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METHOD

Following the community development of SIHUMIx – a new intestinal *in vitro* model for bioreactor use

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ABSTRACT

Diverse intestinal microbiota is frequently used in *in vitro* bioreactor models to study the effects of diet, chemical contaminations, or medication. However, the reproducible cultivation of fecal microbiota is challenging and the resultant communities behave highly dynamic. To approach the issue of reproducibility in *in vitro* models, we established an intestinal microbiota model community of reduced complexity, SIHUMIx, as a valuable model for *in vitro* use.

The development of the SIHUMIx community was monitored over time with methods covering the cellular and the molecular level. We used microbial flow cytometry, intact protein profiling and terminal restriction fragment length polymorphism analysis to assess community structure. In parallel, we analyzed the functional level by targeted analysis of short-chain fatty acids and untargeted metabolomics. The stability properties constancy, resistance, and resilience were approached both on the structural and functional level of the community. We show that the SIHUMIx community is highly reproducible and constant since day 5 of cultivation. Furthermore, SIHUMIx has the ability to resist and recover from a pulsed perturbation, with changes in community structure recovered earlier than functional changes.

Since community structure and function changed divergently, both levels need to be monitored at the same time to gain a full overview of the community development. All five methods are highly suitable to follow the community dynamics of SIHUMIx and indicated stability on day five. This makes SIHUMIx a suitable *in vitro* model to investigate the effects of e.g. medical, chemical, or dietary interventions.

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Introduction

The human intestinal microbiota is essential to human health and is even considered as an extra organ, as it provides enzymes for nutrient breakdown and produces essential nutrients, such as short-chain fatty acids (SCFA) and vitamins.[1–3] It was also shown that the intestinal microbiota modulates the immune system.[4,5] Due to the importance of the intestinal microbiota to human health and well-being, the effects of environmental stress on the intestinal microbiota can have serious consequences. For example, pesticides, but also other chemicals, like perfluoroalkyl acids or plasticizers, mainly enter the human body via the oral route thus potentially affecting the intestinal microbiota.[6,7]

Testing the impact of environmental influences (e.g., pesticides, nutrients) on the intestinal microbiota cannot be accomplished with the required throughput in animal models due to ethical and practical reasons. Thus, the development of suitable models is essential. The cultivation of intestinal bacteria under gut-like conditions is a relevant approach to gain insight into the bacterial response to environmental influences. The effect upon various treatments, e.g. of dietary compounds, the impact of pathogenic microorganisms on the intestinal microbiota or the response to chemicals has already been investigated in

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in vitro bioreactor systems.[8,9] Most often fecal bacteria are cultivated in these bioreactor systems.[8–11] However, true experimental replication is hard to achieve due to the intra-individual and longitudinal heterogeneity of fecal samples.[12] Additionally, a variety of methods, like denaturing gradient gel electrophoresis (DGGE),[13] short chain fatty acid[14] and next-generation 16S rRNA sequencing analysis,[10] have been used to define constant *in vitro* community states, when the community remains unchanged (often referred to as stable state). This state is essential to start the treatment, since a starting point is a pivotal element for the experimental set up.[15,16] Depending on the method different results regarding the time of cultivation to reach such a state have been obtained.[16,17] Proceeding from this and from an ecological point of view the term *stability* is ambiguous.[18] Grimm and Wissel (1997) point out that *stability* itself is not a stability property and therefore extracted main stability properties.[19] Some of these are *constancy, resilience* and *resistance*. E.g., (1) *constancy* means that a system remains essentially unchanged, (2) *resistance* resembles the ability of a system to remain unchanged despite a perturbation and (3) *resilience* describes the ability of a system to recover to a reference state after a perturbation.[19]

To circumvent challenges regarding the community reproducibility and the identification of suitable states to introduce a treatment, we established the extended simplified human microbiota (SIHUMIx) as a model community for *in vitro* use. SIHUMIx comprises of eight bacterial species, *Anaerostipes caccae, Bifidobacterium longum, Bacteroides thetaiotaomicron, Blautia producta, Clostridium butyricum, Clostridium ramosum, Escherichia coli* and *Lactobacillus plantarum*, and covers the genera *Firmicutes, Bacteroidetes* and *Proteobacteria* that are dominant in human feces.[20] We tracked the dynamics during community adaptation with a multi-method approach, which combined standard fingerprinting and OMICs techniques. Methods like terminal restriction fragment length polymorphism[21] analysis, flow cytometric fingerprinting[22] and short chain fatty acid analysis[23] are widely applied in microbial community characterization. Moreover, we evaluated untargeted metabolomics and intact protein profiling,[24] as these methods have the potential to depict community development on the metabolic and structural level, respectively, even though these methods have not been used to follow community development yet.

Acidification of the colon lumen by a decreased luminal pH resembles a severe disturbance and has been reported from patients with active ulcerative colitis.[25] For those reasons, we on the one hand cultivated the SIHUMIx community unimpededly, and on the other hand perturbed the community with a decrease of pH during cultivation. The aims of our study were to clarify if the SIHUMIx community (i) behaves in a reproducible way, (ii) establishes a constant state, (iii) is sensitive and responsive to perturbations, and (iv) if such a perturbation can be traced both on the structural (FC) and metabolic level (SCFA).

Results

Development of SIHUMIx under defined chemostat conditions

In order to follow the community dynamics of SIHUMIx, pre-cultivated bacteria were inoculated into three parallel bioreactors A, B and C (day 0) and grown on complex intestinal medium (CIM, supplemental file 1: Table S1) at a dilution rate of $D = 0.04\ d^{-1}$. After an initial establishing phase of 24 h (day 1) the continuous cultivation was started and community structure and function were monitored daily in a 24 h interval until day 7 (Figure 1a). Samples were taken for flow cytometric fingerprinting analysis (FC), intact protein profiling (IPP) and t-RFLP profiling, to study possible variations on the cell, protein, and DNA level from biomass, respectively. In addition, community function was assessed with untargeted metabolomics and short chain fatty acid (SCFA) analysis from the culture supernatant.

