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Ecophysiology of sulfoquinovose-metabolizing gut bacteria in humans, mice,
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The thesis underwent language improvement with Chat-GPT 4.o and Grammarly,
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Summary

Sulfoquinovose (SQ) is a sulfonated glucose and component of the thylakoid membrane lipid sulfoquinovosyl diacylglycerol (SQDG) in photosynthetic organisms. Despite its abundance in the environment, the dynamics of SQ metabolism in complex microbial communities remain poorly understood. In anoxic environments such as the human gut, specialized bacteria degrade SQ via interspecies metabolite transfer to sulfonate-respiring bacteria, resulting in the production of hydrogen sulfide with concentration-dependent health outcomes. The role of mammalian cells in SQ degradation remains unknown. In this thesis, SQ degradation and its ecological significance in different mammals were investigated through *in vivo* studies in germ-free and conventional mice and *in vitro* microcosm experiments with human feces, mouse gut content, and cow rumen fluid. Our study found that SQ is not metabolized by human cells or germ-free mice, indicating that it serves solely as a microbial substrate in these hosts. We established a relevant SQ dose range in human fecal microcosms. Although the mouse microbiota possessed the genetic capacity to degrade SQ and its metabolite, 2,3-dihydroxy-1-propanesulfonate (DHPS), complete SQ degradation, as observed in human fecal microcosms, did not occur in mouse gut content microcosms. The detection of DHPS in feces of mice after SQ administration proved microbial conversion of SQ *in vivo* for the first time. Our results elucidated the role of human and mouse cells in SQ degradation and showed fundamental differences in the SQ metabolism in the human gut and the wild type laboratory mice gut. We further developed an amplicon sequencing-based approach for diversity studies targeting *yihQ*, which encodes a sulfoquinovosidase that catalyzes the cleavage of SQ from SQDG and SQ glycosides in most known SQ degraders. We found that the richness of SQ degraders was low in the human and mouse gut but very high in the cow rumen. In rumen fluid microcosms, SQ was degraded to isethionate by novel uncultured members of *Caproiciproducens* (*Acutalibacteraceae*), *Limivacinus* (*Oscillospiraceae*), and *Sphaerochaetaceae*. Isethionate was then respired to sulfide, likely by *Mailhella* (*Desulfovibrionaceae*). Our results suggest that SQ(DG) plays an important role in the ruminant diet and microbial fermentation processes within the rumen. Overall, this work provides new insights into the role of SQ in the microbial sulfur metabolism in the animal gut.

Zusammenfassung

Sulfoquinovose (SQ) ist eine sulfonierte Glukose und Bestandteil des Thylakoidmembranlipids Sulfoquinovosyldiacylglycerol (SQDG) in photosynthetischen Organismen. Trotz der weiten Verbreitung in der Umwelt sind die Stoffwechselprozesse von SQ in komplexen mikrobiellen Gemeinschaften bislang nur unzureichend verstanden. In anoxischen Umgebungen wie dem menschlichen Darm wird SQ von spezialisierten Bakterien durch interspezifischen Metabolittransfer an Sulfonat-respirierende Bakterien abgebaut. Als Endprodukt entsteht hierbei Schwefelwasserstoff, welches konzentrationsabhängige, gesundheitliche Auswirkungen haben kann. Die Rolle von Säugetierzellen im SQ-Abbau ist bislang unbekannt. Diese Arbeit untersucht den SQ-Abbau und seine ökologische Bedeutung in verschiedenen Säugetieren anhand von *In-vivo*-Studien mit keimfreien und konventionellen Mäusen sowie *In-vitro*-Mikrokosmosexperimenten mit menschlichen Fäkalien, Maudarminhalt und Rinderpansenflüssigkeit. Unsere Studie ergab, dass SQ weder von menschlichen Zellen noch von keimfreien Mäusen metabolisiert wird, was darauf hindeutet, dass es in diesen Systemen ausschließlich als mikrobielles Substrat dient. Wir ermittelten einen relevanten SQ-Dosisbereich in menschlichen fäkalen Mikrokosmen. Obwohl die Mikrobiota der Maus die genetische Kapazität zur Verwertung von SQ und seines Metaboliten 2,3-Dihydroxy-1-propansulfonat (DHPS) besaß, fand in Maudarm-Mikrokosmen kein vollständiger SQ-Abbau statt, wie er in humanen Fäzes-Mikrokosmen beobachtet wurde. Der Nachweis von DHPS im Kot von Mäusen nach SQ-Gabe belegte erstmals die mikrobielle Umsetzung von SQ *in vivo*. Unsere Ergebnisse verdeutlichen die Rolle menschlicher und muriner Zellen im SQ-Abbau und zeigen grundlegende Unterschiede im SQ-Metabolismus im menschlichen Darm und im Darm von Wildtyp-Labor-Mäusen. Des Weiteren entwickelten wir einen auf Amplikonsequenzierung basierenden Ansatz zur Diversitätsanalyse von SQ-Abbauern, der auf das *yihQ*-Gen ausgerichtet ist. Dieses kodiert eine konservierte Sulfoquinovosidase, die SQ von SQDG und SQ-Glykosiden in den meisten bekannten SQ-Abbauern abspaltet. Wir fanden eine geringe Diversität an SQ-Abbauern im menschlichen und Maus-Darm, jedoch eine sehr hohe Vielfalt im Pansen von Rindern. In Mikrokosmen mit Pansenflüssigkeit wurde SQ durch nicht kultivierte Mitglieder von *Caproiciproducens* (*Acutalibacteraceae*), *Limivacinus* (*Oscillospiraceae*) und *Sphaerochaetaceae* zu Isethionat abgebaut. Isethionat wurde dann zu Sulfid veratmet,

wahrscheinlich von *Mailhella* (*Desulfovibrionaceae*). Unsere Ergebnisse deuten darauf hin, dass SQ(DG) eine wichtige Rolle in der Ernährung von Wiederkäuern und bei mikrobiellen Fermentationsprozessen im Pansen spielt. Insgesamt liefert diese Arbeit neue Einblicke in die Rolle von SQ im mikrobiellen Schwefelmetabolismus im tierischen Darm.

Introduction

Sulfur plays an essential role in various biological processes, including protein synthesis, enzyme function, and cellular respiration[1–3]. In the human diet, abundant organic sulfur compounds include the sulfur-containing amino acids cysteine, homocysteine, methionine, and taurine as well as glucosinolates and sulfolipids [4–6]. Dietary sources of sulfur-containing amino acids are animal products such as meat, fish, and eggs, while glucosinolates and sulfolipids are found in *Brassica* vegetables and green plants and algae, respectively [6–9]. On the other hand, inorganic sulfur compounds like sulfates are found in mineral water and various other beverages, while sulfites are additionally used as food preservatives [10]. Once ingested, sulfur compounds undergo transformation and cycling within the gastrointestinal tract, mediated by the host and its gut microbiota. The mammalian gut microbiome, comprising around 100 trillion microbial cells and a few hundred different bacterial and archaeal species, harbors a diverse set of microorganisms capable of metabolizing ingested sulfur compounds through a variety of biochemical pathways [11,12]. These pathways include sulfur assimilation, reduction, and oxidation processes, all of which contribute to the local sulfur cycle [13].

The sulfur metabolism in the human gut

Within the inorganic sulfur metabolism in the human gut, dissimilatory sulfite reduction is a central metabolic pathway that is fueled by various organic and inorganic sulfur compounds (**Figure 1**, **Figure 2**). Sulfate-reducing bacteria, such as *Desulfovibrio* spp., contribute to this process by first reducing sulfate to sulfite, which is then further reduced to hydrogen sulfide (H_2S). Similarly, sulfite-reducing bacteria, such as *Bilophila wadsworthia*, utilize sulfite as an electron acceptor during anaerobic respiration, also producing H_2S [14]. Human mucosal cells oxidize H_2S to thiosulfate, which may be further oxidized to tetrathionate by reactive oxygen species during inflammation [15–18]. Tetrathionate and thiosulfate can subsequently serve as an electron acceptor for intestinal bacteria regenerating H_2S [13,15,18,19]. Additionally, bacteria assimilate inorganic sulfur into organic compounds (**Figure 1**). In assimilatory sulfate reduction, sulfate is reduced to H_2S , which is then incorporated into cysteine via a reaction with O-acetylserine [20].

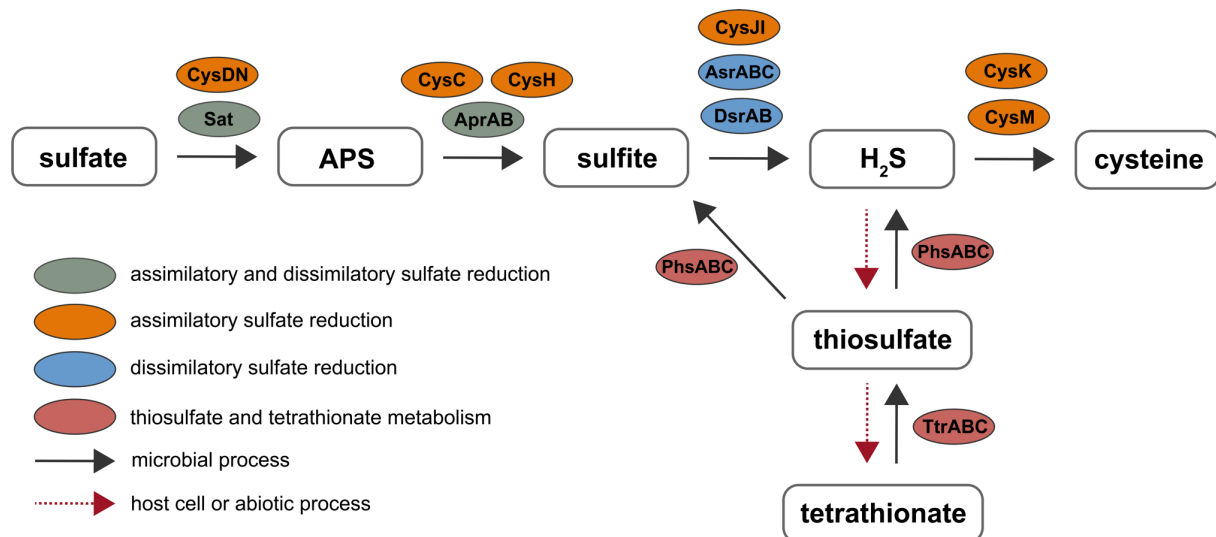


Figure 1. Inorganic sulfur metabolism in the human gut. Enzymes are categorized by function: assimilatory and dissimilatory sulfate reduction (green), assimilatory sulfate reduction (orange), dissimilatory sulfate reduction (blue), and thiosulfate/tetrathionate metabolism (red). Black arrows represent microbial processes, whereas red arrows indicate host cell or abiotic reactions. Not all intermediate steps are shown, and only key sulfur reactants and products are depicted. CysDN and Sat, sulfate adenylyltransferase; CysC, adenylyl-sulfate kinase; CysH, phosphoadenosine 5'-phosphosulfate reductase; Apr, adenylyl-sulfate reductase; CysJI, sulfite reductase NADPH flavoprotein; AsrABC, anaerobic sulfite reductase; DsrAB, dissimilatory sulfite reductase; CysK, cysteine synthase A; CysM, cysteine synthase B; PhsABC, thiosulfate reductase; TtrABC, tetrathionate reductase; H₂S, hydrogen sulfide; APS, adenosine 5'-phosphosulfate.

Dietary sulfur-containing amino acids can reach the colon and undergo bacterial degradation (**Figure 2**). These organic sulfur compounds contribute to sulfite reduction and concomitant H₂S production in the human gut as their inorganic sulfur moiety serves as an electron acceptor while their organic moiety provides carbon [13]. Cysteine is either directly degraded to H₂S or via the intermediates cystathionine and homocysteine. Methionine, in contrast, is initially converted to homocysteine before further degradation to H₂S [13]. In addition to dietary taurine, taurine is also released in the small intestine as a component of endogenously produced conjugated bile acids during enterohepatic circulation [13]. Bacterial bile acid hydrolases cleave these conjugates, generating free taurine and unconjugated bile acids, the latter of which are reabsorbed by the host [21]. Free taurine is then metabolized by the gut microbiota, first to sulfoacetaldehyde, which is either desulfonated to sulfite or first converted to isethionate before undergoing desulfonation [13,22]. Beyond dietary sulfur compounds, endogenous organosulfur compounds such as sulfated bile, chondroitin sulfate, heparin, and sulfomucins also serve as sulfur sources for the gut microbiota, further contributing to the gut sulfur metabolism [23].

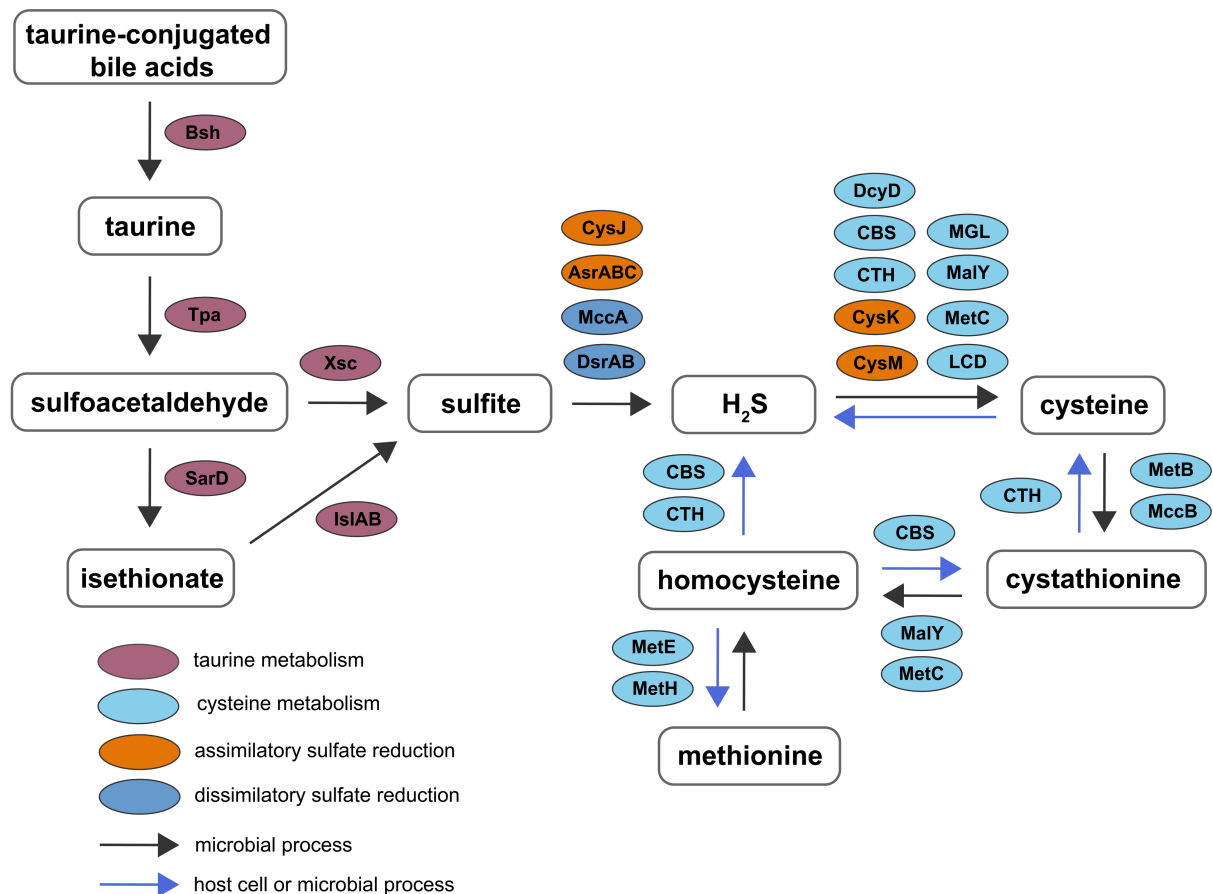


Figure 2. Organic sulfur metabolism in the human gut. Enzymes are categorized by function: taurine metabolism (purple), cysteine metabolism (light blue), assimilatory sulfate reduction (orange), and dissimilatory sulfate reduction (blue). Black arrows represent microbial processes, whereas blue arrows indicate host cell or microbial reactions. Not all intermediate steps are shown, and only key sulfur reactants and products are depicted. Bsh, bile salt hydrolase; Tpa, taurine: pyruvate aminotransferase; SarD, sulfoacetaldehyde reductase; IslAB, isethionate sulfite-lyase; Xsc, sulfoacetaldehyde transferase; CysJ, sulfite reductase NADPH flavoprotein; AsrABC, anaerobic sulfite reductase; MccA, cytochrome c sulfite reductase; DsrAB, dissimilatory sulfite reductase; DcyD, D-cysteine desulfhydrase; CBS and MetB, cystathionine β -synthase; CTH and MccB, cystathionine γ -lyase; CysK, cysteine synthase A; CysM, cysteine synthase B; MGL, methionine γ -lyase; MalY and MetC, cystathionine β -lyase; LCD, L-cysteine desulfhydrase; MetE and Meth, methionine synthase; H_2S , hydrogen sulfide.

More recently, sulfoquinovose (6-deoxy-6-sulfoglucose, SQ) — a sulfonated dietary hexose— has been recognized as a previously underexplored contributor to the gut sulfur metabolism [6,24]. Given the prevalence of plant-derived compounds in the human diet, SQ may play a significant role in sulfur turnover within the gut [25]. Its degradation by specialized gut bacteria not only contributes to sulfur cycling but may also have important implications for host-microbiome interactions, for example, through the modulation of microbial community composition and through the production of sulfur-containing metabolites and short-chain fatty acids.

Sulfoquinovose

SQ is commonly found as the polar headgroup of the sulfolipid sulfoquinovosyl diacylglycerol (SQDG) and its metabolites in photosynthetic organisms such as plants, algae, and cyanobacteria [6]. SQDG resides in thylakoid membranes and supports the structural integrity and activity of photosystem II [26,27]. Beyond photosynthetic organisms, SQ has also been detected in certain archaeal glycoproteins and glycans [28–30]. With an estimated annual production of approximately 10^{13} tons, SQ and SQDG play a key role in the biogeochemical sulfur cycle, with production levels comparable to those of the sulfur-containing amino acids cysteine and methionine [6,31].

Bacterial degradation of sulfoquinovosyl diacylglycerol to sulfoquinovose

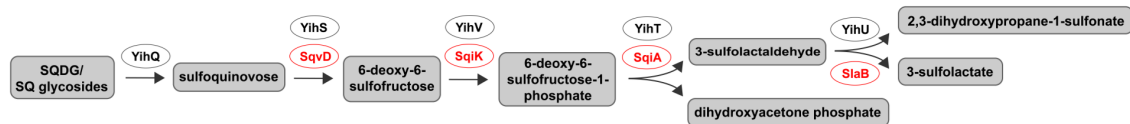
SQ glycosides are formed through the delipidation of SQDG, whereas free SQ results from delipidation and subsequent glycoside hydrolysis. Although known bacterial gene clusters for SQDG degradation lack genes that encode lipases for SQDG delipidation, two glycoside hydrolases have been identified to hydrolyze SQ glycosides [32,33]. The first discovered sulfoquinovosidase, YihQ, was found in *Escherichia coli* and belongs to glycoside hydrolase family 31 [32,34]. Notably, YihQ hydrolyzes both α -sulfoquinovosyl glycerol and α -SQDG, demonstrating that glycoside hydrolysis does not necessarily require prior delipidation [6]. Homologs of YihQ were found in most currently known gene clusters associated with SQ degradation (**Figure 3**) and are collectively referred to as YihQ in this work. However, some bacteria have gene clusters involved in SQ degradation that lack a YihQ homolog [35–37]. Instead, they possess the nicotinamide adenine dinucleotide (NAD⁺)-dependent glycoside hydrolase family 188 sulfoquinovosidase SsqA, which hydrolyzes both α - and β - sulfoquinovosyl glycerol through an oxidoreductive mechanism [33]. SsqA is abundant in marine bacteria but was also found in eukaryotes and archaea [33].

Bacterial sulfoquinovose degradation

Once SQ glycosides are hydrolyzed to SQ, sulfoglycolytic bacteria degrade SQ via different pathways (**Figure 3**). A first sulfoglycolytic pathway resembling the Embden-Meyerhof-Parnas pathway was discovered in *E. coli* K-12. In this pathway, SQ is degraded aerobically or anaerobically to two C₃-moieties, dihydroxyacetone phosphate, which enters the central carbon metabolism, and 2,3-dihydroxypropane-1-sulfonate (DHPS), which is excreted [38,39]. In a variation of this pathway, the

intermediate metabolite 3-sulfolactaldehyde is not reduced to DHPS but instead oxidized to 3-sulfolactate [40]. In the sulfo-Enter-Doudoroff pathway, discovered in *Pseudomonas putida*, SQ is aerobically degraded to the two C₃-moieties 3-sulfolactaldehyde and pyruvate. Pyruvate enters the central carbon metabolism while 3-sulfolactaldehyde is oxidized to 3-sulfolactate [24,41]. The sulfofructose transaldolase pathway, resembling the pentose-phosphate pathway, was discovered in *Bacillus aryabhattai*, *Otoolea symbiosa* (formerly *Clostridium symbiosum*), and *Priestia megaterium* (formerly *Bacillus megaterium*) [42,43]. SQ is cleaved to 3-sulfolactaldehyde and a non-sulfonated C₃-moiety. The latter is transferred onto glyceraldehyde-3-phosphate, which is subsequently used as a carbon and energy source [24,42,43]. 3-sulfolactaldehyde is either oxidized to 3-sulfolactate or reduced to DHPS [42]. Many strictly fermenting bacteria, including *Clostridium* sp., use the sulfo-transketolase pathway for SQ degradation, which resembles the pentose-phosphate pathway [40]. SQ is degraded through the sequential transfers of two non-sulfonated C₂-moieties onto two glycerol-3-phosphate molecules. The resulting xylose-5 phosphates go into the central carbon metabolism while the sulfonated C₂-moiety sulfoacetaldehyde is reduced to isethionate or oxidized to sulfoacetate [40,44]. In a bifurcated sulfoglycolytic transketolase/transaldolase pathway, SQ is cleaved into C₃- and C₂-moieties, resulting in isethionate, DHPS, and 3-sulfolactate formation [45]. In the monooxygenase pathway for SQ degradation in *Novosphingobium aromaticivorans* and *Agrobacterium tumefaciens*, a flavin- and oxygen-dependent alkanesulfonate monooxygenase facilitates the cleavage of the C-S bond into 6-dehydroglucose and sulfite [40,46]. Similarly, an iron-dependent and α -ketoglutarate-dependent dioxygenase cleaves the C-S bond into 6-dehydroglucose and sulfite in *Marinomonas ushuaiensis* [36].

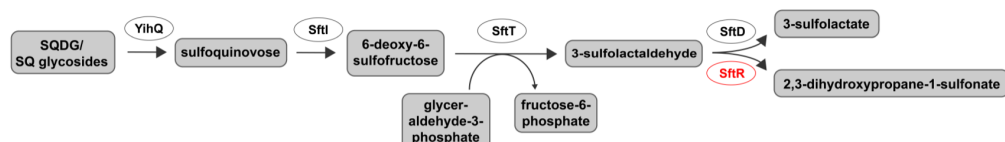
Sulfo-Embden-Meyerhof-Parnas pathway (sulfo-EMP /-EMP2)



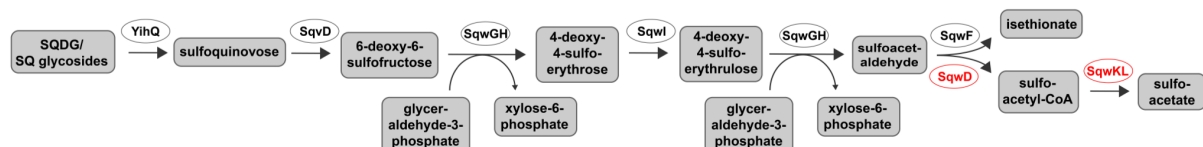
Sulfo-Entner-Doudoroff pathway



Sulfofructose transaldolase pathway (SFT/SFT2)



Sulfo-transketolase pathway (TK/TK2)



SQ oxygenase pathway (ASMO/ASDO)

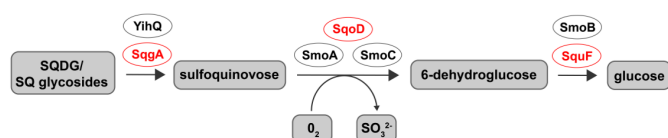


Figure 3. SQ degradation pathways. Currently known SQ degradation pathways are the sulfo-Embden-Meyerhof-Parnas (sulfo-EMP) pathway, the sulfo-Entner-Doudoroff pathway, the sulfofructose transaldolase (SFT) pathway, the sulfo-transketolase (TK) pathway, and the SQ oxygenase (ASMO, alkanesulfonate monooxygenase; ASDO, alkanesulfonate dioxygenase) pathway. Some pathways have two variations, with one variation highlighted in red (-EMP2, SFT2, TK2, ASDO). Enzymes involved in these variations are also marked in red. Metabolites are shown in grey rounded rectangles, while enzymes are in ovals. Not all intermediate steps are shown, and only key sulfur reactants and products are depicted. YihQ and SqgA, sulfoquinovosidase; YihS, SqvD, and SftI, sulfoquinovose isomerase; YihV and Sqik, sulfofructose kinase; YihT and Sqia, sulfofructosephosphate aldolase; YihU and SftR, 3-sulfolactaldehyde reductase; SlaB, Sladh, and SftD, 3-sulfolactaldehyde dehydrogenase; Sqdh, sulfoquinovose dehydrogenase; Sgl, 6-deoxy-6-sulfofoglucolactone lactonase; Sgdh, 6-deoxy-6-sulfofoglucuronate dehydratase; Sgald, 2-keto-3,6-dideoxy-6-sulfofoglucuronate aldolase; SftT, 6-deoxy-6-sulfo-D-fructose transaldolase; SqwGH, 6-deoxy-6-sulfo-D-fructose transketolase; Sqwl, 4-deoxy-4-sulfo-D-erythrose isomerase; SqwF, sulfoacetaldehyde reductase; SqwD, sulfoacetaldehyde dehydrogenase; SqwKL, sulfoacetate-CoA ligase; SqoD and SmoC, alkanesulfonate monooxygenase; SmoA, flavin reductase; SmoB and SqwF, 6-dehydroglucose reductase; SQDG, sulfoquinovosyl diacylglycerol.

In four SQ degradation pathways, C₂- or C₃-intermediates are produced and released [38,40–42]. For complete SQ degradation, these organosulfonates are further degraded by syntrophic second-tier degraders, which usually cleave the C-S bond, resulting in a carbon backbone and sulfite. While the carbon moiety enters the central

carbon metabolism, sulfite is used as an electron donor or acceptor, depending on the environment. In aerobic environments, sulfite is usually oxidized to sulfate [47–50], while in anaerobic environments, sulfite is used as a terminal electron acceptor and is reduced to H_2S [39,51–53]. In aerobic environments, DHPS is oxidized to 3-sulfolactate, which is then cleaved to pyruvate and sulfite via the sulfolactate-sulfolase SuyAB, with sulfite being subsequently oxidized to sulfate [47]. Alternatively, 3-sulfolactate is oxidized to 3-sulfoxypropionate and then bifurcated to cysteate, with subsequent C–S cleavage to pyruvate and sulfite, and to sulfoacetaldehyde, followed by C–S cleavage to acetyl-CoA and sulfite. Sulfite is then oxidized to sulfate [48]. In anaerobic environments, several distinct pathways for DHPS degradation have been identified (see Figure 1b in Chapter 1). *B. wadsworthia* and other sulfite-reducing bacteria convert DHPS to hydroxyacetone and sulfite, with the latter being reduced to H_2S [25,39,51]. In *Desulfovibrio* sp. DF1, however, DHPS instead undergoes cleavage via 3-sulfolactaldehyde and 3-sulfolactate, yielding pyruvate and sulfite. Sulfite is then further reduced to H_2S [39]. Certain fermenting bacteria metabolize DHPS via sulfoxypropionaldehyde, which is further reduced to 3-hydroxypropane-1-sulfonate [51]. Alternatively, DHPS is bifurcated to 3-hydroxypropane-1-sulfonate and 3-sulfoxypropionate in *Enterococcus gilvus* [54]. While many SQ-degrading bacteria rely on cooperative degradation of released organosulfonates, some SQ-degrading bacteria possess so-called ‘extended sulfoglycolytic’ pathways. These pathways not only enable first-tier SQ degradation but also allow for further metabolism of intermediate C_2 - and C_3 -organosulfonates within a single organism [51,55].

Sulfoquinovose in the human gut

As a component of plant and algal thylakoid membranes, SQDG is frequently ingested with the human diet. Spinach was the vegetable with the highest amount of SQDG, with 824 $\mu\text{g/g}$ of dried vegetable, and also had the highest percentage of SQDG in all glycolipids, with 35.8% [56]. Other tested vegetables with high amounts of SQDG were parsley, green onion, and chives [56]. In algae, SQDG is within the top three most abundant glycolipids [57]. SQ is a substrate for a few specialized bacteria in the human gut [25]. In human fecal microcosms, *Agathobacter rectalis*, formerly known as *Eubacterium rectale*, degraded SQ to formate, acetate, and DHPS, the latter of which was excreted. *B. wadsworthia* subsequently degraded DHPS further into hydroxyacetone and H_2S (Figure 4) [25]. In human gut metagenome-assembled genomes, 44% of *A. rectalis* metagenome-assembled genomes contained the

sulfofructose transaldolase pathway. In contrast, only 0.5% of all species-level genome bins harbored the sulfofructose transaldolase pathway [25]. Nevertheless, SQ degradation was considered a core function of the intestinal microbiota as the sulfofructose transaldolase pathway ranked among the top third most expressed pathways in a metatranscriptomic analysis of human fecal samples. While individual samples were mostly dominated by only one SQ degrader, other bacteria, such as *Faecalibacterium prausnitzii*, *Lachnoclostridium* sp., and *Roseburia* sp. also encoded the sulfofructose transaldolase pathway [25]. *A. rectalis*, *F. prausnitzii*, *Lachnoclostridium* spp., and *Roseburia* spp. are butyrate producers in the human gut [58–61]. Butyrate is the preferred energy source of the colonocytes and has been linked to the prevention of colitis and colorectal cancer [62,63]. Additionally, the growth of *A. rectalis* was associated with lower postprandial glycemic response, and *F. prausnitzii* delayed or prevented tumorigenesis in animal experiments [64–66]. Acetate, one of the main SQ degradation products, has been shown to support epithelial maintenance, promote wound healing, and enhance gut barrier integrity [67–70]. Additionally, acetate reduced colonic inflammation in mice and protected against enteropathogens [71,72]. Acetate also serves as the substrate for butyrate production for *A. rectalis*, *F. prausnitzii*, and *Roseburia* spp. [73–75]. While these first-tier SQ degraders are generally associated with gut health and positive health outcomes, second-tier SQ degradation by *B. wadsworthia* and production of H₂S have ambivalent and dose-dependent health effects [76]. H₂S may play a role in limiting pathogen colonization by impairing their aerobic respiration [77], may preserve T cell viability and function [78], and contributes to mitochondrial energy metabolism, acts as an intracellular antioxidant, and functions as a gasotransmitter at low nano- to micromolar levels [79]. In contrast, at micro- to millimolar concentrations, H₂S can compromise the intestinal mucosal barrier by breaking disulfide bonds, potentially allowing bacteria to breach this barrier [80]. Elevated levels of H₂S can additionally inhibit cytochrome c and lead to apoptosis [81]. High levels of sulfite-reducing bacteria, such as *B. wadsworthia*, are also considered potential contributors to the onset of gut inflammation [14,80,82,83].

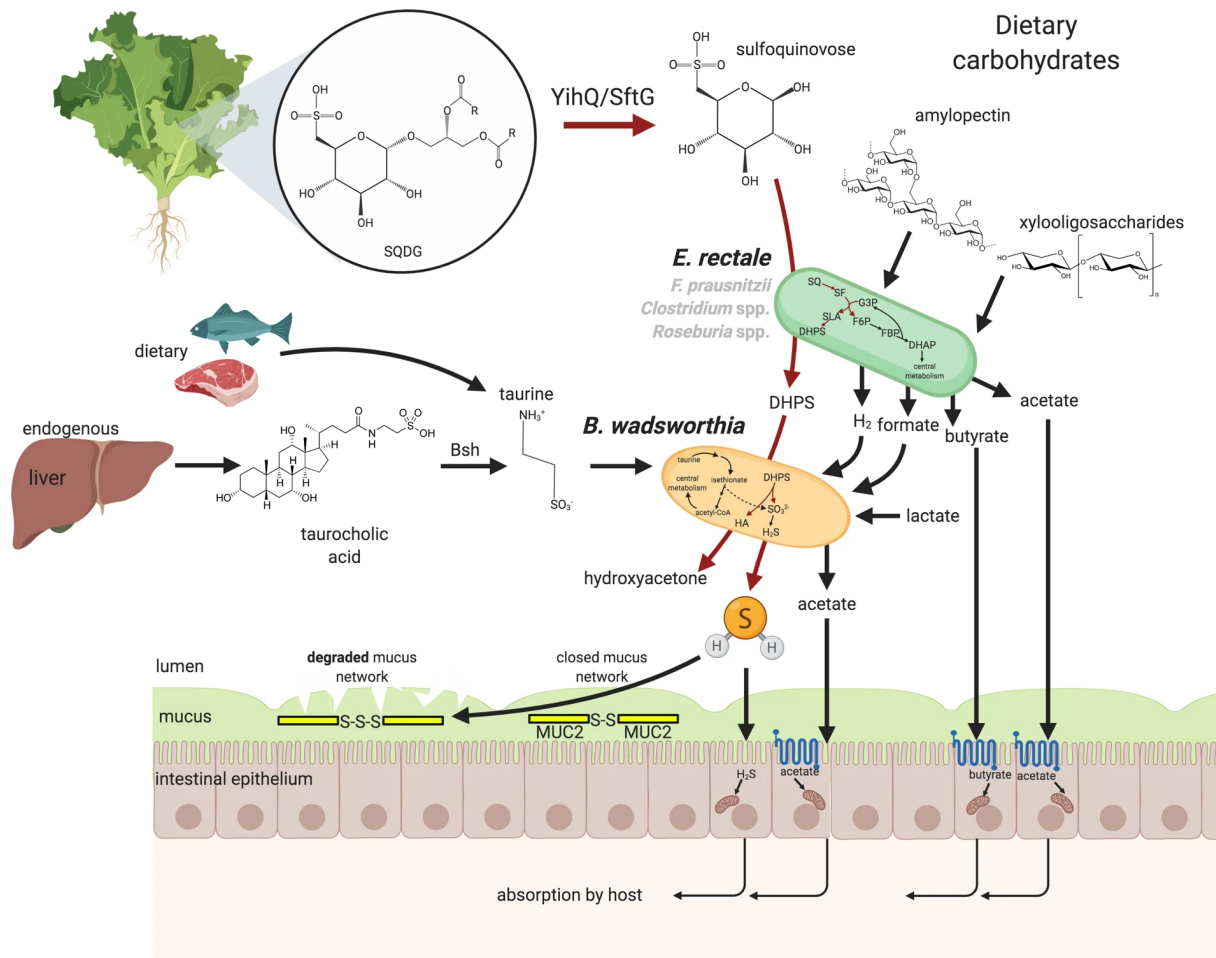


Figure 4. SQ metabolism in the human gut. Figure is reproduced from Hanson *et al.*, The ISME Journal (2021), under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>) [25]. No changes were made. Dietary SQDG reaches the gut, where bacterial YihQ sulfoquinovosidases cleave SQ from the lipid moiety. In most individuals, SQ is degraded via interspecies metabolite transfer between *A. rectalis* and *B. wadsworthia*. *A. rectalis* ferments SQ via the sulfofructose transaldolase pathway, resulting in significant DHPS, acetate, and formate formation. *A. rectalis* also metabolizes dietary carbohydrates like amylopectin and xylooligosaccharides. The sulfofructose transaldolase pathway can also be facilitated by other bacteria, including members of *Faecalibacterium*, *Roseburia*, and *Clostridium*. *B. wadsworthia* further degrades DHPS by cleavage of the carbon–sulfur bond to release sulfite, which is further reduced to H₂S. Additionally, hydroxyacetone is released. Taurine, derived from the diet or the bile acid metabolism via bile salt hydrolases (Bsh), is another sulfonate source for H₂S production by *B. wadsworthia*. H₂S and short-chain fatty acids serve as energy substrates and signaling molecules for the colonic epithelium and are absorbed [79,84,85]. H₂S can disrupt the colonic mucus network by reducing its disulfide bonds to trisulfides [80]. Established pathways are indicated in black, while newly identified pathways by Hanson *et al.* are highlighted in red [25]. SQDG sulfoquinovosyl diacylglycerol, SQ, sulfoquinovose; SF, 6-deoxy-6-sulfofructose; G3P, glyceraldehyde-3-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; SLA, 3-sulfolactaldehyde; DHPS, 2,3-dihydroxypropane-1-sulfonate; HA, hydroxyacetone.

