteria, *Bifidobacterium* spp., *Bacteroides* spp., *Faecalibacterium* spp., *Akkermansia* spp., *Blautia* spp., *Butyricicoccus* spp., and *A. caccae*, are summarized in Supplementary Table 1^{58–65}.

Given the inherent limitations of 16S rRNA-targeted primers in universally encompassing highly diverse or recently expanded taxonomic clades, we strategically employed established species-specific amplicons as reliable ecological proxies for broader genus-level dynamics. For *Blautia* spp., owing to the absence of a perfectly specific pan-genus primer lacking cross-reactivity with other Lachnospiraceae, we utilized previously validated primers explicitly targeting its two dominant human gut representatives (*Blautia wexlerae* and *Blautia luti*)⁶³. Similarly, for *Faecalibacterium* spp. and *Akkermansia* spp., we employed primers originally designed for *Faecalibacterium prausnitzii*⁶¹ and *Akkermansia muciniphila*⁶², respectively. As these foundational species historically defined their genera and fundamentally dominate their respective niches in the human gut microbiota, their targeted quantification provides a high-fidelity proxy for monitoring global genus-level population shifts within the S-KUHIMM platform without compromising phylogenetic stringency.

For the precise tracking of cross-feeding networks involving lactate/acetate-utilizing butyrate producers, we specifically targeted *A. caccae*. This focused selection was empirically driven by our 16S rRNA sequencing data, which identified *A. caccae* as the functionally dominant responder within its genus (> 84% relative dominance among assigned species). To ensure high specificity and complete methodological transparency, we utilized the previously validated species-specific primers for *A. caccae*⁶⁵.

SCFA analysis

Organic acids (succinate, lactate, formate, acetate, propionate, and butyrate) were quantified using ultra-performance liquid chromatography (Nexera Organic Acid Analysis System; Shimadzu Corporation, Kyoto, Japan), equipped with a CBM-40 system controller, CDD-10AVP conductivity detector, SIL-40C autosampler, CTO-40C column oven, and LabSolutions Version 5.110. Cultures were centrifuged at 4 °C using an Allegra V-15R Benchtop Refrigerated Centrifuge (Beckman Coulter, Brea, CA, USA; V-15R). Supernatants were filtered through a FastRemover MF (GL Sciences, Tokyo, Japan) or a Mini-UniPrep 0.2 µm PTFE filter (Cytiva, Marlborough, MA, USA). Separation was achieved on a Shim-pack Fast-OA column (Shimadzu Corporation) at 40 °C using p-toluenesulfonic acid as the mobile phase and Bis-

Tris/EDTA for pH buffering (Reagents kit for Organic Acid Analysis; Shimadzu Corporation). Quantification was performed using standard curves generated from analytical-grade standards of each organic acid.

Statistical analysis

Statistical analyses and visualizations were performed using custom Python 3 scripts executed in a Jupyter Notebook environment (Google Colaboratory), R (version 4.3.2)⁶⁶, and GraphPad Prism 10. The net increase (Delta, Δ) of SCFA concentrations and microbial counts was calculated by subtracting the baseline values (mucin-only control) from the values after substrate intervention. These Δ values inherently reflect the net balance between primary metabolite formation and cross-feeding consumption over the fermentation period, providing a robust endpoint evaluation of community metabolic shifts rather than continuous kinetic fluxes.

For two-group comparisons of continuous variables, the two-sided Mann–Whitney U test (for independent samples) or the two-sided Wilcoxon signed-rank test (for paired samples) was employed. Differences among three or more groups (e.g., ecological stratification tiers) were evaluated using the Kruskal–Wallis test, followed by Dunn’s multiple comparisons test. To establish these ecological stratification tiers for balanced multi-group comparisons, the cohort was partitioned into approximate numerical thirds (tertiles) based on the continuous distribution of ΔpH during inulin fermentation (severe: $\Delta\text{pH} \leq -0.80$, moderate: $-0.80 < \Delta\text{pH} < -0.44$, mild: $\Delta\text{pH} \geq -0.44$). Relationships between continuous variables (e.g., metabolic output, microbial expansion, and pH dynamics) were assessed using Pearson’s correlation coefficients (r) or Spearman’s rank correlation coefficients (ρ) as appropriate. To rigorously control for Type I errors across large-scale screenings (e.g., 177 ingredients, 16S rRNA genus-level profiling), raw P values were adjusted using the Benjamini–Hochberg FDR method⁶⁷. An FDR-corrected q -value < 0.05 was considered statistically significant unless otherwise stated. Taxa with $P < 0.05$ are listed.

To maintain strict scientific integrity, missing values or qPCR measurements below the detection limit (undetermined) were explicitly excluded strictly pairwise from correlational analyses. Arbitrary data imputation (e.g., zero-imputation or mean substitution) was strictly prohibited to prevent the introduction of mathematical artifacts.

To estimate the ETP, a segmented (piecewise) linear regression model was applied to the ΔpH and $\Delta\text{butyrate}$ dataset. Non-linear optimization methods (e.g., Levenberg–Marquardt) are susceptible to being trapped in local minima (e.g., near ΔpH

= -0.34). To rigorously identify the true global minimum, we performed optimization across a comprehensive grid of biologically plausible initial bounds (ranging from -1.5 to -0.1). The optimal breakpoint (estimated at $\Delta\text{pH} = -0.81$ for inulin) was identified by minimizing the sum of squared residuals, ensuring that the optimization is strictly data-driven and independent of initial parameter assumptions. Furthermore, to evaluate the stability and uncertainty of this estimate against inter-individual biological variation, a non-parametric bootstrap resampling procedure (n=1,000 iterations) was performed to calculate the 95% confidence interval. Because individual estimates contain inherent variance, the global ecological stability margin was ultimately defined by integrating the convergence across 5 distinct bifidogenic substrates including inulin.

Data availability

The 16S rRNA gene sequencing data generated in this study have been deposited in the DDBJ Sequence Read Archive under accession code PRJDB42381 (DRR1058518-DRR1059258). Source data for all figures and the supplementary table are provided with this paper. Additionally, the processed source data (including absolute quantitative values of SCFAs, pH, and qPCR measurements) required to reproduce the statistical analyses and figures are available in the GitHub/Zenodo repository (DOI: 10.5281/zenodo.21128628).

