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Research Paper

Dissemination of sulfonamide resistance genes in digester microbiome during anaerobic digestion of food waste leachate

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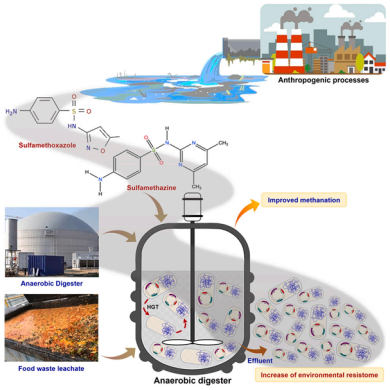
HIGHLIGHTS

- Cumulative methane yields enhanced by 37 % under SAs loading compared to FWL alone.
- Methanosarcinales-led acetoclastic methanogenesis played a predominant role in methanation.
- Dissemination of *sul1* depended on *intl1*-based HGT.
- Transmission of *sul2* was *intl1*-independent.
- Methanogenesis was uninterrupted by the influence of SAs or ARGs.

GRAPHICAL ABSTRACT



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ABSTRACT

The preeminence of sulfonamide drug resistance genes in food waste (FW) and the increased utilization of high-strength organic FW in anaerobic digestion (AD) to enhance methane production have raised severe public health concerns in wastewater treatment plants worldwide. In this regard, the dissemination patterns of different sulfonamide resistance genes (*sul1* and *sul2*) and their impact on the digester core microbiota during AD of FW leachate (FWL) were evaluated. The presence of various sulfonamide antibiotics (SAs) in FWL digesters improved the final methane yield by 37 % during AD compared with FWL digesters without SAs. Microbial population shifts towards hydrolytic, acidogenic, and acetogenic bacteria in the phyla Actinobacteriota, Bacteroidota,

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Chloroflexi, Firmicutes, Proteobacteria, and Synergistota occurred due to SA induced substrate digestion and absorption through active transport; butanoate, propanoate, and pyruvate metabolism; glycolysis; gluconeogenesis; the citrate cycle; and pentose phosphate pathway. The initial dominance of *Methanosaeta* (89–96 %) declined to 47–53 % as AD progressed and shifted towards *Methanosarcina* (40 %) in digesters with the highest SA concentrations at the end of AD. Dissemination of *sul1* depended on class 1 integron gene (*intl1*)-based horizontal gene transfer to pathogenic members of Chloroflexi, Firmicutes, and Patescibacteria, whereas *sul2* was transmitted to Synergistota independent of *intl1*. Low susceptibility and ability to utilize SAs during methanogenesis shielded methanogenic archaea against selection pressure, thus preventing them from interacting with *sul* or *intl1* genes, thereby minimizing the risk of antibiotic resistance development. The observed emergence of cationic antimicrobial peptide, vancomycin, and β-lactam resistance in the core microbiota during AD of FWL in the presence of SAs suggests that multidrug resistance caused by bacterial transformation could lead to an increase in the environmental resistome through wastewater sludge treatment.

1. Introduction

Dependence on synthetic and semi-synthetic antibiotics to treat infectious diseases in humans and animals has led to the widespread dispersal of undegraded or partially metabolized antibiotics into waste streams of municipal and industrial sewage systems. This makes wastewater treatment plants (WWTPs) worldwide multidrug reservoirs [40,54]. It has been reported that the Covid-19 pandemic might be encouraging the overuse of antibiotics due to misinformation about antibiotics' benefits in patients with Covid-19 infection and the estimated global antibiotics use in human and terrestrial and aquatic food-producing animal sectors could reach 236,757 tons annually by 2030 [16,52]. Approximately 30–90 % of administered antibiotics are excreted through feces or urine as a cocktail of parent compounds and their metabolites due to incomplete metabolism by humans or animals [30,56]. The concentrations of different antibiotics and their metabolites vary considerably in WWTP influents and effluents, depending on the source of influent wastewater and the environmental persistence of antibiotics [55]. The accumulation of antibiotics in WWTPs creates selection pressure towards vertical and horizontal gene transfer (VGT and HGT, respectively) among and across bacterial populations and intensifies the prevalence of antibiotic-resistant microorganisms (ARMs) in natural wastewater microbiota, resulting in serious public health risks [62,63]. Antibiotic resistance genes (ARGs), mobile genetic elements (MGEs), and ARMs were more abundant in WWTP effluents than influents in Germany and China owing to favorable selection conditions for HGT among bacteria, resulting in ARMs that were resistant even to chlorination and UV treatment [23]. Therefore, understanding the fate of antibiotics, ARGs, MGEs, and ARMs during wastewater treatment is essential considering the environmental consequences affecting downstream surface water, soil, and groundwater.