While the bacterial degradation of dietary SQDG/SQ in the human gut has been studied, the role of mammalian cells in SQDG degradation has only been partially elucidated. Pancreatic lipases hydrolyze SQDG to sulfoquinovosyl glycerol [86,87], but it is unclear if mammalian cells can metabolize sulfoquinovosyl glycerol or SQ.

Previous work with SQDG and mammalian cells focused on the potential health implications of SQDG. SQDG had an antiproliferative effect on different human cancer cell lines due to the targeted inhibition of the mammalian DNA polymerase and the cellular tumor antigen p53 [56,88–93]. Additionally, SQDG exhibited an anti-inflammatory effect by inhibiting nitric oxide on LPS-induced murine macrophage cells [94].

Sulfoquinovose in other environments

SQDG/SQ may also be a key compound in the intestinal sulfur metabolism of other mammals, especially those that ingest a lot of green plants, such as cows or rabbits. Apart from the mammalian intestine, SQDG may play a role in terrestrial, freshwater, and marine environments. Since chloroplast destruction in senescent leaves is not thought to involve SQDG degradation [43], SQDG remains in the soil when plant materials fall to the ground and may be degraded by soil bacteria [43,55]. Notably, SQ degradation pathways were identified in some soil-derived bacterial isolates. *P. megaterium* uses the sulfofructose transaldolase pathway [43], while *P. putida* metabolizes SQ via the sulfoglycolytic Entner-Doudoroff pathway [41], and *Klebsiella oxytoca* degrades SQ via the sulfoglycolytic Embden-Meyerhof-Parnas pathway [95].

In marine environments, sulfolipids are abundant in membranes of photosynthetic marine microorganisms and cyanobacterial vesicles. The high sulfate concentration of ~28 mM in the ocean is primarily derived from inorganic sources [55,96,97] and provides an ample sulfur supply for the synthesis of sulfonates like SQ. The production of these sulfonates by phototrophs and their degradation by heterotrophs represent an important component of the carbon flux in this ecosystem [55,96]. Bacteria of the genera *Roseobacter* and *Marinomonas* were identified as microbial SQ degraders in marine ecosystems by using the alkanesulfonate monooxygenase or the alkanesulfonate dioxygenase pathway, respectively [35,36].

Research objectives and approaches

SQ is commonly consumed in the human diet, but its degradation in mammals remains poorly understood, particularly regarding the role of mammalian host cells. While *in vitro* studies have shown that SQ serves as a substrate for select gut bacteria, contributes to H₂S production, and stimulates *B. wadsworthia* growth, the potential health impact of SQ on the host requires further investigation. The first study of my

PhD aimed to develop a better understanding of the SQ metabolism in humans and evaluate wild type laboratory mice as a model organism for studying the host impact of SQ. This involved examining SQ degradation *in vitro* in human colon cancer cells and *in vivo* in germ-free mice using ^{13}C -SQ stable isotope probing, studying the effect of SQ on microbiota abundance and activity in human fecal microcosms, and assessing SQ metabolism in wild type laboratory mice through *in vivo* and *in vitro* using metabolite analyses and microbiome profiling.

SQDG is a ubiquitous substrate in different ecosystems, and while we know some SQ degraders from *in vitro* studies with environmental isolates, we lack knowledge about the diversity and dynamics of SQ degraders and their metabolism across various environments. The second study of my PhD, therefore, aimed to establish a *yihQ*-targeted amplicon sequencing approach to assess the diversity of SQ degraders across different environments. For this, several degenerate primer variants were designed from a *YihQ/yihQ* reference database and initially tested with pure culture and environmental DNA. Two primer sets were further evaluated for amplicon sequencing using samples from human feces, mouse intestinal contents, cow rumen fluid, and marine sediment. Amplicon sequencing data were analyzed to show the specificity of primer pairs and diversity of SQ degraders in various environments. The newly developed amplicon sequencing assay was applied to SQ-amended rumen fluid microcosms. Additionally, SQ degradation dynamics within the microcosms were studied through metabolite analysis, 16S rRNA gene sequencing, and metagenomic sequencing.

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Chapter 1

Sulfoquinovose is exclusively metabolized by the gut microbiota and degraded differently in mice and humans

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Author contributions

J.K. and A.L. conceived the study. J.K., B.T.H., A.S.W., S.B., M.L., and G.A. performed experiments and analyzed data. A.R., D.B., D.M., D.S., and B.S. contributed essential experimental infrastructure and analytical instrumentation, and expert advice. J.K., T.S.T., and B.H. performed bioinformatic analyses. J.K., B.T.H., A.S.W., S.B., T.S.T., M.M., and A.L. interpreted the data. J.K., M.M., and A.L. wrote the article. All authors revised and approved the manuscript.

Abstract

Background

Sulfoquinovose (SQ) is a green-diet-derived sulfonated glucose and a selective substrate for few human gut bacteria. Complete anaerobic SQ degradation via interspecies metabolite transfer to sulfonate-respiring bacteria produces hydrogen sulfide, which has dose- and context-dependent health effects. Here, we studied potential SQ degradation by the mammalian host and the impact of SQ supplementation on human and murine gut microbiota diversity and metabolism.

Results

$^{13}\text{CO}_2$ breath tests with germ-free C57BL/6 mice gavaged with ^{13}C -SQ were negative. Also, SQ was not degraded by human intestinal cells *in vitro*, indicating that SQ is not directly metabolized by mice and humans. Addition of increasing SQ concentrations to human fecal microcosms revealed dose-dependent responses of the microbiota and corroborated the relevance of *Agathobacter rectalis* and *Bilophila wadsworthia* in cooperative degradation of SQ to hydrogen sulfide via interspecies transfer of 2,3-dihydroxy-1-propanesulfonate (DHPS). Similar to the human gut microbiome, the genetic capacity for SQ or DHPS degradation is sparsely distributed among bacterial species in the mouse gut. *Escherichia coli* and *Enterocloster clostridioformis* were identified as primary SQ degraders in the mouse gut. SQ and DHPS supplementation experiments with conventional laboratory mice and their intestinal contents showed that SQ was incompletely catabolized to DHPS. Although some *E. clostridioformis* genomes encode an extended sulfoglycolytic pathway for both SQ and DHPS fermentation, SQ was only degraded to DHPS by a mouse-derived *E. clostridioformis* strain.

Conclusions

Our findings suggest that SQ is solely a nutrient for the gut microbiota and not for mice and humans, emphasizing its potential as a prebiotic. SQ degradation by the microbiota of conventional laboratory mice differs from the human gut microbiota by absence of DHPS degradation activity. Hence, the microbiota of conventional laboratory mice does not fully represent the SQ metabolism in humans, indicating the

need for alternative model systems to assess the impact of SQ on human health. This study advances our understanding of how individual dietary compounds shape the microbial community structure and metabolism in the gut and thereby potentially influence host health.

Key words

Sulfoquinovose, prebiotic, hydrogen sulfide, organosulfonate, sulfur metabolism, gut microbiome

Introduction

The sulfonated glucose sulfoquinovose (6-deoxy-6-sulfo-D-glucose, SQ) is an abundant sulfur molecule in the global sulfur cycle. As the polar headgroup of the sulfonolipid sulfoquinovosyldiacylglycerol (SQDG), an estimated 10 billion tons of SQ is synthesized annually by photosynthetic organisms such as plants, algae, and cyanobacteria [1]. SQDG is located in thylakoid membranes and maintains the activity and structural integrity of photosystem II [2–5]. Sulfoglycolytic microorganisms can cleave SQ from SQDG or other SQ glycosides via a sulfoquinovosidase. Two types of sulfoquinovosidase enzymes are known. The YihQ sulfoquinovosidase belongs to glycoside hydrolase family 31 [6] and the NAD⁺-dependent SqqA sulfoquinovosidase belongs to glycoside hydrolase family 188 [7]. To date, six microbial SQ degradation pathways have been described. In two pathways, SQ is catabolized aerobically to sulfite by individual organisms using an alkanesulfonate monooxygenase [8] or an iron- and alpha-ketoglutarate-dependent alkanesulfonate dioxygenase [9]. The other pathways require syntrophic interspecies metabolite transfer of C₂- or C₃-organosulfonates for complete degradation. The sulfo-Embden-Meyerhof-Parnas pathway generates the C₃-organosulfonates 2,3-dihydroxypropane-1-sulfonate (DHPS) or 3-sulfolactate (SL) during aerobic or anaerobic growth [10,11]. The sulfo-Entner-Doudoroff-pathway degrades SQ aerobically to SL [12]. The sulfofructose-transaldolase pathway produces SL aerobically and SL or DHPS anaerobically [13,14]. The sulfo-transketolase pathway degrades SQ to two molecules of the C₂-organosulfonates isethionate or, putatively, sulfoacetate [11]. In these pathways, the residual carbon skeleton (either C₃ or C₂) enters the central carbon metabolism while organosulfonates are excreted.

DHPS is a key metabolite of SQ-degrading human gut bacteria (Figure 1 a) [15]. Four pathways for anaerobic DHPS degradation are currently known (Figure 1 b). Two of these pathways are utilized by select sulfite-reducing bacteria (*Desulfovibrio*, *Bilophila*) and result in the intracellular formation of sulfite, which is subsequently respired to hydrogen sulfide (H₂S). In *Desulfovibrio* strain DF1, DHPS is degraded to pyruvate and sulfite via 3-sulfolactaldehyde and SL [16]. In *Bilophila wadsworthia*, DHPS is cleaved to hydroxyacetone and sulfite [15,17]. Some fermenting bacteria, such as *Klebsiella oxytoca* and *Hungatella hathewayi*, can degrade DHPS by reducing

it to 3-hydroxypropane-1-sulfonate (3-HPS) via sulfopropionaldehyde in a third pathway [17]. In a fourth, bifurcated pathway in *Enterococcus gilvus*, DHPS is converted to both 3-HPS and 3-sulfopropionate [18]. In the human gut, *B. wadsworthia* may use 3-sulfopropionate, but not 3-HPS, as a terminal electron acceptor resulting again in H₂S formation [18]. The strictly anaerobic mouse gut bacterium *Taurinivorans muris* encodes another potential DHPS degradation pathway, but did not degrade DHPS in growth tests [19]. However, this pathway was thus far only demonstrated in aerobic bacteria, which have the sulfopropanediol-3-dehydrogenase HpsN to oxidize (R)-DHPS to (R)-sulfolactate [20], which is then cleaved to pyruvate and sulfite by sulfolactate-sulfolylase SuyAB [21].

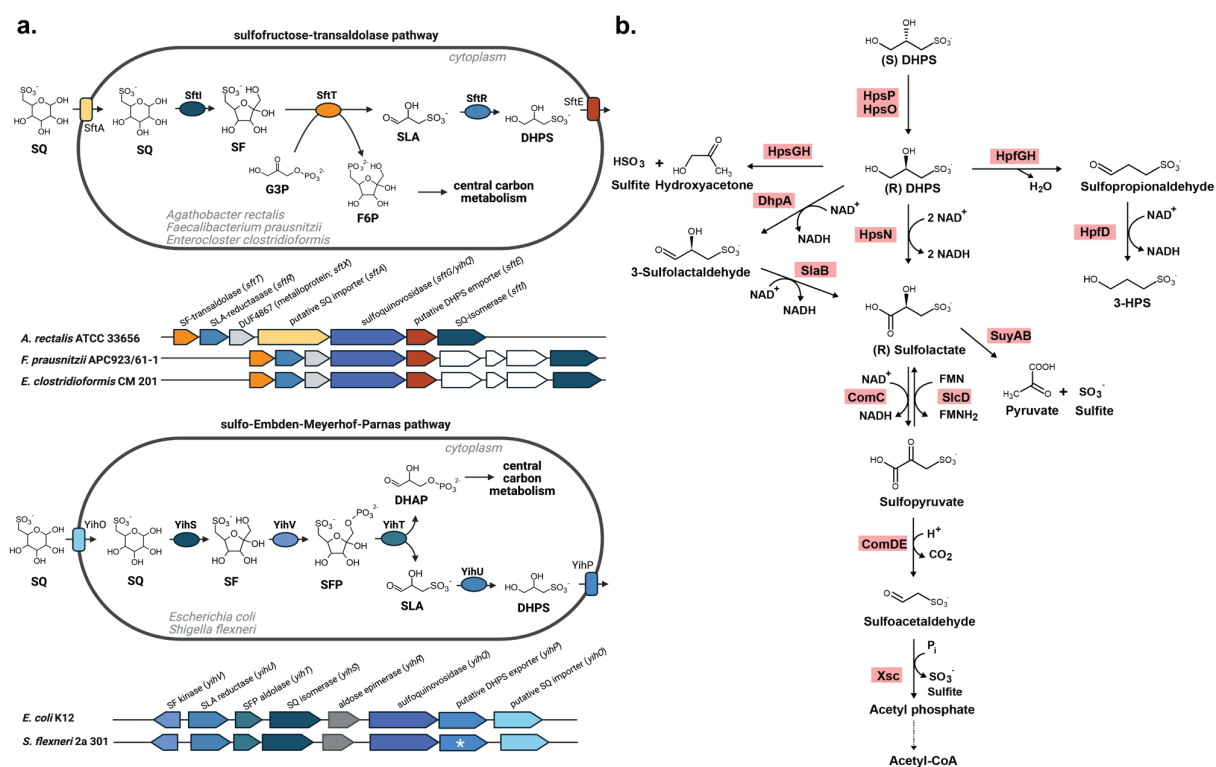


Figure 1: Prevalent SQ degradation pathways in the human gut and overview of enzymatic DHPS degradation reactions. SQ is degraded to DHPS via the sulfofructose-transaldolase pathway in *Agathobacter rectalis* (GCF_000020605.1), *Faecalibacterium prausnitzii* (GCF_003287495.1), and *Enterocloster clostridioformis* (GCF_000371405.1) or via the sulfo-Emden-Meyerhof-Parnas pathway in *Escherichia coli* (GCF_000005845.2) and *Shigella flexneri* (GCF_000006925.2) (created with biorender.com) (a). Genes depicted in white have no predicted function. The white asterisk indicates that the *yihP* homolog of *S. flexneri* contains a frameshift and is thus a potential pseudogene. Overview of known DHPS degradation pathways (b). HpsP, DHPS-2-dehydrogenase; HpsO, DHPS-2-dehydrogenase; HpsG, DHPS-sulfolylase; HpsH, DHPS-sulfolylase activating enzyme; HpfG, DHPS-dehydratase; HpfH, DHPS-dehydratase activating enzyme; HpfD, sulfopropionaldehyde reductase; HpsN, DHPS-1-dehydrogenase; DhpA, dehydrogenase; SlaB, dehydrogenase; SuyAB sulfolactate-sulfolylase; ComC, sulfolactate dehydrogenase; SlcD, sulfolactate dehydrogenase; ComDE, 3-sulfolpyruvate decarboxylase; Xsc, sulfoacetaldehyde acetyltransferase. SQ, sulfoquinovose; SF, 6-

deoxy-6-sulfofructose; SLA, 3-sulfolactaldehyde; DHPS, 2,3-dihydroxypropane-1-sulfonate; G3P, glyceraldehyde-3-phosphate; F6P, fructose-6-phosphate; SFP, 6-deoxy-6-sulfofructose phosphate; DHAP, dihydroxyacetone phosphate.

As a component of edible green plants and algae, SQDG is part of the human and animal diet. While the capability of mammalian cells to metabolize SQ remains unclear, SQ is a selective substrate for a few but abundant human gut bacteria [15]. *Agathobacter rectalis* (formerly *Eubacterium rectale*) and *B. wadsworthia* were identified as main primary and secondary SQ degraders in human fecal microcosms, respectively. Nearly half of the high-quality *A. rectalis* genomes from a human microbiome database [22] encoded SQ degradation via the sulfofructose-transaldolase pathway, though this potential was rare among other human gut bacteria, with only 0.5% of species-level genome bins containing the pathway. Despite its limited genomic prevalence, the sulfofructose-transaldolase pathway ranked among the top third most abundant microbial pathways in fecal metatranscriptome datasets of four human cohorts. This highlights SQ degradation as a core function of the human gut microbiome, with *A. rectalis* and *Faecalibacterium prausnitzii* contributing most to its transcription [15]. Primary SQ degraders are generally associated with beneficial host effects [23,24] and thus SQ is a candidate prebiotic. However, excessive stimulation of secondary, H₂S-producing SQ degraders may be harmful as H₂S has dose-dependent impacts on host health [25]. At low nano- to micromolar concentrations, H₂S has predominantly beneficial effects [26]. H₂S serves as an energy source for mitochondria, as a cellular antioxidant, and as a gasotransmitter in mammals [27]. Additionally, H₂S may prevent colonization by pathogens by inhibiting their aerobic respiration [28]. At high micro- to millimolar concentrations produced by the gut microbiota [26], however, H₂S may disrupt disulfide bonds in the intestinal mucosal layer, thus enabling bacterial penetration [29], and can lead to cell death by inhibiting cytochrome c [30]. Overabundance of H₂S-producing bacteria, such as *B. wadsworthia*, is considered as a potential driver of gut inflammation [29,31,32].

Here, we assessed the prebiotic potential of SQ by studying its degradation and metabolism in humans and mice using both human and mouse gut content microcosm experiments, as well as *in vivo* interventions in conventional and germ-free mice. We elucidated the role of mammalian cells in SQ metabolism through *in vitro* cultures and

stable isotope-tracing in germ-free mice. We show that SQ is not degraded by the host, establish a minimum SQ concentration that impacts the human gut microbiota, and uncover fundamental differences in the bacterial SQ metabolism between wild type mice and humans.

Materials and Methods

Human cell culture

HT-29, a human colorectal adenocarcinoma cell line (German Collection of Microorganisms and Cell Cultures, Germany), was cultured in Dulbecco's Modified Eagles Medium (DMEM), which contains 25 mM glucose, supplemented with 10% (v/v) fetal calf serum and 1% (v/v) penicillin/streptomycin at 37°C and 5% CO₂ under humidified conditions. All components were purchased from Invitrogen™ Life Technologies (Germany). Cells were regularly monitored for mycoplasma contamination.

To investigate whether human intestinal cells degrade SQ, HT-29 cells were grown in DMEM or in glucose-free DMEM either supplemented with 25 mM glucose, 25 mM SQ, or left unamended. Supernatants were sampled after 0, 1, 6, 24, and 48 h and stored at -20°C for metabolite analysis by capillary electrophoresis.

To study the potential inhibitory effect of SQ on the growth of HT-29 cells, 7,500 cells were seeded per well and incubated in DMEM medium supplemented with glucose or SQ at three concentrations (2.5 mM, 25 mM, 250 mM). In three independent experiments, duplicate incubations (n = 6) were performed for all treatments. Protein production was measured as a proxy for growth after 3, 5, and 7 days of incubation using sulforhodamine B colorimetric assays as described previously [33]. Briefly, plates were washed with 1x PBS twice and cells were fixed with a 5% trichloroacetic acid solution. Plates were dried overnight at room temperature. Then, the plates were stained with 0.4% (w/v) sulforhodamine B for 1 h. After washing twice with water and twice with 1% (v/v) acetic acid, the stain was eluted using a 10 mM Tris buffer (pH 10). Absorbance was measured at 570 nm and normalized to the solvent control (1% v/v DMSO) to assess the impact of treatments on cell growth.

Germ-free mouse experiment and $^{13}\text{CO}_2$ breath test

Six germ-free male C57BL/6 mice (6–20 weeks old), were housed under sterile conditions in flexible film isolators (North Kent Plastic Cages, England) or in Hangnotocages (ZOONLAB GmbH, Germany). Mice were kept on a twelve-hour light-dark cycle at 20.5–23.5°C and 45–65% humidity, with autoclaved water and Mouse-Breeding complete feed (Ssniff, Germany) provided *ad libitum*. Mice were scored twice daily for their health status, and sterility was checked monthly during housing, before the experiment, and during the experiment using qPCR with universal bacterial 16S rRNA gene primers [34,35]. Mice were randomly assigned to two treatment groups and kept in separate cages. The administered substrates were either 10 mg of fully ^{13}C -labeled D-glucose (Sigma-Aldrich, Germany) or 10 mg of fully ^{13}C -labeled SQ, which was synthesized from fully ^{13}C -labeled D-glucose following an established protocol [36]. Each treatment group received their substrate dissolved in 100 μl sterile water (Ampuwa; Fresenius Kabi, Germany) via gavage. Respired air from individual mice was sampled for CO_2 stable isotope analysis prior to gavage (baseline) and at 1, 3, 6, 9, 12, and 24 h post-gavage. For breath air sampling, individual mice were placed in an airtight one-liter container sealed with a lid fitted with a rubber stopper for approximately 2 minutes. Separate containers were used for each treatment group to avoid cross-contamination between groups. Gas samples (15 ml) were taken at 30 and 90 seconds and injected entirely into pre-vacuumed exetainers (12 ml) creating an overpressure to prevent leakage from ambient air into the exetainers. Ambient air samples were taken before the experiment for blank measurements. If feasible, fecal samples were collected to validate sterility of mice and for metabolite analysis. $^{13}\text{CO}_2$ concentrations were measured in the gas samples using isotope ratio mass spectrometry (GasBench II headspace sampler connected to a Delta V Advantage isotope mass spectrometer; Thermo Fisher Scientific, Germany). $^{13}\text{CO}_2$ production rates per minute were calculated from the differences in $^{13}\text{CO}_2$ (atom percent excess) between the two samples taken at 30 s and 90 s.

Anoxic *in vitro* gut microcosm incubations and pure culture growth tests

All anoxic incubations and growth tests were performed in an anaerobic tent (Coy Laboratory Products, United States) operated with a gas mix of 85% N_2 , 10% CO_2 and 5% H_2 . The H_2 levels in the tent atmosphere ranged between 1.5 and 2.5%.

Fecal samples were collected from five human individuals, who had not taken antibiotics in the three months before sampling. Samples were stored at 4°C for maximally 24 h before processing. All samples were pooled, and 3.9 g of fecal mass was homogenized in oxygen-free 30 mM bicarbonate buffer (pH 6.8). This fecal slurry (1.32 mg/ml) was distributed into 20 ml Hungate tubes (final volume: 15 ml) and sealed with butyl-rubber stoppers and screw-on caps. Triplicate microcosms were amended with 10 mM, 1 mM, 0.1 mM, or 0.01 mM SQ (MCAT GmbH, Germany). One microcosm was left unamended (negative control). Tubes were incubated at 37°C and subsamples (1 ml) were collected after 0, 3, 6, 9, 12, 16, and 24 h. From each subsample, 20 µl was used for sulfide quantification. The remaining subsample was centrifuged at 11,000 x g for 5 min at 4°C. Biomass-free supernatants were stored at -20°C for subsequent capillary electrophoresis. Cell pellets were frozen at -80°C for DNA extraction.

In two independent experiments, we sampled intestinal contents of conventional C57BL/6 wild type mice to create mouse gut content slurries. Mice were housed under specific-pathogen-free conditions with standard chow diet (Ssniff V1534, Ssniff, Germany) and water *ad libitum*, in compliance with Federation of European Laboratory Animal Science Associations guidelines [37]. Specific pathogen-free status was confirmed through regular screening for viral, bacterial, mycoplasmal, and parasitic agents such as murine norovirus, mouse hepatitis virus, *Helicobacter* species, *Salmonella* species, *Streptococcus* species, protozoa, and helminths. After euthanasia, mice were dissected in an anaerobic chamber.

In the first experiment, ten mice (4 females, 6 males, 8–10 weeks old) were dissected, and their small intestinal, cecal and large intestinal contents were pooled and homogenized with oxygen-free 1x PBS (pH 7.4). The gut content slurry (5.25 mg/ml) was distributed into 20 ml Hungate tubes (final volume: 10 ml). Mouse gut microcosms were left unamended or amended with 10 mM SQ or 10 mM DHPS. All treatments were performed in triplicate. Hungate tubes were sealed with butyl-rubber stoppers and screw-on caps and incubated at 37°C. Subsamples were collected at 0, 24, 48, and 96 h, and sulfide was quantified in 20 µl of subsample.

In the second experiment, eight mice (4 females, 4 males, 8–10 weeks old) were dissected, and their small intestinal (2.2 g) and cecal contents (5.9 g) were separately

pooled. Small intestine and cecal contents were homogenized with oxygen-free 1x PBS (pH 7.4) and transferred into 20 ml Hungate tubes (final volume: 15 ml) with a concentration of 4.9 mg/ml and 5.15 mg/ml of small intestinal and cecal content, respectively. Both sets of microcosms were left unamended or amended with 10 mM SQ, and sealed with butyl-rubber stoppers and screw-on lids for incubation at 37°C. All treatments were performed in triplicate. Subsamples (1 ml) were taken at 0, 24, 120, and 220 h. In both experiments, subsamples were centrifuged at 11,000 x g for 5 min at 4°C. Pellets were stored for DNA extraction at -80°C and biomass-free supernatants were stored at -20°C for metabolite analyses.

Enterocloster clostridioformis YL32 (DSM 26114) and *Faecalibaculum rodentium* ALO17 (DSM 103405) were each grown in triplicate cultures in 100% and 30% brain heart infusion broth with or without 10 mM SQ at 37°C in gas-tight Hungate tubes. Anoxic and autoclaved ultrapure water was used for dilution of the medium. Growth was monitored by measuring optical density at 600 nm. Identities of grown strains were confirmed by direct Sanger sequencing (Microsynth GmbH, Austria) of 16S rRNA gene amplicons.

Conventional wild type mouse experiment

Fifteen conventional C57BL/6 wild type mice (6-8 weeks) were bred and housed under specific pathogen-free conditions in accordance with the guidelines of the Federation of European Laboratory Animal Science Associations [37]. As aforementioned, specific pathogen-free status was confirmed through regular screening. Mice were maintained with *ad libitum* water and autoclaved mouse chow (Ssniff V1534, Ssniff, Germany). The day/night cycle was 14/10 hours, and environmental conditions were maintained at 20–24°C with 45–65% humidity. After one week of acclimatization, mice were randomly assigned to three groups (two males and three females each) with no significant differences in weight between groups. Two treatment groups were gavaged with 1 mg or 10 mg SQ in 100 µl aqueous solutions, while the third, control group was gavaged with 100 µl autoclaved tap water. Fecal samples were collected 0, 3, 6, 9, 12, and 24 h post-gavage. General discomfort of treated mice was assessed by observing them after gavage and throughout the experiment for signs of reduced mobility, scruffy fur, hunched posture or changes in

facial expression. Mice were sacrificed and dissected after 24 h. Fecal samples and intestinal contents were immediately stored at -80°C for metabolite analysis and 16S rRNA gene amplicon sequencing.

Metabolite analysis

For sulfide quantification, 20 µl subsamples were added to 300 µl 2 g/l zinc acetate solution in the anaerobic tent. Fixed samples were quantified colorimetrically using the Infinite M Nano+ microplate reader (Tecan, Austria), as described previously [38].

Capillary electrophoresis was used to quantify metabolites in the supernatants of human cell cultures, in gut microcosms, in bacterial pure cultures, and in the fecal samples of germ-free and conventional mice. Concentrations of SQ, SL, DHPS, lactate, and the short-chain fatty acids (SCFAs) formate, succinate, acetate, propionate, butyrate, and valerate were quantified using a 7100 capillary electrophoresis system (Agilent, United States). Mouse fecal samples were prepared by adding 40 µl of ultrapure water to 0.01 g of feces. After mixing, samples were centrifuged at 11,000 x g for 10 min at 4°C and supernatants were stored at -20°C. For analysis, culture supernatants or metabolite extracts from fecal samples were diluted 1:5-1:20 in ultrapure water containing 0.1 mM of an internal standard. Caproate, valerate or succinate was used as an internal standard and was selected based on the sample background. Analyte standard mixtures were prepared in concentrations of 0.025 mM, 0.05 mM, 0.1 mM, 0.25 mM, 0.5 mM and 1 mM. An organic acid buffer (Agilent, United States) was used as a running buffer. The instrument's bare fused silica capillary (75 µm inner diameter, 72 cm effective length; Agilent, United States) was flushed within the system for four minutes as part of the preconditioning process before each measurement. Samples were then injected at 50 mbar for five seconds. Measurements were performed at -25 kV, 100 µA, and 6 W.

SQ and its degradation products DHPS, SL, isethionate, 3-HPS, 3-sulfopropionate, and sulfoacetate in mouse gut content microcosms, *E. clostridioformis* pure cultures, and in fecal samples of germ-free and conventional mice were additionally quantified using liquid chromatography-mass spectrometry. Samples were prepared by centrifuging at 18,000 x g for 5 minutes at 4°C. The supernatants were then diluted 1:10 with ultrapure water and mixed at a 7:3 (v/v) ratio with ≥ 99.9%

acetonitrile containing 6.7 mM para-toluenesulfonate as internal standard. Samples were quantified using a LCMS-2020 single quadrupole mass spectrometer system (Shimadzu, Japan) consisting of LC-40B XR pumps, a SIL-40C XR autosampler, a CTO-40S column oven (heated to 30°C) and a MS-2020 electrospray ionization mass spectrometer. Organosulfonate separation was carried out on an iHILIC-Fusion(+) column (100 × 2.1 mm, 3.5 µm, 100 Å), as described previously [39].

16S rRNA gene amplicon sequencing and real-time PCR

DNA extraction and 16S rRNA gene amplicon sequencing was performed by the Joint Microbiome Facility of the Medical University of Vienna and the University of Vienna under the project numbers JMF-2012-06 (human fecal microcosms), JMF-2108-01 (conventional mouse experiment), JMF-2212-09 (mouse gut content microcosms). DNA was extracted with the QIAamp Fast DNA Stool Mini Kit according to the manufacturer's protocol (Qiagen GmbH, Austria). Extraction contamination was controlled for using blank extractions containing kit-provided buffers alone. An established two-step PCR-barcoding method (H_515F_mod-5'-(head) GCT ATG CGC GAG CTG C - GTG YCA GCM GCC GCG GTA A and H_806R_mod-5'-(head) TAG CGC ACA CCT GGT A - GGA CTA CNV GGG TWT CTA AT) was used for 16S rRNA gene amplicon sequencing of bacteria and archaea [40]. Prior to preparing the sequencing libraries, barcoded amplicon libraries were normalized with the SequelPrep normalization plate kit and subsequently pooled (TrueSeq Nano Kit; Illumina, United States) [40,41]. Amplicon sequencing was performed on the MiSeq (V3, 600 cycles; Illumina, United States) and results were analyzed according to the procedures outlined previously [40]. Amplicon sequence variants (ASVs) were inferred using the DADA2 workflow [42] and taxonomically classified with the SILVA database (release 138). Sequencing libraries were rarified and analyzed with the software packages phyloseq (v1.42.0) [43] and vegan (v2.6-4) [44] for RStudio (rstudio.com, R version 4.2.2). Principal coordinate analysis was performed using a Bray-Curtis dissimilarity matrix computed with phyloseq. DESeq2 (v1.38.3) was used to perform differential abundance analyses between treatment groups and time points [45].

In the human fecal microcosm experiment, ASV abundance at each time point and treatment was compared to two controls: Its corresponding baseline at 0 h, and to the abundance in the 0.01 mM SQ incubation at the same time point. Notably, the

0.01 mM SQ incubation was used as control because (i) metabolite data showed no significant differences compared to the unamended control and (ii) only one control incubation was sequenced. The baseline at 0 h served as control to identify ASVs with an increased abundance compared to the beginning of the experiment. ASVs significantly enriched in both analyses were considered responsive to the amendment. For the mouse gut content microcosm experiments, each treatment was evaluated against the unamended control at each time point. In the conventional mouse experiment, ASV abundance was compared to the baseline at 0 h for each treatment. However, ASVs were considered significantly responsive to the amendment only, if they were not also significantly enriched in the unamended control over time. Adjusted *p*-values of <0.01 were considered significant. The taxonomic classification of enriched ASVs in all experiments was verified by Blastn against the NCBI 16S rRNA sequences database, excluding uncultured and environmental sample sequences [46].

The total 16S rRNA gene copies were quantified by real-time PCR (CFX96 Real-Time System; Bio-Rad Laboratories Ges.m.b.H, Austria). PCR reactions consisted of 10 µl iQ SyBr Green Mix 2x, 0.8 µl of each primer (0.4 µM), 6.4 µl sterile nuclease-free water, and 2 µl DNA extract. The universal primers 515F (5'-GTG YCA GCM GCC GCG GTA A-3') [47] and 806R (5'-GGA CTA CNV GGG TWT CTA AT-3') [48] targeting bacteria and archaea were used. The PCR program started with an initial denaturation step at 95°C for 5 min followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Final elongation was performed at 72°C for 2 min. A negative control, i.e. water as template, was included in each run. Genomic DNA of *Escherichia coli* K12 was used to prepare a standard serial dilution series of 10⁹ (15.4 ng/µl DNA of the standard) to 10³ copy numbers of the 16S rRNA gene to generate a standard curve, which was linear across the entire dilution series. The 16S rRNA gene copy numbers of selected ASVs were calculated by multiplying their relative abundances with the total 16S rRNA gene abundances determined by real-time PCR.

Analysis of genomes from the mouse microbiome

High-quality mouse microbiota-derived bacterial genomes were downloaded from the Mouse Gastrointestinal Bacteria Catalogue (n=26640) [49]. Open reading frames were determined using prodigal and subsequently annotated via HMSS2 [50].

Additional hidden Markov models were generated for SQDG and DHPS conversion related proteins not represented in HMSS2, as described previously [51]. Among others, this included isethionate reductase *IsrBHDK* [52], DHPS-sulfolase *HpsGH*, DHPS-dehydratase *HpFGH* [17], sulfopropionaldehyde reductase *HpFD*, succinate semialdehyde dehydrogenase *Ssadh* [53], DHPS dehydrogenase *DhpA*, 3-sulfolactaldehyde dehydrogenase *SlaB* [16] and sulfoquinovosidase *SqgA* [7] (Table S1). For all hidden Markov models, a general bitscore cutoff of 100 was chosen to detect homologs. All hits were tested for co-location in the respective genome with a maximum distance of 3500 nucleotides between genes, as described before [51]. The resulting gene clusters were then searched for patterns of genes with the same sequential occurrence using a custom implementation of the colinear syntenic blocks finder algorithm [54], with a tolerance of two gene insertions and a length of four co-located genes. This was done to ensure enzyme function in the respective metabolism by sequence similarity and genetic vicinity.

