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In vitro evaluation of the anti-pathogenic activity of *Okoubaka aubrevillei* on the human gastrointestinal tract

In vitro Untersuchung der antipathogenen Effekte von *Okoubaka aubrevillei* auf den menschlichen Gastrointestinaltrakt

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Schlüsselwörter

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ZUSAMMENFASSUNG

Hintergrund *Okoubaka aubrevillei* wird in der westafrikanischen Ethnomedizin und in der Homöopathie zur Behandlung und Vorbeugung verschiedener gastrointestinaler Probleme genutzt. Ziel der Studie – durchgeführt mit einem SHIME-System (Simulator of the Human Intestinal Microbial Ecosystem) – war die Untersuchung des Einflusses wiederholter Gaben von *Okoubaka* als Urtinktur (10 %ige ethanolische Tinktur der Rinde; UT) oder 3. Dezimalpotenz (D3) auf die Aktivität des gesunden Dickdarm-Mikrobioms und eines prophylaktischen Effekts gegen Durchfallerreger.

Methoden Die Stuhlflora eines gesunden Spenders wurde in einem SHIME-System mit 4 parallelen proximalen Colon-Armen entweder mit *Okoubaka* UT, D3, Ethanol oder nicht für 7 Tage behandelt. Das *Okoubaka*-behandelte Mikrobiom aus dem SHIME-System wurde anschließend für „Challenge-Tests" mit enterotoxischem *Escherichia coli* (ETEC) und *Salmonella enteritidis* in 4 verschiedenen Konzentrationen (10^3–10^8 CFU; unter Beachtung der typischen Infektionsdosen) genutzt. Dabei wurden die Pathogenkonzentrationen, kurz- und verzweigtkettige Fettsäuren (SCFA/b-SCFA) als Dreifachbestimmung nach 0, 24 und 48 h gemessen.

Ergebnisse In den Challenge-Tests konnten beide *Okoubaka*-Produkte die Vermehrung von ETEC und *Salmonella* für 3 der 4 Pathogenkonzentrationen (ausgenommen die höchsten) reduzieren, mit besseren Ergebnissen für die UT, welche eine Verminderung des Wachstums um bis zu 2 log-Einheiten für ETEC ($p < 0,0001$) und 1,1 log-Einheiten für *Salmonella* ($p < 0,0001$) herbeiführte. Die Gesamt-SCFA-Konzentration blieb unbeeinflusst, jedoch stieg die Butyratkonzentration während der ersten 24 h an ($p < 0,0001$ für ETEC), zeitgleich mit einem Abfall der Acetatkonzentration.

Schlussfolgerung In diesem Modellsystem konnte ein aktivierender Einfluss von *Okoubaka* auf die Abwehrmechanismen eines körpereigenen Mikrobioms beobachtet werden, welcher zu einem antipathogenen Effekt (speziell gegen ETEC) führte. Darauf basierend stellen wir die Hypothese auf, dass die Wirkung von *Okoubaka in vivo* ebenfalls auf einem systemisch-regulativen Effekt beruht.

ABSTRACT

Background *Okoubaka aubrevillei* is used in traditional West African medicine and in homeopathy for treatment and prevention of several gastrointestinal problems. The aim of this *in vitro* study was to evaluate the effect of repeated doses of two *Okoubaka* products (10 % ethanolic tincture, mother tincture (MT); 3rd decimal potency, 3X) on the microbial activity of physiological human colon microbiota using a Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) and to investigate any preventive effect against infections with diarrhea-causing pathogens.

Methods Upon inoculation with fecal microbiota from a healthy donor, 4 parallel proximal colon compartments of the SHIME were treated either with *Okoubaka* MT, *Okoubaka* 3X, ethanol control or blank control for 7 days. Using the *Okoubaka*-adapted microbial community from SHIME, 48 h challenge tests were performed with enterotoxigenic *Escherichia coli* (ETEC) and *Salmonella enteritidis* in 4 different doses

(10^3–10^8 colony forming units as typical *in vivo* infectious doses). Pathogen concentrations, short-chain fatty acids (SCFAs) and branched SCFA production were measured in triplicate at 0, 24 and 48 h.

Results In the challenge tests, both *Okoubaka* products were able to restrict the colonization of ETEC and *Salmonella* at 3 of the 4 pathogen doses (except the highest doses), with a stronger anti-pathogenic effect for MT, which included a reduction of 2.0 log-units of ETEC ($p < 0.0001$) and 1.1 log-units of *Salmonella* ($p < 0.0001$). Total SCFA levels remained unaffected, but butyrate increased during the first 24 h ($p < 0.0001$ for ETEC), accompanied by decreased acetate production.

Conclusion We observed *in vitro* a systemic activating effect of *Okoubaka* on intestinal microbiome resistance, which resulted in an anti-pathogenic effect, especially against ETEC. We hypothesize that the mode of action *in vivo* is also based on systemic regulative effects.

Introduction

Okoubaka (*Okoubaka aubrevillei* Pellegr. et Normand) is a West African tree from the plant family Santalaceae. The bark was initially used as a remedy in traditional West African medicine, e. g., to prevent food poisoning [1]. As an herbal preparation, it is an ingredient of malaria remedies used in African ethnomedicine [2].

Magdalena Kunst, a German doctor, brought *Okoubaka* bark from one of her journeys in 1972 back to Germany [1]. *Okoubaka* developed into an established and registered homeopathic medicinal product over the last 40 years. The raw material is monographed in the German Homeopathic Pharmacopoeia (GHP) [3], and a commission D (advisory board of the German competent authority, Federal Institute for Drugs and Medical Devices) monograph was published in 1989 [4].

Okoubaka is known to act on the gastrointestinal tract [5–8] and is used, usually as mother tincture (MT, 10 % ethanolic tincture of the bark, prepared according to the European Pharmacopoeia method 1.1.8) or in low homeopathic potencies (e. g., 3X, corresponding to a 10^{-3} dilution) in the prevention and treatment of traveler's diarrhea [9].

Due to its tannins and catechins content, the bark acts as a tanning agent with its typical astringent effect and probably also by adsorbing toxins and bacterial enterotoxins. Further immunomodulating effects, e. g., an increase of about 20 % of phagocytosis in granulocytes tests, were observed for the macerates in water and ethanol and for *Okoubaka* MT [10, 11]. Kreutzkamp [10] tested various fractions of the separated bark (watery macerate; ethyl acetate fraction of a complex extraction) against gram-positive and gram-negative bacteria (e. g., *E. coli*) in an agar dilution test. The author concluded that probably no antibiotic substances were present in *Okoubaka* as the evaluated minimum inhibitor concentrations (between 256 and 1024 µg/ml) were too high compared with typical antibacterial drugs (approximately 1 µg/ml) [10].

Because of the unique homeopathic manufacturing methods (i. e., potentization by stepwise dilution and succussion), the final concentration of active substances in *Okoubaka* potencies is rather low (dilution of active substances 10^{-3} for 3X) and cannot explain its adsorbing effects and its resulting prophylactic property – even for the MT, which includes 10 % of starting material. It is assumed that homeopathic medicinal products do not act in a pharmacological dose-dependent way but as a stimulation and regulation therapy by activating the body's self-healing capacities [12–15]. According to the similarity principle ("Similia similibus curentur"), homeopathic substances treat complaints characterized by signs and symptoms that are similar to those that these substances would induce in a healthy subject [16].

Okoubaka is used according to its homeopathic "drug picture" for the treatment of several health problems and diseases [6, 8, 17]. However, the main focus of the remedy is on gastrointestinal problems, as mentioned above [4].

Our study aimed to evaluate the effect of homeopathically prepared *Okoubaka* as MT and potency (3X). For this purpose, an established *in vitro* model of the human intestinal tract was used (Simulator of the Human Intestinal Microbial Ecosystem, SHIME®) [18]. Our working hypothesis was that the intestinal microbial community works as a self-regulating system and that treatment with *Okoubaka* products increases intestinal microbiome resistance and the bacterial defense/immune mechanism against pathogens.