Code availability

The custom Python and R scripts used for the S-KUHIMM data integration, stratification analysis (including cumulative responder rate calculations), and visualization are available at GitHub (https://github.com/S-KUHIMM/S-KUHIMM_microbiome_analysis ver. 1.0.1) and permanently archived on Zenodo (DOI: 10.5281/zenodo.21784716). The repository includes a figure-to-script manifest, software environment specifications, and the input and output files required to reproduce each main and supplementary figure.

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Author contributions

D.S. and Y.M. conceived and designed the study. T.S. established the KULFFI library. D.S. and T.S. performed the in vitro fermentation experiments and biochemical analyses. D.S. conducted the bioinformatic and statistical analyses. A.K. supervised the

project and provided essential resources. D.S. wrote the manuscript. All authors discussed the results, reviewed, and approved the final version of the manuscript.

Competing interests

D.S. is employed by Bacchus-Bio innovation under a cross-appointment agreement and holds an academic position at Kobe University. A.K. is the Chief Executive Officer (CEO) of Bacchus-Bio innovation and serves as a Guest Professor at Kobe University.

All authors are inventors on pending patent applications concerning the S-KUHIMM platform (Application Nos. PCT/JP2024/040978, PCT/JP2024/040979, and PCT/JP2024/040980), which have been licensed to Bacchus-Bio innovation Co., Ltd.

Furthermore, D.S. and Y.M. are inventors on a pending patent application filed by Kobe University concerning the specific metabolic stratification and precision substrate optimization strategies reported in this manuscript.

Materials & Correspondence

Correspondence and requests for materials should be addressed to Daisuke Sasaki.

Figure Legends

Fig. 1 | Establishment and scalable miniaturization of the S-KUHIMM platform for high-fidelity gut microbiome remodeling. **a**, Schematic overview of the in vitro screening and evaluation strategy. **b**, Impact of basal media formulations on microbiome profile stability (Pearson correlation coefficient r of centered log-ratio [CLR]-transformed genus-level abundances versus 0 h native feces, top) and total bacterial load at 48 h (bottom). **c**, Effect of medium supplements on ecological stability (r ; top) and alpha diversity (maximum Shannon index; bottom) at 48 h. The optimized condition (S-KUHIMM, 0.8% mucin) was compared against the baseline KUHIMM (Gifu Anaerobic Medium [GAM], modified GAM [mGAM], Reinforced Clostridial Medium [RCM], Yeast extract–Casitone–Fatty Acid [YCFA], supplemented Tryptic Soy Broth [sTSB], Brain Heart Infusion [BHI], Tryptic Soy Broth [TSB], Nutrient Broth [NB], de Man–Rogosa–Sharpe [MRS], and Todd–Hewitt Broth [THB]). **d**, Time-course community stability over 72 h evaluating conventional (200 μ L), scaled-up (500 μ L), and mucin-primed (S-KUHIMM) inoculation strategies. **e**, Relative abundances of key taxonomic guilds at 72 h across distinct inoculation strategies relative to the original fecal inoculum. **f**, Temporal tracking accuracy (r) over 96 h demonstrating the equivalence of the 96-well microplate format (Incubators A and B) against the conventional 100 mL jar system. **g**, Functional and ecological equivalence validation of the miniaturized 96-well system at 72 h, comparing Shannon index, total bacterial load, total short-chain fatty acid production, and pH against those of the jar system, which was used as a benchmark. For all panels, $n = 6$ biologically independent human donors per group. In all box plots (**b–e**, **g**), the central horizontal line indicates the median, box limits represent the upper and lower quartiles (25th and 75th percentiles), and whiskers extend to $1.5 \times$ the interquartile range. Individual data points are overlaid. For line plots (**f**), data are the mean $\pm$ s.d. Statistical significance was determined using two-sided Wilcoxon signed-rank tests (**b–e**) or two-sided Mann–Whitney U tests (**g**). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ns, not significant. All P values are nominal and were calculated without adjustment for multiple comparisons.

Fig. 2 | High-throughput metabolic screening platform and ecosystem reproducibility. **a**, Schematic representation of the S-KUHIMM/KULFFI platform. The high-throughput in vitro ecosystem model utilizes a 96-well anaerobic cultivation system to screen 177 functional ingredients across 52 human fecal donors. **b**, Global metabolic landscape and analytical reproducibility. Box plots illustrating the

concentrations of major short-chain fatty acids (acetate, propionate, butyrate) and total organic acids produced across the 177 tested ingredients. The coefficient of variation (CV) for the unsupplemented control group is shown to evaluate analytical platform stability. **c**, Microbiome profile stability and ingredient-specific perturbation. Pearson correlation coefficients (r) of centered log-ratio (CLR)-transformed genus-level abundances between the in vitro cultured ecosystems and their respective native inoculum feces. Data are shown for the unsupplemented control and representative functional ingredients (resistant maltodextrin, resistant starch, D-mannose, inulin, xylitol, and erythritol). For the metabolic profiling (**b**), $n = 52$ biologically independent human donors were analyzed per ingredient. For the microbiome community analysis (**c**), $n = 48$ biologically independent human donors were evaluated. In all box plots (**b**, **c**), the central horizontal line indicates the median, box limits represent the upper and lower quartiles (25th and 75th percentiles), and whiskers extend to $1.5 \times$ the interquartile range. Individual data points are overlaid.