Results

SQ is not degraded by human HT-29 cells *in vitro*

To test whether SQ is degraded by human intestinal cells, we incubated HT-29 human colorectal cancer cells with and without SQ. SQ concentrations in cell culture supernatants from both SQ-supplemented DMEM and glucose-free DMEM remained unchanged throughout the experiment (Figure 2 a, b). Lactate was only produced in glucose-containing incubations (Figure 2 c, d). The effect of increasing glucose or SQ concentrations on HT-29 cell growth in DMEM was similar for both substrates. Protein production was fully inhibited only in cultures treated with very high, non-physiological concentrations (250 mM) of glucose or SQ (Figure 2 e, f). Overall, these findings suggested that SQ is not degraded and metabolized by human cells *in vitro*. A potential limitation of using cancer cells is that their metabolism does not necessarily reflect the metabolism of primary cells [55].

^{13}C -labeled SQ is not respired to ^{13}C -CO₂ by germ-free mice

Next, we employed stable isotope tracing with ^{13}C -SQ in germ-free mice to study potential SQ degradation by mammalian cells *in vivo*. Germ-free mice received a single dose (10 mg) of either fully labeled ^{13}C -SQ or ^{13}C -glucose and their respired air and fecal samples were subsequently collected over a 24-hour period for $^{13}\text{CO}_2$ breath tests and metabolite analysis, respectively. Mice administered ^{13}C -SQ did not produce $^{13}\text{CO}_2$. Contrarily, mice receiving ^{13}C -glucose produced up to 117 μg $^{13}\text{CO}_2$ /min at one hour post-gavage (Figure 2 g). In mice gavaged with ^{13}C -SQ, SQ was detected in fecal samples already one hour after its administration (Figure 2 h). Yet, SQ degradation metabolites were not detected in the feces.

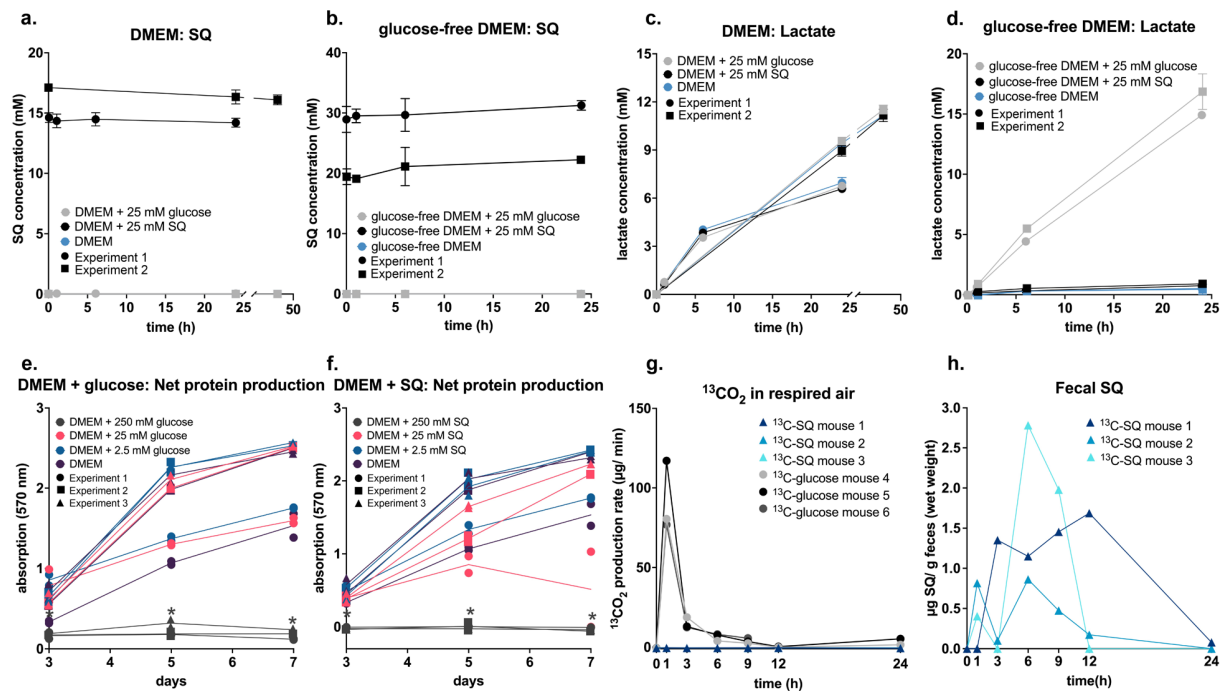


Figure 2: SQ was not metabolized by human intestinal cells and germ-free mice. SQ was not degraded by HT-29 cells in DMEM medium amended with 25 mM SQ (shown in black) (a). SQ was not degraded by HT-29 cells in glucose-free DMEM medium amended with 25 mM SQ (shown in black) (b). HT-29 cells produced lactate independent of the treatment in DMEM medium (c). In glucose-free DMEM, HT-29 cells produced lactate only in incubations amended with 25 mM glucose (shown in grey) (d). Protein production was inhibited in incubations with 250 mM glucose (shown in dark grey), but not in incubations with 25 mM glucose (shown in pink) and 2.5 mM glucose (shown in blue) (e). Protein production was inhibited in incubations with 250 mM SQ (shown in dark grey), but not in incubations with 25 mM SQ (shown in pink) and 2.5 mM SQ (shown in blue) (f). Each germ-free mouse (n = 3 per group) was gavaged with 10 mg of fully labeled ^{13}C -glucose or ^{13}C -SQ. (g). Glucose, but not SQ, was respired by germ-free mice as shown by $^{13}\text{CO}_2$ production via breath air isotope analysis. SQ was repeatedly detected in the feces of all mice gavaged with ^{13}C -SQ within 24 h after the gavage (h). In a., b., c., and d., lines show averages of triplicate incubations with error bars representing one standard deviation. Two experiments were conducted varying only slightly in the sampling time points. Different

data point shapes indicate the two experiments. In e. and f., lines show averages of duplicate measurements and data points show individual values. Different data point shapes represent the three conducted experiments. Asterisks mark significant differences between a treatment group and the control (DMEM medium without amendment) at each time point. Significant differences were determined by ANOVA with Dunnett's post hoc test. Adjusted *p*-values smaller than 0.05 were regarded as significant. SQ, sulfoquinovose; DMEM, Dulbecco's Modified Eagle Medium.

Response of the microbiota to different doses of SQ in human fecal microcosms

We performed anoxic incubations with human fecal microcosms to assess the dose-dependent effects of SQ (0.01 mM, 0.1 mM, 1 mM, and 10 mM) on metabolite production and gut microbiota composition (Figure 3 a). Only the microcosms with high SQ concentrations (1 mM, 10 mM) consistently showed significant differences in metabolite levels compared to the unamended control (Figure S1). The temporal dynamics of SQ degradation and the production of metabolic products were consistent between the 1 mM and 10 mM SQ treatments (Figure 3 b, c). SQ was mostly degraded within 12 h, coinciding with the production of DHPS. In comparison, SL and sulfide were produced later and represented only a smaller fraction of the produced sulfur metabolites within the 24 h incubation. SQ, DHPS, and SL were not detected in the unamended control incubation (Figure 3 d). The production of acetate and butyrate, known SQ degradation products [15,16,39], increased significantly in the 1 mM and 10 mM SQ treatments compared to the unamended control (Figure 3 e, f).

Changes in total microbial abundance during the 24 h incubation period were quantified by 16S rRNA gene-targeted real-time PCR (Figure 3 g). Gene copy numbers decreased in microcosms without SQ and with 0.01 or 0.1 mM SQ and remained transiently constant in microcosms with 1 mM SQ. A significant increase in gene copy numbers was only observed in microcosms with 10 mM SQ.

We performed 16S rRNA gene amplicon sequencing and differential abundance analyses to identify amplicon sequence variants (ASVs) with increased relative abundance in SQ-amended incubations and thus a possible role in SQ degradation. Significantly enriched ASVs were only detected in incubations with concentrations of 0.1 mM SQ or higher; one, two, and fourteen ASVs were enriched in microcosms with 0.1, 1, and 10 mM SQ, respectively (Table S2, Figure S2). *Escherichia/Shigella* ASV 8 was enriched at all three SQ concentrations, *Walteria intestinalis* ASV 7 was enriched at 1 and 10 mM SQ, while the following ASVs were only enriched at 10 mM SQ: *A. rectalis* ASV 1, *Alistipes shahii* ASV 2, *Ruminococcus bromii* ASV 3,

Oscillospiraceae ASV 5, *B. wadsworthia* ASV 6, *Enterocloster citroniae* ASV 9, *Phocaeicola dorei* ASV 4, *Phocaeicola massiliensis* ASV 12, *Phocaeicola vulgatus* ASV 14, *Bacteroides xylanisolvens* ASV 10, *Bacteroides caccae* ASV 11, and *Bacteroides ovatus* ASV 13. Variations in the dose-response patterns of SQ-responsive microorganisms are not yet fully explained but may result from differences in SQ metabolic energetics and enzyme kinetics. Close relatives of some of the enriched ASVs (Figure 3 h, i, j) have been physiologically confirmed to degrade SQ (*A. rectalis*, *E. coli*) or DHPS (*B. wadsworthia*) [10,15]. Furthermore, members of the genera *Enterocloster* and *Ruminococcus* encode the sulfofructose-transaldolase pathway [15]. Together with a member of *Alistipes*, members of these genera/species have also previously been significantly enriched in human fecal microcosms amended with 10 mM SQ [15]. ASVs related to these taxa generally showed a continuous increase in absolute abundance over the 24 h incubation, which coincided with the kinetics of SQ disappearance and DHPS production (Figures 3 and S2). In contrast, ASVs of *Phocaeicola* and *Bacteroides* showed an initial spike in absolute abundances at 6 h followed by a decline, suggesting they are not directly involved in SQ degradation (Table S2, Figure S2). Type strain genomes of the most closely related *Alistipes*, *Waltera*, *Bacteroides* or *Phocaeicola* species do not encode known SQ or DHPS degradation pathways.

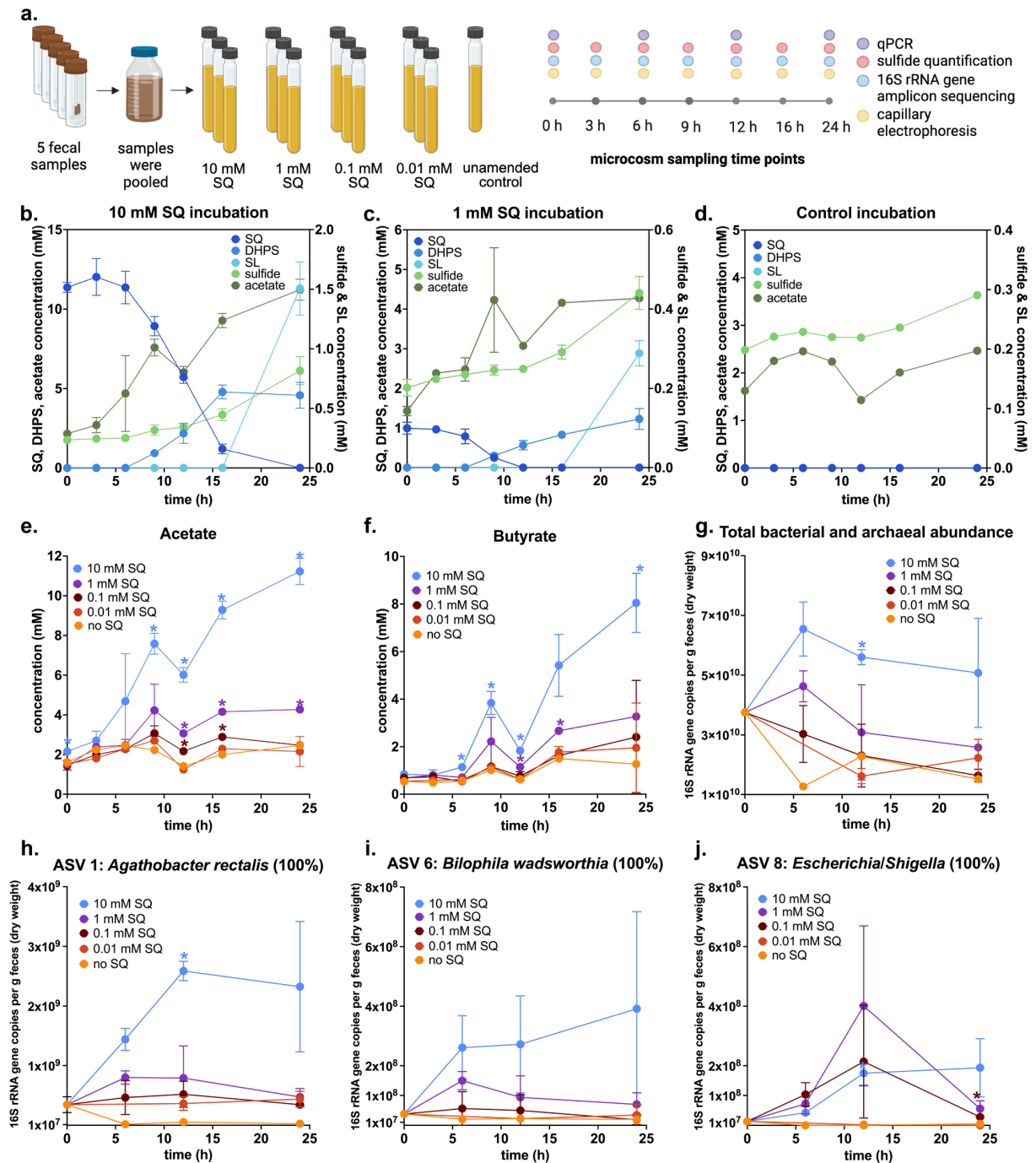


Figure 3: Metabolite dynamics and differential responses of microbiota members to different doses of SQ in 24 h human fecal microcosm incubations. Overview of the experimental and analytical approach (created with biorender.com) (a). Sulfur metabolite dynamics in incubations amended with 10 mM SQ (b), 1 mM SQ (c), and in the unamended control (d). SQ, DHPS, and SL were not detected in unamended control microcosms. Higher doses of SQ led to increased concentrations of acetate (e) and butyrate (f). No significant differences in acetate concentrations were detected between incubations with 0.01 mM SQ (shown in red) and unamended controls (shown in orange). No significant differences in butyrate concentrations were detected between incubations with 0.1 mM SQ (shown in dark red) or 0.01 mM SQ (shown in red) and unamended controls (shown in orange). Changes in absolute bacterial and archaeal 16S rRNA gene copy numbers in the microcosm incubations with different SQ doses (g). Changes in the absolute abundance of *A. rectalis* ASV 1 (h), *B. wadsworthia* ASV 6 (i), and *Escherichia/Shigella* ASV 8 (j). The 16S rRNA gene sequence similarity (%) to the closest, named relative is shown for each ASV. In b., c., e., f., g., h., i., and j., the lines show the average of

triplicate incubations with error bars representing one standard deviation. Only a single incubation was performed for the unamended control microcosm. Coloured asterisks show significant differences between the respective treatment ($n = 3$) and the retrospectively designated control incubations ($n = 4$, consisting of three 0.01 mM SQ incubations and one unamended control) at individual time points. Significant differences were determined by ANOVA with Dunnett's post hoc test. Adjusted p -values smaller than 0.05 were regarded as significant. SQ, sulfoquinovose; DHPS, 2,3-dihydroxypropane-1-sulfonate; SL, 3-sulfolactate; ASV, amplicon sequencing variant.

Incomplete SQ degradation in mouse gut content microcosms

Two independent microcosm experiments were performed with pooled gut contents of mice and amended with SQ or DHPS to investigate the SQ metabolism (Figure 4 a). In experiment 1, microcosms containing entire gut contents were incubated with either 10 mM SQ or 10 mM DHPS. Following a lag phase of over 24 hours, SQ degradation to DHPS was observed in SQ-amended incubations at a near 1:1 ratio, as measured by liquid chromatography-mass spectrometry (Figure 4 b). SL, sulfoacetate, and 3-HPS were not detected. Yet, small amounts of isethionate (0.01 mM after 48 h and 0.02 mM after 96 h) were detected (Figure S3). Sulfide concentrations in SQ-amended microcosms increased slightly, remaining below 1 mM, and did not correlate with SQ degradation in timing or stoichiometry. Capillary electrophoresis analysis of SCFAs showed an increase in acetate production in SQ-amended incubations, from 5 mM to 11.1 mM over 96 h, compared to the control incubation, from 2.6 mM to 4.3 mM (Figure 4 b). Concentrations of DHPS and acetate remained constant in DHPS-amended incubations (Figure 4 c). Sulfide concentrations were significantly higher in DHPS-amended incubations compared to SQ-amended incubations and unamended controls. However, the small increase from 0.2 mM to 0.5-0.6 mM sulfide in the DHPS-amended incubations compared to 0.3-0.4 mM sulfide in the unamended control did not suggest a quantitative turnover of DHPS to sulfide (Figure 4 c). SQ and DHPS were not detected in the unamended control incubations, and sulfide concentrations remained below 0.4 mM (Figure 4 d).

Microbial abundances decreased over time in all microcosms, but remained significantly higher in SQ incubations from 48 h onwards (Figure S4). Microbial abundances decreased from $1.8 \pm 0.7 \times 10^{10}$ 16S rRNA gene copies/g dry weight to $5.2 \pm 1.0 \times 10^9$ 16S rRNA gene copies/g dry weight in SQ-amended microcosms and to $5.3 \pm 1.4 \times 10^8$ 16S rRNA gene copies/g dry weight in the unamended controls.

Differential abundance analysis of 16S rRNA gene amplicon sequencing data identified nine ASVs enriched in the 10 mM SQ incubations compared to unamended controls (Figure S5). *Escherichia/Shigella* ASV 8 had the highest log₂ fold change of all enriched ASVs and its absolute abundance dynamic corresponded to the turnover of SQ into DHPS (Figure 4 b, e). Other enriched ASVs included *Lachnospiraceae* ASV 15, *Bacteroides acidifaciens* ASV 16, *Bacteroides* ASV 19, *F. rodentium* ASV 17, *Coprobacillaceae* ASV 18, *Ruminococcus* ASV 20, *Prevotellaceae* ASV 21, and *P. vulgatus* ASV 14 (Table S3). Notably, *Escherichia/Shigella* ASV 8 and *P. vulgatus* ASV 14 were enriched in both human fecal microcosms and mouse gut content microcosms with SQ. In contrast to SQ-amended incubations, temporal changes in microbial community composition in the DHPS-amended incubations were indistinguishable from control incubations and no ASVs were identified as enriched (Figure 4 f).

For experiment 2, separate microcosms for small intestinal contents and cecal contents were each incubated with 10 mM SQ and analyzed for changes in microbial community composition compared to unamended control incubations. Microbial 16S rRNA gene copy numbers decreased in control incubations, while remaining rather constant in cecal incubations with SQ or decreased less in small intestinal incubations with SQ compared to the control (Figure S4). Seven ASVs were significantly enriched in small intestinal incubations and four ASVs in cecal incubations. ASVs in small intestinal incubations were *Enterococcus* ASV 22 and 23, *Enterocloster* ASV 24, 25, and 26, *Ligilactobacillus* ASV 27, and *Akkermansia muciniphila* ASV 28 (Figure 4 g, Table S3). In cecal incubations, enriched ASVs belonged to *Escherichia/Shigella* ASV 8, *Parabacteroides distasonis* ASV 29 and 30 and, *Enterocloster* ASV 25 (Figure 4 h, i, Table S3). In contrast to the other ASVs, the absolute abundances of *Ligilactobacillus* ASV 27 and *A. muciniphila* ASV 28 decreased during the incubation and thus are unlikely to be involved in primary SQ degradation (Figure S6).

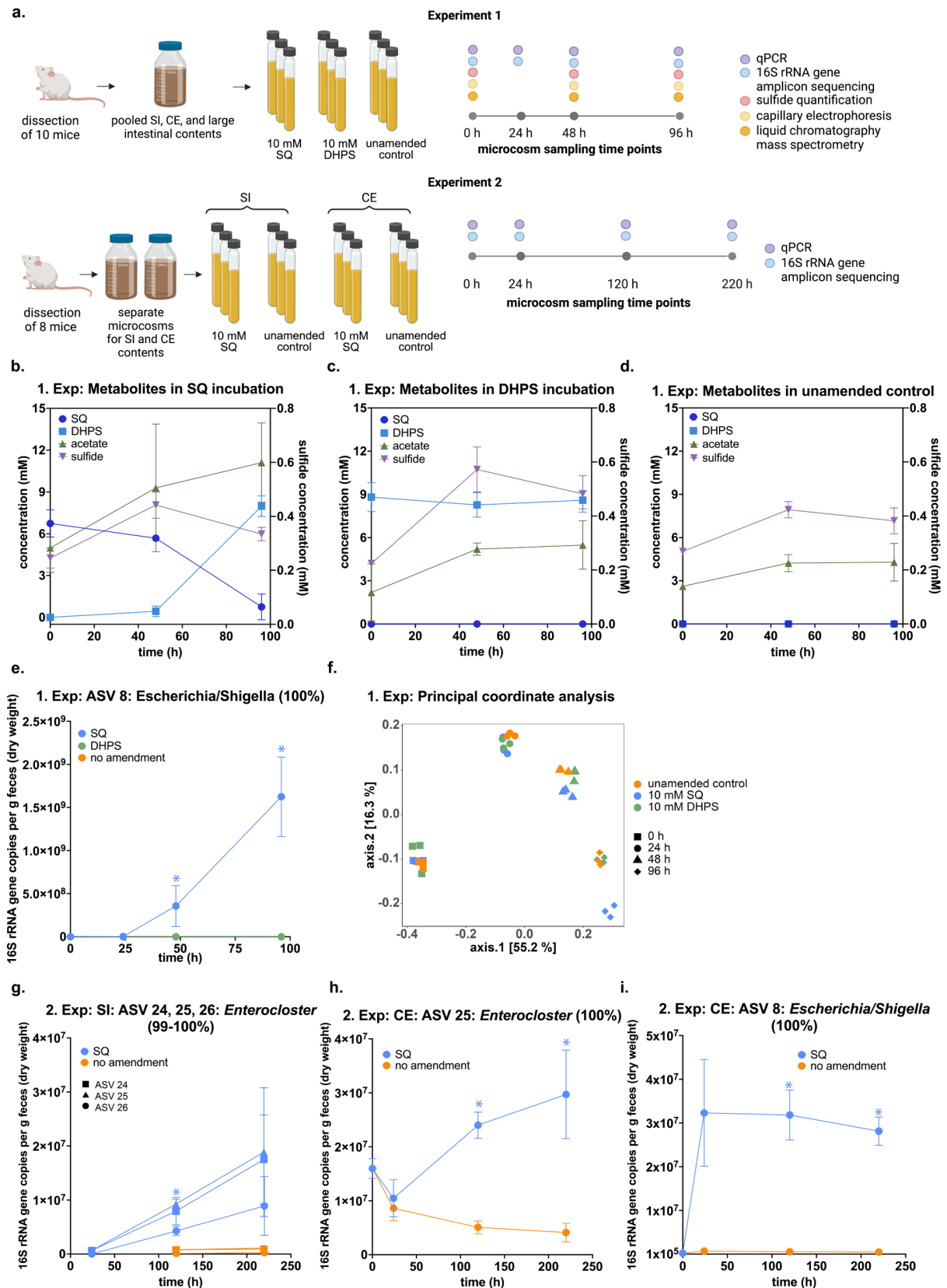


Figure 4: Metabolite dynamics and differential responses of microbiota showed that SQ, but not DHPS, was degraded in murine gut content microcosms. Overview of the experimental and analytical approach (created with biorender.com) (a). Metabolite dynamics in gut content microcosms (Experiment 1: pooled small intestinal, cecal, and large intestinal contents) incubated with 10 mM SQ (b), 10 mM DHPS (c), and without amendment (control) (d). SQ and DHPS were not detected in control

incubations. Calculated absolute 16S rRNA gene abundance of *Escherichia/Shigella* ASV 8 in incubations of experiment 1 (e). Principal coordinate analysis plot of beta diversity (Bray-Curtis dissimilarity) of 16S rRNA gene amplicon data from incubations of experiment 1 (f). Calculated absolute 16S rRNA gene abundance of selected ASVs that were significantly enriched in SQ-amended mouse small intestinal content and cecum content incubations of experiment 2 (g-i). The 16S rRNA gene sequence similarity (%) to the closest, named relative is shown for each ASV. The lines in b., c., d., e., g., h. and i. show averages of triplicate incubations with error bars representing one standard deviation. Coloured asterisks show significant differences between the respective treatment and the unamended control at individual time points. Significant differences were either determined by two-way ANOVA with Dunnett post hoc test (1. experiment: pooled gut content) or by multiple unpaired t-tests with Holm-Šidák post hoc test (2. experiment: small intestine and cecum). Adjusted *p*-values smaller than 0.05 were regarded as significant. SQ, sulfoquinovose; DHPS, 2,3-dihydroxypropane-1-sulfonate; Exp, experiment; SI, small intestine; CE, cecum; ASV, amplicon sequencing variant.

SQ is degraded to DHPS in conventional wild type mice

To investigate SQ metabolism by the mouse gut microbiota in vivo, we gavaged a single dose of either 1 mg SQ or 10 mg SQ to conventional wild type laboratory mice and collected fecal samples up to 24 h post-gavage (Figure 5 a). Mice fed with SQ showed no signs of digestive problems or general discomfort. While the water content of fecal pellets was not measured, the consistency of fecal pellets remained stable throughout the experiment, with no visible difference between the tap water control and SQ-treated mice. Fecal metabolites were analyzed via capillary electrophoresis as well as liquid chromatography-mass spectrometry (Table S4). SQ was detected in mice receiving 10 mg SQ between 3 and 6 h post-gavage, while DHPS appeared at 6 h and remained detectable in some mice until the end of the experiment (Figure 5 b, Figure S7). Additionally, taurine as well as small amounts of 3-sulfolactate, sulfoacetate, and isethionate were measured via liquid chromatography-mass spectrometry in mice receiving 10 mg SQ (Table S4). Taurine and isethionate were detected already at the beginning of the experiment, indicating that the presence of these metabolites was independent from SQ addition. Temporal dynamics of SCFA concentrations, total 16S rRNA gene copy numbers, and microbial composition were not significantly different between the three treatment groups (Figure 5 c - g).

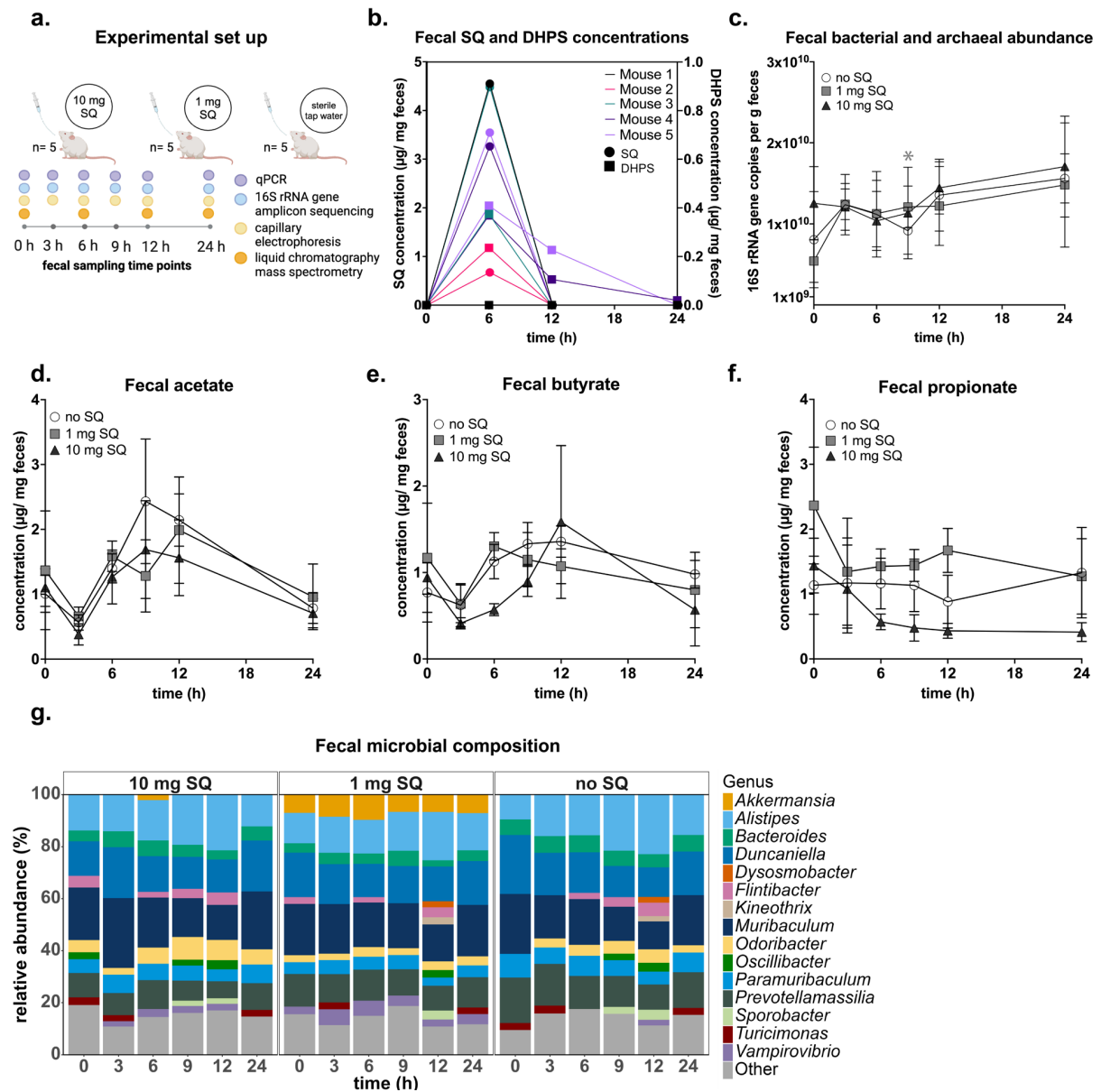


Figure 5: Fecal metabolite and microbial community dynamics of conventional wild type mice over 24 h after receiving a single dose of SQ. Overview of the experimental and analytical approach (created with biorender.com) (a). Concentrations of SQ (circles) and DHPS (boxes), as analyzed by liquid chromatography-mass spectrometry, in feces of mice (n = 5) that each received a single dose of 10 mg SQ by gavage (b). Metabolite concentrations of all treatment groups, as analyzed by capillary electrophoresis, are depicted in Supplementary Table S4 and Figure S7. Total bacterial and archaeal 16S rRNA gene copy numbers in feces of the three treatment groups (c). Five biological replicates were measured in triplicate qPCR assays. The line shows the mean and the error bars represent one standard deviation. Concentrations of acetate (d), butyrate (e), and propionate (f) in feces of the three treatment groups. Mean values of five biological replicates are shown and error bars depict one standard deviation. Mean relative 16S rRNA gene abundance of dominant bacterial genera in feces of the three treatment groups (g). All genera below an abundance of 2% are summarized in 'Other'. All data refer to the wet weight of the feces. Asterisks show significant differences between the respective treatment and the tap water control at individual time points. Significant differences in c., d., e., and f. were determined by ANOVA with Dunnett's post hoc test. Adjusted *p*-values smaller than 0.05 were regarded as significant. SQ sulfoquinovose, DHPS 2,3-dihydroxypropane-1-sulfonate.

SQDG/SQ and DHPS degradation pathways are encoded in few bacterial genomes from the mouse gut

We comprehensively examined the genetic potential for SQDG/SQ and DHPS degradation in the mouse microbiota by analyzing the collection of 26640 metagenome-assembled genomes (MAGs) from the Mouse Gastrointestinal Bacteria Catalogue for presence of the respective pathways [49]. Our analysis revealed SQDG/SQ and DHPS degradation pathways in only 0.3% (n=76) and 0.2 % (n=48) of all MAGs and in 16 (1.5%) and 8 (0.7%) of the 1094 species-level genome bins, respectively (Figure 6 a, b). The sulfofructose-transaldolase pathway was encoded in 57 MAGs, primarily from *E. clostridioformis*, *Otoolea symbiosa* (formerly *Clostridium symbiosum*), and *E. aldenensis*, and represented the most prevalent SQDG/SQ degradation pathway in the mouse microbiome (Figure 6 a). The sulfo-Embden-Meyerhof-Parnas pathway was encoded in 13 *Enterobacteriaceae* MAGs. The sulfo-transketolase pathway was encoded in 6 *Oscillibacter* MAGs. Key enzymes in DHPS degradation pathways (Figure 1 b) were encoded in 48 MAGs across five genera. These included 16 MAGs with *hpsN*, 12 MAGs with *dhpA*, 10 MAGs with *hpfGH*, 8 MAGs with *hpsGH*, and 2 MAGs with both *hpsGH* and *dhpA* (Figure 6 b). These pathways could generate sulfite intracellularly and were present in the sulfidogenic genera *Desulfovibrio*, *Taurinivorans*, *Mailhella*, and *Bilophila* that employ a DsrAB-dissimilatory sulfite reductase to respire sulfite to H₂S [15,16,19]. Nine *E. clostridioformis* MAGs and one *E. aldenensis* MAG encoded the fermentative *hpfGHD* pathway along with the sulfofructose-transaldolase pathway, suggesting these bacteria may ferment SQ via DHPS also to 3-HPS [17].