Changes in microbial activity result in changes in the production of short-chain fatty acids (SCFAs, concentrations of acetate, propionate, and butyrate) and branched SCFAs (b-SCFAs). Therefore we measured propionate and butyrate, which contribute to salt and water homeostasis in the colon and a healthy large bowel epithelium [19]. Furthermore, acetate and lactate, which some bacteria convert to butyrate ("cross-feeding") [20], as well as b-SCFAs (the sum of isobutyrate, isovalerate, and isocaproate) and

ammonium, which are signs of the proteolytic activity (protein fermentation) of the gut microbiota [21], were measured.

In a second step, we aimed to simulate, by challenge tests, an infection that might occur, for instance, during acute diarrhea while traveling. Therefore the *Okoubaka*-adapted microbial community from the SHIME® was used and challenged with *Salmonella enteritidis* or enterotoxigenic *Escherichia coli* (ETEC), as both pathogens are known for causing traveler's diarrhea [22, 23]. Pathogen concentrations and SCFA and b-SCFA production were measured during the 48-hour challenge tests.

Material and methods

All experiments were performed in the laboratories of ProDigest (Gent, Belgium), a research organization specializing in pre-clinical gastrointestinal research.

In vitro modeling of the gastrointestinal tract

The SHIME system simulates the different parts of the gastrointestinal tract of the adult human in 5 reactors and was first described by Molly et al. [24]. The first 2 reactors represent the stomach and small intestine. Special conditions of food intake and digestion are simulated with peristaltic pumps, and a defined amount of SHIME feed and pancreatic and bile juices are added. Further, the reactors are emptied after defined time intervals ("fill and draw" principle). The next 3 reactors simulate the 3 colon compartments of the large intestine and contain a fecal sample from a healthy donor. The reactors are continuously stirred, contain a constant volume, and are pH-controlled. Inoculum preparation, retention times, pH, temperature settings, and SHIME feed composition represent *in vivo* conditions and have been previously described by Possemiers et al. [25].

For the present experiments, an adapted SHIME system with a simulation of 4 parallel running proximal colon compartments was used (▶ **Fig. 1**). This part of the colon was chosen because it is closest to the small intestine, where the infections with the pathogens causing diarrhea (*Salmonella* and ETEC) occur.

The colon reactors of each arm were inoculated with a fresh fecal sample from a healthy donor (who had not taken antibiotic treatment during the previous 6 months, did not have a history of gastrointestinal disorders, and had a BMI below 25) and simulated the conditions in the proximal colon (pH 5.6–5.9; retention time 20 hours; volume 500 ml; 12 % concentration of fecal inoculum).

During the experiment, the following parameters were monitored to identify changes in microbial community composition and activity:

Acid/base consumption

pH was continuously monitored during the SHIME experiment to ensure the pH range required in the SHIME reactors (pH 5.6–5.9 for proximal colon). During the treatment period, bacteria might produce increased amounts of SCFAs, resulting in acidification of the reactor. To keep the pH within the pre-set intervals, a base was added that further led to increased base consumption. As a possible rough indicator for changes in the microbial fermentative activity, acid/base consumption and overall fermentative activity were continuously monitored during the SHIME experiment by the built-in SHIME software. Data were exported and used for further evaluation at the time points on which samples for the other parameters mentioned below were taken to get corresponding consumption values for these time points.

SCFA and lactate concentration

Changes in microbial activity result in changes in SCFA production and lactate concentration. SCFAs were monitored by gas chromatography with flame ionization detection (GC-FID, as described by De Weirdt et al. [26]) as a concentration of acetate, propionate, and butyrate on days 2, 4, and 7 of the treatment period and in triplicate. Lactic acid determination was conducted at the same time points using a commercially available enzymatic assay kit (Enzytec™ D-/L-Lactic Acid; R-Biopharm, Darmstadt, Germany), according to the manufacturer's instructions.

b-SCFA and ammonium concentration

b-SCFAs (sum of isobutyrate, isovalerate, and isocaproate) were monitored by GC-FID as described by De Weirdt et al. [26] on days 2, 4, and 7 of the treatment period, in triplicate. Ammonium concentrations were determined by a steam distillation approach followed by titration, as previously described by Duysburgh et al. [21].

Experimental design

The following experimental procedure was performed (▶ **Fig. 1**):
1. 2-week stabilization period;
2. 1-week "prophylactic" treatment period in SHIME system, followed by
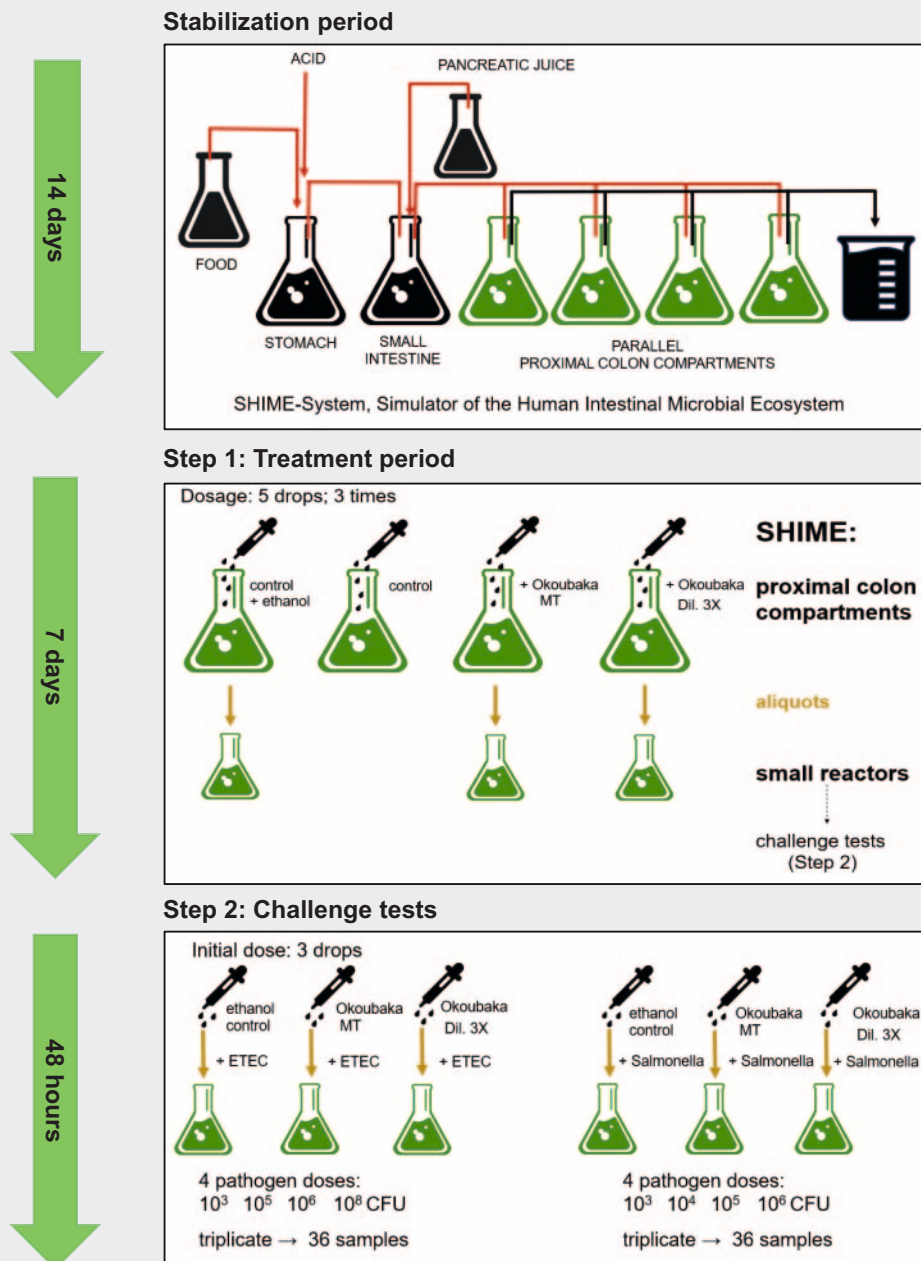3. 48-hour challenge tests with 2 pathogens in different doses.