By non-metric multidimensional scaling (NMDS), we visualized upcoming community variations in the three parallel bioreactors (Figure 1b, Supplemental file 2: input data for NMDS analysis). All methods, FC (1), IPP (2), t-RFLP profiling (3), untargeted metabolome analysis (4), and SCFA analysis (5) revealed the same development pattern of the SIHUMIx community for all reactors. The community structure and function during the first two days of cultivation were similar

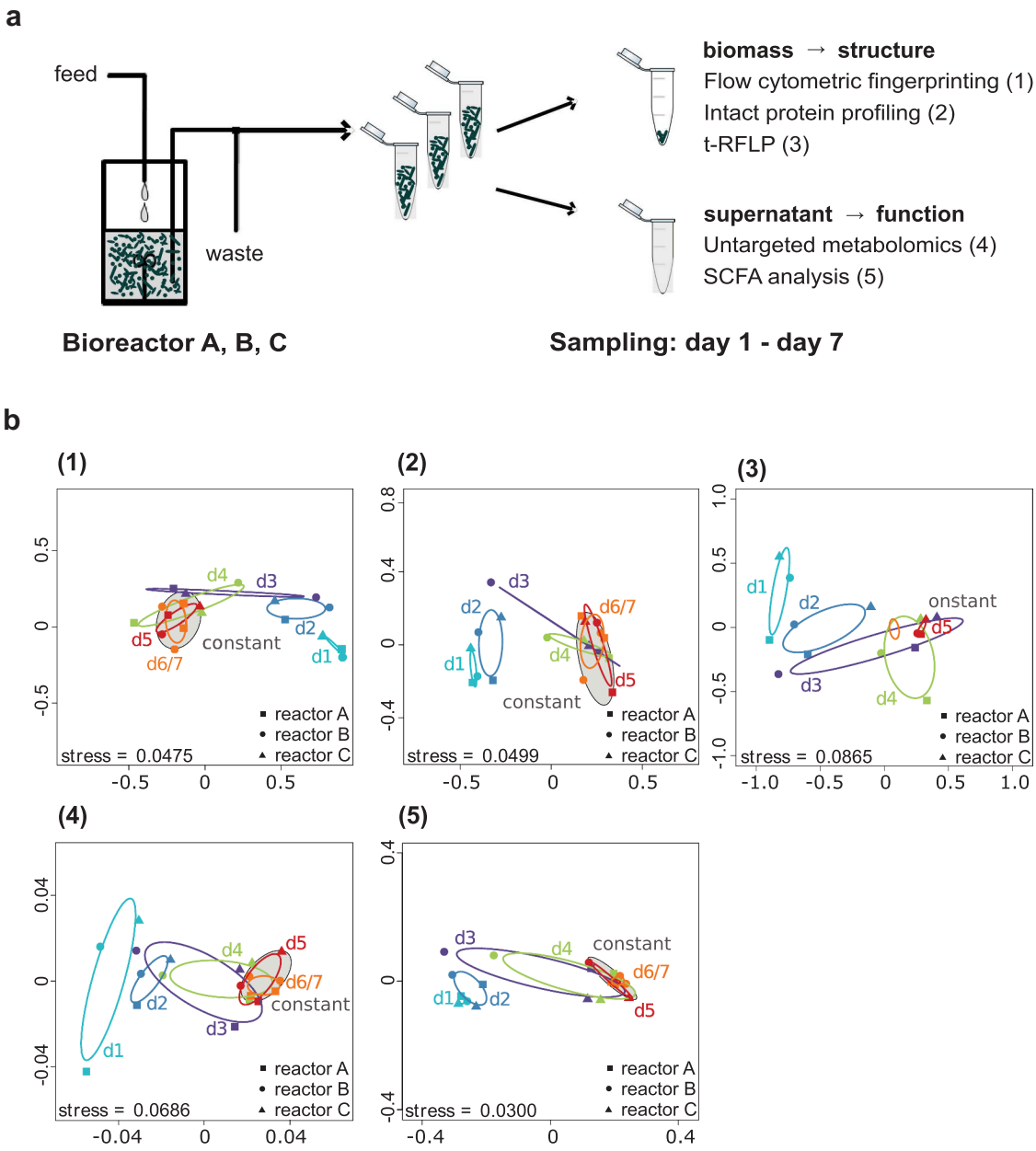

Figure 1. Experimental-set-up and development of the SIHUMIx community. (a) The SIHUMIx community was cultivated *in vitro*. After a sterile run the bioreactors A, B and C were inoculated (day 0) and after 24 h establishing time continuous cultivation started. Daily samples were taken from day 1 to day 7. Bacteria pellets were analyzed by flow cytometric fingerprinting (FC), t-RFLP and intact protein profiling (IPP) to investigate community structure. Culture supernatants were used for functional analysis namely SCFA analysis and untargeted metabolomics. (b) Community development was visualized by NMDS: (1) FC, (2) IPP, (3) t-RFLP, (4) SCFA, (5) untargeted metabolomics. Grouping was based on the day of cultivation.

and significantly different from the states of the communities at the end of the cultivation (p-value 0.03, pairwise Permanova, Supplemental file 1: Table S2, Table S3). The states of the communities on day 3 and day 4 lay between the states of the early communities and the late communities, thus mapping transitional states. Finally, the community on day 5 of all bioreactor replicates A, B and C was indistinguishable from the communities on day 6/7 (pairwise Permanova, Supplemental file 1: Table S2, Table S3).