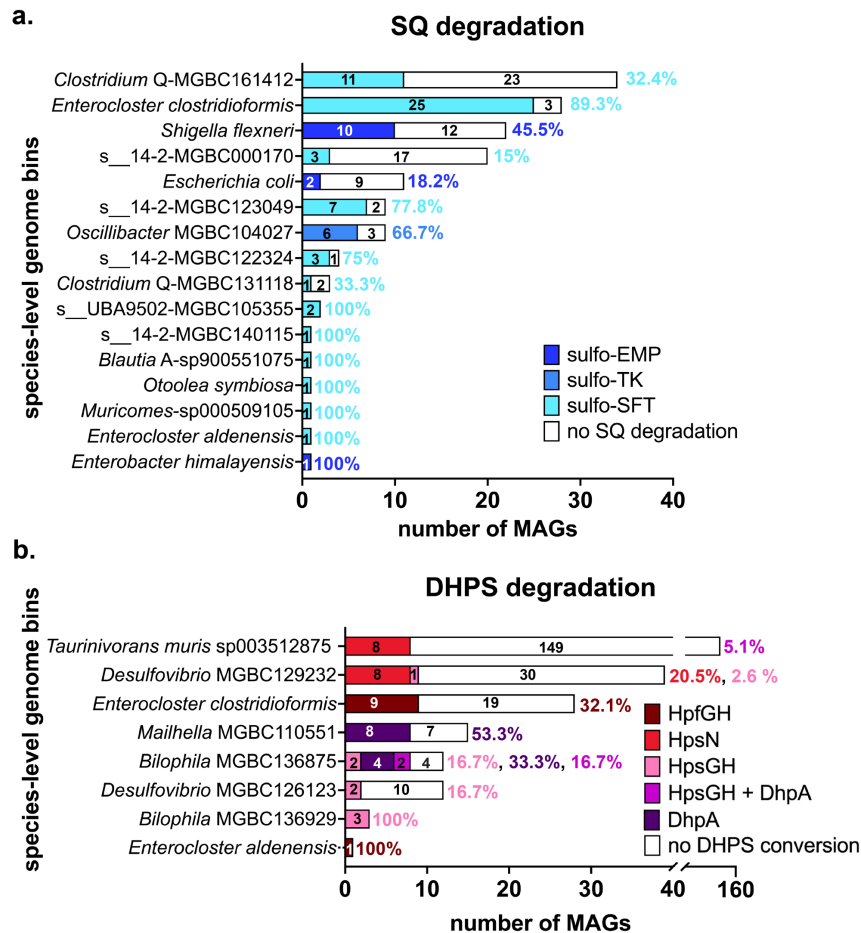


Figure 6: Analysis of microbial genomes revealed genetic SQDG/SQ and DHPS degradation potential is sparsely distributed in the mouse microbiome. Distribution of SQDG/SQ (a) and DHPS (b) degradation potential across a collection of 26,640 microbial MAGs from the mouse intestinal tract. Species-level genome bins are ordered based on the total number of genomes. The total and relative (%) number of genomes that encode a specific SQDG/SQ or DHPS pathway (shown in different colors) is indicated for each species-level genome bin. Sixteen (represented by 76 MAGs) and eight (represented by 48 MAGs) species-level genome bins, contained complete gene clusters for SQDG/SQ and DHPS degradation, respectively. Some MAGs of *E. clostridioformis* and *E. aldenensis* encoded both SQDG/SQ and DHPS degradation pathways. MAG, metagenome-assembled genome; sulfo-EMP, sulfoglycolytic Embden-Meyerhof-Parnas pathway; sulfo-ED, sulfoglycolytic Entner–Doudoroff pathway; sulfo-SFT, sulfoglycolytic sulfofructose-transaldolase pathway; SQ, sulfoquinovose; DHPS, 2,3-dihydroxypropane-1-sulfonate; HpsG, DHPS-sulfolylase; HpsH, DHPS-sulfolylase activating enzyme; HpfG, DHPS-dehydratase; HpfH, DHPS-dehydratase activating enzyme; HpsN, DHPS-1-dehydrogenase; DhpA, dehydrogenase.

***Enterocloster clostridioformis* degrades SQ to DHPS in pure culture**

Our mouse gut content microcosm experiments and genome analyses revealed members of the genus *Enterocloster* as SQ degraders. We thus performed anaerobic growth tests with the mouse strain *E. clostridioformis* YL32, which shares 100%, 99.6%, and 99.2% 16S rRNA gene sequence identity with the enriched ASVs 25, 24, and 26 in the mouse gut microcosms, respectively. Initial growth of strain YL32 in

diluted or undiluted rich medium did not differ, regardless of SQ supplementation. However, cultures supplemented with SQ exhibited a secondary growth phase during the late stationary phase, achieving significantly higher optical densities than cultures without SQ. This secondary growth was more pronounced in diluted rich medium (Figure 7 a). Diauxic growth in SQ-supplemented cultures indicated that SQ supported growth only after energetically more favorable substrates in the rich medium were consumed. This was supported by the timing of SQ degradation, which concurred with the second growth phase after 100 h. SQ was degraded nearly stoichiometrically to DHPS (Figure 7 b) while sulfide production did not differ significantly between cultures with and without SQ (Figure 7 c). Although *E. clostridioformis* YL32 possesses the genetic potential to ferment DHPS further (Figure 7 d), the DHPS fermentation product, 3-HPS, was not detected.

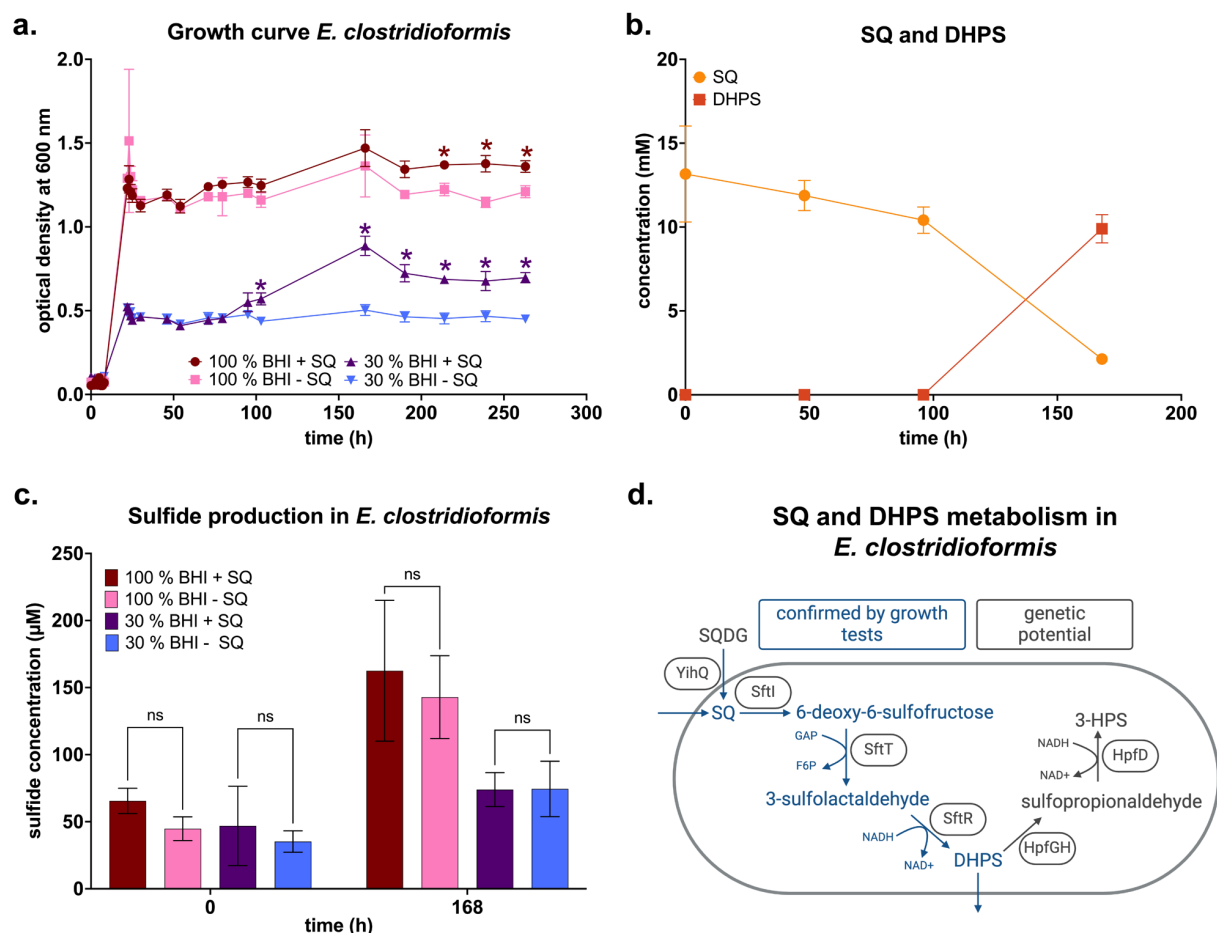


Figure 7: A pure culture of *Enterocloster clostridioformis* anaerobically degraded SQ to DHPS. Anaerobic growth tests of *E. clostridioformis* strain YL32 with 10 mM SQ (a). SQ triggered a second growth phase of *E. clostridioformis* in diluted (30%) rich medium (a) that coincided with a decrease of SQ and an increase of DHPS at a stoichiometry of about 1:1 (b). Small amounts of sulfide were produced in all treatments but there were no significant differences between the sulfide produced in incubations

amended with SQ compared to unamended control incubations (c). Schematic overview of the proposed SQ and DHPS pathways of *E. clostridioformis* YL32 (genome assembly number GCA_016696785) based on the growth tests and genome analysis (created with biorender.com) (d). SQ can either be imported via the transporter SftA or cleaved from SQDG by the sulfoquinovosidase YihQ (gene locus tag I5Q83_09300). SQ is converted to 6-deoxy-6-sulfofructose via the SQ isomerase SftI (gene locus tag I5Q83_09270). The 6-deoxy-6-sulfofructose transaldolase SftT (gene locus tag I5Q83_09315) catalyzes the transfer of a C3-moiety from 6-deoxy-6-sulfofructose to glyceraldehyde-3-phosphate (GAP), forming fructose 6-phosphate (F6P). The resulting 3-sulfolactaldehyde is then reduced to DHPS by the 3-sulfolactaldehyde reductase SftR (gene locus tag I5Q83_09310). The DHPS dehydratase HpfG (gene locus tag I5Q83_10645) and its activating enzyme HpfH (gene locus tag I5Q83_10640) catalyze the cleavage of the CO-bond of DHPS to form sulfofropionaldehyde, which is then reduced by the sulfofropionaldehyde reductase HpfD (gene locus tag I5Q83_10625) to 3-HPS. In a., b., c., lines/bars show averages of triplicate incubations with error bars representing one standard deviation. In a., coloured asterisks show significant differences between treatment groups and their respective control groups without SQ. In c., brackets show non-significant results in pairwise comparisons of treatment groups and their respective unamended control. Significance was tested using ANOVA with Tukey's post hoc test. Adjusted *p*-values smaller than 0.05 were regarded as significant. SQ, sulfoquinovose; BHI, brain-heart infusion medium; DHPS, 2,3-dihydroxypropane-1-sulfonate; 3-HPS, 3-hydroxypropane-1-sulfonate; ns, not significant; SQDG, sulfoquinovosyldiacylglycerol.

Additionally, we tested SQ degradation by the mouse strain *F. rodentium* ALO17, which shares 100% identity with an ASV enriched in the mouse gut microcosms. However, growth of strain ALO17 was not significantly different between cultures with and without SQ. Also, SQ degradation and formation of degradation products were not detected (Figure S8). These results are in line with the absence of known SQ and DHPS pathway genes in the *F. rodentium* ALO17 genome.

Discussion

SQ in the gut exclusively derives from dietary intake, primarily of green edible plants (e.g. spinach, green onions) and edible micro- and macroalgae (e.g. spirulina, nori) that contain the ubiquitous thylakoid membrane lipid SQDG. In the mammalian gut, pancreatic lipases hydrolyze SQDG to sulfoquinovosyl glycerol [56,57]. Subsequently, SQ is cleaved off from SQDG or sulfoquinovosyl glycerol by sulfoquinovosidases [6,7], key enzymes of certain gut bacteria that take up SQ for further respiration or fermentation. SQ is structurally analogous to glucose-6-phosphate and was initially proposed to be degraded via a pathway similar to glycolysis [58,59]. Since the first discovery of sulfoglycolysis in bacteria [10], further sulfoglycolytic pathways have been described in different bacterial taxa [8,9,11–14].

While plants [58] and algae [60] can also degrade SQ via yet unknown pathways, it was unclear if animals are capable of SQ degradation and if mammalian cells are involved in intestinal SQ metabolism. Here, we show that SQ is neither metabolized by human colorectal adenocarcinoma (HT-29) cells *in vitro* nor respired to CO₂ by germ-free mice *in vivo*. This suggests that SQ cannot be metabolized by mammals, and instead serves exclusively as a nutrient and growth substrate for specialized bacteria in the animal gut.

A. rectalis was previously shown to be the most dominant SQ-degrading species in human fecal samples. Other species, including *F. prausnitzii*, *E. aldenensis* (formerly *Clostridium aldenense*), *E. clostridioformis* (formerly *Clostridium clostridioforme*), and *Roseburia* sp. AM16-25 also contributed to transcription of sulfofructose-transaldolase pathway genes in the fecal microbiome [15]. Transcription of the sulfo-Embden-Meyerhof-Parnas pathway of *Enterobacteriaceae* such as *E. coli* was considerably less important. Additionally, *B. wadsworthia* was identified as the dominant degrader of DHPS, which is produced by the primary SQ fermenters [15]. Our human fecal microcosm experiments with different SQ doses corroborated the relevance of *A. rectalis* and *B. wadsworthia* for complete SQ degradation to H₂S in the human gut. We further revealed that a minimum concentration of 0.1 mM SQ was sufficient to induce significant changes in the absolute abundance of selected microbiota members. The total amount of SQ in 10 ml microcosms amended with 0.1 mM SQ corresponds to the SQ content in about 11 g of fresh spinach (Table S5), the edible plant with the highest known SQDG concentration [3,61]. These results align with previous findings showing that SQ degradation ranks among the top third of all transcribed metabolic pathways of the fecal microbiome, establishing it as a microbial core function in the human gut [15]. Intestinal SQ levels reached by regular dietary intake of green vegetables may thus impact gut microbiota composition and potentially affect human health via SQ degradation metabolites [15]. The main SQ fermentation products are formate, acetate, and DHPS [10,13,16,62]. Besides acetate formation, our human fecal microcosm experiments also revealed a significantly higher butyrate concentration in microcosms amended with 10 mM SQ compared to the control (Figure 3 f). Increased butyrate production during SQ degradation has not been observed in previous SQ-amended human fecal microcosms [15], which suggests it may be specific to the mixture of human fecal samples used in our study. While the reason for the increased

butyrate production remains unknown, it is likely produced by *A. rectalis* and other bacteria that were enriched in the SQ-amended microcosms. *A. rectalis* and relatives of other enriched ASVs (e.g. *W. intestinalis*) can produce butyrate from fermentation of carbohydrates [63–65]. Butyrate production by *A. rectalis* involves the acetyl-CoA pathway, with butyryl-CoA:acetate CoA-transferase (But) as the key terminal enzyme that generates butyrate from two acetyl-CoA molecules and acetate as co-substrate [66–69]. *Faecalicatena* sp. DSM22707, an isolate from petroleum reservoir produced water, produces butyrate and other metabolites from SQ, highlighting butyrate as a new SQ fermentation product [39]. Production of SCFAs, particularly of butyrate, is considered a beneficial function of the gut microbiota and a prominent effect of dietary supplementation with known prebiotics such as resistant starch, inulin-type fructans, and fructooligosaccharides [70–74]. Butyrate is the preferred energy source of the colonocytes [75] and has been linked to the prevention and treatment of colitis [76,77] and colorectal cancer [78]. Supporting the prebiotic potential of SQ, *A. rectalis* maintained stable abundances when switching from a high-fiber to a low-fiber diet supplemented with SQ in a humanized gnotobiotic mouse model colonized with fourteen fiber- and mucin-degrading bacterial strains from the human gut. Without SQ, its abundance dropped approximately 1000-fold after switching to the low-fiber diet. This effect was specific to *A. rectalis* and not observed for *O. symbiosa*, another SQ degrader in the consortium [79].

The identity and metabolic features of microorganisms involved in SQ degradation in the gut of non-human animals is unknown. Laboratory mice are important models of biomedicine and nutrition research. Here we demonstrate that, similar to the human gut, the capacity for SQ degradation is restricted to few members of the murine microbiota. For example, the sulfofructose-transaldolase pathway, the most prevalent SQ degradation pathway in both systems, is found in only 0.5% of human and 1.1% of mouse gut microbiota species-level genome bins, underscoring its limited distribution (Figure 6) [15]. SQ is mainly degraded via the sulfo-Embden-Meyerhof-Parnas pathway and the sulfofructose-transaldolase pathway to DHPS. Members of the genera *Escherichia* and *Enterocloster* are primary SQ degraders in the mouse gut. The more than 24 h lag phase till onset of SQ degradation in mouse gut content microcosms and the absence of a significant shift in fecal microbiota composition upon single-dose gavage of SQ to mice suggested that microbial SQ

metabolism was not active before SQ supplementation. The SQDG contents of mouse chows are not known. However, the absence of SQ in feces before gavaging (Figures 2 h and 5 b) may indicate that the standard mouse chow used in this study, and possibly also in other mouse facilities, is not composed of foods with high SQDG content [3,80,81]. The single 1 mg and 10 mg SQ doses gavaged to mice correspond to the SQ content in approximately 0.37 g and 3.7 g of fresh spinach, which would represent about 7.5% and 75% of the total daily food intake in grams of a mouse, respectively (Table S5). Mice showed no adverse effects after ingesting a high SQ dose. Our results support previous findings from oral SQ supplementation experiments in susceptible IL-10-deficient mice [82]. Daily gavage with 0.45 mg SQ per g body weight, which corresponds roughly to the 10 mg SQ dose used in our study, over three weeks did not induce changes in microbial beta-diversity and intestinal inflammation in the IL-10-deficient mice [82].

Although some mouse microbiota members encode the capacity to either respire DHPS to H₂S (*T. muris*, *Desulfovibrio* species) or ferment DHPS to 3-HPS (*E. clostridioformis*), experiments with gut content microcosms, with conventional laboratory mice, and mouse gut-derived bacterial strains did not provide evidence for active degradation of DHPS. *T. muris* did not utilize DHPS in pure culture [19]. Further, *B. wadsworthia*, a confirmed DHPS degrader in the human gut [15], is not widely distributed among mice and, if present, mostly derived from inoculation of a human strain or human feces transplants [19]. Among the DHPS degradation pathway-encoding taxa in the mouse microbiota, only *E. clostridioformis* was significantly enriched in the mouse gut content microcosms. While we showed that the murine *E. clostridioformis* strain YL32 ferments SQ to DHPS for growth, further degradation of DHPS to 3-HPS was not observed. So-called 'extended sulfoglycolytic pathways' include genes for degradation of SQ and SQ degradation products such as DHPS or sulfolactate [83]. For example, *E. gilvus* contains the sulfofructose-transaldolase pathway and the bifurcated HpfGHD-HpfXYZ pathway for SQ and DHPS degradation [18]. *F. prausnitzii* is thought to also possess an extended sulfoglycolytic pathway, as its genome encodes the sulfofructose-transaldolase pathway along with a putative *hpfG* [17]. The HpfGHD pathway and the HpfXYZ pathway both do not produce H₂S from DHPS [17,18]. The HpfGHD pathway does not conserve energy, but regenerates NAD⁺ [17], while ATP is generated in the bifurcated HpfXYZ pathway [18]. Our results

indicate that DHPS is not a relevant growth substrate for the gut microbiota of conventional laboratory mice. Unlike the human gut, where SQ degradation proceeds beyond DHPS, SQ degradation stopped at the level of DHPS in the mouse gut and did not contribute to sulfide production. However, a previous study reported significant sulfide production after 168 h incubation of anoxic microcosms with feces of IL-10-deficient mice and 4 mM SQ, which indicated complete SQ degradation by the microbiota of this mouse model [82]. Microbiota composition was not analyzed in the fecal microcosms of IL-10-deficient mice. Yet, the discrepancy between the findings of this previous study and our study may be attributed to differences in microbial community structure and in incubation times. H₂S-producing bacteria of the family *Desulfovibrionaceae* (genera *Taurinivorans*, *Desulfovibrio*, and *Mailhella*) seem to be generally more abundant in wild mice compared to laboratory mice [19,28,84]. Whether the absence of an active DHPS metabolism observed in the laboratory mice in our study reflects a host-specific microbial adaptation that is conserved across diverse wild and laboratory mouse lineages [85] or results from the specific diet and housing of laboratory mice remains unclear.

Conclusions

Our work demonstrates that dietary SQ is exclusively degraded by the gut microbiota and not directly metabolized by the human and murine host. We observed the enrichment of commensal bacteria and significantly increased acetate and butyrate production in human fecal microcosms at high physiological SQ concentrations. These results align with key criteria for prebiotics [70]. SQ degradation by the gut microbiota of conventional wild type laboratory mice differed fundamentally from humans, primarily by the lack of DHPS degradation. Gnotobiotic animals colonized with defined consortia of microbial strains, including primary and secondary SQ degraders, may be better models to study effects of complete SQ degradation on the host [79,82]. Our study provides fundamental insight into SQ degradation in the mammalian gut, and has implications for potential application of SQDG-containing foods or SQ in dietary and prebiotic interventions.

Declarations

Ethics approval and consent to participate

Sampling and microbial analyses of feces from healthy human adults was approved by the ethics committee of the University of Vienna, Austria (reference numbers 00161 and 00714). Experiments with wild type mice were approved by the national authorities in Austria (Bundesministerium für Bildung, Wissenschaft und Forschung, 2022-0.745.974). Experiments with germ-free mice were approved by the local authorities in Germany (Regierung von Oberbayern, ROB-55.2-2532.Vet_02-19-53).

Consent for publication

Not applicable.

Availability of data and material

All sequence data generated in this project is available at NCBI under BioProject ID PRJNA1198716. The hidden Markov models developed in this project (Table S1) are available as Additional File 1.

Competing interests

The authors declare that they have no competing interests.

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Sulfoquinovose is exclusively metabolized by the gut microbiota and degraded differently in mice and humans

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Supplementary Figures

Figure S1. Sulfur metabolites and short-chain fatty acid dynamics in 24 h human fecal microcosm incubations with different SQ concentrations.

Figure S2. Enriched ASVs in SQ-amended human fecal microcosm incubations.

Figure S3. Isethionate production in mouse gut content microcosms amended with 10 mM SQ (Experiment 1).

Figure S4. Changes in total bacterial and archaeal abundance in mouse gut content microcosms amended with 10 mM SQ or 10 mM DHPS.

Figure S5. Enriched ASVs in mouse gut content microcosm incubations amended with 10 mM SQ (Experiment 1).

Figure S6. Enriched ASVs in mouse gut content microcosm incubations amended with 10 mM SQ (Experiment 2).

Figure S7. Fecal SQ and DHPS concentrations in conventional mice after oral SQ supplementation as measured by capillary electrophoresis.

Figure S8. *Faecalibaculum rodentium* did not degrade SQ in both rich and diluted rich medium.

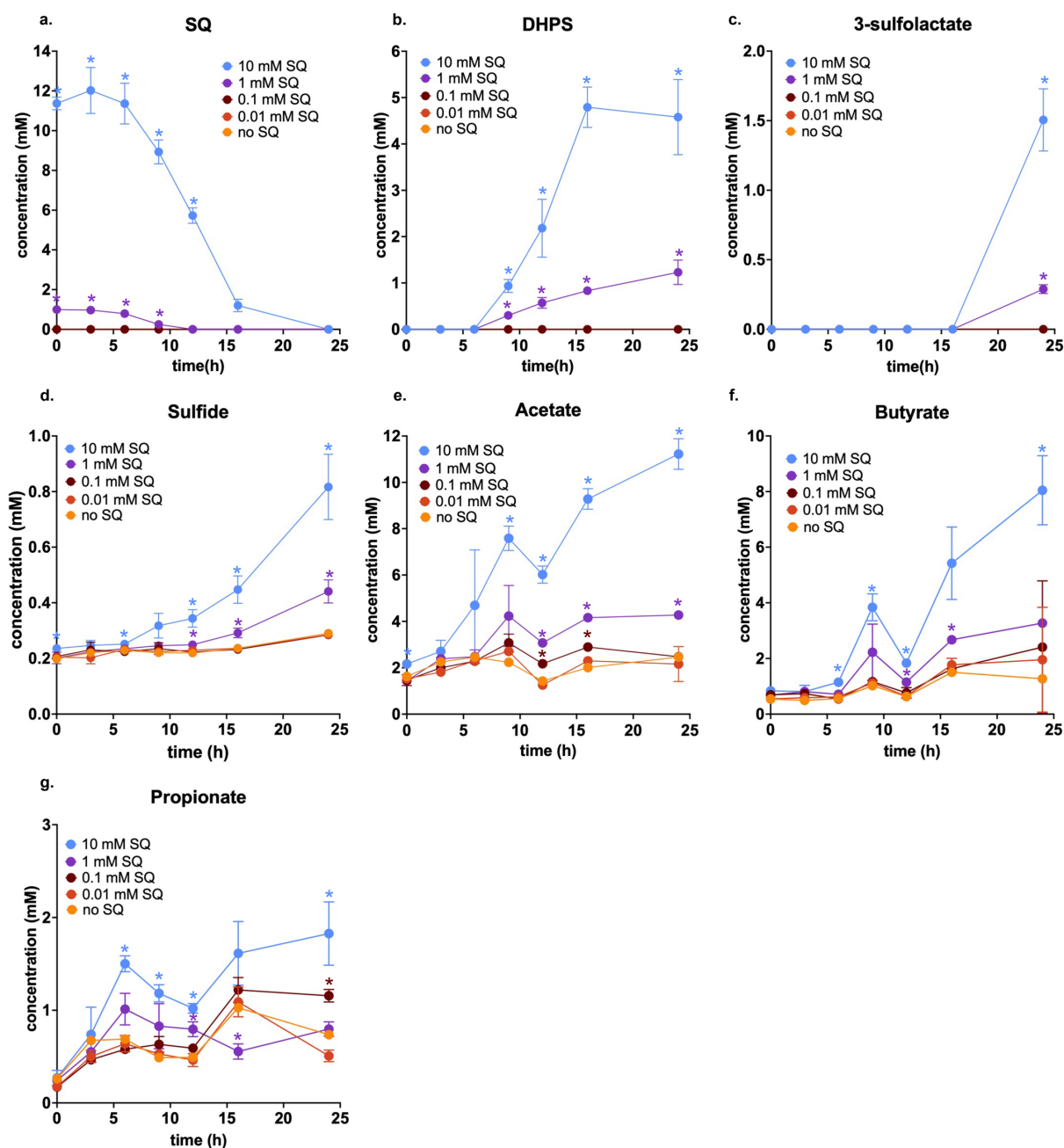


Figure S1. Sulfur metabolites and short-chain fatty acid dynamics in 24 h human fecal microcosm incubations with different SQ concentrations. Dynamics of the sulfur metabolites SQ (a), DHPS (b), 3-sulfolactate (c), sulfide (d), and the short-chain fatty acids acetate (e), butyrate (f), and propionate (g) in incubations with different concentrations of SQ (10 mM, 1 mM, 0.1 mM, 0.01 mM), as determined by capillary electrophoresis. Data points represent the average of triplicate incubations and the error bars represent one standard deviation. Only one unamended control incubation was performed. Coloured asterisks indicate significant differences between treatment groups and the pooled control group (unamended control + 0.01 mM SQ treatment), at individual time points, based on ANOVA with Dunnett's post hoc test. Adjusted *p*-values below 0.05 are considered significant. SQ, sulfoquinovose; DHPS, 2,3-dihydroxypropane-1-sulfonate.

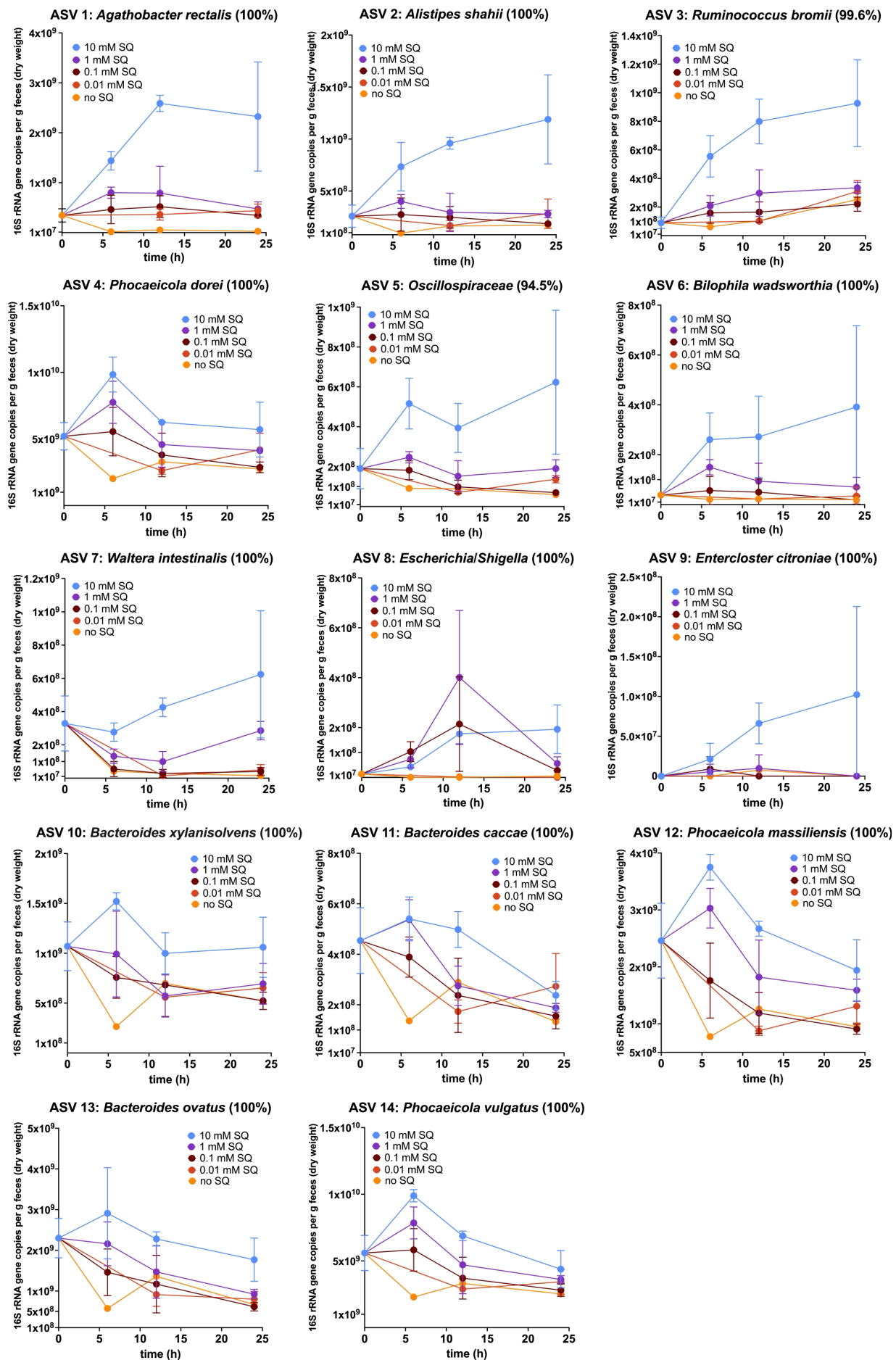


Figure S2. Enriched ASVs in SQ-amended human fecal microcosm incubations. Changes in absolute abundance of the fourteen significantly enriched ASVs in incubations amended with 10 mM SQ, 1 mM SQ and/or 0.1 mM SQ, as identified via differential abundance analysis. ASVs are shown in order of their average increase in absolute abundance after 24 h of incubation. Points represent averages of three replicates, except for the unamended control, of which only one replicate was sequenced. Error bars represent one standard deviation. Taxonomic classification and the 16S rRNA gene sequence similarity to the closest relative (in brackets) is shown for each ASV. ASV, amplicon sequencing variant; SQ, sulfoquinovose.

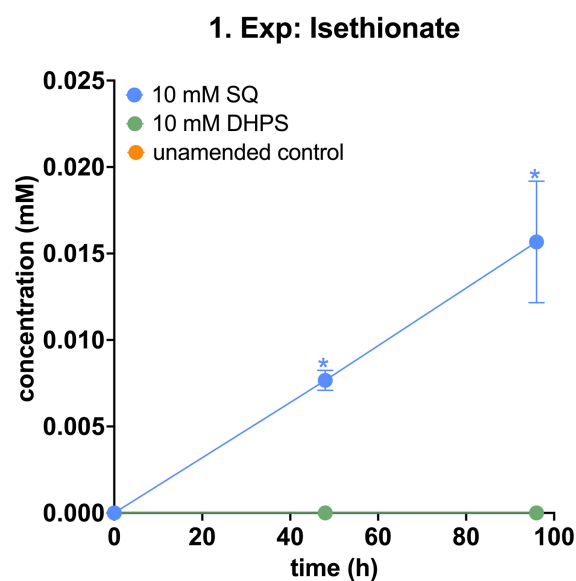


Figure S3. Isethionate production in mouse gut content microcosms amended with 10 mM SQ (Experiment 1). Small amounts of isethionate were only produced in SQ-amended microcosms, as determined via liquid chromatography-mass spectrometry. Lines show the average of triplicate measurements and error bars represent one standard deviation. Asterisks show significant differences between treatment groups and the unamended control at individual time points determined using ANOVA with Dunnett's post hoc test. Adjusted p -values below 0.05 were considered significant. Exp, experiment; SQ, sulfoquinovose; DHPS, 2,3-dihydroxypropane-1-sulfonate.

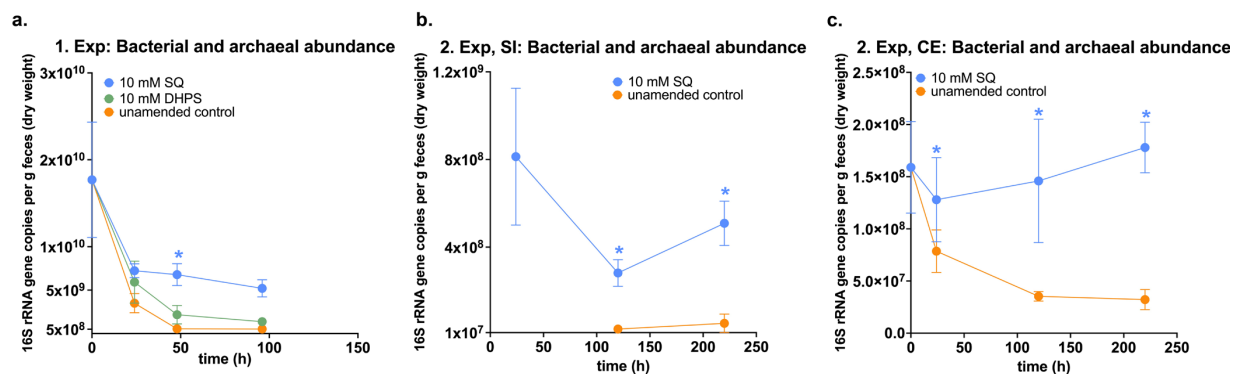


Figure S4. Changes in total bacterial and archaeal abundance in mouse gut content microcosms amended with 10 mM SQ or 10 mM DHPS. Dynamics of bacterial and archaeal 16S rRNA gene copy numbers in SQ-amended mouse gut content microcosms from pooled gut contents (**a**), small intestinal contents (**b**), and cecal contents (**c**). In **b.**, quantification of samples from time point zero h failed. All lines show the average of triplicate measurements and error bars represent one standard deviation. In **a.**, coloured asterisks show significant differences between treatment groups and their respective control groups without SQ at individual time points, as determined using ANOVA with Dunnett's post hoc test. In **b.** and **c.**, significant differences between SQ-amended incubations and the unamended control were determined at each time point via t-test and are depicted as asterisks. Adjusted *p*-values below 0.05 were considered significant. Exp, experiment; SQ, sulfoquinovose; DHPS, 2,3-dihydroxypropane-1-sulfonate; SI, small intestine; CE, cecum.