Sulfonamide antibiotics (SAs) are potent antibiotics used to treat a variety of bacterial and protozoan infections in humans, livestocks, and aquaculture species [21,59]. SAs are frequently detected in WWTP influents and effluents worldwide because of their low susceptibility to chemical and enzymatic hydrolysis, in contrast to other antibiotics such as β-lactams, tetracyclines, chloramphenicol, and vancomycin [55]. Concentrations of SAs in wastewater streams vary between a few ng L^{-1} and a few tens of μg L^{-1}, leading to the enrichment of these antibiotics in wastewater sludge (0.04–665 μg kg^{-1}) [40,57]. SAs are the most frequently detected antibiotics in wastewater containing animal manure from the swine industry, with a concentration range of 91–235 mg L^{-1} [19]. Concentrations of SAs in hospital and livestock wastewater range between 5029 and 142,900 ng L^{-1}. Nevertheless, the processing of food waste leachate (FWL) at WWTPs before its application in anaerobic digestion (AD) caused the accumulation of sulfamethoxazole in processed FWL (8817 ng L^{-1}) from its initial concentration of 0.34 ng L^{-1} [15]. The short hydraulic retention time (HRT) of typical WWTPs limits the biodegradation of antibiotics, especially SAs, resulting in their accumulation in activated sludge [34,61]. The hydrophobicity of some antibiotic residues favors a higher affinity to solids during primary and secondary treatments, which in turn leads to accumulation in organic-rich activated sludge [38]. The high generation rate in WWTPs favored by short HRT intensifies this accumulation of antibiotics in activated sludge [14]. In recent studies, the high concentrations of SAs (~2.45 mg•kg^{-1}) found in soil adjacent to poultry farms where manure/sludge (0.14–18 mg kg^{-1} SAs) was applied as organic fertilizer has raised serious environmental concerns for the dissemination of ARGs [40,43]. Furthermore, the persistence of certain SAs, such as sulfamethizole and sulfadiazine, during anaerobic digestion of pig manure over a period of 40 days demonstrates their recalcitrant nature compared to other SAs and suggests possible enrichment of soil microbiota with ARMs after the application of digested sludge as fertilizer in agricultural fields [11].