For SCFA analysis, the concentrations of non-branched SCFA acetate, propionate, butyrate, valerate and caproate and the branched SCFA isobutyrate, isovalerate, isocaproate and 2-methyl

butyrate were measured. As implied from the NMDS plot (Figure 1b (4)), the absolute SCFA production changed notably during the time of cultivation (Figure 2b, supplemental file 1: Table S4). The dominating SCFA detected in the culture supernatant of SIHUMIx were acetate, propionate and butyrate. The acetate and butyrate concentrations increased, whereas the concentration of butyrate decreased during the time of cultivation. The concentration of 2-methylbutyate, isobutyrate and isovalerate also rose during the cultivation of SIHUMIx until day 7. Moreover, we observed an increasing total SCFA concentration during the time of cultivation. Clustering analysis based on the development of SCFA production revealed that data points related to the bioreactor samples clearly arranged in two major clusters with one cluster comprising all replicates A, B and C of day 1 and day 2 and the other cluster containing all replicates of day 5 and day 6/7 (Figure 2b). In the first days of cultivation, the communities produced above-average levels of acetate and butyrate and below-average levels of valerate, isobutyrate, propionate, 2-methyl-butyrate, and isovalerate. During the days 5, 6 and 7 of cultivation this SCFA pattern inversed. Caproate and isocaproate were not detected or below the detection limit. The SCFA production patterns of SIHUMIx was similar in all three bioreactors.

SIHUMIx shows a high reproducibility

To assess the reproducibility of the SIHUMIx community, two batches of single strain pre-cultures were used. Bioreactors A and B were inoculated with one and bioreactor C with the other batch of pre-cultures (day 0). During NMDS analysis Bray-Curtis (BC) distances were calculated for each method (Supplemental file 3: BC distance matrices). Based on the BC

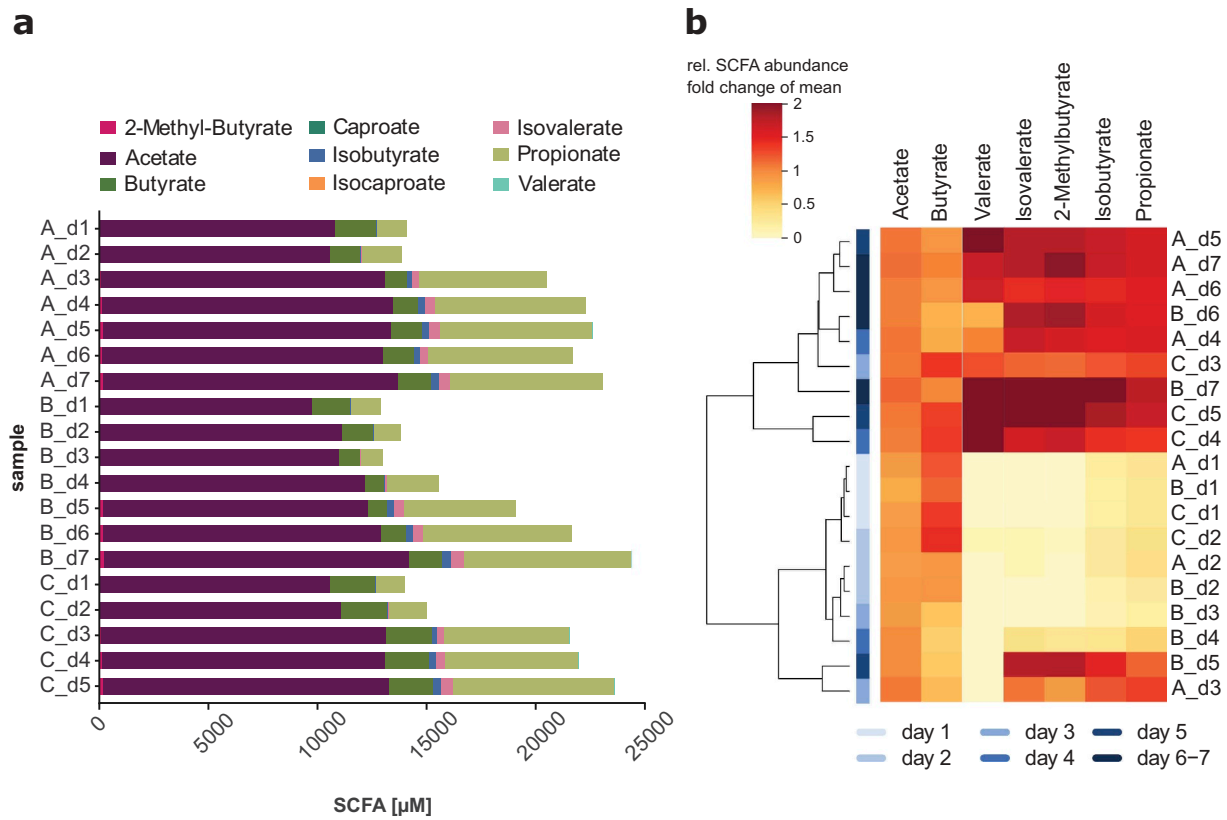

Figure 2. Short chain fatty acid (SCFA) production of SIHUMIx in three independent, parallel bioreactors A, B and C from day 1 to day 7. (a) The absolute SCFA concentrations were determined with targeted SCFA mass spectrometry metabolomics in the culture supernatant of SIHUMIx during cultivation. (b) Heatmap and hierarchical clustering of bioreactor samples based on the absolute SCFA production of SIHUMIx. Mean normalized absolute SCFA concentrations were used for hierarchical clustering. Above mean values are colored in red, mean values in orange and below mean values in yellow. The color code in hierarchical clustering analysis shows light blue color for early days with increasing saturation for later days of cultivation.

distances, the average BC similarity among replicates was determined after the initial establishing phase directly after inoculation (day 1) and at a later time point (day 5) (Figure 3a, Supplemental file 1: Table S5). For all the individual methods but t-RFLP (p-value <0.0001), the replicate BC similarity on day 1 and day 5 was very similar (two-way ANOVA, Supplemental file 1: Table S5). Structural analyses showed a lower BC similarity (FC: 0.89 and 0.93, IPP: 0.83 and 0.75, t-RFLP: 0.67 and 0.88) than metabolic analyses (SCFA: 0.98 and 0.89, untargeted metabolomics: 0.94 and 0.96) on day 1 and day 5, respectively.