Fig. 3 | Inter-individual metabolic stratification and the quantitative association between *Bacteroides* spp. expansion and propionate production. **a**, Heatmap showing the absolute increment (Δ mM) of propionate production across 52 healthy human donors in response to six representative functional ingredients after 72 h in vitro cultivation. **b**, Overview of the propionate response magnitude across the evaluated ingredients. Gray and colored points indicate non-significant and significant responses relative to the control, respectively. **c–e**, Waterfall plots illustrating the ranked inter-individual heterogeneity of propionate production (Δ mM) in response to resistant maltodextrin (RMD) (**c**), resistant starch (RS) (**d**), and D-mannose (**e**). **f**, Specific expansion of *Bacteroides* spp. Box plots display the absolute abundance change (Δ Log10 copies/mL) of *Bacteroides* spp. following cultivation with RMD, RS, and D-mannose. **g–i**, Scatter plots demonstrating the strong quantitative association between the dynamic expansion of *Bacteroides* spp. (Δ Log10 copies/mL) and propionate increment (Δ mM) for RMD (**g**), RS (**h**), and D-mannose (**i**). For all panels, $n = 52$ biologically independent human donors were analyzed. In the box plots (**f**), the central horizontal line indicates the median, box limits represent the upper and lower quartiles (25th and 75th percentiles), and whiskers extend to $1.5 \times$ the interquartile range, with individual data points overlaid. Statistical significance was determined using two-sided Wilcoxon signed-rank tests followed by Benjamini–Hochberg false discovery rate (FDR) correction (**b**, **f**). Asterisks denote statistical significance versus the baseline control ($*q < 0.05$, $**q < 0.01$). For the scatter plots (**g–i**), solid lines represent the

linear regression fit. Statistical significance was evaluated using Pearson's correlation coefficient (r), and q denotes the FDR-corrected q -value across the multiple comparisons.

Fig. 4 | Global metabolic landscape of SCFA induction and component-specific microbial expansions driven by KULFFI. **a, b**, Hierarchical clustering heatmaps detailing the absolute increment (Δ mM) of acetate (**a**) and butyrate (**b**) production across 52 healthy human donors following 72 h in vitro cultivation with 177 distinct functional ingredients. The ingredients are categorized based on their nutritional classification. **c, d**, Volcano plots providing a global overview of the effect size (Log2 fold change) and statistical significance ($-\text{Log}_{10}$ q -value) for acetate (**c**) and butyrate (**d**) induction across the evaluated ingredients. Gray and colored points indicate non-significant and significant responses relative to the unsupplemented control, respectively. **e**, Waterfall plot demonstrating the ranked inter-individual heterogeneity of butyrate production (Δ mM) in response to inulin supplementation. **f**, Specific expansion of key taxonomic groups. Box plots display the absolute abundance change (ΔLog_{10} copies/mL) of representative bacterial taxa following cultivation with inulin. **g–i**, Scatter plots illustrating the strong quantitative associations between *Bifidobacterium* spp. expansion and acetate increment (**g**), *Faecalibacterium* spp. expansion and butyrate increment (**h**), and the inter-metabolite correlation between acetate and butyrate production (**i**) induced by inulin. For all panels, $n = 52$ biologically independent human donors were analyzed per group. In the heatmaps (**a, b**), statistical significance was determined using two-sided paired t -tests followed by Benjamini–Hochberg false discovery rate (FDR) correction, with significance defined as $q < 0.05$. In the volcano plots (**c, d**) and box plots (**f**), statistical significance was assessed using two-sided Wilcoxon signed-rank tests followed by FDR correction. In the box plots (**f**), the central horizontal line indicates the median, box limits represent the upper and lower quartiles (25th and 75th percentiles), and whiskers extend to $1.5 \times$ the interquartile range, with individual data points overlaid. Asterisks denote statistical significance versus the baseline control ($**q < 0.01$, $***q < 0.001$). For the scatter plots (**g–i**), solid lines represent the linear regression fit. Statistical significance was evaluated using Pearson's correlation coefficient (r), and q denotes the FDR-corrected q -value.

Fig. 5 | Stoichiometric cross-feeding networks and dynamic taxonomic shifts determine personalized butyrate production. **a**, Inter-individual variability in butyrate production from fermentation with resistant starch (RS) and sugar alcohols

following S-KUHIMM. **b–d**, Relationships between acetate increment (Δ acetate) and butyrate increment (Δ butyrate). Sugar alcohols exhibit a strong negative correlation, demonstrating a direct stoichiometric metabolic “baton pass” (**c, d**), whereas RS shows no such simple linear correlation, reflecting a more complex cross-feeding network (**b**). **e**, Significant increases in specific butyrate-producing taxa following substrate interventions. **f, g**, Lack of correlation between the absolute increase of specific taxa and total butyrate production for *Butyricicoccus* spp. in RS (**f**) and *Anaerostipes caccae* in erythritol (**g**). **h, i**, Correlation between the dynamic shift of targeted taxa and Δ butyrate. The panels show the lack of predictive value for baseline (Initial) and post-treatment metrics, whereas the dynamic shift (Δ) strongly correlates with butyrate output for *Anaerostipes* spp. (%) in the xylitol intervention (**h**) and *Butyricicoccus* spp. (Log10 copies/mL) in the erythritol intervention (**i**). For the metabolic profiling (**a–d**), $n = 52$ biologically independent human donors were analyzed. For 16S rRNA gene sequencing data (**h**), analysis was performed on $n = 48$ independent donors. For qPCR and correlation analyses of specific low-abundance taxa (**e–g, i**), samples with undetectable targets at either baseline or post-treatment were strictly excluded to prevent artificial zero-imputation, resulting in adjusted sample sizes (*Butyricicoccus* spp., $n = 44$; *A. caccae*, $n = 39$). In the box plots (**a, e**), the central horizontal line indicates the median, box limits represent the upper and lower quartiles (25th and 75th percentiles), and whiskers extend to $1.5 \times$ the interquartile range, with individual data points overlaid. Statistical significance for box plots was determined using two-sided Wilcoxon signed-rank tests followed by Benjamini–Hochberg false discovery rate (FDR) correction ($*q < 0.05$, $**q < 0.01$, $***q < 0.001$). For scatter plots (**b–d, f–i**), the solid lines indicate linear regression, and shaded areas represent 95% confidence intervals. Statistical significance for correlations was evaluated using Pearson’s correlation coefficient (r), followed by FDR correction (q -values).