Prolonged antibiotic exposure has induced the evolution and proliferation of over 1000 subtypes of ARGs and decreased sludge microbial community richness [6,60]. Notably, densely populated sludge microbiota provides optimal conditions for HGT among bacterial species, which could lead to an increase in ARM up to 10^6–10^{10} copies g^{-1} of sludge [23,33]. Substantially higher concentrations of the tetracycline ARGs *tet*(O) and *tet*(W) and the sulfonamide drug resistance gene *sul1* (3-fold) were observed in treated sludge compared to the effluent at a WWTP in Michigan, which were responsible for the proliferation of tetracycline and sulfamethoxazole resistant bacteria in sludge up to 7.08×10^6 and 3.09×10^8 CFU g^{-1}, respectively [12]. Manure soil applications induced a microbial shift towards ARMs through the HGT of 260 unique ARGs and MGEs [13]. Land application of biosolids from WWTPs elevated the concentrations of various ARGs, including *tet*(O), *tet*(W), and *sul1* [33]. The abundance of the sulfonamide drug resistance genes *sul1* and *sul2* (10^6–10^8 copies g^{-1}) and MGE class 1 integron gene *intl1* (10^5–10^7 copies g^{-1}) in the wetland sediments of Sub-Saharan Africa suggests HGT and ARM enrichment due to anthropogenic wastewater discharge [1]. In contrast, the enrichment of agricultural soil with 108 unique ARGs and MGEs after the application of sewage sludge and chicken manure as fertilizer increased soil bacterial diversity by > 10 % and the dissemination of ARGs in the soil induced population shifts to the phyla Bacteroidota, Chloroflexi, Firmicutes, Gemmatimonadetes, and Planctomycetes [9]. The presence of a large number of pathogenic genera (*Arcobacter*, *Comamonas*, *Flavobacterium*, and *Rhodococcus*) in the effluents of anaerobic membrane bioreactors indicates the occurrence of HGT during anaerobic treatment, resulting in the dissemination of *sul1* and *intl1* [63]. Furthermore, a significant increase (10^5-fold) in *sul1* and *intl1* occurred in comparison to tetracycline ARGs after land application of chicken manure-based mineral fertilizer [29]. This apparent close association between *sul1* and *intl1* could contribute to the generation of an HGT-mediated resistome and induce environmental pathogenicity in bacteria.

Dissemination of ARGs through food waste (FW) during FW treatment at WWTPs poses a serious threat to public health as utilization of high-strength organic waste (*i.e.,* FW) for the improvement of biogas production has attracted considerable attention worldwide owing to its high organic content [32,41,7]. A high incidence (88–90 %) of MGEs and sulfonamide drug resistance genes, especially *sul1* and *sul2*, observed in 18 sulfonamide-resistant *E. coli* isolates from whiteleg

shrimp and pork samples indicated a high probability of HGT with serious consequences to public health [21]. The dominance of *sul1* in various Korean organic solid wastes, such as FW-recycling wastewater, sewage sludge, and manure, and its correlation with *intl1* indicate a high potential for HGT [28]. A strong positive association between *intl1* and various ARGs in the effluents of full-scale FW digesters contributed to the induction of HGT [37]. In this regard, AD of FWL was conducted in the presence of SAs to evaluate the fate of antibiotics, ARGs, and MGEs; the diversity of ARMs in digesters; and the functional potential of the core microbiome. A correlation matrix was generated to determine the dissemination patterns of different ARGs and MGEs among the digester microbiota during AD to raise public awareness.

2. Materials and methods

2.1. Preparation of substrate, antibiotics, and inoculum

Food waste leachate was collected from the cafeteria of Hanyang University, Seoul, Republic of Korea and characterized following the methods described in our previous study [48]. Sulfamethoxazole and sulfamethazine (≥98.0 % purity, HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as representative SAs. Anaerobic digester sludge (ADS) from Jungnang Sewage Treatment Center, Seoul, Republic of Korea was characterized as described above and used as inoculum for AD. Characteristics of FWL (Table 1) resembled the typical Korean FWL [27].

2.2. Anaerobic digestion

Anaerobic digestion of FWL in the presence of two different SAs (sulfamethoxazole and sulfamethazine) was performed in digesters with 550 mL capacity at mesophilic temperature (37 °C) and 150 rpm. The experiments were conducted in triplicate. Digester inoculation was performed under a > 99 % N_2 atmosphere in a Vinyl Anaerobic Chamber, Type B (Coy Laboratory Products, Inc., Grass Lake, MI, USA) using ADS and with 45 % headspace. The concentration of FWL (21 g total solid L^{-1}) in the digesters was maintained at 49 % of the total volatile solids (VS) by adding the inoculum to the FWL at a ratio of 1:1 (VS basis). Sodium bicarbonate (5 g L^{-1}), L-cysteine–HCl (0.5 g L^{-1}), cobalt sulfate (2.6 mg L^{-1}), and nickel chloride (2.2 mg L^{-1}) were added to the digesters to promote anaerobic microbial growth [49,51]. The initial pH was adjusted to 7.10 using a 5 N HCl solution. SAs were added to digesters with FWL at concentrations of 0.1 mg L^{-1} and 1.0 mg L^{-1} each. Sets of positive and negative controls were run with and without the addition of FWL, respectively. AD continued until the cessation of biogas production.