The reproducibility of SIHUMIx was proved by metaproteomics and SCFA analysis. Microbial composition of SIHUMIx was analyzed based on the abundance of species-specific proteins.[26,27] The relative species abundances of SIHUMIx from bioreactor A and B on day 1 and day 5, respectively, were very similar (Figure 3b, Supplemental file 1: Table S6). The communities on day 1 mainly comprised of *E. coli, B. producta, A. caccae* and *B. thetaiotaomicron*. The remaining strains, *C. butyricum, C. ramosum, B. longum* and *L. plantarum*, contributed to less than 1% relative species abundance. On day 5 to day 7, the communities had changed considerably and comprised of more than ~60% *B. thetaiotaomicron*, ~10% *B. producta* and *E. coli* and ~2% *A. caccae*. Proteins of all other strains (*C. ramosum, C. butyricum, L. plantarum*, and *B. longum)* were detected, but they contributed to less than 2% species abundance (Supplemental file 1: Table S6). Furthermore, SCFA analysis revealed highly similar relative SCFA concentrations within the replicate bioreactors but the concentrations changed over time (Figure 3c). On day 1, the most abundant SCFAs in the supernatant were acetate,

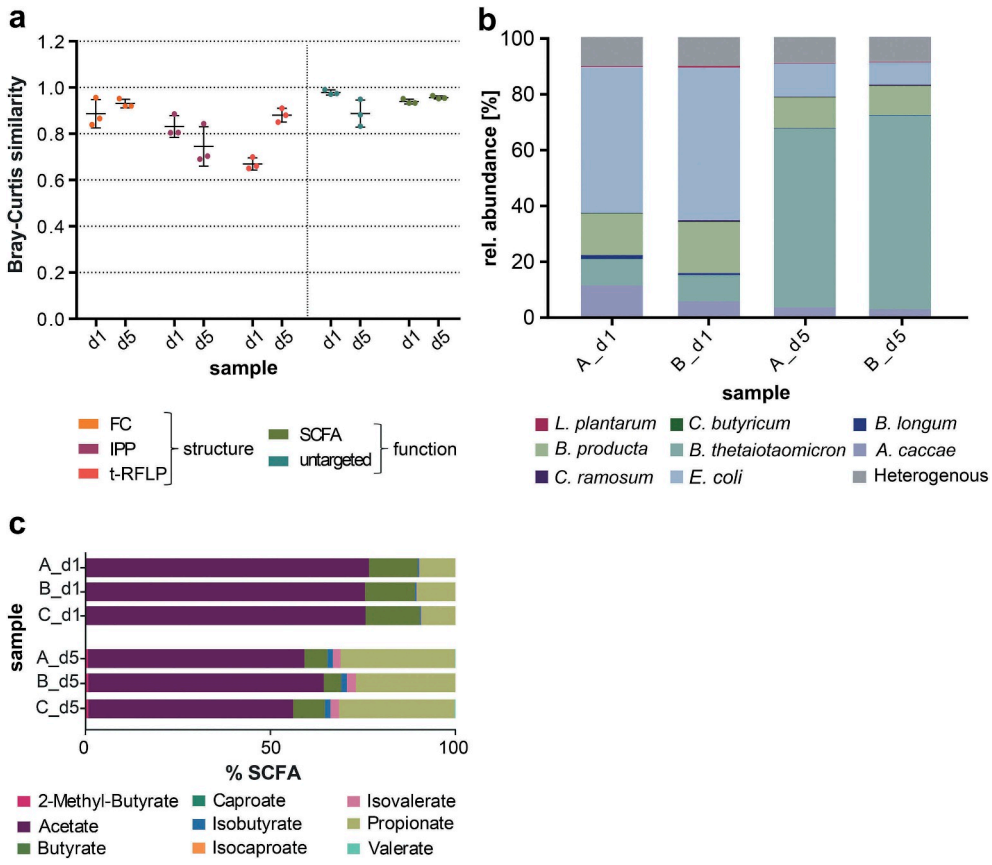

Figure 3. Reproducibility of SIHUMIx. (a) To analyze the reproducibility of SIHUMIx the Bray-Curtis (BC) similarity of bioreactor replicates A, B and C on day 1 and on day 5 (constant state) were determined. Calculations were based on different methods: flow cytometric fingerprinting analysis (FC), intact protein profiling (IPP), t-RFLP, SCFA analysis, and untargeted metabolomics. Data are shown as the mean ± standard deviation of triplicates. (b) Community structure was in detail analyzed by metaproteomics revealing the relative species abundances of the communities on day 1 and day 5 in bioreactors A and B. (c) SCFA production of the SIHUMIx communities was analyzed for the replicate bioreactors A, B and C on day 1 and day 5, respectively.

butyrate, and propionate in decreasing order. On day 5, the realtive SCFA composition was clearly different from that on day 1. The concentrations of acetate and butyrate decreased, but the concentration of propionate, isobutyrate, and isovalerate increased clearly on day 5 (Supplemental file 1: Table S7). Our results prove that SIHUMIx is a highly reproducible community in our bioreactor system, since community adaptation on taxonomy and SCFA levels were similar for all inocula.

SIHUMIx shows constant growth state starting on day 5

To evaluate the ability of SIHUMIx to reach a constant state, the Bray-Curtis (BC) distance values were used as described previously.[10] Therefore, the BC distances were calculated based on the analysis of cell abundances per cell population (technically termed as gate in FC method, Supplemental file 1: Figure S1), relative abundances of proteins, metabolites or SCFAs (IPP, untargeted and SCFA analysis, respectively) and relative abundance of terminal restriction fragments (t-RFLP, Supplemental file 3: BC distance matrices). The average BC similarity of all replicate samples (A, B, and C) compared to the replicates on days 6 and 7 (A and B) from day 1 until day 6 and 7 were visualized (Figure 4a). For all methods, the BC similarities followed the same trend: On day 1 and day 2, BC similarities were significantly lower compared to day 6/7 (pairwise PERMANOVA, p-values < 0.0001, Supplemental file 1: Table S8 and S9) but BC similarity increased steadily over time until day 5 (Supplemental file 1: Table S8 and S9). The average BC similarity on day 5 and day 6/7 compared to day 6/7 was