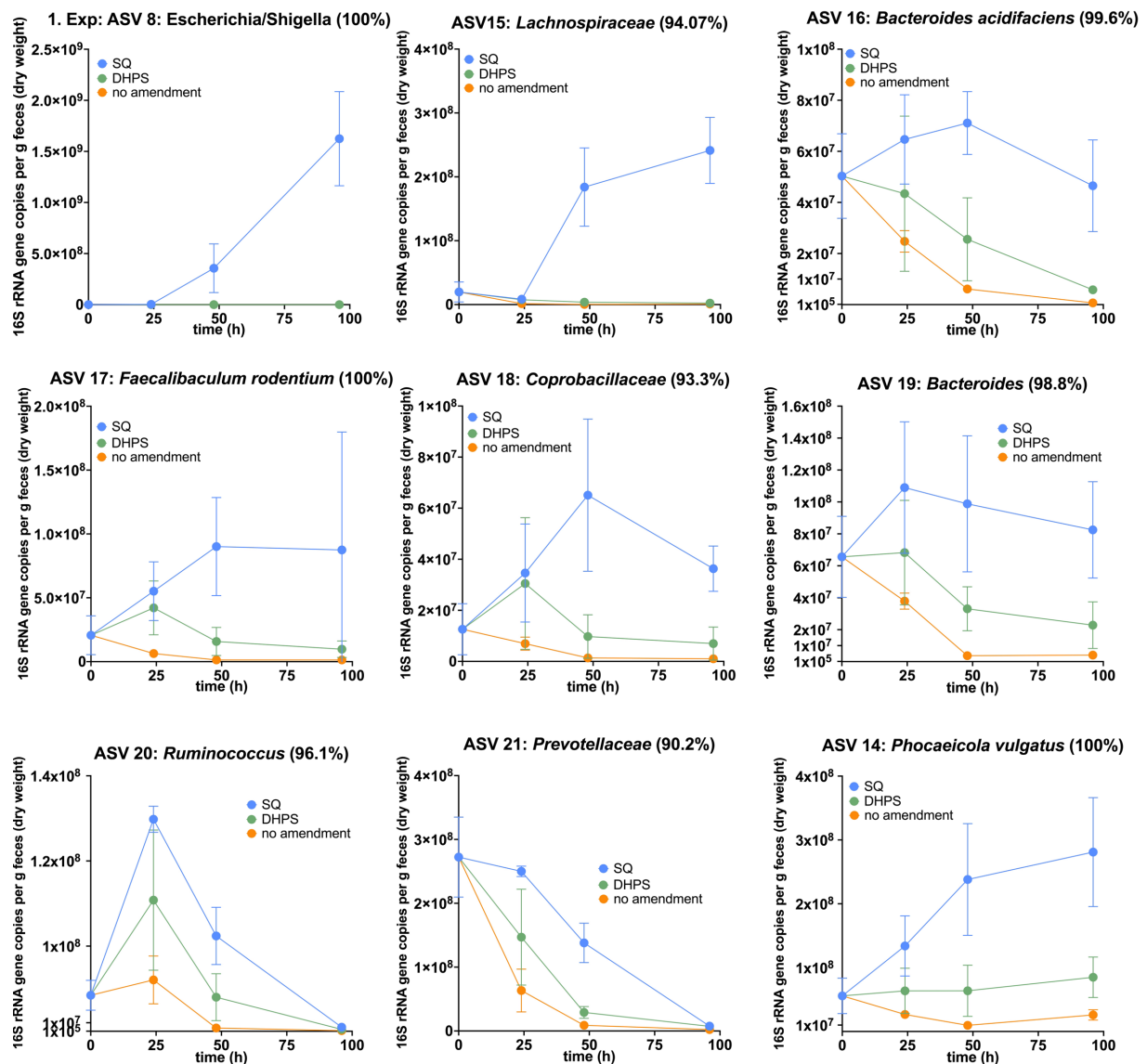


Figure S5. Enriched ASVs in mouse gut content microcosm incubations amended with 10 mM SQ (Experiment 1). Changes in absolute abundance of the nine significantly enriched ASVs in incubations amended with 10 mM SQ, as identified via differential abundance analysis. No ASVs were significantly enriched in DHPS-amended incubations. ASVs are shown in order of their $\log_2$ fold change between SQ-amended incubations and the unamended control. Data points represent averages of three replicates and error bars represent one standard deviation. Taxonomic classification and the 16S rRNA gene sequence similarity to the closest relative (in brackets) is shown for each ASV. ASV, amplicon sequencing variant; SQ, sulfoquinovose; DHPS, 2,3-dihydroxypropane-1-sulfonate.

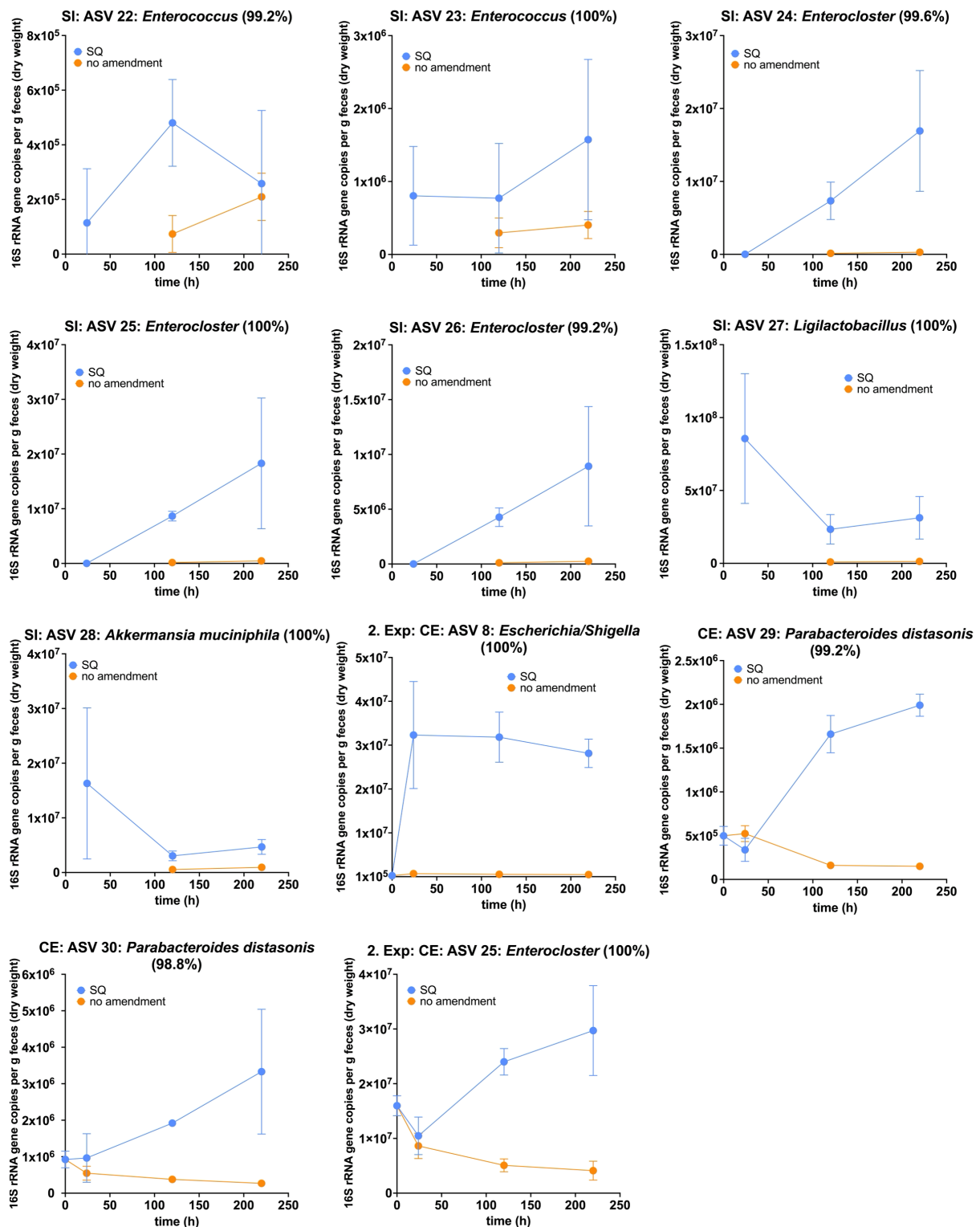


Figure S6. Enriched ASVs in mouse gut content microcosm incubations amended with 10 mM SQ (Experiment 2). Changes in absolute abundance of significantly enriched ASVs in SQ-amended incubations, with seven enriched ASVs in incubations with small intestinal contents and four enriched ASVs in incubations with cecal contents, as identified via differential abundance analysis. *Enterocloster* ASV 25 was enriched in the SQ-amended small intestinal content incubations and cecal content incubations. ASVs are shown in order of their log₂ fold change between SQ-amended incubations and the unamended control. Points represent averages of three replicates. Error bars represent one standard deviation. Taxonomic classification and the 16S rRNA gene sequence similarity to the closest relative (in brackets) is shown for each ASV. SI, small intestine; CE, cecum; ASV, amplicon sequencing variant; SQ, sulfoquinovose.

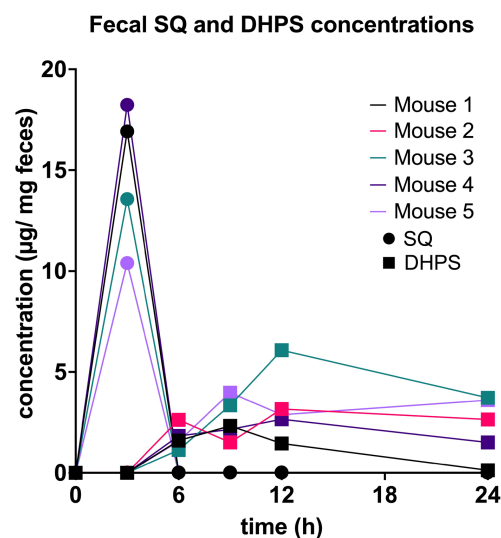


Figure S7. Fecal SQ and DHPS concentrations in conventional mice after oral SQ supplementation as measured by capillary electrophoresis. Ten mg SQ was metabolized within six hours to DHPS in conventional C57BL/6 mice. DHPS was detected between 6 h and 24 h post-gavage. Metabolite dynamics were additionally measured using liquid chromatography-mass spectrometry (Figure 5 b, Table S4). The discrepancies between the two methods are primarily due to (i) the lower sensitivity and specificity of capillary electrophoresis, as well as technical variability between the methods, and (ii) the fact that only a subset of the samples analyzed by capillary electrophoresis were also examined using liquid chromatography-mass spectrometry. SQ, sulfoquinovose; DHPS, 2,3-dihydroxypropane-1-sulfonate.

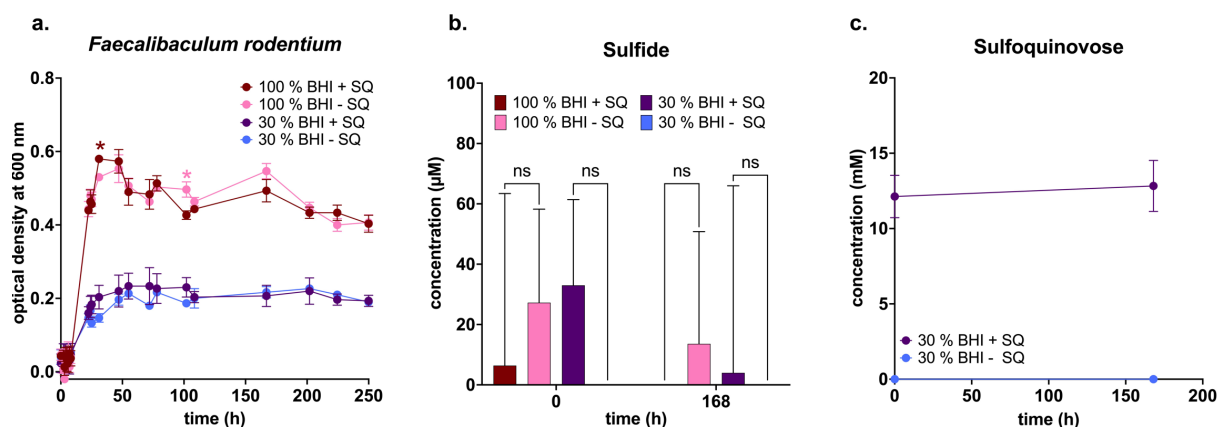


Figure S8. *Faecalibaculum rodentium* did not degrade SQ in rich medium and diluted rich medium. Growth dynamics of *F. rodentium* in undiluted (100%) and diluted (30%) BHI medium with and without 10 mM SQ (a). Sulfide (b) and sulfoquinovose (c) concentrations in *F. rodentium* cultures with or without SQ. SQ concentrations were measured in diluted BHI medium via capillary electrophoresis. SQ addition did not lead to growth stimulation, sulfide production or SQ degradation. Data points in a. and c. show averages of triplicate cultures with error bars representing one standard deviation. In a., coloured asterisks show significant differences between treatment groups and their respective control groups without SQ at individual time points. Bars in b. represent average sulfide concentrations and error bars represent one standard deviation. Values that were negative after subtraction of the blank were set to zero. Brackets show results of pairwise comparisons between treatment groups and their respective unamended control. Significant differences were determined using ANOVA with Tukey's post hoc test. In c., SQ concentrations at 0 h were compared to SQ concentrations at 168 h via t-test. Adjusted *p*-values below 0.05 were considered significant. SQ, sulfoquinovose; BHI, brain-heart infusion; ns, not significant.

Chapter 2

Extensive richness and novel taxa of sulfoquinovose-degrading bacteria in the cow rumen

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Author contributions

J.K. and A.L. conceived the study, with support from M.M.. J.K., H.S., P.M., S.B., and R.S. performed experiments and analyzed data. D.R.S., E.S., W.W., and D.S. contributed essential samples or data, experimental infrastructure, analytical instrumentation and/or expert advice. J.K., S.C., T.S.T., J.O., J.S., and B.H. performed bioinformatic analyses. J.K., S.C., P.M, S.B., R.S., M.M., and A.L. interpreted the data. J.K. and A.L. wrote the article. All authors revised and approved the manuscript.

Abstract

Sulfoquinovose (SQ), a sulfonated sugar derived from the thylakoid membrane lipid sulfoquinovosyl diacylglycerol (SQDG), is abundant in photosynthetic organisms and plays a key role in global sulfur cycling. Its degradation in nature is mediated by specialized bacteria, many of which rely on the enzyme sulfoquinovosidase (YihQ) to release SQ from SQDG. Despite its ecological importance, the diversity and functional roles of SQ-degrading microorganisms remain poorly characterized in natural environments. Here, we developed a *yihQ*-targeted amplicon sequencing approach to investigate the richness and distribution of SQ-degrading bacteria across selected environments, including marine sediments and the mammalian gut. We revealed particularly high richness of *yihQ*-containing microorganisms in cow rumen, far exceeding that observed in human and mouse gut microbiomes, suggesting an important role of SQ metabolism in ruminant digestion. Anaerobic microcosm experiments with SQ-amended rumen fluid revealed cooperative microbial degradation of SQ to sulfide via isethionate cross-feeding. Amplicon sequencing and genome-resolved metagenomics identified novel uncultured SQ-degrading taxa, including members of *Caproiciproducens* (*Acutalibacteraceae*), *Limivacinus* (*Oscillospiraceae*), and *Sphaerochaetaceae*, which encode the sulfo-transketolase pathway, along with *Mailhella* (*Desulfovibrionaceae*), a likely isethionate-respiring bacterium. This study presents the first functional gene-based assay for tracking environmental *yihQ* diversity, highlights SQ degradation as a central metabolic process in the cow rumen, describes novel SQ-metabolizing bacteria, and advances understanding of sulfur physiology in complex microbial communities.

Key words

Sulfoquinovose, sulfur cycle, organosulfur, sulfolipid, gut microbiome, rumen, marine sediment, functional gene, sulfoquinovosidase

Introduction

Sulfoquinovose (SQ; 6-deoxy-6-sulfo-D-glucose) is a sulfonated hexose, which is ubiquitous in the environment. Approximately 10 billion tons of SQ are produced globally per year, comparable to the amino acids methionine and cysteine. SQ is an integral component of the biogeochemical sulfur cycle and the human and animal diet [1,2]. While free SQ appears to be rare in nature, it is primarily present as the head group of the sulfolipid sulfoquinovosyl diacylglycerol (SQDG) in the biomass of photosynthetic organisms such as plants, algae, and cyanobacteria [2–6]. SQDG resides in the thylakoid membrane, where it supports the activity and structural integrity of photosystem II [2,7,8].

To date, several pathways for SQ degradation have been identified in bacteria. Some pathways require inter-species transfer of organosulfonates for complete SQ mineralization to sulfate or hydrogen sulfide [9–15]. In contrast, the SQ monooxygenase pathway in *Agrobacterium tumefaciens* and the *Roseobacter* clade and the SQ dioxygenase pathway in *Marinomonas ushuaiensis* aerobically cleave the C-S bond of SQ and directly catabolize SQ to sulfite and a deoxy-hexose [16–18]. Since SQDG is the main natural source of SQ, all known pathways require the enzymatic release of SQ from SQDG or sulfoquinovosyl glycerol [4,19]. In five of the six physiologically and biochemically characterized SQ degradation pathways, this cleavage is catalyzed by sulfoquinovosidases of the glycoside hydrolase family 31 [4,10,11,13,14,16,17,19–21]. These include YihQ in *Escherichia coli* [10], *Akalicoccus urumqiensis* (formerly *Bacillus urumqiensis*) [14], *Clostridium* sp. [14], and *Novosphingobium aromaticivorans* [14], PpSQ1_00094 in *Pseudomonas putida* [11], SftG in *Bacillus aryabhattai* [13], SqvC in *Priestia megaterium* (formerly *Bacillus megaterium*) [21], and Smol in *A. tumefaciens* [16]. These sulfoquinovosidases have conserved structural and functional features and form a monophyletic orthologous group in the glycoside hydrolase family 31 tree [4,19]. We therefore refer to members of this orthologous group as YihQ sulfoquinovosidases. YihQ from *E. coli* and *A. tumefaciens* hydrolyzed both SQDG and sulfoquinovosyl glycerol *in vitro* [4,19]. *E. coli* cultures supplemented with sulfoquinovosyl glycerol or glucose grew to the same optical density. In these growth experiments sulfoquinovosyl glycerol was metabolized faster than glycerol and free sulfoquinovose, but later than glucose.

Growth rates of cultures with sulfoquinovosyl glycerol were also higher than those of cultures grown solely with SQ or glycerol alone, indicating that sulfoquinovosyl glycerol is the preferred substrate for *E. coli* [19].

Genes of *YihQ* are widespread among bacteria that encode SQ-degradation pathways. Some SQ-degrading bacteria, such as *Roseobacter* spp., *Arthrobacter* spp., and *Marinomonas* sp., lack a *yihQ* homolog [17,18,22]. However, a new family of NAD⁺-dependent sulfoquinovosidases was recently discovered and is widely distributed in bacteria that were previously thought to lack a sulfoquinovosidase [23].

SQDG and SQ are integral parts of the sulfur cycle, yet the diversity of SQ-degrading microorganisms across different environments remains largely unexplored, with studies to date limited to human and mouse gut microbiomes and seawater [18,24,25]. The presence of *yihQ* homologs in five of the six SQ degradation pathways suggests that this gene represents a suitable functional marker for many SQDG/SQ-degrading microorganisms. To this end, we developed two *yihQ*-targeted degenerate primer sets and evaluated their use for analyzing the *yihQ* sequence diversity in samples from various environments (soil, marine sediments) and host-associated gut microbiomes (human, cow, and mouse). Furthermore, the *yihQ* amplicon sequencing approach was combined with 16S rRNA gene amplicon sequencing, metagenomics, and metabolite analysis to study, for the first time, SQ degradation and the involved microorganisms and pathways in cow rumen fluid microcosms. We show that the SQ-degrading community in the cow rumen differs from those in the mouse and human gut by comprising several hundred distinct *yihQ* sequences, consisting of undescribed species, and predominantly utilising the sulfo-transketolase pathway for SQ degradation to isethionate.

Materials and Methods

Supplementary Materials and Methods provide further details on the methods described below.

Design of *yihQ*-targeted PCR assays

A YihQ reference database was created using sequences of biochemically validated YihQ sulfoquinovosidases and homologs of functionally different enzymes of the glycoside hydrolase family 31 as queries for Blast against the KEGG prokaryotes database [26] (Table S1). An alignment of representative sequences was used for calculation of a maximum-likelihood YihQ tree (Fig. S1) and for the design of degenerate *yihQ*-targeted primers. Genomic DNA of the *yihQ*-encoding strains *Escherichia coli* K12, *Agathobacter rectalis* A1-86, and *Enterocloster clostridioformis* YL32 were used as positive controls for PCR. DNA of *Segatella copri* DSM 18205 and ultrapure water served as non-target control and negative control, respectively. Primer pairs targeting different *yihQ* regions were initially tested using pure culture DNA for temperature gradient PCR and further optimized by touchdown PCR. The optimized PCR program (Table S2) was subsequently tested with DNA from several environmental and intestinal samples.

Sampling and ethics

Marine coastal surface sediment samples were collected from Canada (Vancouver: 49°17'57.7"N, 123°10'34.6"W), Italy (Fetovia: 42°43'57.2"N, 10°9'15.6"E), Mauritania (Dakhlet Nouadhibou: 20°36'52.7"N, 16°30'35.9"W), and Australia (Casuarina Beach: 12°21'39.96"S, 130°51'56.7"E). A soil sample was collected from the acidic peatland Schlöppnerbrunnen II, Germany (50°07'54.8"N, 11°52'51.8"E), as previously described [27].

A human fecal sample mix was created by pooling freshly collected feces from five adult volunteers. Sampling and microbiota analyses of human feces was approved by the ethics commission of the University of Vienna (reference numbers 00161 and 00714). A mouse gut content sample mix was created by pooling small intestinal and cecal contents from eight conventional C57BL/6 wild type mice (4 females, 4 males,

aged 8–10 weeks). Mice were housed under specific-pathogen-free conditions in compliance with the Federation of European Laboratory Animal Science Associations guidelines, with *ad libitum* access to a standard chow diet (Ssniff V1534, Ssniff, Germany) and water. Rumen fluid was collected at an International Featured Standard-Food certified slaughterhouse in Lower Austria. Cows originated from the same farm, were fed hay and grass silage *ad libitum*, with a maximum of 0.5 kg/day of commercial concentrate (KuhKorn PLUS Energie; Garant-Tiernahrung GmbH, Austria), and had free access to water, which is common practice in the region. Post-mortem, the mechanical isolation procedure and gastrointestinal tract removal were performed by licensed veterinarians following standard operating protocols to minimize contamination and preserve microbial composition. A volume of 50 ml of rumen fluid was obtained from each rumen under controlled hygienic conditions. Samples were immediately transported to the laboratory and processed. During the first collection, a sample was obtained from one cow and used for quantifying SQDG and for *yihQ*-amplicon sequencing alongside with other environmental samples. During the second collection, one sample each was obtained from three cows and was used for the rumen fluid microcosm experiment. As the mouse gut and rumen fluid samples were obtained post-mortem, no ethical approval was required for this study.

Rumen fluid microcosms

Anoxic rumen fluid microcosms supplemented with 10 mM SQ (MCAT GmbH, Germany) were used to investigate SQ metabolism by the cow gut microbiome (Supplementary Materials & Methods). Microcosms were subsampled over 168 hours for metabolite quantification and microbial community analyses.

Metabolite analysis

Ultra-high-performance reverse phase liquid chromatography combined with mass spectrometry was used to quantify and identify all molecular species of SQDG in rumen fluid (Supplementary Materials & Methods). Sulfide was quantified colorimetrically as previously described [28]. Formate, succinate, acetate, propionate, butyrate, valerate, and lactate were quantified by capillary electrophoresis. SQ and its metabolites isethionate, 2,3-dihydroxypropane-1-sulfonate (DHPS), 3-

hydroxypropane-1-sulfonate, 3-sulfolactate, and 3-sulfopropionate were quantified by liquid chromatography–mass spectrometry as described previously (Supplementary Materials & Methods) [29].

16S rRNA gene and *yihQ* amplicon sequencing and analyses

Genomic DNA from environmental, intestinal, and rumen fluid microcosm samples was isolated using the DNeasy PowerSoil Pro Kit (Qiagen, Austria). Amplicon sequencing on the MiSeq platform (V3, 600 cycles, Illumina, USA) was conducted by the Joint Microbiome Facility (University of Vienna, Medical University of Vienna) under project IDs JMF-2310-13 and JMF-2303-02. DNA from each environmental sample was sequenced in triplicates within the same sequencing run. A two-step PCR method [30,31] and primer pairs YIHQa (YIHQ1201Fa/YIHQ1526Ra) and YIHQb (YIHQ1201Fb/YIHQ1526Rb) were used to amplify and barcode an approximately 340 bp region of the *yihQ* gene (Fig. 1, Table S2). Samples from the rumen fluid microcosm experiment were analyzed by amplicon sequencing with primer pair YIHQb and with 16S rRNA gene-targeted primers for bacteria and archaea [30,31]. Amplicon sequence variants (ASVs) were inferred using DADA2 [32]. FASTQ reads 1 and 2 were trimmed at 220 nt and 150 nt, respectively, with allowed expected errors of 2. 16S rRNA gene-ASVs were classified using DADA2 and the SILVA 16S rRNA sequence database (release 138.1) [33]. A workflow was established for analysis of sequences obtained with *yihQ*-targeted primers, including removal of non-*yihQ* sequences and phylogenetic placement in the YihQ reference tree (Fig. S2, Supplementary Materials & Methods). Verified *yihQ* ASV sequences were further clustered into operational taxonomic units (OTUs) using vsearch [34] with a 90% sequence identity threshold. Alpha diversity metrics (observed number of ASVs and OTUs, Shannon diversity index, and Simpson diversity index) were calculated for subsampled (100, 1000, and 10000 reads) *yihQ* sequence libraries. Verified *yihQ* ASV sequences generated with the YIHQb amplicon sequencing assay were additionally classified using the GlobDB genome database (version r220) [35].

Significantly enriched 16S rRNA gene- and *yihQ*-OTUs/ASVs in SQ-supplemented rumen fluid microcosms were identified by DESeq2 (v1.38.3) as described previously [36]. Enriched ASVs/OTUs were taxonomically classified by Blast against the NCBI 16S rRNA sequence database and the GlobDB database [35,37].

Metagenomics and genome analysis

Metagenomic sequencing was performed on a NovaSeq6000 (1/2 SP flow cell, 2×100 bp reads; Illumina, USA) with DNA extracts from SQ-amended (n = 2) and unamended control (n = 1) microcosms of rumen fluid, taken after 168 hours of incubation. Metagenome-assembled genomes (MAGs) were dereplicated and taxonomically classified using GTDB-Tk [38]. Average amino acid identity (AAI) was calculated using the Enveomics Collection [39]. Whole-genome average nucleotide identity (ANI) was calculated using FastANI (version 1.33) [40].

New Hidden Markov Models (HMMs) were generated for the detection of enzymes and transport proteins of sulfoquinovose, isethionate, and DHPS degradation pathways (Table S3). HMMs were integrated into HMSS2 [41], which was then used to identify sulfur metabolism genes in the recovered MAGs and GlobDB genomes (Supplementary Materials & Methods).

Data and code availability

All sequence data generated in this project are available at NCBI under BioProject ID PRJNA1242727. The YihQ reference database, the amino acid alignment of YihQ reference sequences, the YihQ reference tree, and further information are available in Additional Files 1-7. HMMs for microbial organosulfur metabolism proteins are available at github.com/TSTanabe/HMSS2/tree/main/Hidden_Markov_Models.

Results and Discussion

Development of PCR assays for broad coverage of *yihQ* sequence diversity

Based on a curated YihQ sequence database and a YihQ reference tree (Fig. S1), we designed ten degenerate primers that target different conserved regions in the *yihQ* alignment (Fig. 1, Table S4). Of the four tested primer combinations, only two primer pairs, YIHQa (YIHQ1201Fa/YIHQ1526Ra) and YIHQb (YIHQ1201Fb/YIHQ1526Rb) resulted in PCR products of the expected size with DNA templates of three *yihQ*-containing strains in the temperature gradient PCR tests. Primer pairs YIHQa and YIHQb were further tested in a touchdown PCR approach for enhanced specificity. The final protocol for both primer pairs used an initial annealing temperature of 63°C, which decreased by 1°C per cycle over 10 cycles, followed by 20 cycles at 53°C. Subsequent magnesium chloride concentration tests showed that 4 mM magnesium chloride in the PCR buffer consistently produced the strongest bands for the target sequences, while the negative and non-target-DNA controls did not yield a visible PCR product (Table S2). Sanger sequencing of the PCR products confirmed specific amplification of the *yihQ* target gene in all three organisms.

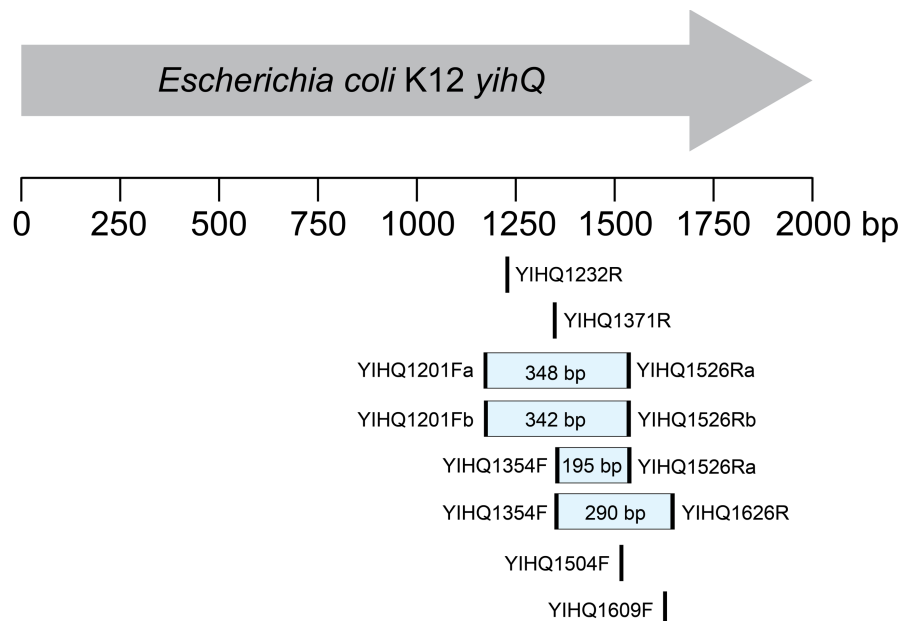


Fig. 1. *yihQ*-targeted primers designed in this study. Primer names refer to the target gene *yihQ* and the binding position in the *yihQ* sequence of *E. coli* K12 (2037 bp, NCBI gene ID: 948376). Bars indicate the predicted length of the *yihQ* PCR product in *E. coli* K12. Primers without bars were not tested. bp = base pairs.

The primers YIHQ1201Fb and YIHQ1526Rb were designed as shorter and less degenerate alternatives to YIHQ1201Fa and YIHQ1526Ra. Thus, primer pairs YIHQa and YIHQb amplify the same, approximately 340 bp region of *yihQ*, yet differ in degeneracy, predicted melting temperatures, and sequence coverage (Table S4), which may influence PCR amplification efficiency and specificity [42]. The YIHQa and YIHQb primer pairs perfectly match 81.8% and 89.8% of the *yihQ* sequences in the reference database, respectively. We thus applied and compared both primer pairs for amplification of DNA from various intestinal and environmental samples. These included pooled mouse gut content and human feces, which are known to harbor *yihQ*-containing bacteria [24,25], and rumen fluid, soil, and diverse marine sediments. The latter are environments that have not yet been investigated for their SQ degradation capacity. Both PCR assays successfully amplified a single band of the expected size with DNA from all intestinal samples. PCRs with peat soil DNA were negative. PCRs with primer pair YIHQa produced faint PCR products with DNA from Canadian marine sediment, but no PCR products with DNA from Italian, Mauritanian, and Australian marine sediments. In contrast, PCRs with primer pair YIHQb generated bands for all marine sediment samples.

Optimized *yihQ* amplicon sequencing reveals highest *yihQ* richness in cow rumen

We optimized the YIHQa and YIHQb PCR assays for amplicon sequencing on the Illumina MiSeq platform. Both primer pairs contained a generic 16 bp 5'-head sequence for two-step PCR barcoding [30,31] (Table S2). The optimized PCR protocol was applied for amplification of DNA from the intestinal and environmental samples. Amplicons of the correct size were obtained with DNA from rumen fluid, mouse gut content, human feces, and Canadian sediment with both PCR assays. Additional amplicons with DNA from Mauritanian and Italian sediment were only obtained with the YIHQb assay, which suggests it has higher sensitivity and/or coverage. For technical replication, triplicate amplicons of each DNA sample that yielded a PCR product with both primer pairs were sequenced on the Illumina MiSeq platform.

Next, we established a computational workflow that allowed us to (i) assess the specificity of the YIHQa and YIHQb PCR assays and (ii) efficiently remove non-*yihQ* sequences from the dataset prior to further diversity analysis (Fig. S2). The workflow consisted of translating nucleic acid sequences into protein sequences, BlastP against the YihQ reference database, and phylogenetic placement in the YihQ reference tree using the evolutionary placement algorithm in RAXML, which shows high accuracy for classification of functional gene sequences [43]. Both primer pairs amplified non-target sequences across all tested samples, but their relative abundance in the sequence libraries varied with sample type (Fig. 2A and B, Table S5). PCR specificity was highest for intestinal samples, particularly for rumen fluid samples, with >98% of reads and >97% of ASVs verified as *yihQ* in both PCR assays. In contrast, less than 35% of reads and 40% of ASVs that were recovered with the YIHQa assay from the Canadian sediment sample were verified as *yihQ*. Hardly any verified *yihQ* reads and ASVs were recovered from sediment using the YIHQb assay. Overall, both YIHQa and YIHQb assays provided high specificity for *yihQ* sequence amplification from intestinal DNA samples but showed considerably reduced reliability with marine sediment DNA.

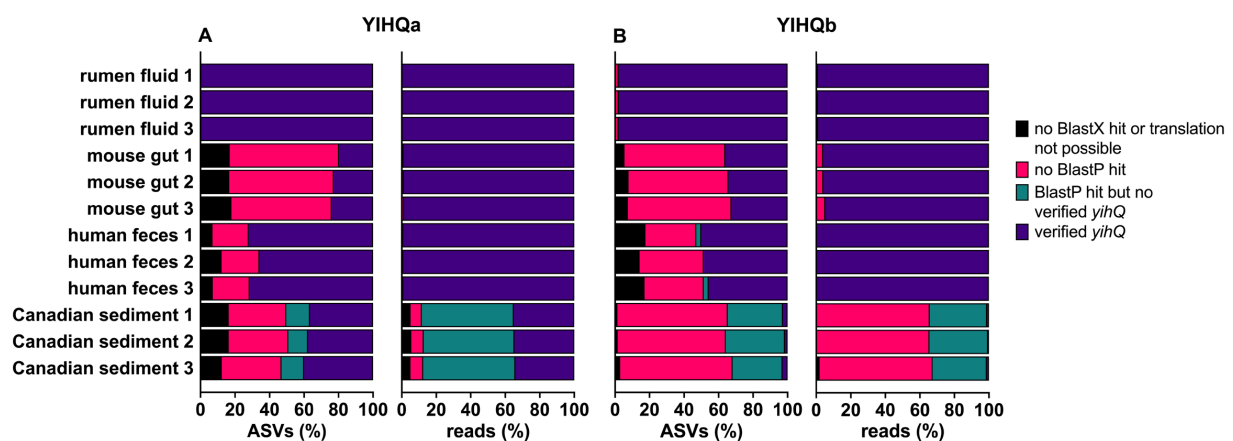


Fig. 2. Specificity of the YIHQa and YIHQb amplicon sequencing assays is sample-dependent. Bar plots display the proportion of ASVs or reads (%) recovered from YIHQa (A) and YIHQb (B) amplicon sequencing assays that were verified as *yihQ* or excluded from further analysis (Fig. S2, Table S5). Each sample was sequenced in triplicates.