Table 1
Characteristics of inoculum and food waste leachate (FWL). ND: not detected.

Properties		Inoculum	FWL
Proximate analysis	Total solid (wt%)	2.59 ± 0.005	6.17 ± 0.001
	Volatile solid (dry wt%)	67.21 ± 0.09	82.12 ± 0.004
	Ash (wt%)	0.85 ± 0.0004	1.10 ± 0.0001
	Fixed carbon (dry wt%)	31.94 ± 0.085	16.78 ± 0.004
	Total carbohydrate (dry wt %)	34.14 ± 5.48	19.14 ± 0.24
	Total lipid (dry wt%)	ND	36.35 ± 0.004
	Total protein (dry wt%)	30.64 ± 0.32	26.01 ± 0.36
Ultimate analysis	Total carbon (dry wt%)	32.31 ± 0.17	45.35 ± 0.17
	Total nitrogen (dry wt%)	4.90 ± 0.06	4.16 ± 0.06
	Total hydrogen (dry wt%)	5.09 ± 0.06	6.76 ± 0.06
	Total sulfur (dry wt%)	0.79 ± 0.12	0.29 ± 0.12
	Total oxygen (dry wt%) (by difference)	56.89 ± 0.22	43.44 ± 0.22
	C/N ratio	6.59	10.89
Physical properties	pH	7.45 ± 0.01	4.65 ± 0.01
	Calorific value (kJ g^{-1})	20.38 ± 0.02	17.41 ± 0.001

2.3. Analytical methods

The methane content of the biogas produced in the headspace of the digesters was quantified each day using a gas chromatography system (6890N, Agilent Technologies, Palo Alto, CA, USA) equipped with a Molecular Sieve 5A column. The modified Gompertz equation (GraphPad Prism version 9.2.0; GraphPad Software, San Diego, CA, USA) was used to calculate the methane production potential (M_{max}), maximum methane production rate (R_m), and lag phase (λ) [50]. The intermediate concentrations of volatile fatty acids (VFAs) and SAs were estimated using the above GC equipped with an HPINNOWax column and an HPLC (Alliance 2695 system, Waters, USA) equipped with a C18 column, respectively [48,59].

2.4. Microbial assessment and quantification of key genes during co-digestion

The taxonomic diversity of the digester microbiome was evaluated through high-throughput sequencing of 16S rRNA amplicons using the Ion GeneStudio™ S5 System (catalog number: A38194; Thermo Fisher Scientific, Waltham, MA, USA). Total microbial DNA was extracted using a DNeasy PowerSoil Pro Kit (catalog number: 47016; Qiagen, Hilden, Germany) at different time periods during co-digestion and used as a template for polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR). PCR amplification of the extracted DNA was performed in a MiniAmp™ Plus Thermal Cycler (catalog number: A37835; Thermo Fisher Scientific) prior to library preparation using the prokaryote-specific forward 341f and reverse 805r primer pair designed based on the variable regions (V3–V4) of 16S rRNA genes (Table S1) due to its overall coverage of total microbial diversity [20,24]. Although, the use of a primer pair combination (344f-1041r/519f-806r) is considered superior for the archaeome analysis [42]. Amplified DNA samples were quantified using the Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific) and processed for library preparation and chip loading using the Ion Chef™ Instrument (catalog number: 4484177; Thermo Fisher Scientific). The operational taxonomic units representing each read were clustered by 99 % similarity against the SILVA 138 16S rRNA reference database using a confidence threshold of 0.8 to identify different microorganisms. The high-throughput sequencing data was deposited to NCBI under Bioproject: PRJNA931785 (Table S3). The 16S rRNA data were processed to compare and visualize the functional potential of the core microbiota in the exponential phase of growth based on the KEGG ortholog [51].