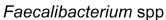
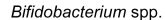
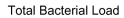
Fig. 6 | Universal pH-induced metabolic stall and ecological stratification. a, Functional decoupling across 177 ingredients. Scatter plot of median absolute *Bifidobacterium* spp. expansion (Δ Log10 copies/mL) versus median butyrate production (Δ butyrate, mM). Highlighted substrates (inulin, purple; RS, cyan; xylitol, green; erythritol, red) demonstrate distinct decoupling behaviors. **b**, pH-driven functional decoupling during inulin fermentation. Scatter plot of Δ butyrate versus Δ Log10 *Bifidobacterium* spp. across 52 donors. Points colored by final pH (blue, highly acidic; red, mildly acidic) indicate butyrate suppression at low pH despite substantial *Bifidobacterium* spp. expansion. **c**, Universal ecological tipping point for functional

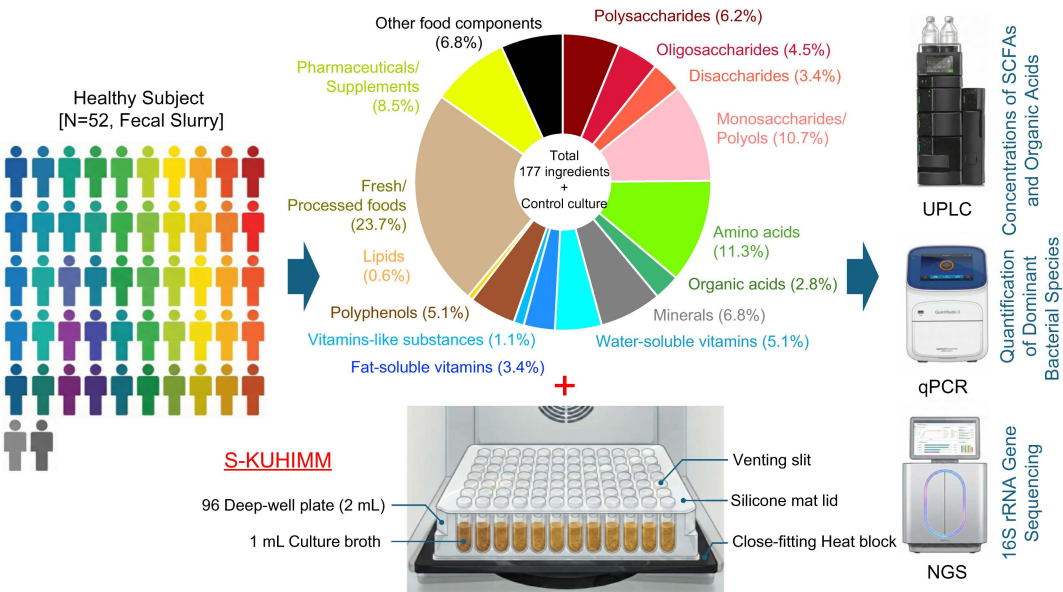
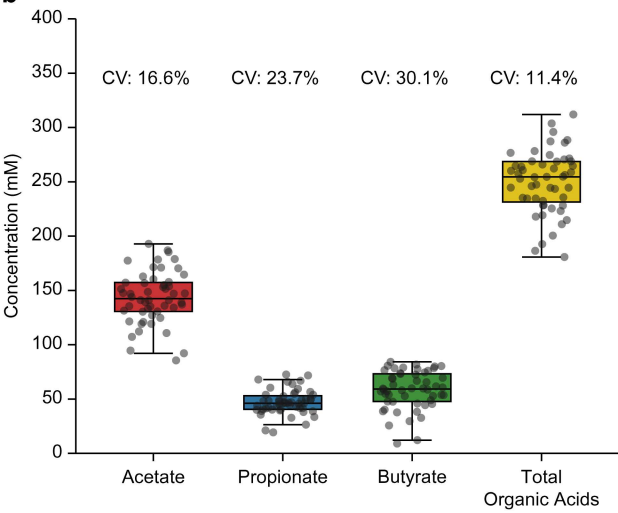
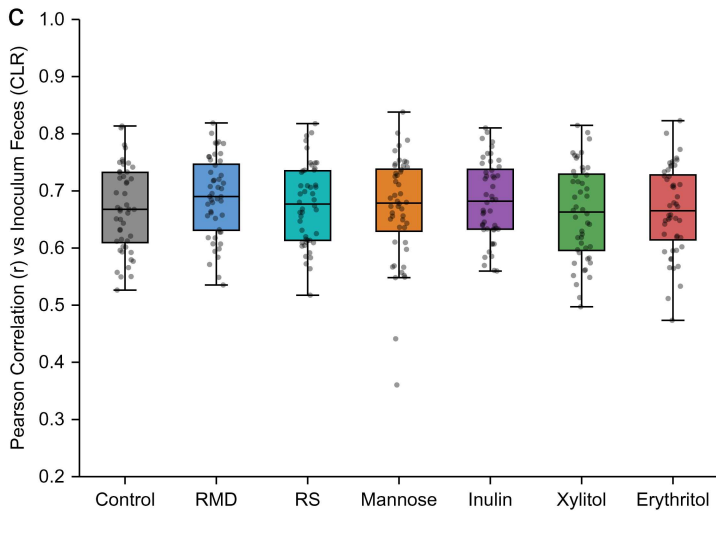
decoupling. Pairwise distribution of ΔpH and $\Delta\text{butyrate}$ across donor-substrate combinations reveals a consistent ecological tipping point where rapid acidification causes metabolic stagnation. **d**, Macroscopic landscape of ecosystem trajectories. Median ΔpH versus median $\Delta\text{butyrate}$ for 177 substrates. **e**, Ecological stratification of donors based on buffering capacity. Waterfall plot of ΔpH following inulin fermentation across 52 donors, sorted by acidification magnitude. Dashed lines indicate predefined thresholds for ecotype classification based on tertile distribution: severe ($\Delta\text{pH} \leq -0.80$, blue), moderate ($-0.80 < \Delta\text{pH} < -0.44$, grey), and mild ($\Delta\text{pH} \geq -0.44$, red). **f**, pH-dependent metabolic stall in inulin fermentation. Pearson correlation between ΔpH and $\Delta\text{butyrate}$ under inulin treatment. **g–j**, Ecological mechanisms of functional decoupling. Comparison of $\Delta\text{butyrate}$ (**g**), $\Delta\text{acetate}$ (**h**), ΔLog10 *Bifidobacterium* spp. (**i**), and ΔLog10 *Faecalibacterium* spp. (**j**) across the three ecotypes. Sample sizes in **g–j**: severe ($n = 17$), moderate ($n = 17$), mild ($n = 18$). For other panels, $n = 52$ biologically independent donors were analyzed. Error bars in **d** represent the interquartile range (IQR). For **f**, the solid line indicates linear regression; significance was evaluated via Pearson's correlation (r) and false discovery rate (FDR) correction (q). In the box plots (**g–j**), the central horizontal line indicates the median, box limits represent the upper and lower quartiles (25th and 75th percentiles), and whiskers extend to $1.5 \times$ the interquartile range, with individual data points overlaid. Significance between ecotypes was determined via two-sided Mann–Whitney U tests with FDR correction (* $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$).