Sequences were further clustered into OTUs using a 90% similarity threshold, which has been used for other functional genes, such as *dsrA* and *dsrB* encoding dissimilatory sulfite reductase subunits, for approximate assignment into species-level

groups [43]. Alpha diversity analysis at varying library sizes demonstrated high reproducibility among technical triplicates (Fig. S3, Table S6). Rumen fluid samples had the highest *yihQ* richness with more than 1,000 and 400 observed ASVs and OTUs, respectively. Contrarily, mouse gut content and human feces samples, which were pooled from several individuals, had a considerably lower *yihQ* richness with only a few tens of observed ASVs and about ten observed OTUs (Fig. S3). The low species number of potential SQ degraders in the murine and human gut is consistent with prior reports [24,25]. The Canadian sediment sample had an intermediate *yihQ* richness with about 50 and 40 observed ASVs and OTUs, respectively. Accordingly, a library depth of only 100 sequences was sufficient to cover >98% of the expected ASV/OTU richness in the *yihQ* PCR product from the human and mouse gut samples, while hundred times more sequences were required to reach the same coverage for the rumen fluid sample (Fig. S3, Table S6).

The phylogenetic placement of the environmental *yihQ* sequences indicated that both assays recovered similar ASVs that were broadly distributed across the reference tree (Fig. 3). Most ASVs from rumen fluid (64% of 1,220 ASVs from the YIHQa library; 47.5% of 1,548 ASVs from the YIHQb library) were placed at a single, long branch of a *YihQ* sequence (protein BK011_07555; AUD65555) of *Tenericutes* bacterium MZ-QX. Most ASVs from human feces (66% of 32 ASVs from the YIHQa library; 87% of 15 ASVs from the YIHQb library) and the mouse gut (71% of 17 ASVs from the YIHQa library; 92% of 13 ASVs from the YIHQb library) were affiliated with a *Clostridia* cluster of known SQ degraders, such as *Agathobacter rectalis* [24] and *Otoolea symbiosa* (formerly *Clostridium symbiosum*) [13]. Many rumen-derived ASVs (27% of 1,220 ASVs from the YIHQa library; 37% of 1,548 ASVs from the YIHQb library) were also affiliated with this cluster. Another subset of ASVs from human feces (19% of 32 ASVs from the YIHQa library; 13% of 15 ASVs from the YIHQb library) and the mouse gut (18% of 17 ASVs from the YIHQa library; 8% of 13 ASVs from the YIHQb library) clustered in the *Gammaproteobacteria*, which include the biochemically validated *YihQ* of *Escherichia coli* [10]. Due to the low coverage of the YIHQb library (only two ASVs were recovered), the phylogenetic placements of ASVs from the two assays could not be compared for the Canadian marine sediment (Fig. 3). However, most marine ASVs from the YIHQa library were placed at a branch with a second *YihQ* gene copy (protein BK011_09290; AUD64050) of *Tenericutes* bacterium MZ-QX (61% of 71 ASVs) or were affiliated with diverse *YihQ* sequences within the

Alphaproteobacteria (27% of 71 ASVs). For refined taxonomic classification, all *yihQ* ASVs generated through the YIHQb assay were additionally matched against GlobDB database, a comprehensive collection of bacterial and archaeal species genomes, using a conservative threshold of 90% *YihQ* sequence identity (Fig. S4, Table S7). Most ASVs were affiliated with *Oscillospiraceae*, *Lachnospiraceae*, and *Acutalibacteraceae* in the class *Clostridia* (Fig. S4).

The phylogenetic and sequence identity-based analysis provides an initial hypothesis for the taxonomic classification of unknown *yihQ*-containing microorganisms in environmental samples, which must be interpreted with caution. Notably, some organisms, such as *Tenericutes* bacterium MZ-QX and some *Limivacinus* species, possess two copies of *yihQ*. Additionally, the phylogeny of *YihQ* does not always align with the taxonomy of the organisms (Fig. 3, Fig. S1). For example, numerous *yihQ* ASVs from cow rumen were assigned to the genus *Limivacinus* but form multiple phylogenetically distinct clades in the *YihQ* tree (Fig. 3, Fig. S4). These potential gene duplications and/or lateral transfers suggest that the evolutionary history of *yihQ* does not necessarily reflect the phylogenetic relationships of the host organisms. Similar to functional marker genes used for other microbial guilds, such as *aprBA*, *dsrAB*, and *amoA* [44–46], this complex evolutionary pattern presents challenges in taxonomic classification but does not preclude the use of *yihQ* as a marker for studying the environmental diversity of SQDG/SQ-degrading microorganisms.

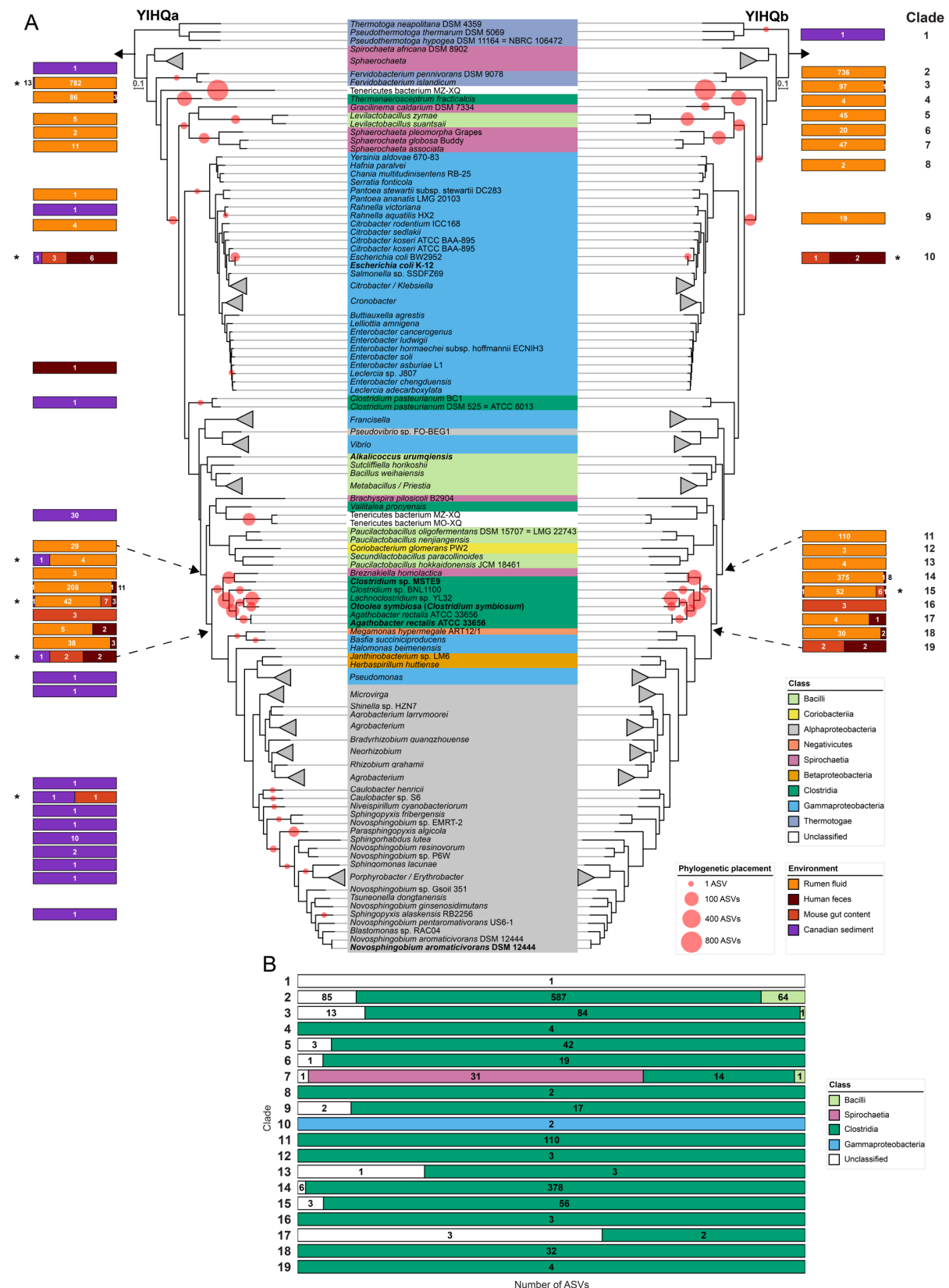


Fig. 3. Phylogeny and taxonomic classification of environmental *yihQ* ASVs. Phylogeny of ASVs obtained from human feces, mouse gut content, rumen fluid, and marine sediment using the YIHQa assay (left tree) and the YIHQb assay (right tree) (**A**). For each ASV, a representative, translated sequence was added to the YihQ reference tree using the evolutionary placement algorithm of RAXML. The scale bar shows 10% sequence divergence. The class-level taxonomy of reference sequences is

indicated in color. Sequence libraries were not rarefied before placement. Phylogenetic placement is indicated with red circles. The size of each circle represents the number of ASVs placed at that position. Adjacent bars show the percentage distribution of ASVs across environments, with numbers indicating the total ASV count separately for each environment. Asterisks indicate identical ASVs found in different environments. In total, 8 ASVs in YIHQa and 2 ASVs in YIHQb were detected across multiple environments (Table S7). ASV clades in the YIHQb tree are numbered next to the bars. Taxonomic composition of *yihQ* ASV clades generated in the YIHQb amplicon sequencing assay (**B**). Translated *yihQ* ASVs were queried against the GlobDB database using BlastP. The top-scoring hit for each ASV was used for taxonomic assignment at the class-level. ASVs with no match or matches below 90% sequence identity were categorized as unclassified (white). Each horizontal bar represents an ASV clade, as defined in **A**, with individual segments showing the percentage distribution of ASVs to taxonomic classes within that clade. Numbers in bar plots refer to the absolute number of ASVs per class within one ASV clade. Taxonomic assignment of ASVs at the family and genus level is shown in Fig. S4 and Table S7.

Novel bacterial species degrade SQ to isethionate via the sulfo-transketolase pathway in cow rumen fluid microcosms

SQDG can be an abundant component of edible green plants and algae [2] and is thus a substrate for the intestinal microbiome. The identity and ecophysiology of intestinal SQDG/SQ-degrading bacteria has so far been only studied for humans [24] and mice [25]. Here, we performed rumen fluid microcosm experiments to investigate microbial SQ degradation in grass- and hay-fed cows, which presumably ingest substantial amounts of SQDG. The SQDG concentration in rumen fluid was 21 µg/g dry weight (Table S8). For comparison, the SQDG concentrations in plants range between 46 µg/g in garlic and 824 µg/g in spinach [47]. SQDG molecules vary in their fatty acid composition, with differences in chain length and degree of saturation. These variations arise because plants produce a diversity of SQDG species depending on their physiology and environmental conditions [48]. In the rumen fluid, the SQDG species 18:3_16:0 represented about 70% of the total SQDG pool, which is similar to the SQDG species composition in green plants such as spinach, basil, and lettuce [49,50].

SQ concentrations decreased only slightly in SQ supplemented rumen fluid microcosms within the first 48 hours of anoxic incubation (Fig. 4A and B). However, SQ was completely metabolized after 168 h. SQ degradation coincided with the production of isethionate while other known organosulfonate SQ degradation products, such as DHPS or 3-sulfolactate [10–13], were not detected. Sulfide concentrations increased across all treatment groups but were significantly higher in SQ-amended microcosms compared to the unamended control (Fig. 4C). Temporal dynamics of acetate, lactate, propionate, and butyrate were not consistently different between SQ-

amended microcosms and unamended controls (Fig. S5). However, the production of the known SQ fermentation products acetate and butyrate [12,24,25,29] may have been masked by the high background concentrations of volatile fatty acids (Fig. S5), which is in agreement with previous studies [51,52]. Despite the high richness of *yihQ*-containing microorganisms in the rumen, which is mostly attributed to members of the genus *Limivacinus* (*Oscillospiraceae*) (Fig. S3, Tables S6 and S9), we identified only a few 16S rRNA gene- and *yihQ*-ASVs/OTUs that were significantly enriched in SQ-amended microcosms compared to unamended controls (Tables S10 and S11). 16S rRNA-ASV 1 and 2 were related to the genera *Caproiciproducens* (*Acutalibacteraceae*) and *Mailhella* (*Desulfovibrionaceae*) and reached a maximal relative abundance of about 2.7% and 0.7% in SQ-amended microcosms, respectively (Fig. 4D and E). Additionally, seven *yihQ*-ASVs that clustered in two OTUs were significantly enriched in SQ-amended microcosms. OTU 1 (ASVs 2-7), classified as *Limivacinus* (*Oscillospiraceae*), and OTU 2 (ASV 1), classified as an unknown *Sphaerochaetaceae* bacterium, reached a maximal relative *yihQ* abundance of 12.6% and 9% in SQ-amended microcosms, respectively (Fig. 4F and G, Fig. S6, Table S11).

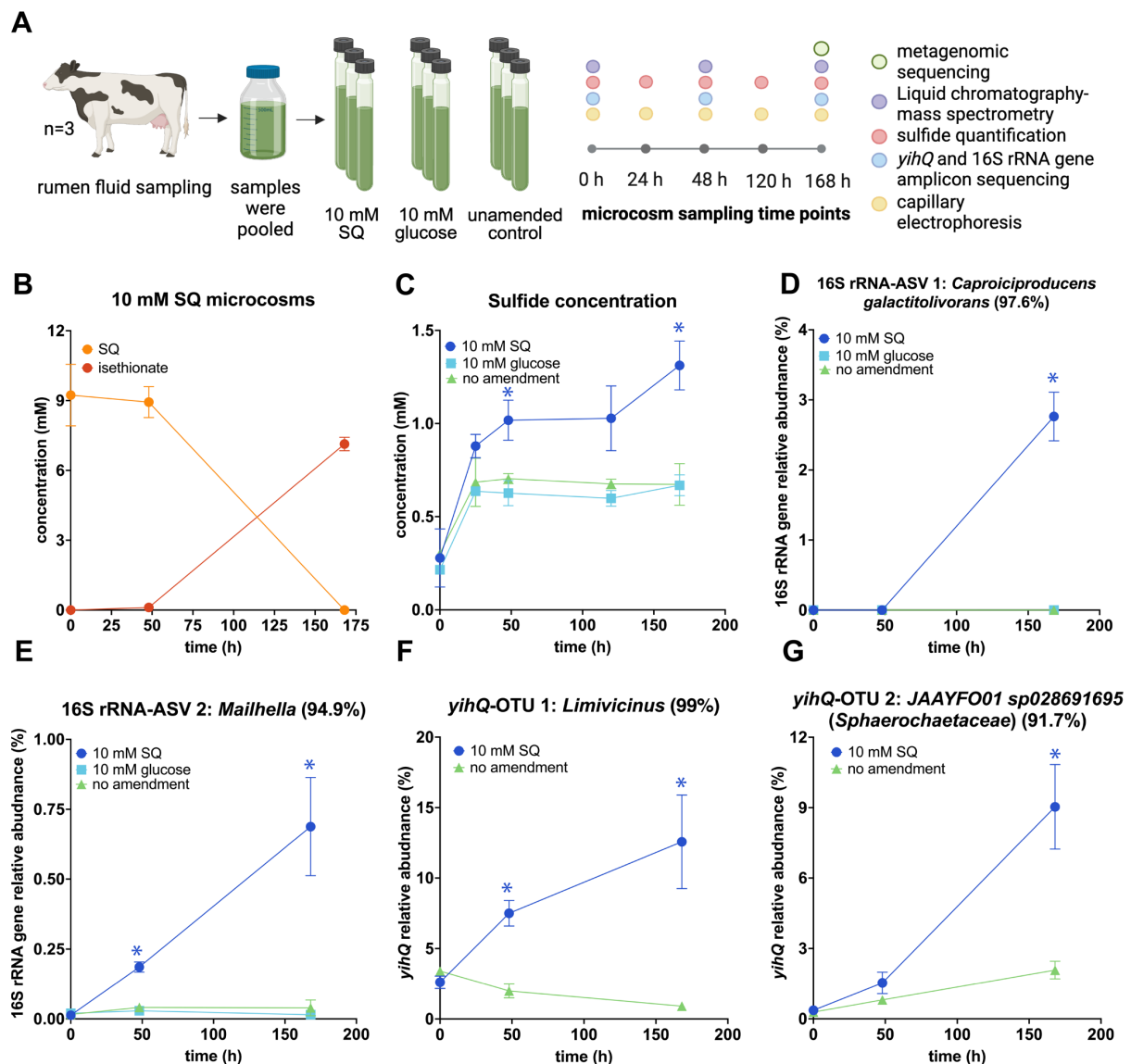


Fig. 4. Yet uncultured *Caproiciproducens*, *Limivacinus*, *Mailhella*, and *Sphaerochaetaceae* species are involved in anaerobic degradation of sulfoquinovose to isethionate and sulfide in cow rumen microcosms. Overview of the rumen fluid microcosm experiment and the analytical approach (created with biorender.com) (A). Microcosms were amended with 10 mM SQ (n = 3) or 10 mM glucose (n = 3). Unamended microcosms (n = 3) served as controls. SQ (orange) degradation coincided with the production of approximately 7 mM isethionate (red) (B). Increase in sulfide concentration was significantly higher in SQ-amended microcosms (dark blue) compared to unamended controls (green) (C). Relative abundance dynamics of 16S rRNA gene-ASVs and *yihQ*-OTUs that were significantly enriched in SQ-amended microcosms compared to unamended controls (D-G). Sequence identity (%) to the next relative is shown in brackets. Data points and error bars represent the average of triplicate measurements and one standard deviation, respectively. Coloured asterisks show significant differences between the respective treatment group and the unamended control at individual time points. Significant differences were determined by ANOVA with Tukey's posthoc test (C-E). Adjusted *p*-values below 0.05 were considered significant. In G and F, significant differences were determined via DeSeq2 analysis with *p*-values of below 0.01 considered significant. SQ, sulfoquinovose; ASV, amplicon sequencing variant; OTU, operational taxonomic unit.

Accordingly, genome-centric metagenomics recovered 25 medium- to high-quality MAGs from the SQ-amended microcosms, including *Caproiciproducens*, *Limivacinus*, *Sphaerochaetaceae*, and *Mailhella* MAGs (Table S12). The latter MAGs represent yet uncultivated and unnamed species and/or genera. *Caproiciproducens* MAGs SQRumen1 and SQRumen2 each represent a different species (93.3% ANI) [40]. *Caproiciproducens* MAG SQRumen2 belongs to the GTDB species *Caproiciproducens* sp000752215 (GCA_000752215) (97.1% ANI), while *Caproiciproducens* MAG SQRumen1 (93.3% ANI to *Caproiciproducens* sp000752215, GCA_000752215) represent a yet unknown species. The two *Limivacinus* MAGs SQRumen19 and SQRumen23 are only about 50% complete and were thus not used for ANI/AAI calculations. *Limivacinus* MAG SQRumen19 was classified as the GTDB species *Limivacinus* sp900317375 (GCA_900317375), while *Limivacinus* MAG SQRumen23 may represent a yet unknown species (Table S12). *Sphaerochaetaceae* MAG SQRumen3 belongs to the GTDB species JAAYFO01 sp028691695 (GCA_028691695) (99% ANI) and has <53% AAI to species outside the GTDB genus JAAYFO01, suggesting it represents a yet undescribed genus [53]. *Mailhella* MAG SQRumen6 belongs to the GTDB species *Mailhella* sp902783285 (GCA_902783285) (98.8% ANI). Consistent with the metabolite data, the *Caproiciproducens* and *Sphaerochaetaceae* MAGs and *Limivacinus* sp900317375 (GCA_900317375) encode the sulfo-transketolase pathway variant for SQ degradation that produces isethionate (Fig. 5A) [14]. Furthermore, a more comprehensive analysis of these taxa revealed that 10.7% of 28 *Caproiciproducens*, 35.5% of 313 *Limivacinus* (4.7% of 3,230 *Oscillospiraceae*), and 11.8% of 246 *Sphaerochaetaceae* genomes in the GlobDB database have the capacity for SQ degradation via this pathway. The three previously available genomes of the *Sphaerochaetaceae* genus JAAYFO01 do not encode the sulfo-transketolase pathway (Table S13). Notably, all sulfo-transketolase pathway-encoding *Caproiciproducens* genomes lacked genes for the two known sulfoquinovosidases YihQ and SqqA [23] (Fig. 5B). This explains the absence of enriched *yihQ*-ASVs of this genus in the SQ-supplemented rumen microcosms (Table S11) and suggests that SQ-degrading *Caproiciproducens* species either rely on other microorganisms for SQ release or harbor a yet unknown sulfoquinovosidase. Sulfo-transketolase pathway-encoding *Caproiciproducens* genomes additionally contained *sftT*, encoding 6-deoxy-6-sulfo-D-fructose transaldolase. However, they lacked genes for the 3-sulfolactaldehyde reductase (*sftR*) and dehydrogenase (*sftD*), indicating an

incomplete sulfo-transaldolase pathway, which is in line with absence of DHPS and 3-sulfolactate in the microcosms [29]. The sulfo-transaldolase and sulfo-Embden-Meyer-Parnas pathways, which are primarily used for SQ fermentation by gut bacteria of humans and laboratory mice [24,25], employ three carbon atoms of SQ for central carbon metabolism and excrete a C₃-sulfonate (DHPS, 3-sulfolactate) [10,13,14]. In comparison, the sulfo-transketolase pathway of the identified cow rumen bacteria utilizes four SQ carbon atoms for the central carbon metabolism, excretes a C₂-sulfonate (isethionate), and may thus maximize the energy yield compared to other SQ fermentation pathways [14,54].

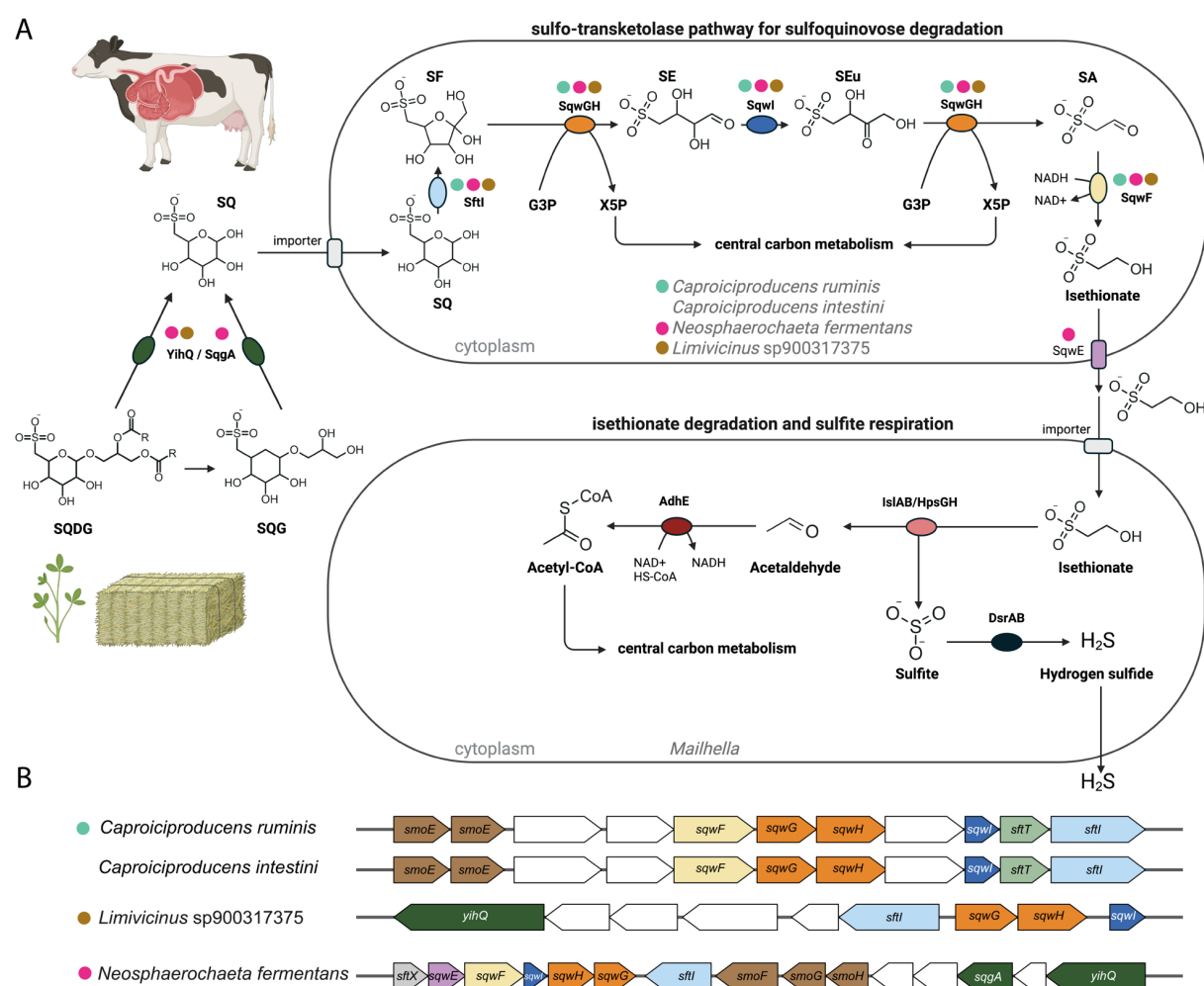


Fig. 5. Sulfoquinovose is cooperatively degraded to sulfide via isethionate cross-feeding from diverse sulfo-transketolase-pathway-encoding *Clostridia* species to organosulfur-respiration-encoding *Mailhella* species. (A) Cows ingest sulfoquinovosyl diacylglycerol (SQDG) through hay, grass or dietary algae supplements. Newly described bacteria from the cow rumen have the capacity for sulfoquinovose (SQ) catabolism. The sulfoquinovosidases YihQ or SqgA initially cleave SQ from sulfoquinovosyl diacylglycerol (SQDG) or sulfoquinovosyl glycerol (SQG). SQ is further catabolized to isethionate by the sulfo-transketolase pathway-encoding rumen species *Caproiciproducens ruminis*

MAG SQrumen1, *Caproiciproducens intestini* MAG SQrumen2, *Neosphaerochaeta fermentans* MAG SQrumen3, and *Limivacinus* sp900317375 (GCA_900317375). Released isethionate is taken up by *Mailhella* species and desulfonated to acetaldehyde and sulfite by the isethionate sulfite-lyase (IslAB) and/or DHPS sulfite-lyase complexes (HpsGH). Sulfite is used as an electron acceptor for anaerobic respiration via the DsrAB-dissimilatory sulfite reductase system. The figure was created with biorender.com. Coloured dots above enzymes indicate presence of the respective gene in the genomes of *Caproiciproducens ruminis* MAG SQrumen1 and *Caproiciproducens intestini* MAG SQrumen2 (turquoise), *Neosphaerochaeta fermentans* MAG SQrumen3 (pink), and *Limivacinus* sp900317375 (brown). Functionally equivalent enzymes are grouped under a single name based on the first characterized homolog (e.g., SftI corresponds to SqvD). (B) Gene clusters for SQ degradation via the sulfo-transketolase pathway in *Caproiciproducens intestini*, *Caproiciproducens ruminis*, *Neosphaerochaeta fermentans* and *Limivacinus* sp900317375 (GCA_900317375). Genes depicted in white have no assigned function in sulfur metabolism. YihQ and SggA, sulfoquinovosidase; SftI, SQ isomerase; SqwGH, 6-deoxy-6-sulfofructose transketolase, Sqwl, 4-deoxy-4-sulfoerythrose isomerase; SqwF, sulfoacetaldehyde reductase; SqwE, isethionate exporter; IslAB, isethionate sulfite-lyase; HpsGH, DHPS sulfite-lyase; DsrAB, dissimilatory sulfite reductase; AdhE, aldehyde-alcohol dehydrogenase; SmoE, sulfoquinovosyl glycerol transport ATP-binding protein; SftT, 6-deoxy-6-sulfofructose transaldolase; SmoFGH, transporter; SftX, DUF4867. SQ, sulfoquinovose; SF, 6-deoxy-6-sulfofructose; SE, 4-deoxy-4-sulfoerythrose; SEu, 4-deoxy-4-sulfoerythrulose; G3P, glycerol-3-phosphate; X5P, xylulose-5-phosphate; SA, sulfoacetate; DHPS, 2,3-dihydroxypropane-1-sulfonate.

The slightly increased sulfide production in SQ-amended microcosms likely resulted from anaerobic respiration of isethionate by a *Mailhella* species (Fig. 4C and 5A). The genus *Mailhella* forms a monophyletic group with the genera *Bilophila* and *Taurinivorans*, which comprise important organosulfonate-respiring bacteria from the human and mouse gut, respectively [55]. *Mailhella* MAG SQrumen6 and 75% of 48 *Mailhella* genomes harbour genes that are homologous to the paralogous genes for DHPS sulfite-lyase (*hpsGH*) and isethionate sulfite-lyase (*islAB*) complexes of *B. wadsworthia* [15,24,56]. Notably, both HpsG and IslA can cleave isethionate to sulfite and acetaldehyde, yet HpsG shows higher activity for DHPS desulfonation [15,56]. Sulfite respiration to sulfide is catalyzed by the dissimilatory sulfite reductase *dsrAB* system, which is evolutionary conserved in *Desulfobacterota* members, including *Mailhella* [55,57].

In summary, our results indicate that diverse, previously undescribed bacterial species in the rumen cooperatively mineralize plant-derived SQ to hydrogen sulfide through isethionate-cross-feeding. These findings carry implications for animal husbandry, as increased sulfite respiration has been associated with decreased activity of methanogenic archaea [58]. Various micro- and macroalgae are tested as feed additives to reduce emissions of the greenhouse gas methane from ruminants

[59,60]. Future research may investigate whether SQDG present in algae contributes to their anti-methanogenic properties [61].

In accordance with the SeqCode [62], we propose the following new taxa for three high-quality MAGs (Fig. 5): *Caproiciproducens ruminis* sp.nov. MAG SQrumen1, *Caproiciproducens intestini* sp.nov. MAG SQrumen2, and *Neosphaerochaeta fermentans* gen. nov., sp.nov. MAG SQrumen3.

Description of *Caproiciproducens ruminis* sp. nov.

Caproiciproducens ruminis sp. nov. (ru'mi.nis. L. gen. neut. n. *ruminis*, of the rumen, the source of the bacterium). The designated DNA sequence is MAG SQrumen1 recovered from cow rumen. This species was enriched in anoxic incubations of rumen fluid with sulfoquinovose. Genome analysis suggests sulfoquinovose is fermented to isethionate via the sulfo-transketolase pathway. The NCBI BioSample number for the genome is SAMN48198398.

Description of *Caproiciproducens intestini* sp. nov.

Caproiciproducens intestini sp. nov. (in.tes.ti'ni. L. gen. neut. n. *intestini*, of the gut, referring to the origin of the bacterium). The designated DNA sequence is MAG SQrumen2 recovered from cow rumen. This species was enriched in anoxic incubations of rumen fluid with sulfoquinovose. Genome analysis suggests sulfoquinovose is fermented to isethionate via the sulfo-transketolase pathway. The NCBI BioSample number for the genome is SAMN48198399.

Description of *Neosphaerochaeta fermentans* sp. nov.

Neosphaerochaeta fermentans sp. nov. (fer.men'tans. L. part. adj. *fermentans*, fermenting) The designated DNA sequence is MAG SQrumen3 recovered from cow rumen. This species was enriched in anoxic incubations of rumen fluid with sulfoquinovose. Genome analysis suggests sulfoquinovose is fermented to isethionate via the sulfo-transketolase pathway. The genome additionally encodes two different sulfoquinovosidases (YihQ and SqqA). The NCBI BioSample number for the genome is SAMN48198401.

Description of *Neosphaerochaeta* gen. nov.

Neosphaerochaeta gen. nov. (Ne.o.sphae.ro.chae'ta. Gr. masc. adj. neos, new; N.L. fem. n. *Sphaerochaeta*, a bacterial genus; N.L. fem. n. *Neosphaerochaeta*, a new genus related to *Sphaerochaeta*). Type species: *Neosphaerochaeta fermentans* sp. nov., family: *Sphaerochaetaceae*.

Conclusions

We developed the first functional gene amplicon sequencing assays for analyzing the diversity of SQDG/SQ-degrading microorganisms in complex samples. The two PCR assays both target the same gene region of the YihQ sulfoquinovosidase and exhibited similar environment-dependent specificity. They were highly specific for *yihQ* in intestinal samples from humans, mice, and cows but amplified a substantial proportion of non-*yihQ* sequences from marine sediments. To ensure accurate *yihQ* diversity analysis, we implemented a computational workflow to efficiently remove non-target sequences. However, the potential amplification of non-target genes may reduce the sensitivity of the PCR assays in certain environments, such as marine sediments. Future improvements to the *yihQ*-targeted PCR assays could address these limitations. Additionally, for a more comprehensive assessment of SQDG/SQ-degrading microorganisms, functional gene amplicon sequencing should be complemented by PCR assays targeting the more recently identified SggA sulfoquinovosidase [23].

Among all analyzed samples, rumen fluid exhibited the highest *yihQ* richness, suggesting that the species/strain-level diversity of potential SQDG/SQ-degraders in the cow gut is one to two orders of magnitude greater than in the human and mouse gut. Furthermore, yet uncultured species were enriched in rumen fluid microcosms supplemented with SQ and identified as primary (*Caproiciproducens*, *Limivacinus*, *Neosphaerochaeta*) or secondary (*Mailhella*) SQDG/SQ-degraders through metabolite analysis, amplicon sequencing, and comparative genomics. This study revealed SQDG/SQ degradation as a relevant metabolic trait of the cow rumen microbiome and provided insights into the diversity and metabolic potential of the newly described taxa involved.

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Competing interests

The authors declare that they have no competing interests.

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Extensive richness and novel taxa of sulfoquinovose-degrading bacteria in the cow rumen

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Figure S1. Phylogeny of YihQ sulfoquinovosidases.

Figure S2. Computational workflow for identification and filtering of *yihQ* sequences.

Figure S3. Alpha diversity analysis of *yihQ* amplicon sequence data shows highly consistent results for all triplicates.

Figure S4. Taxonomic assignment of environmental *yihQ* ASVs.

Figure S5. Dynamics of volatile fatty acids in rumen fluid microcosms.

Figure S6. Relative abundance dynamics of *yihQ*-ASVs that were significantly enriched in SQ-amended rumen fluid microcosms.

References

Supplementary Materials and Methods

Design of *yihQ*-targeted PCR assays

A YihQ reference database was created using sequences of biochemically validated YihQ sulfoquinovosidases and closely-related homologs of functionally different enzymes, such as alpha-xylosidases, of the glycoside hydrolase family 31 as queries for Blast against the KEGG prokaryotes database [1] (Table S1). The retrieved YihQ homologs (n = 500) were de-replicated at 95% protein sequence identity, and the resulting sequences (n = 294) were further aligned with the biochemically validated proteins using MUSCLE [2]. A maximum-likelihood tree was constructed using FastTree [3] with a gamma distribution (-gtr option) to account for different evolution rates at different sites and the Whelan and Goldman protein evolution model (-wag option) [4]. Bootstrap support values for each tree branch was calculated using the CompareToBootstrap.pl script [5]. Homologs (n = 131) that formed a well-supported (>70% bootstrap support) monophyletic clade with biochemically validated YihQ sequences in the tree served as the basis for primer design (Fig. S1). After manually identifying conserved sites in the YihQ sequence alignment, the corresponding sites in the nucleic acids sequence alignment were used for the design of degenerate primers. To further minimize nucleotide degeneracy for some primers, the alignment was examined for each degenerate nucleotide in the primer sequence to determine whether a less degenerate alternative could be used.