Stabilization period

After a 2-week stabilization period, a steady-state status in the SHIME system was reached, controlled by acid/base consumption (pH) and SCFA production (acetate, propionate, and butyrate).

A minimum 20 % coefficient of variation (CV), corresponding to 80 % reproducibility (100 % – CV) [25] of SCFA levels (acetate, propionate, and butyrate) between the different colon units, indicated that the starting microbial community among them was similar, which was important for comparison purposes between the 4 different compartments. We aimed to achieve a reproducibility above 85 %.

"Prophylactic" treatment period

After the stabilization period, one proximal colon compartment of the SHIME reactor was treated with *Okoubaka* MT and one with *Okoubaka* 3X (3rd decimal dilution [potency]) in doses of 5 drops (corresponded with 120 µl) 3 times per day. This dosage regimen corresponds to the recommended dose, adapted to the conditions in the SHIME system, which correspond in turn to half an adult human (the dosage for 1 human consists of a maximum of 5 drops 6 times per day according to the package leaflet). In the remaining 2 proximal colon compartments, 1 control arm with no

Stabilization period

14 days

SHIME-System, Simulator of the Human Intestinal Microbial Ecosystem

Step 1: Treatment period

7 days

Step 2: Challenge tests

48 hours

Endpoints/ Analytical parameters

Acid/ Base con-sumption	Lactate	SCFA -Acetate -Butyrate -Propionate	Branched SCFA	Am-monium	Anti-pathogenic Activity	Toxins	
X	—	X	—	—	—	—	**Stabilization Period**
X	X	X	X	X	—	—	**Treatment period**
—	—	X	X	—	X	X	**Challenge tests ETEC**
—	—	X	X	—	X	—	**Challenge tests *Salmonella***

▶ **Fig. 1** Experimental design.

treatment (blank control) and 1 ethanol control (62 % [m/m]) were included. In all 4 compartments, treatments/controls were given for 1 week.

Challenge tests

After the treatment period, aliquots were taken from both the "*Okoubaka* adapted" (MT and 3X) and "ethanol-controlled" microbial community in SHIME and used for challenge tests with 2 different pathogens in small reactors. An initial dose of 3 drops *Okoubaka* MT/3X or ethanol was added at the beginning of the 48-hour challenge tests in the corresponding sample from the SHIME system.

A 2 % inoculum of overnight grown *Salmonella enteritidis* (brain heart infusion broth as growth medium) or ETEC (Lysogeny broth as growth medium) – both prepared by Oxoid (Cambridge, UK) – were used.

The pathogens were added immediately after the initial dose of the test products and ethanol control in 4 different concentrations: 10^3, 10^5, 10^6, and 10^8 CFUs for ETEC and 10^3, 10^4, 10^5, and 10^6 CFUs for *Salmonella enteritidis*. The typical *in vivo* infectious doses were extrapolated to the conditions in the small reactors (50 ml; 37 °C; under shaking and anaerobic conditions), which correspond to the colon of 1/10 of an adult human.

All experiments were done in triplicate. Anti-pathogenic activity and metabolic activity were determined at time points 0, 24, and 48 hours after taking samples of 2 ml volume. Changes in concentrations of both pathogens were measured during the experiment using different methods of colonization:

Salmonella enteritidis colonization

For the assessment of *Salmonella enteritidis* colonization, selective enumeration on MacConckey agar was used, containing 100 ppm streptomycin, which is selective for gram-negative bacteria. Briefly, samples were collected after 0, 24, and 48 hours to determine the number of CFUs of the bacterial strain tested by spread plating. Ten-fold dilution series were prepared from these samples in PBS, subsequently transferred to Petri dishes containing selective agar media and incubated aerobically at 37 °C for at least 24 hours.

ETEC colonization

For the quantitative assessment of ETEC colonization, the quantitative polymerase chain reaction (qPCR) using a QuantStudio 5 Real-Time PCR system (Applied Biosystems, Foster City, CA USA) was applied, as plating alone is not selective enough for ETEC in the colonic medium. Therefore, CFUs correspond to 16S RNA copies for ETEC.

Cell pellets were generated after 0, 24, and 48 hours of incubation and were subjected to propidium monoazide (PMA) treatment. PMA is an ionic substance that can only cross the damaged cell membranes of non-viable cells and prevent, after UV-induced crosslinking, the amplification of this DNA in qPCR assays. Subsequently, DNA was extracted from PMA-treated cells followed by quantification of their concentration through qPCR using primers that were selective for ETEC [27].

Heat-labile toxin produced by ETEC

ETEC is capable of producing 2 different types of enterotoxins (proteins): 2 heat-labile toxins (LT1 and LT2) and a heat-stable toxin (ST). In our experiments, we measured the production of the heat-labile ETEC-toxin LT1, which acts like the cholera toxin, via ganglioside GM1-enzyme-linked immunosorbent assay (GM1 ELISA). The method described by Salimian et al. [28] was used with the following minor modifications. Both the cell wall-bounded LT1 and the one present in the fermentation liquid (supernatant) were determined. To do so, we centrifuged 1 ml of sample, after which the supernatant and the cell pellet were separated. The cell pellet was resuspended in phosphate-buffered saline (PBS) and sonicated before toxin quantification through GM1 ELISA. All experiments were done in triplicate.

Test products

Okoubaka MT was prepared according to the European Pharmacopoeia method 1.1.8 and method 4a of GHP using ethanol 62 % (m/m) [3, 29]. For the raw material, the "dried bark of the aerial parts of *Okoubaka aubrevillei* Pellegr. et Normand" [3] was used. *Okoubaka* 3X was prepared according to the European Pharmacopoeia monograph 2371, starting from MT (corresponding to the 1st decimal dilution) stepwise to 3X [29]. The potentization steps were done in ethanol 62 % (m/m) according to Hahnemannian principles with "10 vigorous downwards strokes" [30] against an elastic body as described in the original literature [31]. For the ethanol-control, ethanol 62 % (m/m) was used.

All test materials were prepared by Deutsche Homöopathie-Union DHU-Arzneimittel GmbH & Co. KG (Karlsruhe, Germany).

The greatest possible distance between the test products and the ethanol control was ensured during the experiments to prevent interactions between them. Also, the *Okoubaka* test products and the ethanol control were stored in different rooms.

Endpoints

Treatment period

To detect changes in the microbial community during treatment with *Okoubaka* products, the following parameters were measured and normalized to the ethanol control on days 2, 4, and 7 of the treatment period in triplicate: acid/base consumption, SCFAs (acetate, propionate, and butyrate), lactate concentration, b-SCFAs (sum of isobutyrate, isovalerate, and isocaproate), and ammonium concentration.

Challenge tests

The following parameters were evaluated in the challenge tests with the 2 different pathogens (normalized to the ethanol control):
- Direct anti-pathogenic activity of the *Okoubaka*-adapted microbial community was assessed via colonization of both pathogens (*Salmonella enteritidis* and ETEC) 24 and 48 hours after challenge with the pathogens.
- Concentration of the heat-labile ETEC toxin LT1 was evaluated at the time points 0, 24, and 48 hours.
- Changes in microbial metabolic activity were investigated at time points 0, 24, and 48 hours of incubation using an analysis of
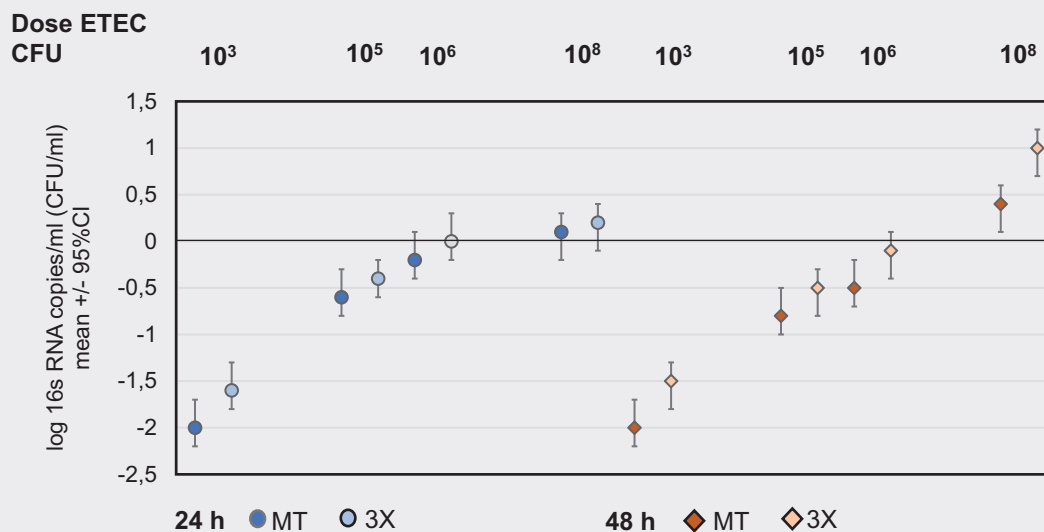
▶ **Fig. 2** Results of the quantitative colonization of ETEC (using qPCR) normalized to ethanol control after 24 and 48 hours of incubation, upon quantification via plating on MacConkey agar supplemented with 100 ppm streptomycin; 16 s RNA copies correspond to CFU.