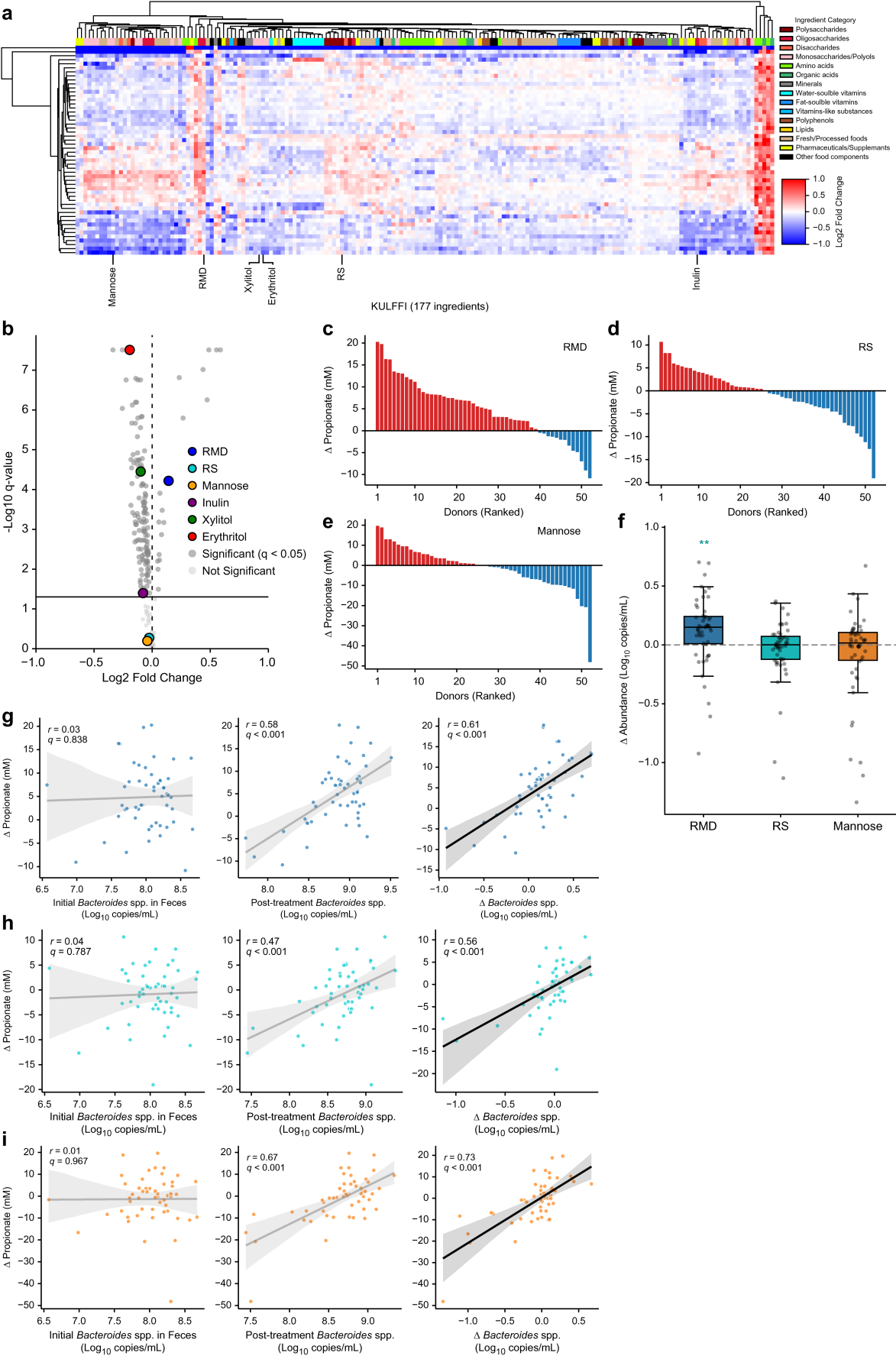
Fig. 7 | Ecological stratification and precision substrate optimization based on the metabolic tipping point. **a**, Segmented (piecewise) linear regression analysis estimating the ecological tipping point (ETP) for inulin across the donor cohort. The critical mathematical breakpoint where the butyrogenic flux collapses (the acid-driven stall) is identified at $\Delta\text{pH} = -0.81$ (dashed red line). **b**, Inter-individual metabolic heterogeneity and trajectory of butyrate production ($\Delta\text{butyrate}$) across four representative substrates (inulin, resistant starch [RS], xylitol, and erythritol). Donors are stratified into three distinct baseline ecotypes based on their inulin-induced acidification: mild ($\Delta\text{pH} \geq -0.44$, red), moderate ($-0.80 < \Delta\text{pH} < -0.44$, grey), and severe ($\Delta\text{pH} \leq -0.80$, blue). Thin lines represent individual donor trajectories, and diamond markers signify the median values for each ecotype. **c**, Stability–efficacy quadrant plots for substrate-specific interventions. The vertical blue dashed line represents the stability margin defined by the ETP ($\Delta\text{pH} = -0.80$), and the horizontal red solid line represents the efficacy threshold ($\Delta\text{butyrate} = 5.0$ mM). Donors are