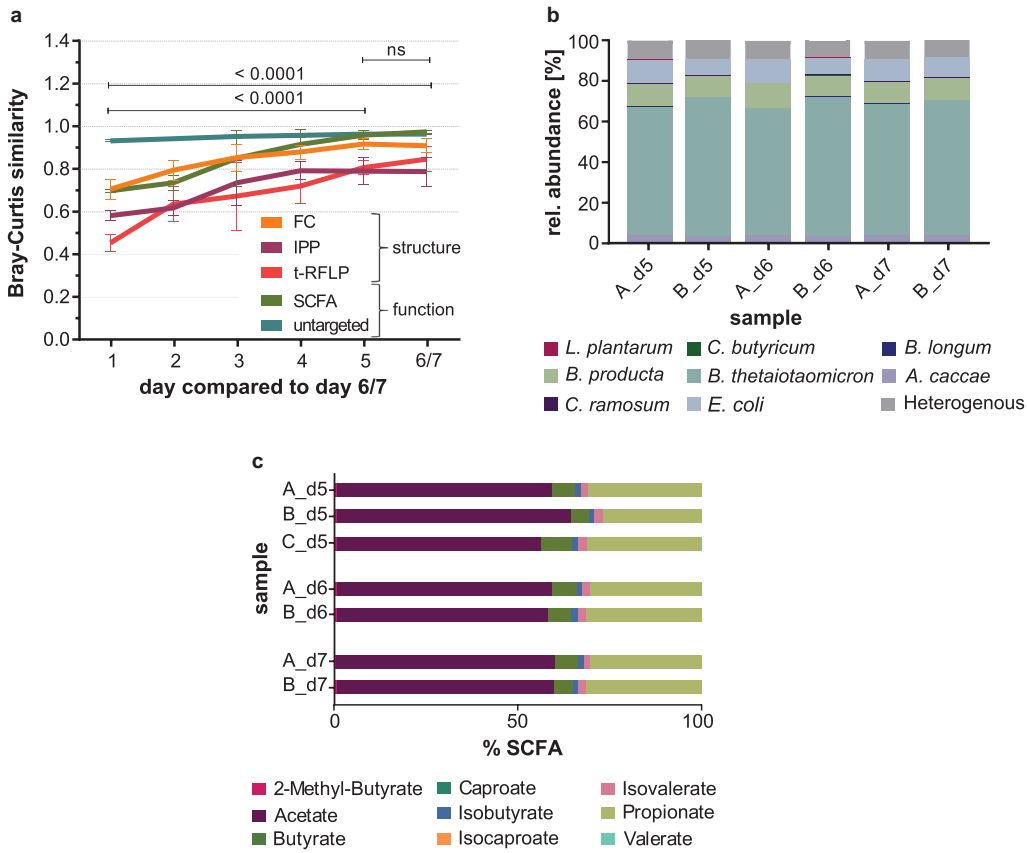

Figure 4. Development of SIHUMIx toward the constant state. (a) The average Bray-Curtis similarity (bioreactors A, B, C) on a given day of cultivation compared to the other bioreactors on day 6 and day 7 is shown for flow cytometric fingerprinting (FC), intact protein profiling (IPP), t-RFLP, SCFA analysis, and untargeted metabolomics. Data are shown as mean ± standard deviation. Comparison of community Bray-Curtis similarity by one-way ANOVA. (b) Species abundances in the SIHUMIx communities on day 5, day 6, and day 7 of bioreactors A and B based on metaproteomics. (c) Relative SCFA composition produced by the SIHUMIx communities in replicate bioreactors A, B and C on day 5, day 6, and day 7.

nearly identical and revealed community similarity on day 5. The BC similarity on day 5 compared to day 6/7 was as high as the replicate BC similarity on day 5, respectively (Supplemental file 1: Table S5). Constancy, respectively the structural and metabolic stability, of SIHUMIx was further analyzed by metaproteomics and SCFA profiles. On day 5, day 6, and day 7 the relative species abundances were very similar (Figure 4b) and SIHUMIx comprised mainly of *B. thetaiotaomicron, B. producta, E. coli* and *A. caccae*. The other four strains, *C. butyricum, C. ramosum, B. longum* and *L. plantarum*, contributed to less than 1% (Supplemental file 1: Table S6). SCFA analysis likewise revealed similar SCFA concentrations on day 5, day 6, and day 7 (Figure 4c). The major SCFAs in the supernatant were acetate, propionate, butyrate, isovalerate, and isobutyrate in decreasing order, whereas 2-methyl butyrate and isovalerate were produced only in very low concentrations (Supplemental file 1: Table S4 and Table S7).

SIHUMIx shows resistance and resilience after pulsed perturbation

Two bioreactors, D and E, were used to examine the dynamics of SIHUMIx when exposed to a pH-caused pulsed perturbation. Therefore, on day 4 the pH was set to 5.5 and reset to 6.5 after 24 h on day 5 (Figure 5a). Community dynamics were visualized in NMDS plots (Figure 5b) on the basis of (1) flow cytometric fingerprinting (FC) and (2) SCFA analysis that represent changes on the structural and functional level, respectively (Figure 5b). For comparison the constant state of SIHUMIx samples from bioreactors A, B, and C were included (gray ellipse, Figure 5b). The reference space defines the normal fluctuation of the constant SIHUMIx community and the threshold (tr, gray dashed line, Figure 5c) indicates the maximum fluctuation of SIHUMIx. The reference state resembles the center of the reference space and represents the mean of all samples used to build the reference space. Structural and functional deviations from the reference state in the disturbed bioreactors were followed. In addition, pH recording is shown to indicate the pH drop (red line). Maximal deviations were used to compute community resistance (RS) values. RS is the ability of a community to remain essentially unchanged in spite of a perturbation. Furthermore, resilience (RE) and elasticity (EL) were calculated. RE is the capability of a community to return to the reference state after a perturbation, and EL is the time it takes for a community to recover to the constant state.