SCFA production (acetate, propionate, and butyrate) and b-SCFA production (sum of isobutyrate, isovalerate, and isocaproate).

Data management/statistical analysis

All variables are described groupwise by mean, median, standard deviation, range (min, max), and 95 % confidence interval (CI), as appropriate.

Data for the anti-pathogenic activity of the challenge tests were statistically analyzed by 2-way analysis of variance (ANOVA) with repeated measurements, with and without interaction terms. Group-specific differences were estimated and expressed as mean ± 95 % CI. A p-value of < 0.05 indicated statistical significance. P-values were not adjusted in terms of multiple testing.

Analyses were performed with software SAS 9.4; in addition, MS Excel was used for some graphical presentations.

Results

Stabilization period: stability of the SHIME setup

SCFA levels of each of the 4 arms of the SHIME system had a reproducibility of 90.1 % (100 % – CV) on average (acetate 91.9 %, propionate 92.7 %, butyrate 85.7 %) at the end of the stabilization period, which was higher than the targeted minimum value of 85 %.

Treatment period: "*Okoubaka*-adapted" microbial community activity

Overall fermentative activity: acid/base consumption

A temporary trend toward increased base consumption was observed at the start (day 2) of the treatment by both *Okoubaka* products (compared to the ethanol control), followed by a decrease at the end (day 7) of the treatment period: 19.4 ml/day for *Okoubaka*

MT and 10.4 ml/day for *Okoubaka* 3X on day 2 of the treatment period; −2.0 ml/day for *Okoubaka* MT and −1.9 ml/day for *Okoubaka* 3X on day 7 of the treatment period.

SCFA production and lactate concentration

Some – but not statistically relevant – changes in the "*Okoubaka*-adapted" microbial community compared to the ethanol control were observed. The changes – determined at days 2, 4, and 7 of the treatment period – concerned the following parameters: acetate increase, trends toward increased propionate levels and decreased butyrate levels, and lactate increase (data not shown).

b-SCFA and ammonium

The levels of the sum of b-SCFAs and ammonium were not strongly affected by the *Okoubaka* treatment. The coefficient of variation (CV) was 5.4 % for b-SCFA levels and 4.5 % for ammonium levels between all 4 arms of the SHIME system (ethanol control, blank control, *Okoubaka* MT, and 3X).

Challenge Tests: anti-pathogenic activity
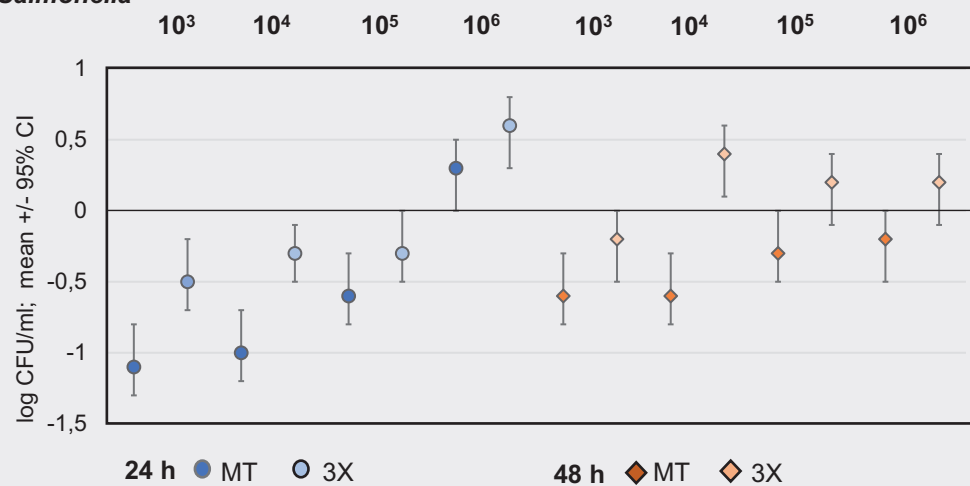
ETEC colonization

▶ **Fig. 2** shows the colonization of ETEC (using qPCR) after 24 and 48 hours of incubation. For graphical representation, test data (done in triplicate) were normalized to the ethanol control.

There was a reduction of colonization compared with ethanol control in 3 out of the 4 pathogen doses for *Okoubaka* MT and 2 out of the 4 pathogen doses for *Okoubaka* 3X. The strongest effects were observed for the lowest ETEC dose (10^3 CFU) with a reduction of 2.0 log-units of ETEC for treatment with MT and of 1.6 log-units of ETEC for 3X after 24 hours. The anti-pathogenic effects were almost similar after 48 hours. At both time points, the effects decreased for higher pathogen doses in a dose-depen-

► **Table 1** Values of the quantitative colonization of ETEC (using qPCR) normalized to ethanol control after 24 and 48 hours of incubation and estimates as developed with a 2-way repeated measurement ANOVA with treatment and dose as fixed factors for ETEC.

	dose 10^3 CFU ETEC		dose 10^5 CFU ETEC		dose 10^6 CFU ETEC		dose 10^8 CFU ETEC	
	MT	3X	MT	3X	MT	3X	MT	3X
log 16S RNA copies /ml (24 h)	−2.0	−1.6	−0.6	−0.4	−0.2	0	0.1	0.2
95 % CI [lower CL; upper CL]; p-value	[−2.2;−1.7] p < 0.0001	[−1.8;−1.3] p < 0.0001	[−0.8;−0.3] p < 0.0001	[−0.6;−0.2] p = 0.0021	[−0.4;0.1] p = 0.1215	[−0.2;0.3] p = 0.8017	[−0.2;0.3] p = 0.4882	[−0.1;0.4] p = 0.1297
log 16S RNA copies /ml (48 h)	−2.0	−1.5	−0.8	−0.5	−0.5	−0.1	0.4	1.0
95 % CI [lower CL; upper CL]; p-value	[−2.2;−1.7] p < 0.0001	[−1.8;−1.3] p < 0.0001	[−1.0;−0.5] p < 0.0001	[−0.8;−0.3] p = 0.0001	[−0.7;−0.2] p = 0.0002	[−0.4;0.1] p = 0.2233	[0.1;0.6] p = 0.0025	[0.7;1.2] p < 0.0001
estimated effects for all pathogen doses for MT	−0.69 [95 % CI: −0.94; −0.44] log-units (log 16S RNA copies)/ ml of ETEC; p < 0.0001							
estimated effects for all pathogen doses for 3 X	−0.38 [95 % CI: −0.63; −0.12] log-units (log 16S RNA copies)/ml of ETEC; p = 0.0041							

► **Fig. 3** Results of the quantitative colonization of *Salmonella enteritidis* normalized to ethanol control after 24 and 48 hours of incubation, upon quantification via plating on MacConkey agar supplemented with 100 ppm streptomycin.

dent way. No anti-pathogenic effects and even a trend towards increased colonization of ETEC were observed at the highest ETEC dose (10^8 CFU). The values are shown in ► **Table 1**.