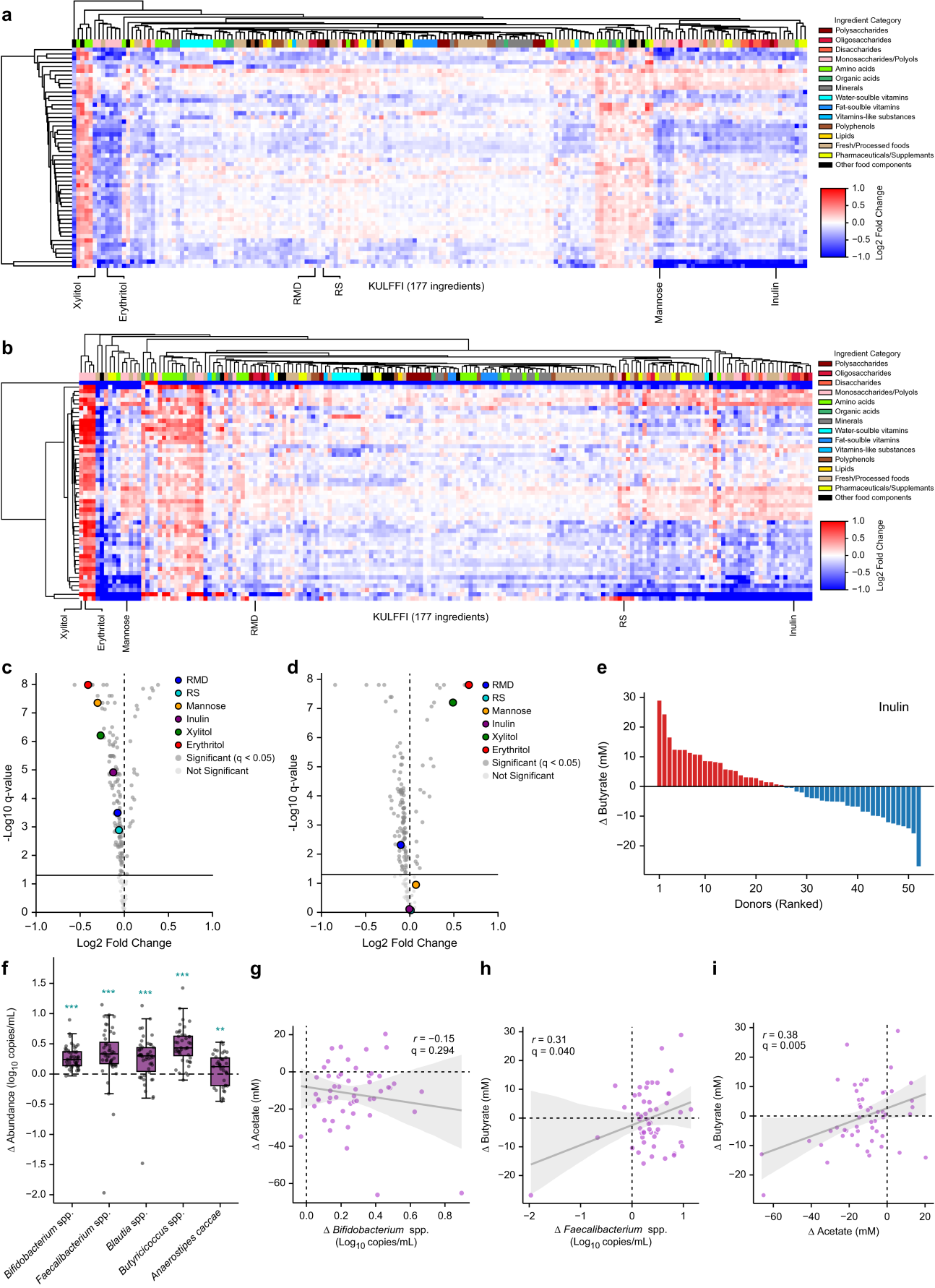
categorized as current responders (filled circles, target zone), prior responders (gray circles, compensated by preceding substrates), or non-responders (black crosses). **d**, Precision stratification framework output illustrating the sequential substrate allocation designed to maximize individual responder rates. Donors are grouped by their optimally assigned substrate (inulin, RS, xylitol, erythritol, or non-responder) and ranked by their maximized Δ butyrate production capacity. Bars are color-coded by the baseline inulin-induced ecotype (mild, red; moderate, grey; severe, blue). Numbers in parentheses indicate the count and percentage of the total donor population assigned to each category. For the segmented regression analysis (**a**) and the total cohort representations (**c**, **d**), $n = 52$ biologically independent human donors were analyzed. The sample sizes for the ecotype groups defined in **b** are severe ($n = 17$), moderate ($n = 17$), and mild ($n = 18$).