After inoculation, the communities in bioreactors D and E were different compared to those from bioreactors A, B and C with regard to (1) community structure and (2) community function (Supplemental file: Figure S2). Nevertheless, these communities developed toward the constant state with respect to both community structure and function until day 3 (Figure 5b). This development was disturbed and immediately mirrored in structural and functional changes (Figure 5b,c). Generally, deviation values for the functional analysis were smaller than those for structural analysis. This became apparent in a smaller threshold value for the reference space (Figure 5c). Nevertheless, since the pH drop on day 5 community structure and function have been influenced

The community structure showed the strongest deviation from the reference state on day 5. The RS of bioreactor D was slightly higher than the RS of bioreactor E, with $RS_D = 0.64$ and $RS_E = 0.54$, respectively. After the pH drop, the community structure developed toward the constant state of the unimpeded community (ref. state) and the deviation values decreased. Samples from both bioreactors were fully recovered to the constant state on day 9, when the deviation values declined below the threshold. During the recovery process, the community in bioreactor E showed a similar resilience ($RL_E = 0.66$) compared to bioreactor D ($RL_D = 0.62$). The constant community state was kept until the end of the experiment on day 14. For bioreactor D the EL value was 0.018 d^{-1} and for bioreactor E 0.019 d^{-1}, respectively.

For community function, changes were also visible directly on day 5, but deviation values peaked on day 7. Here, the RS values were $RS_D = 0.78$ and $RS_E = 0.82$, respectively. After day 7 community functions developed toward the constant state. The community in bioreactor E recovered on day 14 and thus showed functional resilience ($RL_E = 0.60$) to a higher degree compared to the community in

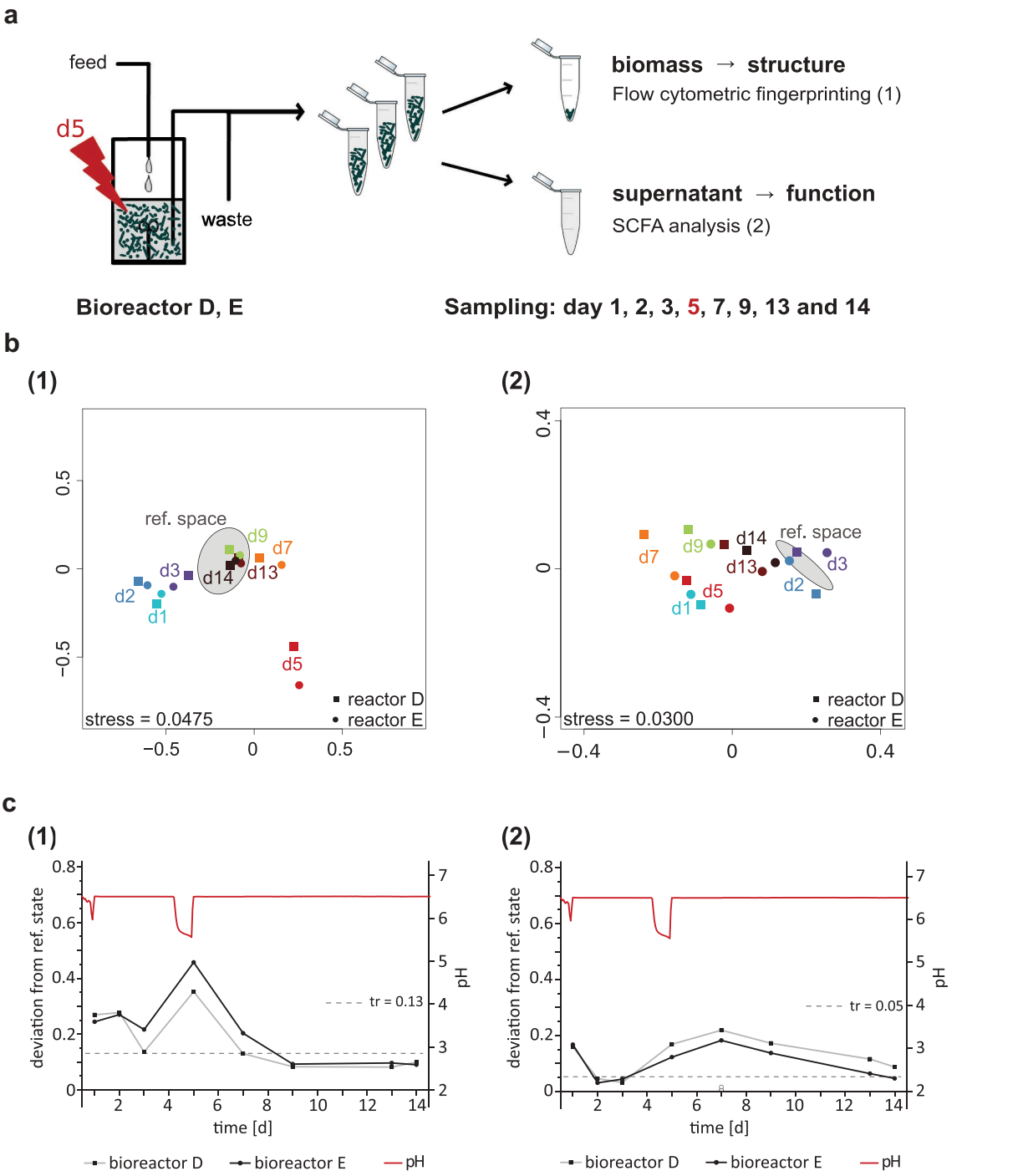

Figure 5. Experimental set-up and development of SIHUMIx before and after a pH drop. (a) The SIHUMIx community was cultivated *in vitro* and two replicate bioreactors D and E were inoculated (day 0). After 24 h the medium feed was turned on. On day 4, the pH was reduced from 6.5 to 5.5. After sampling on day 5, pH was reset to 6.5. Samples were taken on day 1, 2, 3, 5, 7, 9, 13 and 14. (b) Community structure was analyzed by (1) flow cytometric fingerprinting and community function by (2) SCFA analysis. The reference space of the SIHUMIx community resembles the unimpeded community of bioreactors A, B, C on days 5–7. (c) The deviation from the reference state (the center of the reference space) is shown for the communities in bioreactor D and E. The threshold of the reference space indicates the maximal deviation from the reference state in the constant community state. Communities with a deviation below the threshold are similar to the constant community, communities with above-threshold deviation are different from the constant community. The calculations are based on (1) flow cytometric fingerprinting analysis and (2) SCFA analysis.

bioreactor D (RL_D = 0.43). Bioreactor D did not recover until the end of the experiment on day 14. Compared to the recovery of the community structure, community function recovered at a slower velocity.