► **Table 1** also shows the values estimated with a 2-way repeated measurement ANOVA with treatment and dose as fixed factors for ETEC. The estimated effect on colonization reduction for all pathogen doses collectively was significant for *Okoubaka* MT (p < 0.0001) and 3X (p = 0.0041).

Toxin production by ETEC

There were no significant effects of the *Okoubaka* test products on heat-labile ETEC toxin LT1 (results not shown). The measured enterotoxin concentrations after application of either *Okoubaka*

MT or *Okoubaka* 3X were in the range of toxin levels of an active ETEC culture (21.4 ng/mL in the supernatant).

Salmonella enteritidis colonization

► **Fig. 3** shows the colonization of *Salmonella enteritidis* after 24 and 48 hours of incubation.

For graphical representation, test data (done in triplicate) were normalized to the ethanol control.

Both test products (*Okoubaka* MT and 3X) compared with the ethanol control restricted the growth of *Salmonella enteritidis*, except at the highest Salmonella concentration (10^6 CFU), with the strongest anti-pathogenic effect during the first 24 hours of incubation. A stronger anti-pathogenic effect was observed for

▶ **Table 2** Results of the quantitative colonization of *Salmonella enteritidis* normalized to ethanol control after 24 and 48 hours of incubation and estimates as developed with a 2-way repeated measurement ANOVA with treatment and dose as fixed factors for *Salmonella*.

	dose 10^3 CFU *Salmonella*		dose 10^4 CFU *Salmonella*		dose 10^5 CFU *Salmonella*		dose 10^6 CFU *Salmonella*	
	MT	3X	MT	3X	MT	3X	MT	3X
log CFU /ml (24 h)	−1.1	−0.5	−1.0	−0.3	−0.6	−0.3	0.3	0.6
95 % CI [lower CL; upper CL]; p-value	[−1.4;−0.8] p < 0.0001	[−0.8;−0.2] p = 0.0013	[−1.3;−0.7] p < 0.0001	[−0.6;0] p = 0.0543	[−0.9;−0.3] p = 0.0003	[−0.7;0] p = 0.0788	[−0.2;0.7] p = 0.2504	[0.2;1.1] p = 0.0052
log CFU /ml (48 h)	−0.6	−0.2	−0.6	0.4	−0.3	0.2	−0.2	0.2
95 % CI [lower CL; upper CL]; p-value	[−1.0;−0.3] p = 0.0014	[−0.6;0.2] p = 0.3207	[−0.9;−0.2] p = 0.0023	[0;0.7] p = 0.0420	[−0.6;−0.3] p = 0.0392	[−0.2;0.5] p = 0.3223	[−0.5;0.1] p = 0.2093	[−0.1;0.5] p = 0.2169
estimated effects for all pathogen doses for MT	−0.56 [95 % CI: −0.72; −0.39] log-units (log CFU)/ ml of *Salmonella*; p < 0.0001							
estimated effects for all pathogen doses for 3 X	−0.02 [95 % CI: −0.19; 0.15] log-units (log CFU)/ ml of *Salmonella*; p = 0.8406							

▶ **Table 3** Levels of butyrate increase and acetate decrease (difference 24–0 hours) normalized to EtOH during 0–24-hour time interval for MT and 3X during challenge with ETEC.

ETEC dose [CFU]	butyrate levels normalized to EtOH 0–24 hours time interval (Diff. 24 hours–0 hours) [mmol/l]	lower CL [mmol/l]	upper CL [mmol/l]	p-value	acetate levels normalized to EtOH 0–24 hours time interval (Diff. 24 hours–0 hours) [mmol/l]	lower CL [mmol/l]	upper CL [mmol/l]	p-value
MT and 10^3	0.64	0.48	0.79	<0.0001	−1.64	−2.08	−1.84	<0.0001
MT and 10^5	0.62	0.46	0.78	<0.0001	−1.99	−2.43	−1.54	<0.0001
MT and 10^6	0.51	0.36	0.67	<0.0001	−0.44	−0.89	0.00	0.0544
MT and 10^8	0.60	0.44	0.78	<0.0001	0.21	−0.24	0.65	0.3590
3X and 10^3	0.83	0.67	0.98	<0.0001	−0.59	−1.04	−0.15	0.0105
3X and 10^5	0.55	0.40	0.71	<0.0001	−2.28	−2.73	−1.83	<0.0001
3X and 10^6	0.56	0.40	0.71	<0.0001	−1.18	−1.62	−0.73	<0.0001
3X and 10^8	0.42	0.27	0.58	<0.0001	0.15	−0.30	0.59	0.5121

Okoubaka MT with a reduction of 1.1 or 1.0 log-units of *Salmonella* for the 2 lower pathogen doses (10^3 or 10^4 CFU, respectively). The values are shown in ▶ **Table 2**.

▶ **Table 2** also shows the values estimated with a 2-way repeated measurement ANOVA with treatment and dose as fixed factors for *Salmonella*. The estimated effect on colonization reduction for all pathogen doses collectively was significant for *Okoubaka* MT (p < 0.0001).

Challenge Tests: metabolic activity

SCFA and b-SCFA production in ETEC

Total SCFA production – evaluated at time points 0, 24, and 48 hours during the challenge tests – remained essentially unaf-fected after treatment with both *Okoubaka* products, although there were changes in single fatty acid composition.