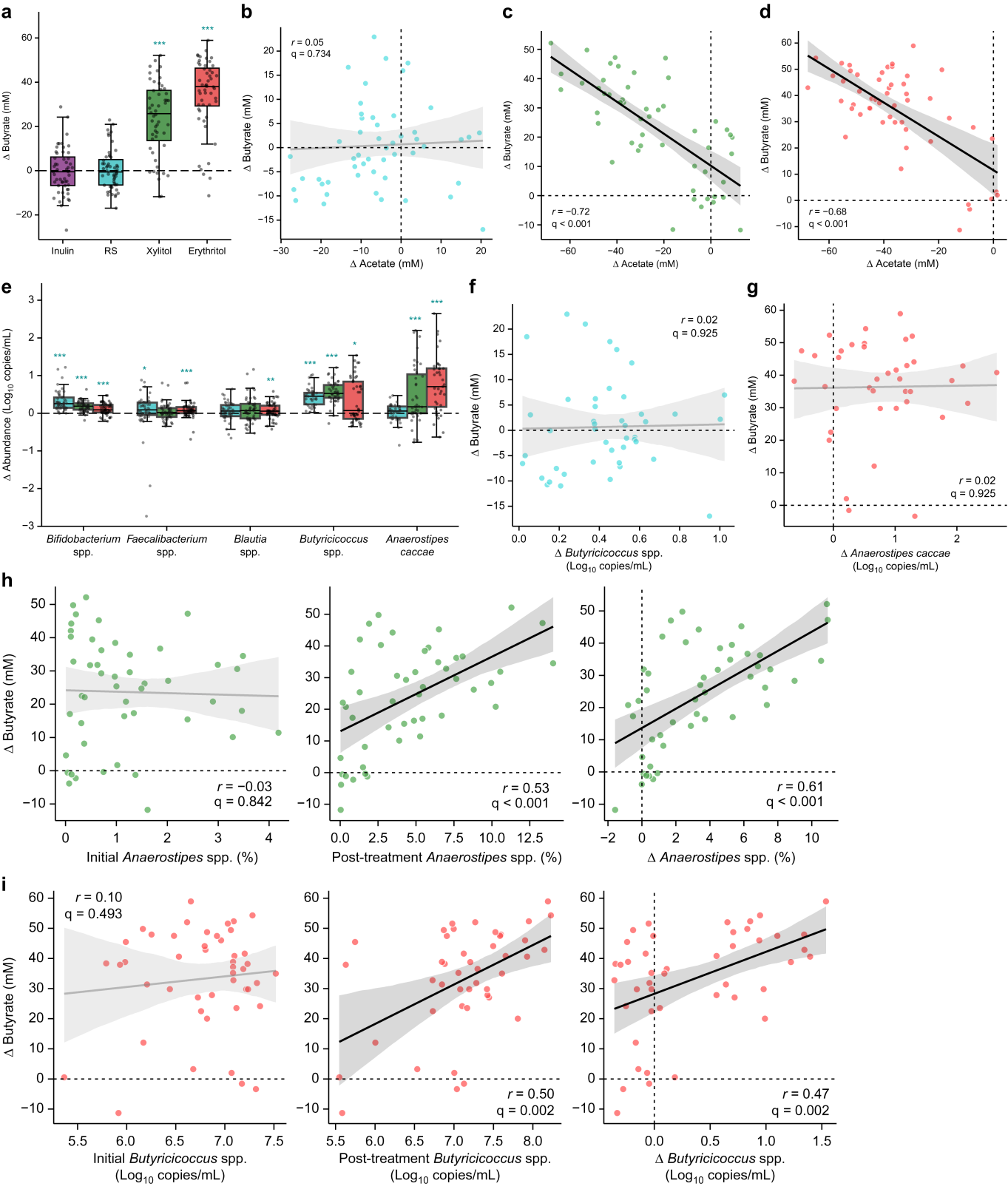
Fig. 8 | Stepwise precision nutrition strategy based on S-KUHIMM-driven ecological stratification. Metabolic route of propionate-driven expansion. The framework stratifies donors based on the proliferative capacity of the *Bacteroides* spp. guild (Δ *Bacteroides* spp. expansion). Responders exhibit specific guild enrichment leading to propionate production (≥ 5.0 mM), whereas non-responders lack this specific proliferation (silent), necessitating the screening of alternative propionate-driving substrates via the S-KUHIMM candidate selection process. Butyrate-driving metabolic route. Donors are stratified based on the ecological tipping point (ETP; Δ pH). Successful cross-feeding between *Bifidobacterium* spp. and *Faecalibacterium* spp. results in butyrate production (≥ 5.0 mM). Conversely, severe acidifiers (excessive acidification) experience functional decoupling and metabolic stall (Δ pH ≤ -0.80), triggering a precision optimization step. This step involves switching to alternative bifidogenic substrates that bypass rapid acidification (e.g., via slow fermentation) to unlock butyrate production.s