Genomic DNA extracts from the *yihQ*-encoding strains *Escherichia coli* K12, *Agathobacter rectalis* A1-86, and *Enterocloster clostridioformis* YL32 were used as positive controls for PCR. DNA of *Segatella copri* DSM 18205 and ultrapure water served as non-target control and negative control, respectively. All strains were grown anaerobically (85% N₂, 10% CO₂, 5% H₂) in brain heart infusion broth at 37°C until the stationary phase, after which genomic DNA was extracted using Chelex 100 Resin (Bio-Rad, Austria), as previously described [6]. Briefly, 200 µl of ultrapure water was added to 200 µl liquid bacterial culture and centrifuged at 15,500 g for 2 min. The supernatant was discarded. 100 µl of 5% Chelex 100 resin was added to the pellet and contents were vortexed until homogenized. Samples were incubated at 100°C for 10 min, then again centrifuged at 15,500 g for 2 min and DNA-containing supernatants were transferred to a new tube. DNA concentration was determined using a QubitTM 3 Fluorometer (Invitrogen, USA). DNA extracts were stored at -20°C using them for

PCR. The absence of PCR-inhibiting substances in the DNA extracts was verified by a positive 16S rRNA gene-targeted PCR with the universal primers 515F (5'-GTG YCA GCM GCC GCG GTA A) and 806R (5'-GGA CTA CNV GGG TWT CTA AT) [7,8].

All *yihQ*-targeted primer pairs were first tested using a temperature gradient PCR. The initial PCR program consisted of denaturation at 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, annealing at temperatures ranging from 40°C to 65°C in ~1°C increments for 30 s, and elongation at 72°C for 45 s, with a final elongation step at 72°C for 10 min. Each 50 µl reaction mixture contained 1× DreamTaq buffer, 0.2 mM dNTPs, 1.25 U recombinant Taq polymerase (5 U/µl), 10 µg bovine serum albumin, 10 µM of each primer, and 1 µl of template DNA. All reagents were sourced from Thermo Fisher Scientific (Germany).

Primer pairs that yielded PCR products of the expected size with positive controls and showed no amplification in negative or non-target controls were validated via Sanger sequencing. To optimize assay conditions, primers that successfully amplified the target gene were further tested using touchdown PCR. Annealing temperatures of 65–55°C, 64–54°C, 63–53°C, 62–52°C, 61–51°C, and 60–50°C were tested. In each condition, the annealing temperature started at the highest value of the respective range (e.g., 65°C for 65–55°C) and decreased by 1°C per cycle over the first 10 cycles. After reaching the lowest temperature of the range (e.g., 55°C for 65–55°C), the reaction continued for an additional 20 cycles at this final annealing temperature. Then, using the determined optimal touchdown temperature range, magnesium chloride concentrations between 0.5–7 mM were tested in 0.5 mM increments. Successful amplification of target genes was confirmed via Sanger sequencing.

The final PCR program was tested with DNA from several environmental and intestinal samples and included an initial denaturation at 95°C for 3 min, followed by 10 cycles of 95°C for 30 s, 63–53°C for 45 s (decreasing 1°C per cycle), and 72°C for 45 s. This was followed by 20 cycles of 95°C for 30 s, 53°C for 45 s, and 72°C for 45 s, with a final elongation at 72°C for 15 min. Each 50 µl reaction contained 1× Taq buffer (with potassium chloride), 0.2 mM dNTPs, 1.25 U Taq Polymerase (recombinant, 5 U/µl), 4 mM magnesium chloride, 10 µM of each primer, and 1 µl of template DNA (Table S2). All reagents were obtained from Thermo Fisher Scientific (Germany).

PCRs with environmental DNA included DNA from a *yihQ*-encoding strain as positive control and DNA from *S. copri* without *yihQ* and ultrapure water as negative controls.

Rumen fluid microcosms

Rumen fluid microcosms were prepared in an anaerobic tent (Coy Laboratory Products, USA) operated with a gas mix of 85% N₂, 10% CO₂, and 5% H₂, maintaining H₂ levels of 1.5–2.5%. Rumen fluid samples from three cows were pooled (1:1:1 ratio, v/v/v) and homogenized. Large particles were left to settle. In each Hungate tube, rumen fluid was mixed 1:1 with 1× PBS (pH 7.4), including the specific amendments, to a final volume of 10 ml. Microcosms were amended with 10 mM SQ (MCAT GmbH, Germany), 10 mM D-glucose (Carl Roth, Germany) or left unamended. Triplicate microcosms were set up for each condition. Tubes were sealed with butyl-rubber stoppers and screw caps. Incubations were conducted at 37°C, with sampling over seven days. Sampling for DNA extraction and metabolite analysis was done after 0, 24, 48, 120, and 168 h. Microcosms were subsampled by retrieving one ml aliquots of fluid, which were centrifuged at 11,000 g for 10 min at 4°C. Pellets were stored at –80°C for DNA extraction. Supernatants were stored at –20°C for metabolite analyses.

Metabolite analysis

Sulfide was quantified in subsamples of 20 µl culture that were fixed by adding 2 g/l zinc acetate in the anaerobic tent. Fixed samples were measured colorimetrically using the Infinite M Nano+ microplate reader (Tecan, Austria), as previously described [9]. Due to the high amount of particles in the rumen fluid microcosms, sampling with needle and syringe was not possible and thus rubber stoppers were briefly removed for sampling. Due to possible leakage, sulfide concentrations might therefore be underestimated in all treatments and time points.

Formate, acetate, propionate, butyrate, valerate, and lactate were quantified in rumen fluid microcosms using an Agilent 7100 Capillary Electrophoresis system (Agilent, USA). Culture supernatants were diluted 1:5 to 1:20 in ultrapure water containing 0.1 mM succinate as an internal standard. Calibration standards for each metabolite were prepared at concentrations of 0.025, 0.05, 0.1, 0.25, 0.5, and 1 mM. An organic acid buffer (Agilent, USA) was used as the running buffer. The bare fused

silica capillary (75 μm inner diameter, 72 cm effective length; Agilent, USA) underwent a 4-minute preconditioning flush before each measurement. After preconditioning, samples were injected at 5 kPa for 5 seconds. Electrophoretic separation was performed at -25 kV with a current of $100\text{ }\mu\text{A}$ and a power of 6 W.

Liquid chromatography–mass spectrometry was used to quantify SQ and its metabolites isethionate, 2,3-dihydroxypropane-1-sulfonate (DHPS), 3-hydroxypropane-1-sulfonate, 3-sulfolactate, and 3-sulfopropionate in supernatants of rumen fluid microcosms. Samples were quantified using a Shimadzu LCMS-2020 single quadrupole mass spectrometer with electron spray ionisation. Separation of organosulfonates was achieved using an iHILIC-Fusion(+) column ($100 \times 2.1\text{ mm}$, $3.5\text{ }\mu\text{m}$, $100\text{ }\text{\AA}$), as described previously [10].

Ultra-high-performance reverse phase liquid chromatography combined with mass spectrometry was used to quantify and identify all molecular species of SQDG in rumen fluid. A rumen fluid sample from one cow was subdivided into two replicates (each 0.1 g) (stored at -80°C) and freeze-dried. Lipid extraction was performed as previously reported [11,12], with few modifications. Briefly, six ml of chloroform-methanol-citrate buffer (0.15 M , $\text{pH } 4.0$, $1:2:0.8\text{ v/v/v}$) was added to freeze-dried samples and mixed in glass vials. Internal standard ($1\text{ }\mu\text{g/ml}$, Equisplash; ^2H -labelled multilipid standard from Avanti Research, USA) was added, vortexed, sonicated for 10 min at room temperature, and incubated overnight in the dark at room temperature. Subsequently, samples were centrifuged ($1,500\text{ g}$ for 10 min) and the supernatant was carefully transferred to a clean vial. The pellet was re-extracted with 2.6 ml of the chloroform-methanol-citrate buffer, vortexed, and centrifuged. The supernatants of both extractions were combined. Then, 1.5 ml of chloroform and 1.5 ml each of citrate buffer were added to the combined supernatants, vortexed, and incubated overnight for phase separation. The lower, lipid-rich organic phase was cautiously transferred to pre-weighed vials. Subsequently, the lipid fraction was dried with N_2 gas and then solubilized in $200\text{ }\mu\text{l}$ of injection solvent (water:acetonitrile:isopropyl alcohol, $1:2:1\text{ v/v/v}$). For liquid chromatography, an Ultimate 3000 Ultra-High-Performance Liquid Chromatography system (Thermo Fisher Scientific, Germany) with an Accucore C30 column ($150 \times 2.1\text{ mm}$; Thermo Fisher Scientific, Germany) and fused-core particles of $2.6\text{ }\mu\text{m}$ diameter were used. The column oven and autosampler were set at 10°C and 40°C , respectively. Solvent A consisted of acetonitrile and water ($3:2\text{ v/v}$) and solvent B of isopropyl alcohol and acetonitrile ($9:1\text{ v/v}$), each amended with 0.1%

formic acid and 10 mM ammonium formate. The flow rate was set to 325 $\mu\text{l}/\text{min}$ with 5 μl injection volume. The gradient profile was as follows: 0-18 minutes, nonlinear increase from 30% to 85% solvent B; 18-20 minutes, ramp to 90% solvent B; 20-24 minutes, hold at 90% solvent B; and 24-28 minutes, linear decrease to 30% solvent B, followed by 4 minutes of re-equilibration at 30% solvent B. Subsequent mass spectrometry was performed with a Q Exactive Orbitrap Mass Spectrometer (Thermo Fisher Scientific, USA) in positive and negative ion modes using a heated electrospray ionisation source. For both polarities, sheath and sweep gas were 40 and 10 (arbitrary units), respectively, and the rate for auxiliary gas was 5 for positive and 7 for negative ionisation modes. The spray voltage was at 3 kV, capillary temperature was at 370°C, the ion transfer tube temperature was at 285°C, for each of the ionisation settings, and the S-lens radio frequency level was adjusted to 50. In separate runs of each sample electrospray ionization-positive and electrospray ionization-negative modes with/without higher-energy collisional dissociation (HCD) fragmentation were measured. The Orbitrap mass analyzer was run at a mass resolving power of 70,000 in positive/negative mode, scan range of 300–1800 m/z , and automatic gain control target of 10^6 . In the HCD mode, the resolving power was set to 17,000, employing HCD fragmentation with stepped normalized collision energy of 20, 25, and 30% (HCD injection time: 100 ms; isolation window: 1 m/z ; automatic gain control target: 10^6), with dynamic exclusion setting of 6 s in the Top5 data-dependent MS2 mode. Raw data were analyzed using the Lipidsearch 5.0 software (Thermo Fisher scientific, USA). SQDG (18:3_16:0) (Sigma-Aldrich, USA) was used as an external standard and class-specific lipid standard for all SQDG-class compounds to quantify SQDG. The Skyline software was used for the identification and quantification of SQDG compounds in the mass spectrometry analysis [13,14].

16S rRNA gene and *yihQ* amplicon sequencing and analyses

Genomic DNA from environmental, intestinal, and rumen fluid microcosm samples was isolated using the DNeasy PowerSoil Pro Kit (Qiagen, Austria). Samples were processed in two ml Lysing Matrix E tubes (MP Biomedicals™, Austria) with CD1 lysis buffer. Samples were incubated at 65°C for 10 minutes and then homogenized using the FastPrep-24 Classic bead-beating system (6 m/s, 2 × 40 seconds; MP Biomedicals™, Austria). DNA was further isolated according to the manufacturer's

standard protocol. The concentration of DNA was determined using a Qubit™ 3 Fluorometer (Invitrogen, USA). DNA samples were stored at -20°C. The absence of PCR-inhibiting substances in the DNA extracts was verified by a positive 16S rRNA gene-targeted PCR with the universal primers 515F and 806R.

Amplicon sequencing was conducted by the Joint Microbiome Facility (University of Vienna, Medical University of Vienna) under project IDs JMF-2310-13 and JMF-2302-02. DNA from each environmental sample was sequenced in triplicates within the same sequencing run. A two-step PCR method [15,16] and primer pairs YIHQa (YIHQ1201Fa/YIHQ1526Ra) and YIHQb (YIHQ1201Fb/YIHQ1526Rb) were used to amplify and barcode an approximately 350 bp region of the *yihQ* gene (Fig. 1). In the first step, amplification was performed with *yihQ*-targeted primers containing 16 bp 5' head adapters (forward: 5'-GCT-ATG-CGC-GAG-CTG-C-3'; reverse: 5'-TAG-CGC-ACA-CCT-GGT-A-3') and with an optimized magnesium chloride concentration of 3 mM. Other reaction components and PCR conditions were as described before (Table S2). In the second step, the individual PCR products were tagged with unique 12 bp barcodes [16].

Samples from the rumen fluid microcosm experiment were analyzed by *yihQ* amplicon sequencing with primer pair YIHQb according to the described protocol and by 16S rRNA gene amplicon sequencing. For 16S rRNA gene amplicon sequencing, the two-step PCR-barcoding method (H_515F_mod-5'-(head) GCT ATG CGC GAG CTG C - GTG YCA GCM GCC GCG GTA A and H_806R_mod-5'-(head) TAG CGC ACA CCT GGT A - GGA CTA CNV GGG TWT CTA AT) was used to target bacteria and archaea [15,16].

Barcoded *yihQ* and 16S rRNA gene amplicon libraries were normalized with the SequelPrep normalisation plate kit and subsequently pooled (TrueSeq Nano Kit, Illumina, USA; modified according to [15]), prior to preparing the sequencing libraries. Samples were sequenced on the MiSeq (V3, 600 cycles, Illumina, USA) and results were analyzed according to the procedures described before [16].

Amplicon sequence variants (ASVs) of 16S rRNA gene sequences were inferred and classified using DADA2 [17] with Release 138 of the SILVA 16S rRNA sequence database [18]. All *yihQ* amplicon sequences were translated into amino acid

sequences (Fig. S2). To achieve this, open reading frames were identified and translated using EMBOSS Transeq [19]. Sequences that generated a stop codon in this approach were instead translated with Prodigal in metagenomic mode, which is suitable for partial sequences that lack start codons [20]. If neither method yielded a stop codon-free translation, a local BlastX search was performed against the YihQ reference database [21]. Sequences with a significant BlastX hit (defined as having a coverage >80% and e-value <10⁻⁴) were translated based on the reading frame of their best BlastX match. Sequences that still retained a stop codon after translation in the suggested BlastX reading frame or lacked a significant BlastX hit in the YihQ reference database were excluded from further analyses. Translated sequences without stop codons from all approaches were compiled and subjected to a BlastP search against the YihQ reference database to ensure that only sequences with significant similarity to sequences in the YihQ reference database were included in the subsequent phylogenetic analysis [21]. Sequences without a significant BlastP hit (coverage >80% and e-value <10⁻⁴) in the YihQ reference database were excluded from further analyses.

All amino acid sequences with a significant BlastP hit in the YihQ reference database were placed into the YihQ reference tree using the evolutionary placement algorithm of RAxML. Phylogenetic placement was performed with the LG substitution model and a gamma distribution to account for rate heterogeneity across sites [22–24]. Sequences that were placed within the YihQ branch of the reference tree with an accumulated likelihood of >0.95 were classified as verified *yihQ* sequences. ASV sequences that did not meet these criteria were considered non-target sequences, removed from the dataset, and excluded from further analysis. All verified *yihQ* ASV sequences were clustered into operational taxonomic units (OTUs) using vsearch [25] with a 90% sequence identity threshold. Translated verified *yihQ* ASV sequences generated with the YIHQb amplicon sequencing assay were additionally subjected to BlastP analysis against all YihQ sequences extracted from the GlobDB database (version r220) for taxonomic classification. GlobDB is a curated, dereplicated collection of representative genomes of all species in the global taxonomy database (GTDB) and of species that are not present in GTDB but in the genomic catalog of earth's microbiomes, the searchable planetary-scale microbiome resource, and the genomic catalog of soil microbiomes [26].

Verified *yihQ* sequences were further analyzed in R Studio (rstudio.com, R version 4.2.2) using the software packages “Phyloseq” (v1.42.0) [27] and “Vegan” (v2.6-4) [28]. Libraries were subsampled at 100, 1000, and 10000 reads to evaluate the impact of sequencing depth on diversity estimates. Alpha diversity was evaluated using the observed number of ASVs and OTUs, Shannon diversity index, and Simpson diversity index.

Differential abundance analysis of 16S rRNA gene sequences and verified *yihQ* sequences from the rumen fluid microcosm experiment was conducted using DESeq2 (v1.38.3) in RStudio, as described previously [29]. To mitigate biases from uneven library sizes, sequencing libraries were rarefied to match the smallest library before DESeq2 analysis. For 16S rRNA gene data, SQ-amended microcosms were compared to unamended controls at 48 h and 168 h of incubation. Relative abundances of *yihQ*-ASVs and OTUs were compared between SQ-amended microcosms and controls at 168 h of incubation. Adjusted *p*-values <0.01 were considered significant. Taxonomic classification of enriched 16S rRNA gene ASVs was verified using BlastN against the NCBI 16S rRNA sequence database, excluding sequences from uncultured organisms and environmental samples [30]. The next related *YihQ*-encoding genome in the GlobDB database of each significantly enriched *yihQ* OTU and ASV was identified by using BlastP.

Metagenomics and genome analysis

Metagenomic sequencing was performed with samples from SQ-amended (n = 2) and unamended control (n = 1) microcosms of rumen fluid, taken after 168 h of incubation. Libraries were prepared using the NEBNext Ultra FS II DNA Library Prep Kit (New England Biolabs, USA) for Illumina and sequenced on a NovaSeq6000 (1/2 SP flow cell, 2×100 bp reads; Illumina, USA). Quality control was conducted using a Snakemake v8.20.3 workflow to trim reads based on quality scores [31]. Reads were quality-checked with FastQC v0.12.1 [32], and summary statistics were compiled using MultiQC v1.21 [33]. Adapter sequences and phiX contamination were removed with BBDuk (BBMap v39.06) [34], retaining reads of at least 50 bp. Right-end k-trimming

was applied (k-mer = 21, minimum k-mer = 11, hamming distance = 2) with "tpe" and "tbo" options, and quality trimming was performed with a Q-score of 15. The quality-controlled read library was quality checked using FastQC v0.12.1 and quality statistics were merged using MultiQC v1.21 for comparison to the non-trimmed quality. BBDMap's reformat.sh was used to interleave the read library.

Reads were assembled with SPAdes v4.0.0 in "meta" mode, using k-mers from 21 to 121 (step size: 10) [35]. Contigs shorter than 1,000 bp were removed using reformat.sh of BBDMap. Metabat v2.15 [36] was used to bin putative metagenome-assembled genomes (MAGs) without coverage data (minimum length: 1,500 bp). Coverage information for the assembly was generated with BBDMap ($\geq 98\%$ identity) using the read library, followed by sorting with SAMtools v1.20 [37]. A depth file was generated using jgi_summarize_bam_contig_depths and the assemblies were binned into putative MAGs again using the depth file in Metabat. Dereplication of putative MAGs was performed with dRep v3.5.0 [38], applying a minimum completeness threshold of 50% and a maximum contamination threshold of 10%. Taxonomic classification of dereplicated MAGs was conducted using GTDB-Tk v2.4.0's classify_wf (database r220, ANI screening skipped) [39]. Barrnap v0.9 was used to identify and recover 5S, 16S, and 23S rRNA gene sequences in the genomes [40]. Coverage and the percentage of reads mapped to each MAG were determined with BBDMap with a mapping identity of 95%. Average amino acid identity (AAI) was calculated using the Enveomics Collection [41]. Whole-genome average nucleotide identity (ANI) was calculated using FastANI (version 1.33) [42].

New Hidden Markov Models (HMMs) were generated for the detection of enzymes and transport proteins of sulfoquinovose, isethionate, and DHPS degradation pathways (Table S3). Initially, the sequences of the biochemically and/or physiologically characterized enzymes and representative sequences of putative transport proteins were used as a query for a Diamond BlastP against the GlobDB (version r220) with a bitscore cutoff of 100. If a protein sequence in GlobDB had hits above the cutoff to more than one query sequence, the query with the highest bitscore was selected and the target sequence in GlobDB was labeled with the name of the query. For all hits in the resulting list of labeled GlobDB sequences the genomic vicinity was analyzed for additional hits to other query sequences. Specifically, for each genome the nucleotide distance between all genes was calculated. Genes with a maximum of 3500 nucleotides of intergenic space were considered to form a gene

cluster, if at least five genes were encoded and if these were homologous to five different query sequences. An exception was made for the isethionate sulfite-lyase IslA, DHPS-sulfite-lyase HpsG, and DHPS-dehydratase HpfG, as these occur in gene clusters of less than five genes [43,44]. Here, only gene cluster arrangements that were specifically described in the respective publication were selected. Across all genomes in GlobDB, the gene clusters encoding the same number and types of homologs were then grouped into unique gene cluster groups. In the next step, a gene cluster group was excluded if it did not contain at least one gene cluster with one encoded protein with 90% identity to the respective query sequence. To generate the final sequence set for each functionally different protein, all homologs encoded in any gene cluster group were merged into an individual sequence collection for each initial reference protein. The protein sequences in each collection were then aligned and used to generate a HMM, with trusted and noise cutoff scores as previously described [45]. HMMs and cutoff scores were integrated into HMSS2 [46], which was then used to identify sulfur metabolism genes in the recovered MAGs and genomes from the families *Sphaerochaetaceae* and *Oscillospiraceae*, the genera *Mailhella*, *Caproiciproducens* and JAAYFO01 in GlobDB. Proteins were only counted as present (i) if they were detected with a bitscore above the respective trusted cutoff or (ii) if they were detected with a bitscore above the noise cutoff and had at least 95% identity to one of the sequences used to generate the HMM.

Supplementary Figures



Figure S1. Phylogeny of YihQ sulfoquinovosidases. The maximum likelihood YihQ reference tree was calculated with 131 YihQ sequences and 163 outgroup sequences from the reference database after de-replication at 95% protein sequence identity. Outgroup sequences are collapsed. Biochemically or physiologically verified YihQ sulfoquinovosidases are highlighted in bold. Colors indicate taxonomic assignment at the class level.

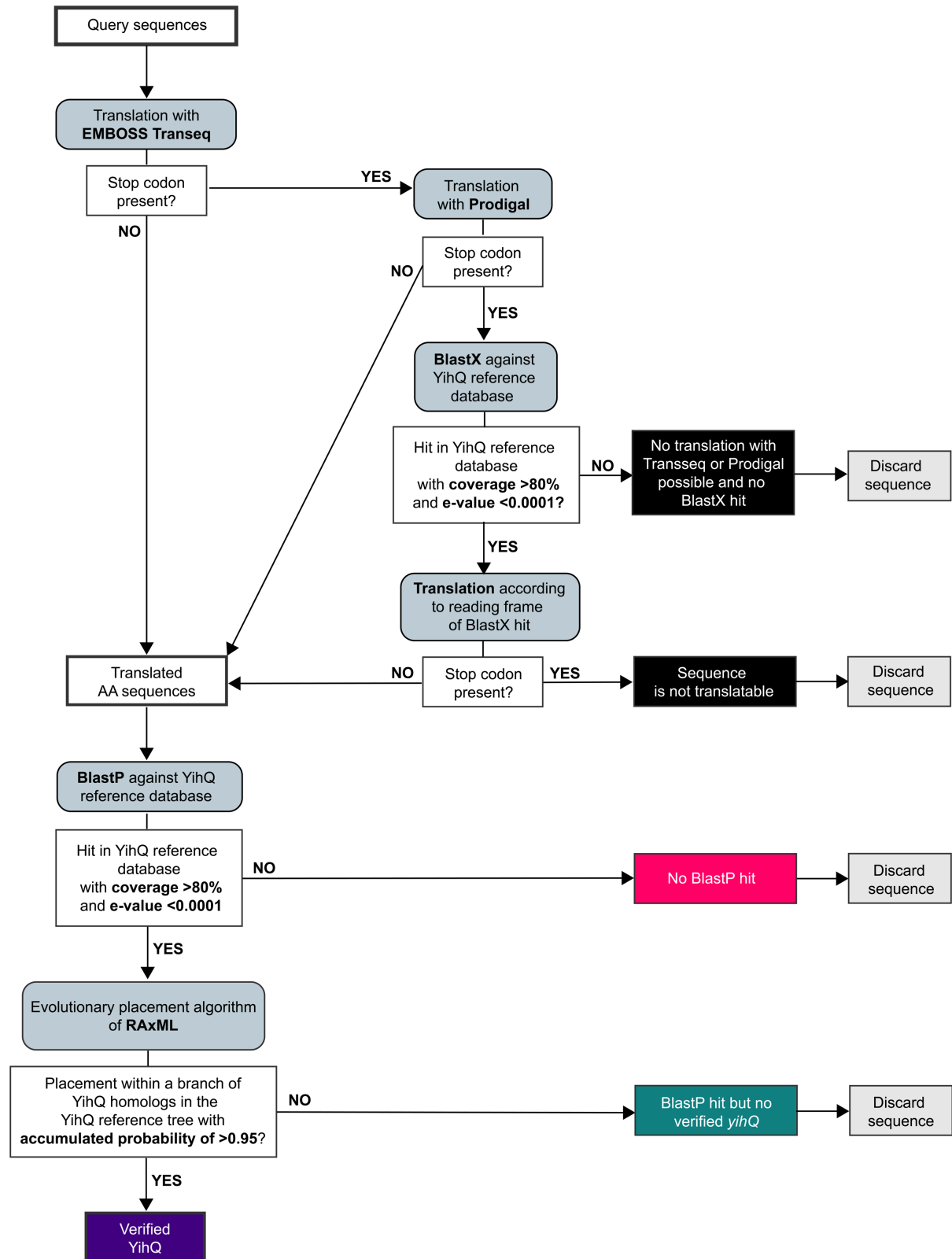


Figure S2. Computational workflow for identification and filtering of *yihQ* sequences.

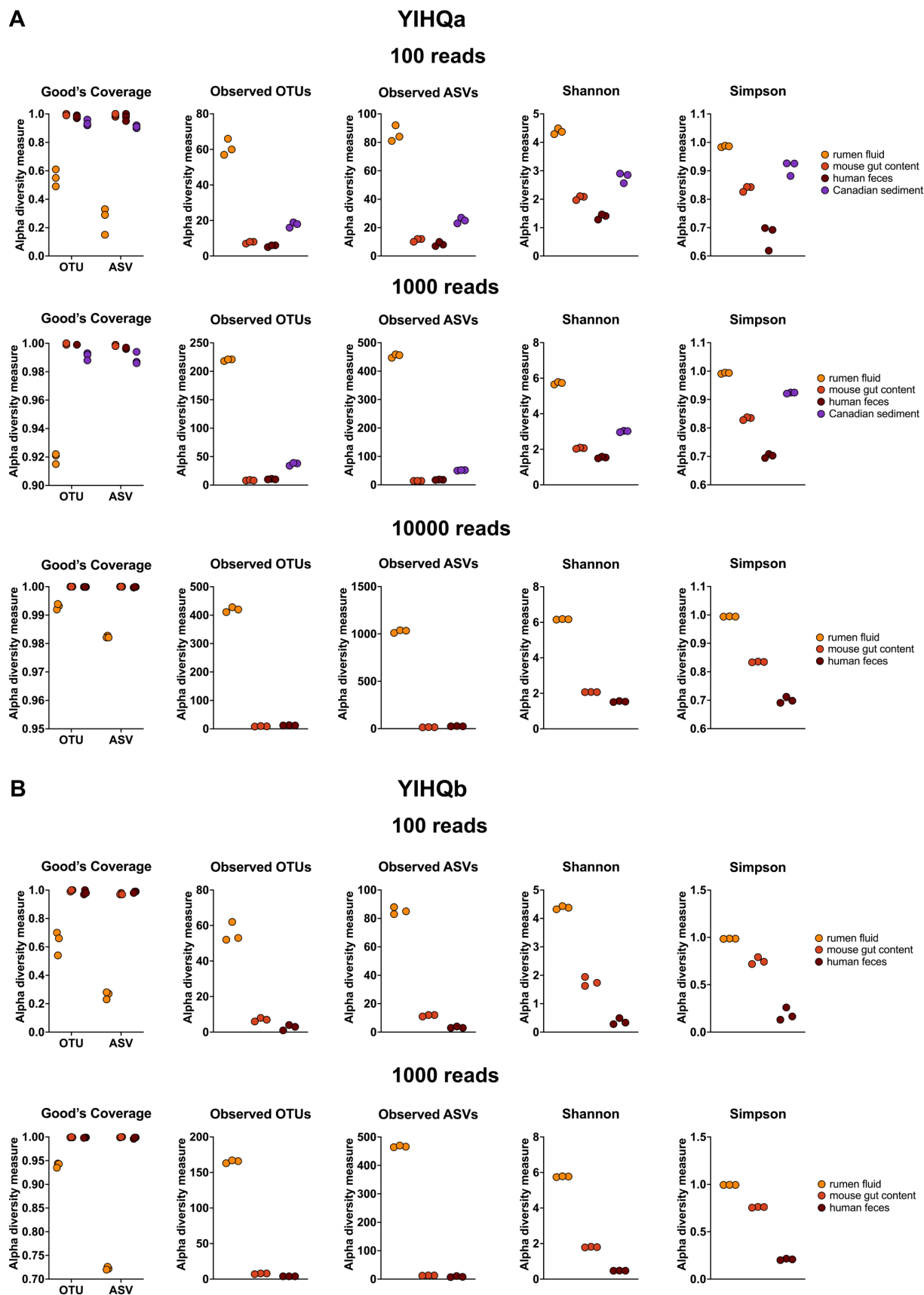


Figure S3. Alpha diversity analysis of *yihQ* amplicon sequence data shows highly consistent results for all triplicates. Good's coverages, numbers of observed OTUs and ASVs, the Shannon diversity index, and the Simpson's diversity index were inferred from *yihQ* amplicon sequence data obtained with the YIHQa assay (**A**) and the YIHQb assay (**B**). Sequencing was performed in technical

triplicates for each environmental and intestinal sample. The analysis was performed with verified *yihQ* sequences. Libraries were re-sampled at 100, 1000, and 10000 reads (Table S6).

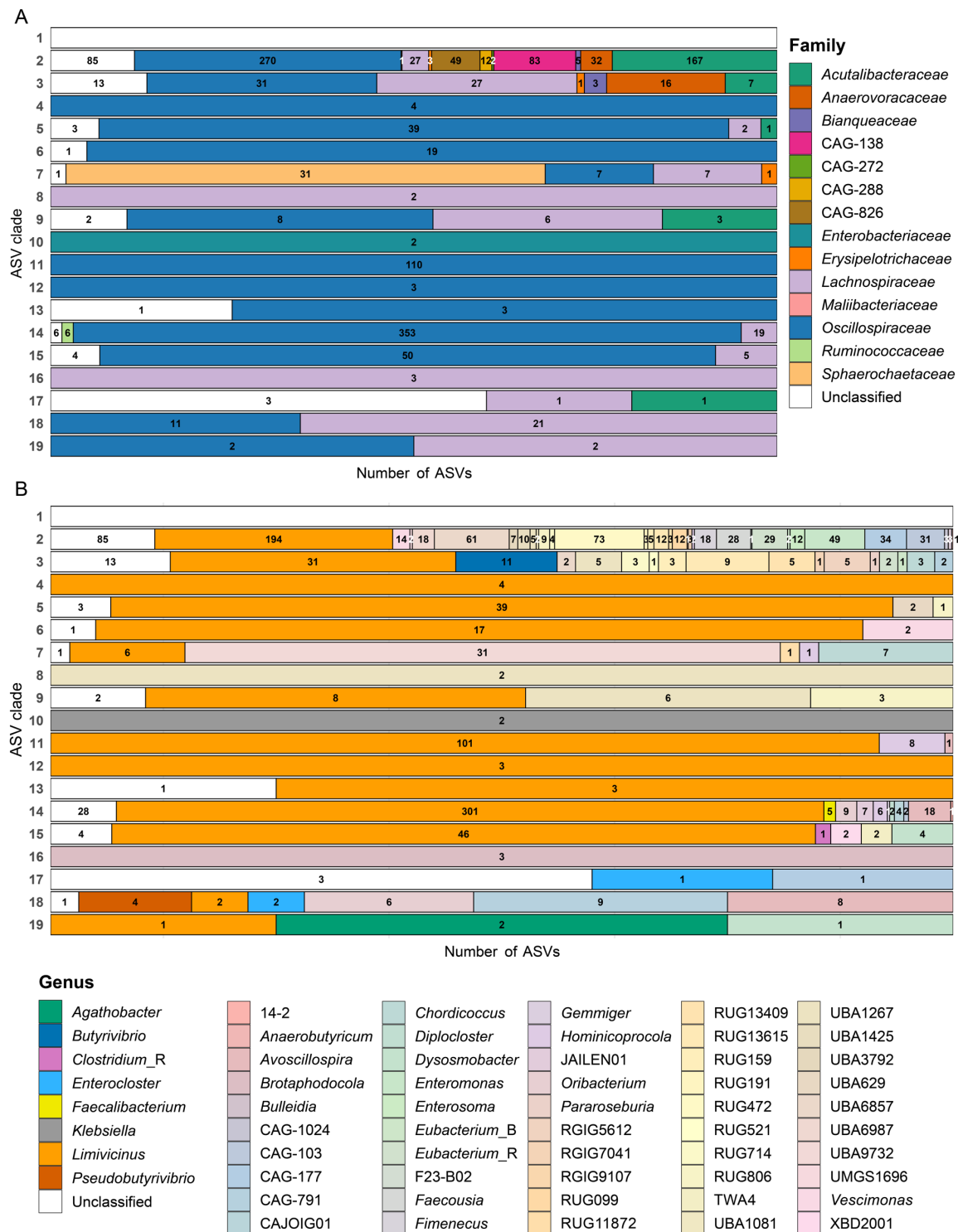


Figure S4. Taxonomic assignment of environmental *yihQ* ASVs. Taxonomic composition of *yihQ* ASV clades from the YIHQB amplicon sequencing assay (Fig. 3) shown at the family (**A**) and genus level (**B**). Translated *yihQ* ASVs were queried against the GlobDB database using BlastP. The two top-scoring hits for each ASV were used for taxonomic assignment. In cases where two equally scoring hits had divergent taxonomic assignments, taxonomy was set to “unclassified” at the conflicting rank. ASVs with no match or matches below 90% sequence identity were also categorized as unclassified (white). Each horizontal bar represents an ASV clade, as defined in Fig. 3A, with segments indicating the percentage of ASVs assigned to each taxon within the clade. Numbers in bar plots refer to the absolute number of ASVs per taxon within one ASV clade. Taxonomic assignment at the class level is presented in Fig. 3B. Full taxonomic details for individual ASVs are available in Table S7.