The changes for butyrate, acetate, propionate, and b-SCFAs during the time intervals 0–24 hours and 24–48 hours are shown in ▶ **Fig. 4**. Increased production of butyrate during the first 24 hours was observed. The butyrate increase for *Okoubaka* MT and 3X, normalized to ethanol control, was statistically significant for the 0–24 hours time interval; data and p-values are shown in ▶ **Table 3**. Butyrate levels remained high during the 24–48 hours time interval. Overall acetate levels increased at the time interval 0–24 hours, followed by a decrease at the 24–48 hours time inter-val. Normalized to ethanol, acetate levels of *Okoubaka* MT and 3X decreased during the 0–24 hours time interval, i. e., reflecting acetate consumption. The decrease was statistically significant for lower doses of ETEC; data and p-values are shown in ▶ **Table 3**.
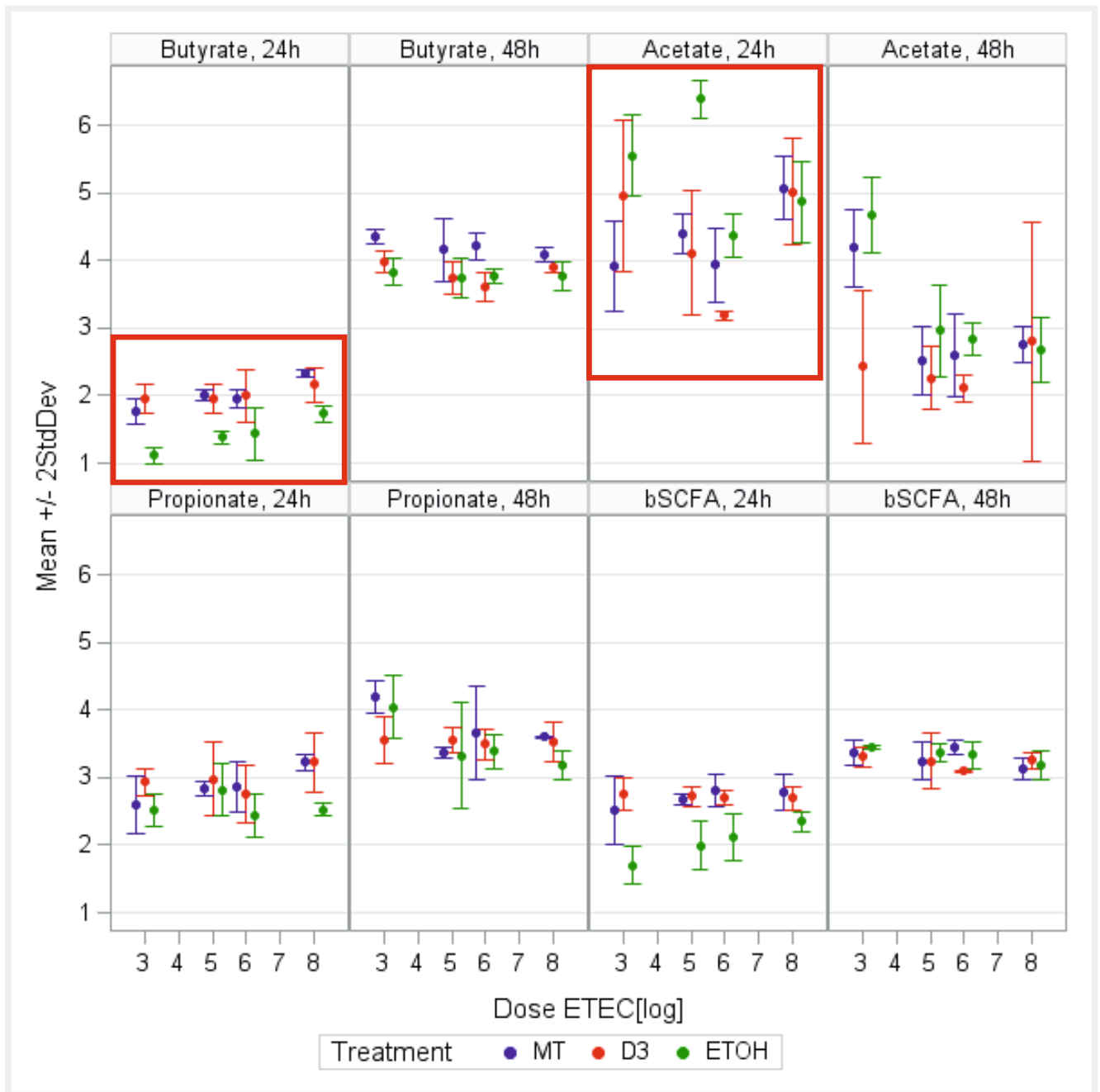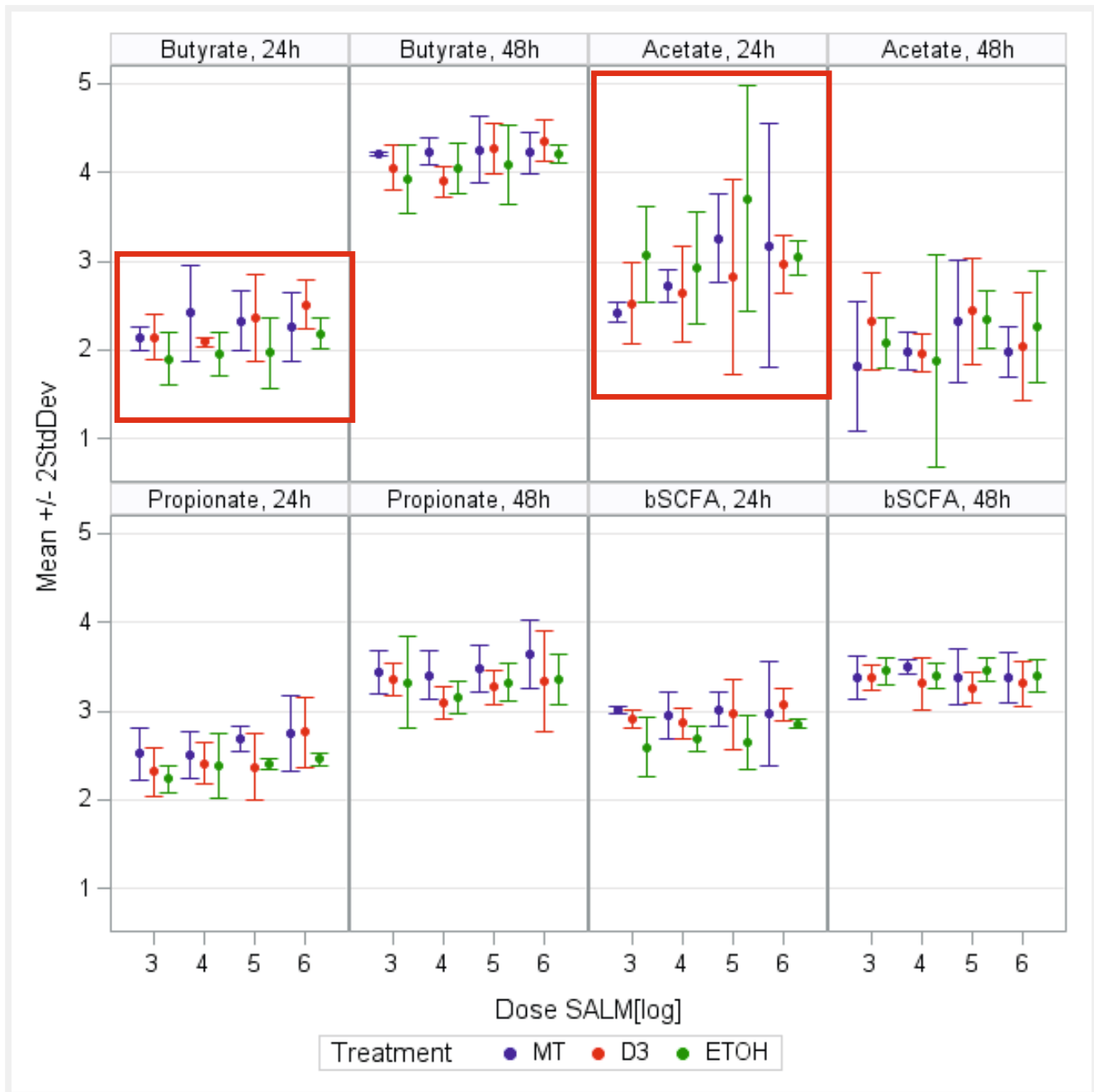
▶ **Fig. 4** Values for butyrate, acetate, propionate, and b-SCFAs [mmol/l] for MT, 3X, and EtOH during challenge with ETEC for the time intervals 0–24 hours and 24–48 hours.

SCFA/b-SCFA production in *Salmonella enteritidis*

Total SCFA production remained unaffected upon treatment with both *Okoubaka* products, but there were changes in single fatty acid composition.

The changes for butyrate, acetate, propionate, and b-SCFAs during 0–24 hours and 24–48 hours time intervals are shown in ▶ **Fig. 5**. Increased production of butyrate during the first 24 hours was observed. The values of butyrate increase for *Okoubaka* MT and 3X, normalized to ethanol control, are shown in ▶ **Table 4** and were less pronounced as compared to ETEC. Butyrate levels remained

high during the 24–48 hours time interval. Overall, acetate levels increased at the time interval 0–24 hours, followed by a decrease at the 24–48 hours time interval. Normalized to ethanol, acetate levels of *Okoubaka* MT and 3X slightly decreased during the 0–24 hours time interval, i. e., reflecting acetate consumption. The postulated cross-feeding effect from acetate to butyrate was less pronounced; data are shown in ▶ **Table 4**.

▶ **Fig. 5** Values for butyrate, acetate, propionate, and b-SCFAs [mmol/ l] for MT, 3X, and EtOH during challenge with *Salmonella enteritidis* for the time intervals 0–24 hours and 24–48 hours.

Discussion

During the experiments, the effect of *Okoubaka* MT and 3X on the human gut microbiota in terms of microbial activity was tested using a long-term SHIME setup to simulate a prophylactic treatment. Subsequently, in short-term challenge tests, the anti-pathogenic activity of the *Okoubaka*-adapted microbial community was evaluated against 2 diarrhea-causing pathogens (ETEC and *Salmonella enteritidis*) in 4 different concentrations.