Discussion

Bioreactor systems are useful tools to investigate effects on the intestinal microbiota *in vitro*.[12,28] Most often complex fecal samples are used to inoculate bioreactors simulating the human intestine, but these complex inocula lack true biological replication.[12,16] It has recently been shown that despite the use of the same homogenous inoculum complex bacterial communities can develop toward completely different communities.[29] Besides, community structure can keep fluctuating over time in complex bacterial communities.[29,30] A variety of methods has been developed to analyze the community dynamics. In principle, the community structure and/or the community function are used to define an unchanged, constant state in complex communities. As *stability* is no stability property itself, we described the community development on the basis of the stability properties *constancy, resistance* and *resilience*.[19]

The major aim of this study was to establish an *in vitro* model community for bioreactor use that reproducibly develops toward a constant community state with regard to both community structure and function. Furthermore, we wanted to determine, if different methods similarly depict the community development. Therefore, we cultivated the SIHUMIx community under defined chemostat conditions and analyzed the community development on the structural and functional level with five different analytical methods.[20] Even though the SIHUMIx community comprised of only eight bacterial species SIHUMIx shares the major SCFA with complex fecal communities.[31] SIHUMIx produces high levels of acetate, propionate and butyrate and low levels of branched SCFA. Additionally, SIHUMIx covers the major phyla *Firmicutes, Bacteroidetes, Proteobacteria*, and *Actinobacteria*. Nevertheless contrasting fecal communities, the model community SIHUMIx is far less diverse. Under balanced state conditions, SIHUMIx was dominated by *B. thetaiotaomicron* (phylum *Bacteroides*). Besides, *E. coli* (phylum *Proteobacteria), B. producta* and *A. caccae* (phylum *Firmicutes*) were found in decreasing order with a relative species abundance >3%. The remaining bacterial strains were present with relative species abundances below 1%. Since we aimed to simulate the colonic environment as close as possible *in vitro*, we used a complex culture medium similar to other culture media used to cultivate fecal microbiota.[13,29,32] The culture medium contains mucin and since *B. thetaiotaomicron* is a known mucin degrader this species gains a growth advantage.[33] Microbial growth of the model community SIHUMI (SIHUMIx without *C. butyricum*) has been modeled in the absence and presence of mucin.[34] Bauer et al. show that in the presence of mucin *B. thetaiotaomicron* will be the dominant species in the community. Nevertheless, it has recently been shown that all SIHUMIx bacterial strains remain in the community during continuous cultivation of SIHUMIx for up to 14 days, even though four strains from the SIHUMIx community are low abundant with less than 1% relative species abundance [Schäpe et al. 2019 in *microorganisms*, accepted for publication].

With respect to community structure analyses, the range of methods used in bioreactor research is considerably large. Analyses like microbial flow cytometry (FC),[35] metaproteomics,[36] and DNA-based methods, such as 16S rRNA gene analysis, terminal restriction fragment length polymorphism (t-RFLP) analysis,[21] next-generation sequencing[10] or denaturing gradient gel electrophoresis (DGGE),[13] have been published. We wanted to analyze the community structure on different cellular levels and therefore applied FC, intact protein profiling (IPP) and t-RFLP to analyze the cell-, protein- and DNA-related structural development. T-RFLP profiling is frequently used to track community dynamics in bacterial communities and the choice of the target gene allows the analysis of distinct microbial domains or phyla.[37] FC has the ability to resolve changes down to the single cell level and is therefore highly sensitive. Furthermore, FC provides the option of next-generation 16S rRNA analysis after sorting of interesting cell populations.[38] In contrast to metaproteomics, the workflow of IPP is much faster and, even though no biological information is

generated, it provides a protein-based fingerprint.[39]

Community function of gut microbiota is routinely assessed using SCFA analysis and provides insights into the metabolic activity and thereby delivers specific information on the fermentation process.[16,17,40] In some studies, besides SCFA, other metabolites have been measured.[14,41] For that reason, we decided to include a more general approach in our analyses, specifically untargeted metabolomics.

To demonstrate the constant growth state of SIHUMIx, we compared the Bray-Curtis (BC) similarity of the communities on each day of cultivation to the communities on day 6/7. With proceeding adaptation, the BC similarity increased and reached a maximum on day 5. Then, the BC similarity on day 5 compare to day 6/7 was identical to the BC similarity of all samples on day 6/7. Thus, our results demonstrate that the SIHUMIx community starts to adapt directly after inoculation and then reaches a constant state with regard to both community structure (bacteria (FC), protein (IPP), and DNA (t-RFLP) composition) and community metabolism (SCFA analysis, untargeted metabolomics). Furthermore, compared to the *in vitro* cultivation of fecal communities the adaptation of SIHUMIx is very fast. This might result from the low diversity and the high abundance of *B. thetaiotaomicron*. McDonald et al. (2013) observed a structure-related constant state after 30 to 36 days of cultivation of fecal communities in their structural analysis with DGGE.[13] Liu et al. (2018) followed the development of their fecal community in the TWIN-SHIME by 16S rRNA and SCFA analyses.[17] In their study, the community structure became constant on day 10, whereas the communities' SCFA metabolism became constant not before day 17. Furthermore, in contrast to the complex fecal community, SIHUMIx develops simultaneously on the structural and metabolic level during adaptation.[17]

During the adaptation of SIHUMIx, all five methods similarly depicted the community development and indicated that the SIHUMIx community reached a constant state on day 5 with regard to community structure, as well as with regard to function. Since FC, t-RFLP and SCFA analyses have already been applied to follow complex community dynamics, we expected them to record the development of SIHUMIx reliably. However, to our knowledge, IPP and untargeted metabolomics have not been used to follow community dynamics, yet. Our results prove that both methods are suitable. For complex fecal communities, their suitability has to be validated.