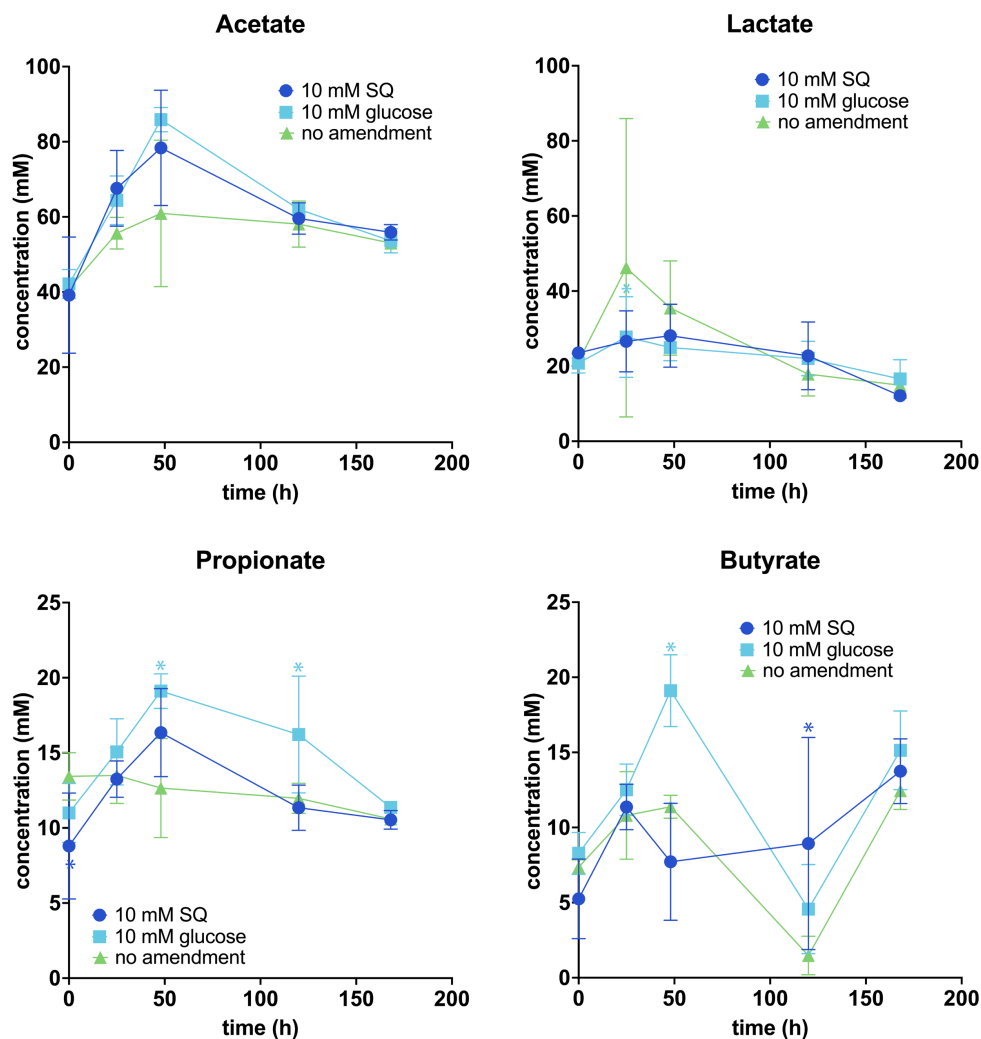


Figure S5. Dynamics of volatile fatty acids in rumen fluid microcosms. Concentrations of volatile fatty acids are represented as the average of triplicate measurements. Error bars represent one standard deviation. Coloured asterisks show significant differences between the respective treatment and the unamended controls at individual time points. Significant differences were determined by ANOVA with Dunnett's post hoc test. Adjusted p -values of smaller than 0.05 were regarded as significant. SQ, sulfoquinovose.

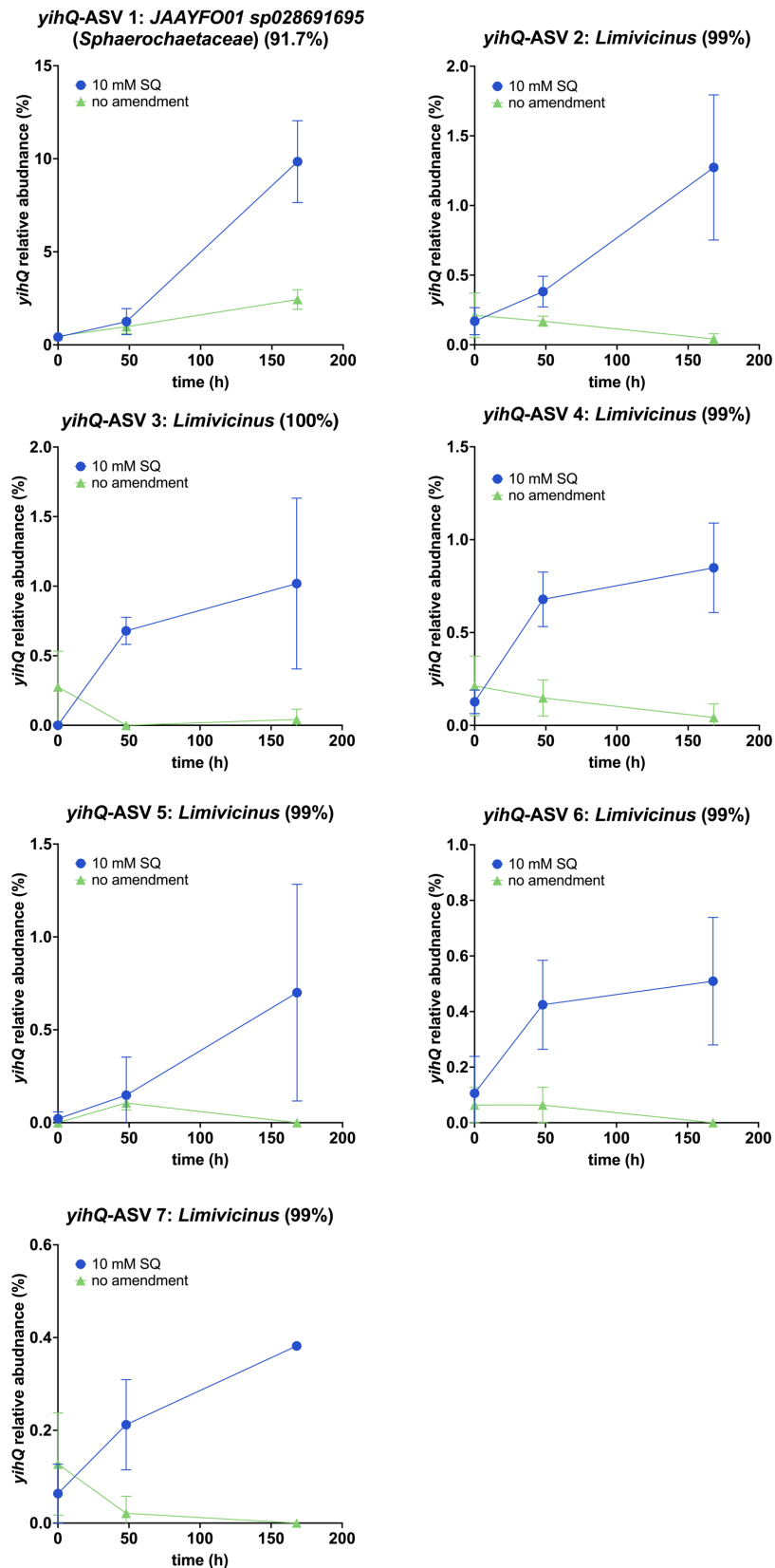


Figure S6. Relative abundance dynamics of *yihQ*-ASVs that were significantly enriched in SQ-amended rumen fluid microcosms. ASVs are shown in order of their average relative abundance increase compared to the unamended control. Points represent averages of three replicates and error bars represent one standard deviation. Taxonomic classification and the *yihQ* sequence similarity to the closest relative (in brackets) is shown for each ASV. ASV, amplicon sequencing variant; SQ, sulfoquinovose.

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Discussion

Although SQ is a ubiquitous molecule in the biogeochemical sulfur cycle, the first sulfoglycolytic pathway was only discovered in 2014 [1,2]. Over the past decade, six SQ degradation pathways have been characterized in bacterial isolates, yet studies on SQ degradation in environmental or host-associated microbial communities remain scarce [1,3–7]. This work expands our understanding of the SQ metabolism in microbial communities by examining its degradation in the digestive tract of three mammals: the human gut, the mouse gut, and the cow rumen. SQ was metabolized in all three systems, but each showed distinct metabolic characteristics (**Figure 1**). In human fecal microcosms, SQ was cooperatively degraded to DHPS, formate, acetate, butyrate, hydroxyacetone, and H₂S within 24 h, mainly by *A. rectalis* and *B. wadsworthia* [8,9]. In contrast, SQ degradation halted at DHPS in mouse gut microcosms [9]. The sulfofructose transaldolase pathway was the most prevalent SQ degradation pathway in both systems, with *A. rectalis* and *E. clostridioformis* as enriched representatives in human fecal and mouse gut microcosms, respectively [8,9]. Although less prevalent, an *Escherichia/Shigella* ASV predicted to use the sulfo-Embden-Meyerhof-Parnas pathway was enriched in human fecal and mouse gut microcosms [8,9]. Consistent with previous findings in human fecal samples [8], only a few bacterial species in the mouse gut microbiota possessed the genetic potential to degrade SQ and DHPS [9]. In contrast to this, *yihQ*-targeted amplicon sequencing of rumen fluid revealed over 2000 *yihQ* ASVs, indicating a remarkable diversity of YihQ sulfoquinovosidases in the cow rumen [10]. Despite this richness, only a few OTUs and ASVs were significantly enriched in SQ-amended rumen fluid microcosms. These included novel uncultured primary SQ degraders of the genera *Limivacinus* (*Oscillospiraceae*), *Caproiciproducens* (*Acutalibacteraceae*), and *Neosphaerachaeta* (*Sphaerochaetaceae*), as well as *Mailhella* (*Desulfovibrionaceae*), a likely secondary SQ degrader [10]. SQ degradation proceeded slowly over 168 hours, with isethionate and sulfide accumulating, indicating SQ degradation via the sulfo-transketolase pathway. Consistent with this, members of *Limivacinus*, *Caproiciproducens*, and *Sphaerochaetaceae* encoded the isethionate-producing sulfo-transketolase pathway, while *Mailhella* species encoded homologs of *islAB* and *hpsGH*, indicating a role in isethionate respiration [10]. The observed differences in SQ degrader diversity and metabolism across the hosts suggest that host-specific factors influence this process.

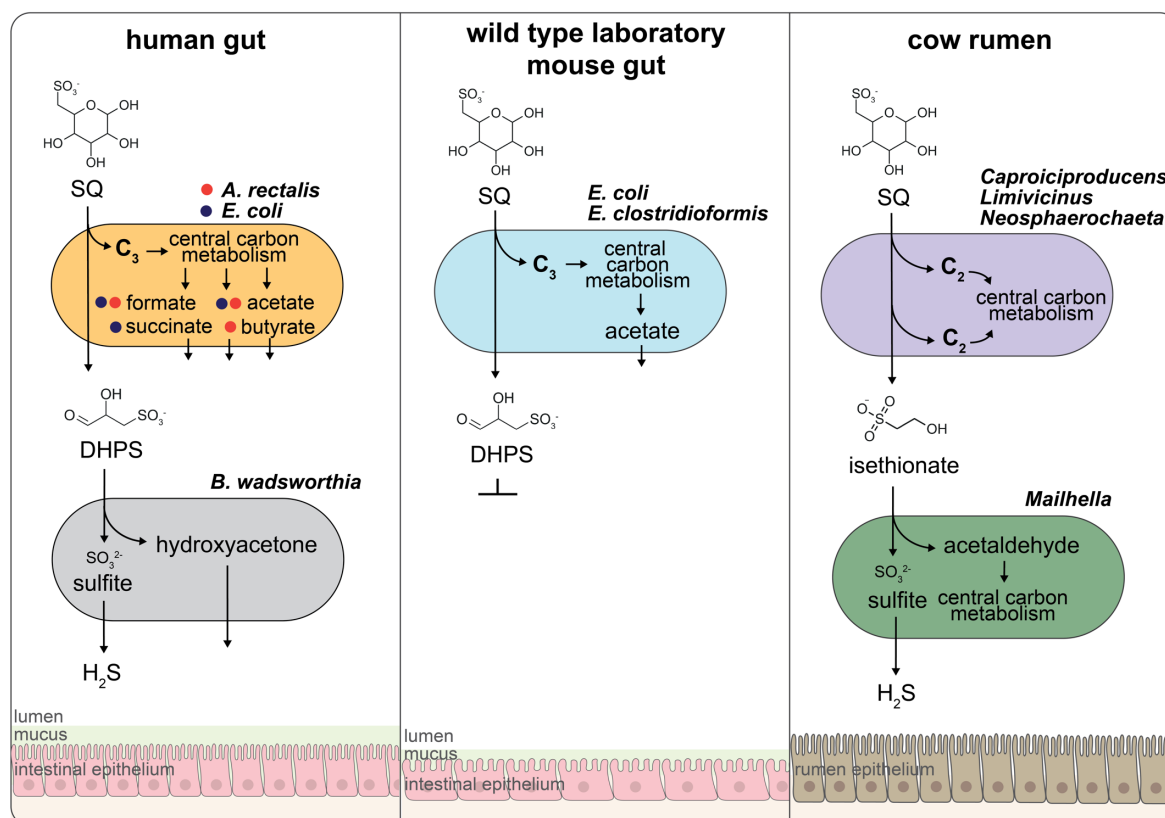


Figure 1. Sulfoquinovose (SQ) degradation pathways in the human gut, mouse gut, and cow rumen. The schematic overview illustrates the microbial SQ metabolism across three hosts. Human gut (left): The main SQ degrader, *A. rectalis*, uses the transaldolase pathway for SQ degradation, producing DHPS, acetate, formate, and butyrate [8,9]; *E. coli* uses the sulfo-Embden-Meyerhof-Parnas pathway, yielding DHPS, acetate, formate, and succinate [11]. The excreted DHPS is desulfonated by *B. wadsworthia* to hydroxyacetone and sulfite, which is reduced to H_2S [8]. Coloured dots indicate detected fermentation products for *A. rectalis* (red) and *E. coli* (blue). Wild type laboratory mouse gut (center): Similarly to the human gut, *E. clostridioformis* and *E. coli* mediate primary SQ degradation via the sulfofructose transaldolase pathway and the sulfo-Embden-Meyerhof-Parnas pathway, respectively. However, DHPS accumulates without further transformation, despite the genetic potential for DHPS in a few bacteria [9]. Cow rumen (right): Members of *Caproiciproducens*, *Limivicinus*, and *Neosphaerochaeta* process SQ via the sulfo-transketolase pathway, releasing isethionate. *Mailhella* species cleave isethionate to sulfite and acetaldehyde. Sulfite is then respired to H_2S [10]. While SQ degradation in the human and mouse gut uses a C_3 -moiety as a carbon and energy source, ruminal SQ degraders use two C_2 -moieties via the transketolase pathway [8–10]. SQ, sulfoquinovose; DHPS, 2,3-dihydroxypropane-1-sulfonate; H_2S , hydrogen sulfide.

Host-specific factors as potential modulators of SQ degradation

Several host-dependent factors, including physiology, microbiota composition and function, and diet, may explain the similarities and differences in the SQ metabolism across the three host-associated systems. Despite key distinctions, such as the fore-stomach and the large cecum as primary fermentation sites in mice [12–15], the human and mouse gut share many structural similarities, whereas the cow's gastrointestinal system functions vastly differently. As foregut fermenters, cows

possess a four-chambered stomach. The rumen, their primary fermentation site, functions as a unit with the adjacent reticulum forming the reticulorumen [16–19]. The reticulorumen facilitates regurgitation, which enhances microbial fermentation efficiency by reducing particle size and increasing surface area [16]. Following fermentation, digesta passes through the omasum for further digestion and absorption [17], before reaching the abomasum, which functions similarly to monogastric stomachs [16,20]. Like in humans and mice, the cow's small intestine facilitates nutrient absorption, while the large intestine supports additional microbial fermentation, energy extraction, water absorption, and feces formation [17]. Gut transit time differs across hosts. In cows, gut transit time ranges between 40 and 52 hours, with feed remaining in the stomach compartments for the majority of this period (28–39 hours) [21]. Humans exhibit a range of 14 to 76 hours, depending on diet and individual variation, whereas mice have a short gut transit time of 6 to 7 hours, which aligns with their higher metabolic rate [22]. The prolonged retention time in the reticulorumen combined with regurgitation maximizes fermentation efficiency and enables cows to derive ~70% of their energy from microbial fermentation [17]. In contrast, humans obtain 2–12% of their energy this way, and laboratory mice 17–25% [23]. The high *yihQ* sequence diversity in the cow rumen suggests that SQ may contribute to this high fermentation efficiency, as the functional redundancy of *yihQ* may enhance the resilience of the sulfoquinovosidase reaction after perturbations [24,25], enable parallel processing of SQ glycosides by multiple taxa, and support higher metabolic flexibility within SQDG degradation [26]. Together, these features may sustain more stable energy extraction from SQDG under fluctuating rumen conditions, thereby supporting the fermentative capacity of the rumen microbiota.

In all three hosts, the fermentation of dietary components resistant to enzymatic digestion by the host, such as certain fibers and polysaccharides, relies entirely on the microbiota [27–29]. Comparisons between the mouse and human microbiota revealed similarities in taxonomic composition even at the genus level, though with quantitative differences [30,31]. Of the 60 genera defined as core genera in the mouse gut, 25 were also part of the core human gut microbiota [30]. Functionally, only 4% of microbial genes in the mouse gut microbiome shared $\geq 95\%$ nucleotide identity and $\geq 90\%$ coverage with genes from the human gut microbiome, yet 95.2% of KEGG orthologous groups overlapped, indicating substantial functional similarity [30]. Consistent with this, genes associated with DHPS degradation were present in both mouse gut and human

gut microbiomes. However, DHPS degradation activity has been observed in human fecal microcosms [8] but not in mouse gut content microcosms [9]. This functional difference may reflect variation in gene expression, community composition, or ecological context. DHPS-degrading isolates from the human gut exist, including *B. wadsworthia* [8], *Desulfovibrio piger* [32], and *Enterococcus gilvus* [33], yet no isolates have been recovered from the mouse gut. Our metagenomic analysis of the mouse microbiota showed genetic capacity for DHPS metabolism in a few genera, such as *Taurinivorans*, *Bilophila*, and *Enterocloster*, though experimental evidence is lacking [9,34]. Notably, *Bilophila* was only found in 7.5% of mouse microbiota amplicon sequencing datasets, of which 82% of the *Bilophila*-positive samples originated from mice colonized with human fecal transplants or defined human strain consortia [34].

The cow rumen microbiota differs substantially from the human and mouse microbiota due to the different physiology and diet. However, the most abundant phyla in the rumen are *Bacteroidota* and *Bacillota*, as in the human and mouse gut [30,35]. The rumen contains approximately 10^{10} to 10^{11} bacterial cells/ g of rumen content, with a significant proportion remaining unclassified [36]. The core rumen microbiome comprises less than 0.25% of all bacterial species but accounts for up to 60% of the relative abundance [37]. Among the dominant core taxa are fiber-degrading genera such as *Fibrobacter*, *Prevotella*, *Butyrivibrio*, *Pseudobutyrvibrio*, and *Ruminococcus*, as well as secondary degraders like *Treponema bryantii* and *Selenomonas ruminantium* [35,37–42]. Members of *Butyrivibrio*, *Pseudobutyrvibrio*, and *Ruminococcus* also encoded the sulfofructose transaldolase pathway [4,8]. Although many rumen bacteria harbored the *yihQ* gene, SQ degradation in rumen fluid microcosms proceeded markedly slower than in human fecal microcosms and only became substantial after 48 h [10]. This delay may reflect the rumen bacteria's specialization in polysaccharide degradation [43], potentially favoring these substrates in the nutrient-rich rumen fluid. Additionally, rumen bacteria are likely more accustomed to metabolizing SQDG or other SQ glycosides derived from plant material, rather than free SQ [2]. Utilization of SQ in our microcosms might therefore have required a metabolic adaptation. The observed timeline in the microcosms suggests that SQ was either accessed only after depletion of preferred nutrients and/or only after SQ transporters and the necessary enzymatic machinery had been induced. The role of free SQ as a substrate *in vivo* remains unclear, but, in contrast to rumen fluid microcosms, alternative nutrients, such as polysaccharides or SQDG and SQ

glycosides, are likely continuously available in well-nourished, healthy cows [44]. Only a limited number of ASVs were enriched under SQ-amended conditions, with primary SQ degradation proceeding via the sulfo-transketolase pathway [10]. This pattern may reflect intense competition among the numerous potential SQ degraders in rumen fluid microcosms, potentially favoring those with the most efficient SQ utilization strategies. The sulfo-transketolase pathway is the anaerobic primary SQ degradation pathway with the highest carbon yield known to date. It retains four carbon atoms for the central carbon metabolism, whereas the sulfofructose transaldolase pathway, predominant in the human and mouse gut, yields three carbon atoms (**Figure 1**, see also Figure 3 in the introduction) [5,8,9].

Microbiota composition and function are shaped by the available substrates [45]. To understand how much SQ(DG) is available to the microbiota of the three hosts, I calculated a realistic range of dietary SQDG intake for humans, cows, and mice based on the lowest and highest known SQDG concentrations in plants and normalized these values by body weight and gut transit time (**Table 1**). SQDG levels vary widely among plants, ranging from 46 µg/g dry weight in garlic to 824 µg/g dry weight in spinach [46]. For my calculations, I used a water content of 92.4% for spinach and 64.4% for garlic [47,48]. Assuming a daily vegetable intake of 200 g, following the recommendations of the World Health Organization [49], human SQDG consumption would range from ~3 mg (with low-SQDG plants like garlic) to ~13 mg (with high-SQDG plants like spinach) per day. The amount of SQDG ingested by laboratory mice with their chow is unknown, but SQ was not detected in feces before gavage with SQ in our experiments, suggesting that the standard mouse chow used in this study, and possibly in other mouse facilities, does not contain high-SQ components [50,51]. However, wild mice may ingest higher amounts of SQDG since they eat a more versatile diet including ~27% plant materials [52–54]. Given a daily feed intake of 5 g [55] with 27 % plant materials [54], this translates to an SQDG intake of 0.02–0.08 mg/day, based on the reported SQDG levels in garlic and spinach. Grass-fed cows primarily consume green plants, which could be rich in SQDG, but data on the SQDG amounts in hay and grass are lacking. In an example by the University of Nebraska-Lincoln, a 544 kg non-lactating cow consumes 10.9 kg (dry weight) of forage [56]. Assuming an SQDG content of 46-824 µg/ g, the estimated intake ranges from ~500 to 9000 mg/day.

Table 1. Estimated SQDG uptake of humans, cows, and wild mice.

	SQDG intake ¹		
	(mg/day) ²	per kg body weight (mg/kg/day) ³	per h gut transit (mg/h) ⁴
human	3.3-12.5	0.05-0.18	0.05-0.89
cow	501.4-8981.6	0.92-16.51	9.64-224.54
wild mice	0.02-0.08	1.1-4.2	0.003-0.010

¹ Calculations assume a low SQDG plant with 46 µg/g dried plant and 64.4% water content, and a high SQDG plant with 824 µg/g dried plant with 92.4% water content [46–48].

² Calculations assume intake of 200 g of vegetables/ day for humans [49], 10.9 kg (dry weight) of forage/ day for cows [56], and 5 g of feed/ day for mice with 27% plant material [54,55].

³ The assumed weight is 70 kg for humans, 544 kg for cows [56], and 20 g for mice [57].

⁴ The gut transit time is assumed to range between 14-76 h in humans [22], 40-52 h in cows [21], and 6-7 h in mice [22].

Absolute SQDG intake is highest in cows, reflecting their large body size and plant-rich diet. Normalization suggests that, even relative to their size and gut transit time, SQDG consumption of cows exceeds the SQDG consumption of humans and wild mice. Repeated exposure of the rumen microbiota to high SQ(DG) concentrations may have selected for microbial populations adapted to SQ(DG) utilization, which may explain the higher prevalence of the *yihQ* gene in rumen microbiota compared to the human and mouse gut microbiota. In contrast, humans and wild mice consume lower amounts of SQDG, which may reduce the selective pressure to maintain this function across taxa, potentially leading to lower functional redundancy of SQ(DG) degraders [58,59].

Contribution of SQ to the microbial sulfur metabolism in the human gut

I estimated that the daily dietary intake of SQDG ranges from 3-13 mg/day in humans, which corresponds to ~0.87-3.77 mg SQ [9]. To better understand the significance of this amount, I compared it to the intake of other organic sulfur sources, such as the abundant sulfur-containing amino acids cysteine, methionine, and taurine (**Table 2**). The World Health Organization recommends a daily intake of 15 mg/kg body weight of the sulfur-containing amino acids cysteine and methionine, equating to 1,050 mg/day for a 70 kg individual [60]. Notably, the actual intake often exceeds these recommendations [61]. Dietary taurine intake varies by diet and population. A British study measured a mean taurine intake of 58 mg/day for omnivores but did not detect

it in the diet of vegans and therefore considered taurine intake negligible for vegans [62]. In contrast, Japanese subjects had a mean taurine intake of 145.6 mg/day, while Korean men and women consumed 327.3 mg/day and 245.1 mg/day, respectively [63,64]. Dietary intake of organic sulfur compounds varies depending on the subject's eating habits, but sulfur amino acid ingestion will, on average, exceed the dietary intake of SQ.

In the next step, I calculated how much of the ingested substrates are available to the gut microbiota. We showed that human colon cancer cells and germ-free mice did not metabolize SQ, suggesting that all ingested SQ is available to the gut microbiota [9]. In contrast, peptides and amino acids undergo absorption in the duodenum and jejunum, preceding most microbial activity [65]. It was estimated that 2-7% of total dietary protein reaches the colon [65], but measurements on dietary sulfur amino acids specifically are lacking. Assuming 2–7% of cysteine, methionine, and taurine reach the microbiota, this would correspond to 21–73.5 mg/day of cysteine and methionine and 1.16–22.91 mg/day of taurine. In addition to these dietary sources, endogenous taurine and cysteine contribute significantly to the sulfur pool in the colon. Around 30-35% of bile acids are conjugated with taurine [66], and 0.2-0.6 g of bile acids per day, corresponding to 5% of the total bile acids, escape their reabsorption in the terminal ileum [67,68]. This would result in 60–210 mg of taurine-conjugated bile acids reaching the colon, where they are deconjugated by bacterial bile salt hydrolases. Assuming complete deconjugation and considering that taurine accounts for 24% of the molecular weight of taurine-conjugated bile acids [69,70], this would release an additional 14.4-50.4 mg of taurine into the colon. Host-derived mucins, which are rich in cysteine, represent a major endogenous cysteine source accessible to the gut microbiota. While the rate of colonic mucus production remains unknown, ~2–3 g of mucins from the upper gastrointestinal tract enter the colon each day [71]. Mucus is composed of ~95% water and 1–10% glycoproteins [71], but data on its cysteine content are lacking. Consequently, a qualified estimate of the contribution of endogenous cysteine to the total colonic cysteine pool cannot be provided.

Table 2. Estimated dietary intake and colonic availability of sulfoquinovose (SQ), cysteine and methionine, and taurine.

	dietary intake (mg/day)	colonic availability (mg/day)		
		from diet	endogenous production	total
sulfoquinovose	0.87-3.77 ¹	0.87-3.77	0	0.87-3.77
cysteine + methionine	1,050 ¹	21-73.5 ²	cysteine: not quantified; methionine: 0 ³	>21-73.5
taurine	58.0-327.3 ¹	1.16-22.91 ²	14.4-50.4 ⁴	15.56-73.31

¹ The estimate of dietary SQ is based on my calculations of SQDG intake. Intake of cysteine and methionine is estimated from the intake recommendation of the World Health Organization for a 70 kg person [60]. Dietary taurine is estimated as the range between the lowest and the highest taurine intake found in the literature [62,64].

² Calculations assume that 2-7% of cysteine, methionine, and taurine reach the colon [65].

³ Methionine is an essential amino acid that cannot be synthesized endogenously [72].

⁴ The amount of bile acids escaping enterohepatic circulation is assumed at 0.2-0.6 g [67,68], 30-35% of which are taurine-conjugated [66]. Taurine makes up 24% of the molecular weight of taurine-conjugated bile acids [69,70].

An analysis of 514 human fecal microbial genomes found that genes involved in cysteine metabolism were predominant, indicating that cysteine metabolism plays a key role in the gut sulfur cycle [73]. Similarly, taurine was the second most abundant free amino acid in colonic mucosa biopsies from healthy individuals [74]. Overall, SQ levels available to the gut microbiota are likely substantially lower than those of the sulfur amino acids cysteine, methionine, and taurine, but dietary habits influence individual variations. Specifically in strict vegetarians and vegans, SQ could be a significant contributor to microbial sulfur metabolism in the gut.

Prebiotic potential of sulfoquinovose

Some primary SQ degraders are associated with beneficial host effects [75,76], suggesting that SQ may have a prebiotic effect. By definition, a prebiotic is “a substrate that is selectively utilized by host microorganisms, conferring a health benefit” [77]. Further studies are required to investigate the health effects of SQ supplementation, particularly regarding the currently unexamined and potentially adverse effects of SQ-induced H₂S formation. In a previous *in vivo* experiment, gavage of 0.45 g/kg body weight SQ to IL-10-deficient conventional mice did not change the composition of the intestinal microbiota or increase the abundance of *B. wadsworthia* [78], indicating that

supplementation of SQ does not cause a bloom of *B. wadsworthia*, as observed for taurine [79]. Additionally, supplementation of SQ could prevent the decrease of the *A. rectalis* abundance when switching from a high-fiber to a low-fiber diet in mice with a synthetic human microbiota [80]. The microbiota consisted of fourteen bacterial species that covered the taxonomical and metabolic diversity of the human microbiota with fiber and host mucosa glycan degrading capacity and that was stable when feeding mice with a high-fiber diet [81]. *A. rectalis* abundances decreased by a factor of 1000 when switching from a high-fiber to a low-fiber diet within one week. This low abundance persisted until the end of the experiment, two weeks after the diet shift. In contrast, when the low-fiber diet was supplemented with SQ, *A. rectalis* abundance remained stable [80]. Apart from *A. rectalis*, this synthetic community contained other SQ-degradation-encoding species, such as the primary degraders *E. coli*, *F. prausnitzii*, *Roseburia intestinalis*, and *O. symbiosa*, as well as the secondary SQ-degrader *Desulfovibrio piger*. However, of these strains, only *O. symbiosa* and *D. piger* contained the full gene cluster for primary and secondary SQ degradation, respectively. Yet, both organisms remained without significant abundance changes when switching from a high-fiber to a low-fiber diet with and without SQ supplementation [80].

Notably, not all primary SQ degraders are associated with beneficial health effects. Although *E. coli* strains are predominantly commensal bacteria in the human gut microbiota that can synthesize vitamins K2 and B2 and inhibit pathogen colonization [82–84], some *E. coli* strains are (opportunistic) intra- or extraintestinal pathogens [85,86]. *S. enterica* is not considered a commensal in the human gut and can cause foodborne gastroenteritis and typhoid fever [87]. Although *S. enterica* and *E. coli* had previously not been enriched in SQ-amended human fecal microcosms [8], an *Escherichia/Shigella* ASV was enriched in our human fecal microcosms amended with 0.1, 1, and 10 mM SQ [9]. While some strains of *E. coli* and *S. enterica* possess the sulfo-EMP pathway for SQ degradation [88,89], it remains unclear how widespread this capacity is among clinical isolates. This uncertainty highlights the necessity for more research in this area.

Perspectives

Building on our findings, several directions emerge for future research (**Table 3**). First, the potential health effects of SQ remain unclear. Mechanistic *in vivo* studies are

essential to address this, but their translatability depends heavily on model selection [90–93]. We showed that conventional laboratory mice poorly represent human SQ metabolism when given a single SQ dose. One approach could be to gradually shift the microbiota composition of laboratory mice by regularly feeding SQ over an extended period to increase the capacity for SQ and DHPS degradation. Alternatively, wild mice, who likely consume more green plant material than laboratory mice [52–54], may harbor a microbiota that is more adjusted to SQ(DG) degradation. Another option may be the use of humanized gnotobiotic mouse models, where germ-free mice are colonized with a microbiota derived from human fecal samples, ranging from simplified consortia to complete fecal transplants [94]. This model may offer greater translatability to humans than the conventional mouse model since the consortium could include primary and secondary SQ degraders from the human gut. Yet, simplified models lack the complexity of the human microbiota, and immunological abnormalities of humanized mice have been observed [94]. To investigate the impact of SQ supplementation and the corresponding sulfide formation under dysbiosis, inflammation models, for example, with dextran sodium sulfate-treated mice supplemented with SQ, may be insightful. Finally, herbivorous animals like rabbits [95], whose microbiota are naturally adapted to SQDG-rich diets, may provide accurate models for *in vivo* microbial SQ degradation.

Second, while SQ degradation has been characterized in the human and mouse gut [9], in the cow rumen [10], and in the ocean [96], its role and the identity of SQ degraders in other environments, such as sediments or soils, remain unknown. SQ-amended microcosm experiments with soil or sediment could help investigate the SQ metabolism in these habitats. Most known SQ degraders possess the YihQ sulfoquinovosidase [1,3–6]. However, the recent discovery of the SggA sulfoquinovosidase showed that our *yihQ*-targeting primers detect only a subset of SQ degraders [10,97]. Notably, all known human and mouse gut SQ degraders possess *yihQ* [1,4,8,11], but many marine degraders possess *sggA* instead [97]. Developing a complementary *sggA*-targeting amplicon sequencing assay is necessary to avoid underestimating the diversity of SQ degraders, particularly in unexplored habitats. Our results also revealed the presence of previously uncultured SQ degraders in the cow rumen [10]. Enrichment cultures with repeated additions of SQ or DHPS could facilitate the isolation of uncultured degraders from the cow rumen, as well as from soils and sediments.

Third, although six major SQ degradation pathways have been identified in recent decades, it remains unclear whether additional metabolic routes exist. Our findings showed that SQ degraders within *Caproiciproducens* lack both known sulfoquinovosidases, YihQ and SggA, suggesting either the utilization of free SQ as a substrate or the presence of a yet unknown sulfoquinovosidase [10]. These findings raise the question of whether current pathway models fully capture the diversity of the SQ metabolism. To uncover alternative pathways, future efforts could focus on isolating and cultivating SQ degraders, applying (meta-)genomics and (meta-)transcriptomics, as well as performing protein purification and enzyme characterization. Collectively, these research directions provide a roadmap toward a mechanistic understanding of SQ metabolism, its distribution across environments, and its impact on host health.

Table 3. Future research questions related to SQ metabolism, their underlying knowledge gaps, and suggested approaches to address them.

research question	research gap	approach
Is SQ a prebiotic?	unclear health effects of SQ in different gut contexts (e.g., healthy vs. inflamed)	preclinical studies with wild mice, humanized mice, rabbits, inflammation models (e.g., with detran sodium sulfate)
What is the diversity of SQ degraders?	unknown diversity of SQ degraders in yet unexplored ecosystems, e.g., soils and sediments	enrichment cultures, <i>sggA</i> -targeted amplicon sequencing, SQ-amended soil and sediment microcosm experiments
Are there more SQ degradation pathways or enzymes?	potential existence of additional SQ degradation pathways or novel enzymes	cultivation of SQ degraders, genomics, transcriptomics, protein purification and characterization

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