SHIME system: reproducibility (comparability) of the 4 arms of the SHIME system

With a 90.1 % reproducibility (calculated as 100 % – CV) of the SCFA levels between the 4 proximal colon units of the SHIME system at the beginning of the treatment period, there were similar starting conditions between the *Okoubaka* treatments, ethanol, and blank control arms. It was higher than the 80 % value generally accepted for the SHIME system established by Possemiers et al. [25] and even higher than the targeted 85 % for the present study. To rule out unequal baseline conditions as far as possible, the sam-

▶ Table 4 Levels of butyrate increase an acetate decrease (difference 24–0 hours) normalized to EtOH during 0–24-hour time interval for MT and 3X during challenge with *Salmonella enteritidis*.

Salmonella dose [CFU]	butyrate levels normalized to EtOH 0–24 hours time interval (Diff. 24 hours–0 hours) [mmol/l]	lower CL [mmol/l]	upper CL [mmol/l]	p-value	acetate levels normalized to EtOH 0–24 hours time interval (Diff. 24 hours–0 hours) [mmol/l]	lower CL [mmol/l]	upper CL [mmol/l]	p-value
MT and 10^3	0.23	0.00	0.46	0.0530	−0.64	−1.12	−0.16	0.0094
MT and 10^4	0.46	0.25	0.67	<0.0001	−0.21	−0.63	0.22	0.3308
MT and 10^5	0.35	0.15	0.56	0.0013	−0.44	−0.87	−0.02	0.0415
MT and 10^6	0.07	−0.14	0.28	0.4869	0.14	−0.29	0.56	0.5192
3X and 10^3	0.24	0.04	0.45	0.0223	−0.55	−0.98	−0.13	0.0123
3X and 10^4	0.14	−0.07	0.35	0.1858	−0.30	−0.72	0.13	0.1690
3X and 10^5	0.40	0.19	0.60	0.0004	−0.87	−1.29	−0.44	0.0002
3X and 10^6	0.32	0.11	0.53	0.0035	−0.08	−0.50	0.35	0.7161

ple with the highest butyrate levels combined with the lowest acetate levels was allocated to the ethanol control. Hence, the ethanol control had the best starting conditions with regard to health-related butyrate of all 4 SHIME units.

In addition, ammonium and b-SCFAs remained quite stable during the 1-week treatment period. These parameters were essentially unaffected by the *Okoubaka* treatment and matched well in the 4 reactors at the 3 measurement time points (4.5 % CV for ammonium and 5.4 % CV for b-SCFAs).

Challenge tests: anti-pathogenic activity

Both *Okoubaka* products were capable of restricting the colonization of ETEC and *Salmonella enteritidis* at 3 pathogen concentrations up to 2.0 log-units for ETEC and 1.1 log-units for *Salmonella*. A reduction of 2.0 log-units implies a 99 % reduction of the pathogen, while a reduction of 1.0 log-unit corresponds to a 90 % reduction of the pathogen.

Some differences in the intensity of the effects shall be discussed in the following.

A stronger anti-pathogenic effect was observed for the MT. One explanation could be that the MT (defined as 1X potency according to European Pharmacopoeia method 1.1.8 [29]) contains 100-fold more starting material (*Okoubaka* bark) compared with 3X. Such a concentration dependence was also observed in *in vitro* tests with other potentiated substances [32, 33]. It could also be explained by the fact that *in vitro* tests – even if the bacterial community tested is a complex self-regulating system – should be interpreted within the boundaries of the models and are therefore not able to reflect the integrated conditions of an entire organism.

The anti-pathogenic effect was stronger for the lower pathogen concentrations, with a reduction of up to 2 log-units of ETEC and 1.1 log-units of *Salmonella* for the lowest pathogen doses (10^3 CFU/10^4 CFU). With increasing doses, the anti-pathogenic activity decreased, however. For ETEC, typical infectious doses be-

tween 10^3 and 10^8 germs are described [22, 34], ingested, for instance, by contaminated water or food. Both ETEC and *Salmonella* are very sensitive to the acid milieu in the stomach. Therefore patients with hypoacidity of the stomach or those treated with proton pump inhibitors are much more sensitive to infections with these pathogens [22, 35]. For those patients, infectious doses of 1000 germs for ETEC and lower are possible. For *Salmonella*, the minimum infectious doses are even lower, between 100 (described for contaminated fatty food) and 10^6 germs [36]. To compare the SHIME *in vitro* model with a human, the typical infectious doses must be extrapolated to the conditions in the proximal colon: taking into account that, in the present *in vitro* model, the acidic gastric compartment has been bypassed and the conditions in the reactors corresponded to 1/10 adult, 100 to 10 000 CFU for ETEC, and 10 to 10 000 CFU for *Salmonella* are needed for them to be infectious. Therefore, the lower pathogen concentrations tested in the study – for which prophylaxis through *Okoubaka* was observed – reflect the typical range of bacterial infections.

For concentrations above about 10^6 to 10^7 CFU for ETEC and about 10^5 CFU for *Salmonella*, which under realistic conditions can only be reached when visibly spoiled food is consumed, the prophylactic administration of *Okoubaka* had no anti-pathogenic effect.

For the highest pathogen doses (10^8 for ETEC and 10^6 for *Salmonella*), a trend for increased growth of the pathogens compared to the ethanol control was observed in the test model. Such stress-dependent effects have also been observed in other experimental systems with potentized remedies, e. g., in the treatment of arsenic- or mercury-stressed duckweeds with potentized *Arsenicum album* or *Mercurius solubilis*. In slightly stressed duckweeds, treatment with the isopathic remedy resulted in a growth enhancement (improvement), whereas in severely stressed duckweeds, the same treatment resulted in growth reduction [37]. Such inverse effects might be related to the specific mode of action of homeopathic remedies: each medicine produces an "artificial illness" (pri-

mary action, phase 1), to which the organism (secondary action, phase 2) reacts accordingly. If the artificial illness is (homeopathically) similar to the disease's symptoms to be treated, there will be an improvement [13]. Thereby the regulation ability of the organism (by activating self-healing capacities) is a prerequisite. If the disease (in our model, the number of pathogens) is too strong, the system's regulation ability may be overstrained. It is also conceivable that phase 1 and phase 2 are delayed: since the time frame in our system was limited to 48 hours, it could be that the secondary action would be observed after 48 hours.

Challenge tests: production of ETEC toxin

The *Okoubaka*-adapted microbial community was able to inhibit the growth of ETEC but could not help the degradation of already existing toxins. Toxin elimination via adsorbing effects through tannins is one of the discussed modes of action for *Okoubaka* bark. However, in the diluted preparations and even in the MT, the tannin concentration is too low to exhibit adsorbing effects.

As the exact tannin content of the tested MT is unknown and analytical testing not available, the tannin content was estimated as follows: by macerating 100 g dried *Okoubaka* bark in 1 liter methanol for 5 hours, Kreutzkamp isolated 3.2 g of the dry residual of catechins corresponding to a yield of 3.2 %. After extracting this residual in ethyl acetate following by distillation, 800 mg of tannins were obtained [10, 11]. In the investigated Okoubala MT (10 % tincture of the bark in 62 % [m/m] ethanol, prepared by percolation), the proportion of dry residual is much lower (i. e., 0.4 %) [3]. A usual tannin-based preparation against diarrhea contains 500 mg tannin albumin per tablet or 250 mg tannins. The recommended dose is 1–2 tablets 4–6 times per day. This corresponds to a minimum of 1000 mg and a maximum 3000 mg tannins per day [38]. The amount of tannins in 3 drops MT is approximately 0.01 mg (rough estimation based on dry residual) and therefore 100 000 orders of magnitude lower. Therefore, our results are plausible and were to be expected.

Mode of action

We observed no relevant effects in the microbial community during treatment with *Okoubaka* products, as monitored on days 2, 4, and 7 of the treatment period regarding acid/base consumption, SCFAs, b-SCFAs, and ammonium. In the challenge tests, both *Okoubaka* products restricted the colonization of ETEC (up to 2.0 log-units) and *Salmonella enteritidis* (up to 1.1 log-units), but no effect was observed on the ETEC toxin.