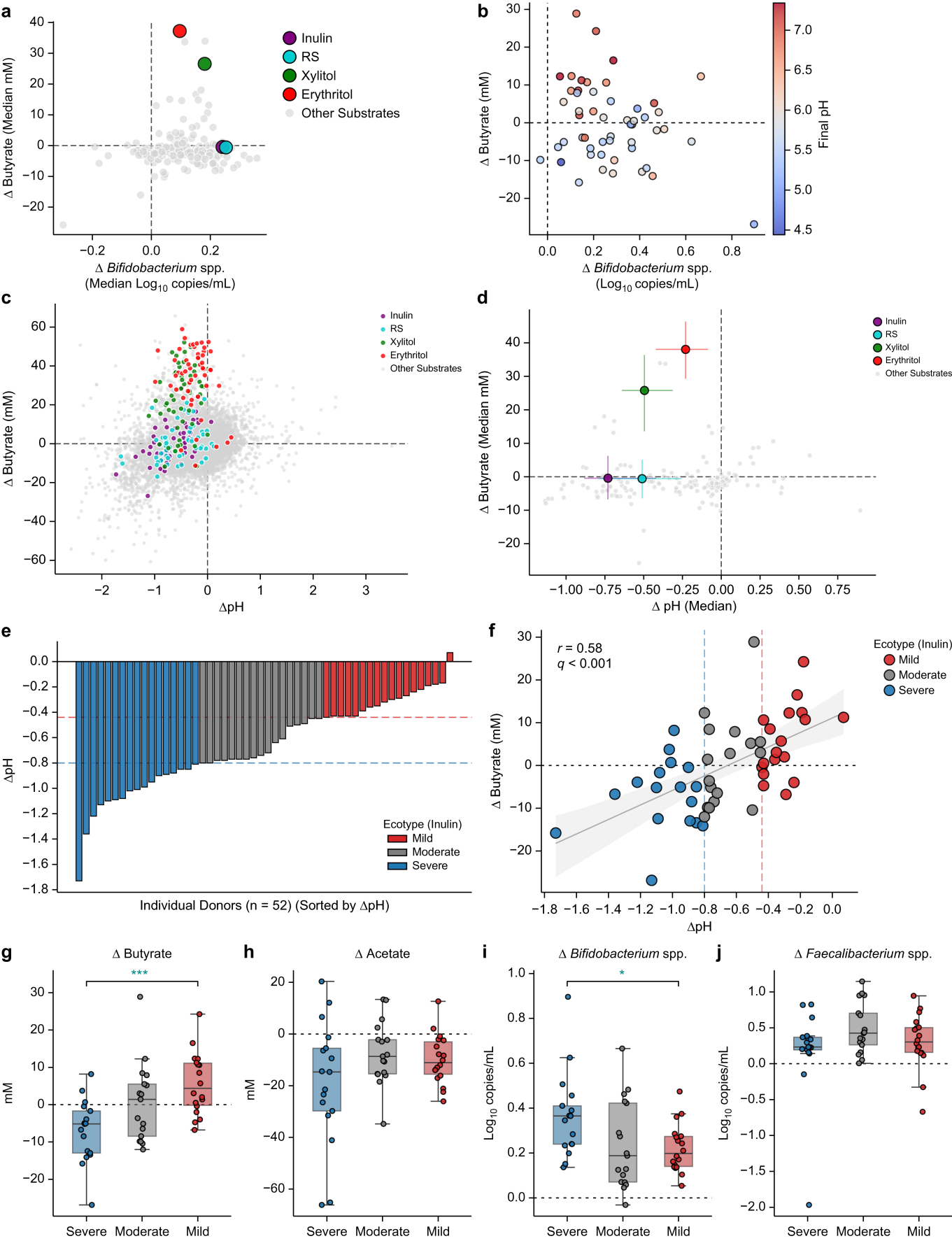


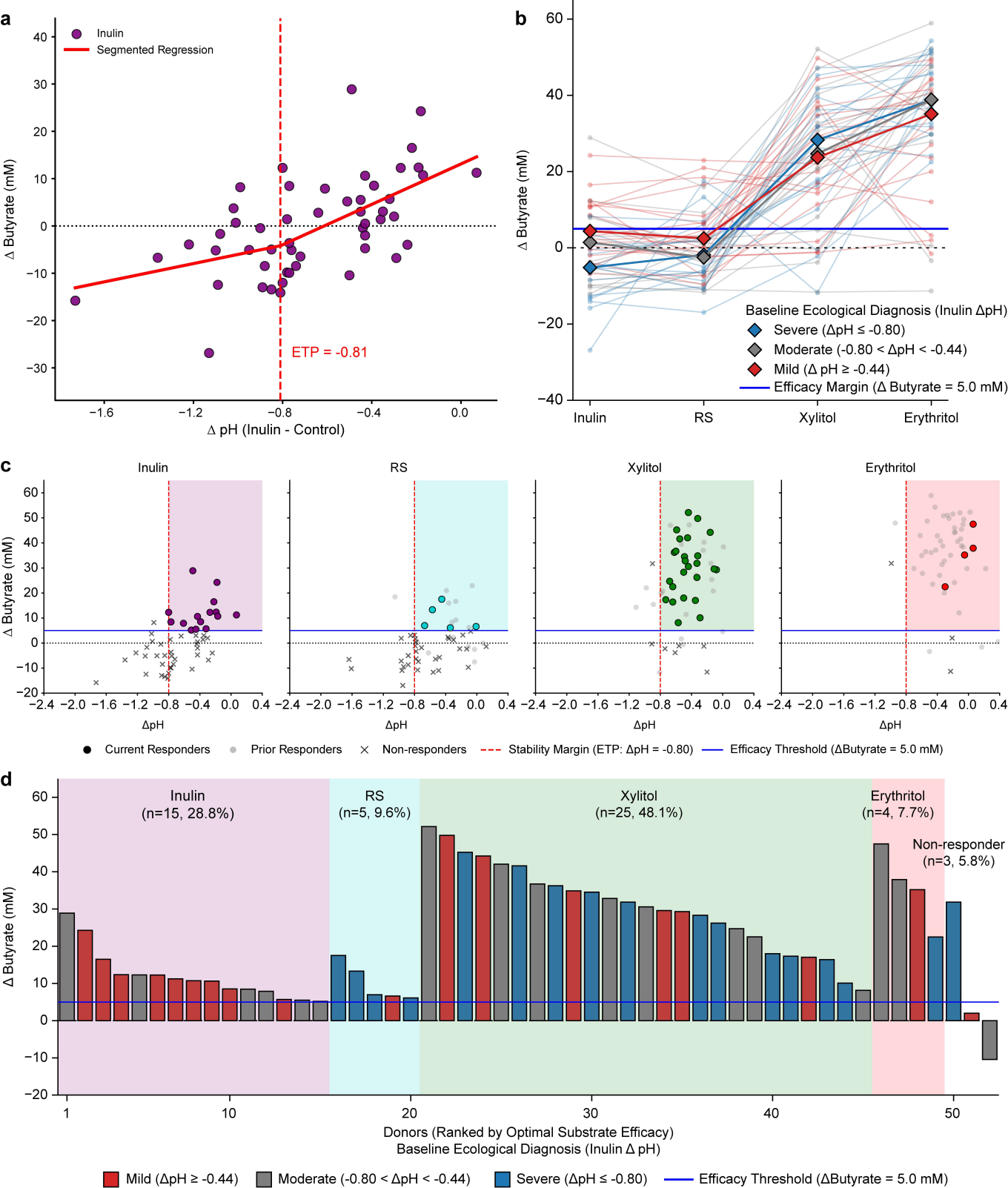
a**b****c**











Target Substrate
(e.g., Prebiotics, Probiotics, Synbiotics, Functional foods, Therapeutics, Herbal medicines, Uncharacterized ingredients)

S-KUHIMM Platform

Dynamic Ecological Stratification

Propionate-driving Route

e.g., RMD, D-Mannose ...

Stratification Marker : Δ Bacteroides spp. expansion

Target output : Δ Propionate ≥ 5 mM

Responder



↑ *Bacteroides* spp.

Non-responder



→ *Bacteroides* spp.

+ Propionate production
(≥ 5 mM)

- Silent heterogeneity

[Optimization Step]

Precision Substrate Allocation

Screening alternative propionate-driving substrates to match individual microbiota.

Butyrate-driving Route

First-line: bifidogenic substrates, e.g., inulin

Stratification Marker : Δ pH ≥ -0.8 (ETP)

Target output : Δ Butyrate ≥ 5 mM

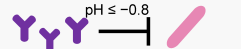
Responder



Bifidobacterium spp. *Faecalibacterium* spp.

+ Butyrate production
(≥ 5 mM)

Non-responder



Bifidobacterium spp. *Faecalibacterium* spp.

- Functional decoupling

[Optimization Step]

Precision Substrate Allocation

Switch to other bifidogenic substrates and sequential switch to niche-specific drivers / Bypassing rapid acidification via slow fermenting

Supplementary Files

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