It was observed that during cultivation microbial communities from the same homogenous inoculum can develop into different communities with regard to community structure.[29] This was not the case for SIHUMIx, which adapted to the system to a similar state in each bioreactor. To prove that for SIHUMIx, we used different batches of bacteria to inoculate replicate bioreactors. SIHUMIx unravels a highly reproducible development toward the same constant state. Surprisingly, directly after inoculation the communities from the disturbed bioreactors (D and E) were different compared to the unimpeded bioreactors. The only difference was that the pre-cultures for bioreactor A, B and C were grown for 72 h, whereas those for bioreactor D and E were cultivated for 48 h. However, the communities reached the same constant community state. This shows that the constant state of SIHUMIx is highly reproducible, but to achieve maximal reproducibility during the adaptation there is a need for standardized inocula. Generally, the BC similarity among replicates was slightly lower for structural analyses compared to functional analyses. Even though it is well known that community function is more stable than community structure,[42] the difference in the range of the BC similarity for structural and functional analyses is likely to result from the sample preparation. The medium background might have elevated the level of replicate BC similarity for metabolome analyses to ~0.95 in comparison to structural analyses that have ~0.80 BC similarity (BC similarity of 1 represents equality), since for structural analyses, bacteria pellets were used to prepare the cell suspensions, protein and DNA extracts. For targeted metabolomics, culture supernatants were used since SCFA are secreted into the medium.[43] Nevertheless, compared to the literature a BC similarity of 0.8 is already high.[10,17] The highly similar and reproducible community development is also visible in the relative species abundances and SCFA concentrations.

It has been found that acidification of the colon occurs in patients with active ulcerative colitis and resembles a severe disturbance.[25] Thus, we validated the responsiveness of the SIHUMIx community to a pH drop for 24 h. Since community structure and function can develop differently,[16,17] we tracked the community dynamics both on a structural and functional level. Since all methods are appropriate, we applied the most informative methods for structural and functional analysis, which are FC and SCFA analysis, respectively. Both methods clearly revealed the consequences of the pH drop and showed the similar behavior of the replicate bioreactors. Community structure responded and recovered faster than community function. This is in accordance with published studies that also found that community structure stabilizes earlier than community function.[16,17] Our data disclose that the SIHUMIx community, in comparison to the complex community of Liu et al., shows a higher resistance against the perturbation.[29] They introduced a long-term temperature perturbation, whereas we exposed the SIHUMIx community only for 24 h to a decreased pH. Nevertheless, the SIHUMIx community possesses the ability to recover to the constant state and therefore is highly resilient. However, to compare the *resistance* and *resilience* of both communities indisputably, the communities have to be exposed to the same perturbation.

SCFA analysis, untargeted metabolomics, FC, IPP, and t-RFLP were evaluated and proven appropriate to trace the development of the SIHUMIx community. Our results suggest that various methods are suitable to follow community dynamics, but it is important to analyze community development both on the structural and functional level at the same time.

Reproducibility with regard to structural and functional development and the ability to reach a reproducible constant state within five days are the major advantages of SIHUMIx. The constant community state of SIHUMIx shares features of a complex community with regard to both community structure and function. The community shows high resistance, even though it responds to perturbations, and has the ability to recover (resilience). These properties allow the investigation of effects on the community that result from environmental factors, like pesticides or plasticizers, but also dietary or medical interventions. Moreover, the usage of microbial model communities, like SIHUMIx, might help to replace animal models in order to evaluate treatment effects. At the same time, the cultivation of small model communities opens the opportunity to study microbial interactions on the species level. Furthermore, the contribution of individual species to resultant changes in the communities' performance is possible as well as the expansion of SIHUMIx to a more complex, but still defined model community. Thus, our results show that SIHUMIx is a suitable model for *in vitro* testing.

Materials and methods

A detailed description of materials and methods is given in the online repository of this journal (Supplemental file 4 – Methods).

Single stage bioreactor – experimental set-up

The cultivation was carried through in a Multifors 2 bioreactor (Infors). The reactors were inoculated with 1×10^9 bacterial cells per strain.

For the establishment of the SIHUMIx community as *in vitro* model, three parallel and independent 250 mL culture vessels (A, B, and C) were modified as chemostat. To investigate the effect of a pH drop on the SIHUMIx community, two parallel and independent bioreactors (D and E) were run for 14 days. The pH was reduced to 5.5 on day 4 and the community was exposed to the low pH for 24 h. After sampling on day 5, the pH was reset to the original pH of 6.5 until the end of the cultivation.

Sampling and sample analyses

Samples were taken in a 24 h interval starting the day after inoculation (d1). For t-RFLP analysis, IPP, SCFA analysis and untargeted metabolomics, samples were centrifuged (5,000 x g, 5 min, and 4° C). Cell pellets without supernatant were stored directly at −20°C for t-RFLP and IPP, whereas the supernatants for metabolome analysis were stored at −80°C. The cells for flow cytometric fingerprinting were harvested by centrifugation (3,200 x g, 10 min, and 4°C) and prepared directly.

Details for flow cytometric fingerprinting, intact protein profiling, metaproteome analysis, targeted short chain fatty acid analysis, untargeted metabolome analysis and terminal restriction fragment length polymorphism are given in the supplementary online repository of this journal.

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Authors' contributions

JLK (Jannike Lea Krause) and SSS (Stephanie Serena Schaepe) performed the bioreactor experiments and evaluated the data. KFW (Katarina Fritz-Wallace), BE (Beatrice Engelmann) performed the metabolome analyses and were supported by URK (Ulrike Rolle-Kampczyk). JLK, FS (Florian Schattenberg) and ZL (Zishu Liu) performed the flow cytometric analyses with support from SM (Susann Mueller). JLK, did the t-RFLP analyses supported by SK (Sabine Kleinsteuber). SSS did metproteomics with support from NJ (Nico Jehmlich). MvB (Martin von Bergen) and GH (Gunda Herberth) conceptualized the study. All authors contributed to and approved the manuscript.

Availability of data and materials

Raw cytometric data can be found at flow repository ID: **FR-FCM-ZYVG** under: **https://flowrepository.org**.

Disclosure of potential conflicts of interest

The authors report no conflict of interest.

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