We essentially see 2 main possibilities to explain these overall findings: by direct phytopharmacological effects and/or by systemic regulative effects on the bacterial community in the SHIME system. As discussed above, direct pharmacological effects by tannins seem rather unlikely as an overall explanation. A further argument for a systemic regulative effect is the comparably small difference for *Okoubaka* MT and 3X in ETEC colonization reduction and the comparable effects on SCFAs and b-SCFAs after 24 hours, though these 2 products differ by a factor of 100 in compound concentration. Though direct pharmacological effects cannot be excluded, we hypothesize based on the results obtained that the

primary mode of action of *Okoubaka* in the present *in vitro* SHIME system is a systemic regulative effect on the bacterial community.

Possible implications for the application of *Okoubaka* in practice: anti-pathogenic activity

Based on the study results concerning anti-pathogenic activity, an extrapolation of the *in vitro* results obtained to *in vivo* conditions would lead to the following considerations. Reducing the pathogen concentration up to 99 % (2 log-units) would give the organism the possibility and time to eliminate the remaining pathogens. Therefore, symptoms of acute diarrhea would possibly not occur, or the course of the disease would be milder. Therefore, our experiments may deliver or be a part of an explanation for the traditional, empirically-based use of *Okoubaka* as prophylaxis before traveling.

Okoubaka seems to work better for prophylaxis of ETEC than for *Salmonella enteritidis* infections. This applies to the extent of the reduction of colonization of the pathogens (log-units), the level of significance of the results, the differences between MT and 3X, and the differences between the first and the second time interval of the challenge tests. Better effects for ETEC were also observed concerning the metabolic activity, especially for butyrate concentration.

One reason for these differences could be the different pathogenic mechanisms of the 2 bacteria. ETEC intoxications are caused by 2 different types of enterotoxins: 2 heat-labile toxins (LT1 and LT2) and a heat-stable toxin (ST). The toxins act in the small intestine supported by "colonization factor antigens" that are responsible for the adhesion of the bacteria to the mucosa of the small intestine. The heat-labile enterotoxin LT1 acts like the cholera toxin in the small intestine by stimulating adenylate cyclase, which leads to the secretion of water and chloride via cAMP-mediated secondary messenger reactions. The heat-stable enterotoxin works as a guanylate cyclase activator in the intestinal mucosa, resulting in cGMP-mediated loss of water and electrolytes [39]. These reactions result in symptoms like watery diarrhea with abdominal cramps; nausea and sub-febrile temperatures are also possible [39].

Salmonella invades the mucosal cells of the small intestine and is phagocytosed by macrophages, which leads to an inflammatory reaction resulting in the excretion of water and electrolytes into the intestinal lumen [39]. Histological investigations show a massive immigration of neutrophils into the intestinal tissue [40]. Elimination of *Salmonella* from the body is difficult due to its penetration into the cells of the intestinal mucosa and its subsequent phagocytosis by the macrophages. Symptoms of *Salmonella* enteritidis infections are diarrhea, headaches, abdominal pain, nausea, and sometimes vomiting. These symptoms are often accompanied by fever. In rare cases, sepsis with high fever has been described [36].

According to the homeopathic similarity principle, based on the various symptoms caused by these 2 pathogens, a different relationship between the 2 different types of enteritis and *Okoubaka* could be assumed, possibly explaining the different effect of *Okoubaka* on the 2 pathogens. For example, "cramping pain in abdomen; diarrhea; loose and frequent stools, as with a virus"

were symptoms experienced during a homeopathic drug proving of *Okoubaka* 12C by Riley in 1990 [41], which fits better to ETEC than to *Salmonella* infections.

Metabolic activity: possible effects of SCFA increase

Changes in microbial activity result in changes in SCFA production. SCFA production of the microbial community is related to various positive health effects.

For SCFAs, diverse effects in the human intestinal tract have been discussed: acetate, butyrate, and also propionate are considered as favorable to health, i. e., with protective effects against inflammation and prevention of the colonization of bacterial pathogens [42]. Butyrate is produced, for example, by members of the Clostridium clusters XIVa (Firmicutes), Faecalibacterium prausnitzii, and Oscillibacter [43], via cross-feeding, which is a process by which some bacteria convert acetate to healthful butyrate along with other substrates [20]. Butyrate plays an important role in the maintenance of the barrier function of the intestine. It reduces intestinal permeability through various mechanisms, closes tight junctions, and initiates repair mechanisms [44, 45].

An increased butyrate production at the expense of acetate (cross-feeding process) was observed during the challenge tests, even for the higher pathogen concentrations. However, there was no significant change in butyrate production during the 7-day prophylaxis. This increase of butyrate, produced by the *Okoubaka*-adapted microbial community during the pathogen challenge, might initiate butyrate-mediated host interactions against diarrhea. These effects might act in addition to the direct anti-pathogenic activity.

Strengths and limitations of this study

One strength of this study is the use of the SHIME technology, which is a validated experimental system [18, 24, 25] and which has been used for an extensive number of research projects in the past, as shown by more than 100 publications in PubMed when searching for the "Simulator of the Human Intestinal Microbial Ecosystem" [46]. In addition, by using a complex *in vitro* model of the human intestinal tract, the intestinal microbial community can be tested as a self-regulating system, thereby exceeding the limits of conventional *in vitro* experiments, such as using single cell lines.

This study also had limitations. Firstly, our results were based on the microbiota of only 1 donor. Further experiments with the microbiome of several donors are needed to investigate the generalizability of the results. Also, even if the SHIME technology is a comprehensive *in vitro* method, it cannot measure the complex interactions of an entire host organism. Furthermore, the time frame for the challenge tests in our system was limited to 48 hours, so possible delayed reactions could not be observed. As the evaluation's main focus was to investigate the influence of the prophylactic administration of *Okoubaka*, only an initial dose (3 drops) of the *Okoubaka* products was applied at the beginning of the challenge tests. In reality, patients would continue treatment in situations of acute diarrhea past the stage of prophylaxis. This may be a plausible explanation for better anti-pathogenic and metabolic activity of *Okoubaka* in our *in vitro* system during the

first 24 hours. Further experiments in which *Okoubaka* products are repeatedly given to the test system during challenge tests may address this hypothesis.

Conclusions

Both *Okoubaka* products had only limited effects on the metabolic activity of the human gut microbiome during the 1-week treatment period in the *in vitro* SHIME system. Statistically significant anti-pathogenic activity against ETEC and *Salmonella enteritidis* was observed in the *in vitro* challenge tests after prophylactic treatment of the microbial community with *Okoubaka* MT and 3X. The effects were more potent for lower pathogen doses, which correspond to typical infectious doses.

The results were concentration-dependent and slightly more pronounced for *Okoubaka* MT compared to the diluted *Okoubaka* 3X preparation. *Okoubaka* treatment seems to better fit for prophylaxis of ETEC infections than for *Salmonella* infections. Slightly positive effects on metabolic activity (change in SCFA production, especially increases in butyrate) were observed. These effects might act in addition to the direct anti-pathogenic activity and initiate effects, such as butyrate-mediated host interactions against diarrhea.

Based on the results obtained in the complex *in vitro* microbial community system, we hypothesize an activating effect on intestinal microbiome resistance for both *Okoubaka* products, which may provide – or be a part of – an explanation for the traditional, empirically-based use of *Okoubaka* as prophylaxis and treatment of traveler's diarrhea in homeopathy.

Contributions

Susann Buchheim-Schmidt had the lead on the conception and the design of the study, contributed to the assembly, analysis, and interpretation of the data, and wrote the manuscript.

Uwe Peters contributed to the conception and the design of the study and data interpretation and reviewed the manuscript.

Cindy Duysburgh, Pieter Van den Abbeele, and Massimo Marzorati had the lead on the generation, collection, and assembly of data; contributed to the analysis and interpretation; and reviewed the manuscript. Cindy Duysburgh had the lead on the practical implementation of the study.

Thomas Keller led the analysis of data, contributed to the interpretation, and reviewed the manuscript.

Stephan Baumgartner, Petra Klement, and David Martin contributed to the conception and the design of the study, the data interpretation, and reviewed the manuscript.

All authors approved the final manuscript.

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Conflict of interest

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