Relative quantification of key genes, such as *sul1*, *sul2*, and *intl1*, was performed using qPCR in an Applied Biosystems StepOne™ Real-Time PCR System (catalog number: 4376373; Thermo Fisher Scientific) using SYBR™ Green PCR Master Mix (catalog number: 4309155; Applied Biosystems, Waltham, MA, USA). Primers for key genes (Table S1) were selected based on a previous study [62] and were obtained from $COSMO_{GENETECH}$ (Seoul, Republic of Korea). Changes in the relative copy numbers of key genes were estimated using the $\Delta\Delta C_T$ method in relation to the 16S rRNA amplicon used as a reference (housekeeping) gene [41].

2.5. Statistical analysis

Statistical significance of the experimental data was assessed using triplicate values, and standard errors of the means were calculated using GraphPad Prism version 9.2.0 for Windows. Differences between variables were detected at a confidence level of $p < 0.05$, and Tukey's multiple comparison test was used for statistical analysis. Correlation

analysis was performed among the major microbial phyla, sulfonamide resistance genes *sul1* and *sul2*, and *intl1* over the digestion period using Pearson coefficient (*r*) analysis conducted using the Cytoscape platform. The Pearson coefficient (*r*) study identified strong and weak correlations as $r > 0.6$ or $r < -0.6$.

3. Results and discussion

3.1. Biogas production

Continuous production of methane and hydrogen from day 1 of AD was observed during AD of FWL for 66 days in the presence of different concentrations (low: 0.1 mg L^{-1} and high: 1.0 mg L^{-1}) of SAs (Fig. 1a, b). Hydrogen production ceased within 7 days of operation and reached a steady state in all digesters (Fig. 1b). An initial decrease in the pH of the suspension (5.8–6.0) on the fourth day in the FWL digesters indicated hydrogen production via rapid hydrolysis and acidogenesis (Fig. S1). A similar observation of an initial decrease in pH was attributed to the production of hydrogen and VFAs due to acidogenesis in previous studies [3,26]. Following the initial decrease, the pH of the suspension increased and stabilized at 7.0–7.8 for the remainder of the digestion process (Fig. S1). The pH (7.1–8.1) in the control digesters remained stable throughout the experiment. The high content of carbonaceous organic matter (carbohydrates and lipids) in FWL (Table 1) stabilizes the pH of the suspension, which is necessary for optimum methanation [49]. Methane production continued for the next 59 d without interruption and reached cumulative values of 2554–3508 mL after utilizing 75–76 % of the total VS in the FWL digesters in the presence or absence of SAs (Fig. 1a, Table 2). A nonlinear regression analysis of M_{max} (R^2: 0.90–0.95) did not reveal any significant differences in R_m among the FWL digesters (p = 0.92–0.99) with or without SAs even at high SA concentrations (three times higher than that of the controls) (Table 2, Table S2). Biogas production and respective methane yields in anaerobic sequencing batch reactors remained stable during the loading of

Table 2
Digestion efficiency and product yield. Entries are given as the mean ± standard error of the mean of triplicate results. ND: not detected.

Parameters	Control	FWL	FWL with SAs	
			0.1 mg L^{-1} SAs	1.0 mg L^{-1} SAs
Total solid utilization (%)	48.43 ± 0.11	62.92 ± 0.13	62.53 ± 0.04	63.82 ± 0.77
Volatile solid utilization (%)	61.30 ± 0.16	75.50 ± 0.27	75.42 ± 0.17	76.28 ± 0.51
Carbohydrate utilization (%)	54.52 ± 0.24	64.80 ± 0.19	68.98 ± 4.56	73.20 ± 0.98
Lipid utilization (%)	ND	68.45 ± 1.23	64.17 ± 3.09	69.25 ± 1.39
Yields of CH_4 (g g^{-1} $VS_{initial}$)	0.04 ± 0003	0.33 ± 0.004	0.44 ± 0.005	0.45 ± 0.006
Percent yields of CH_4	28.38 ± 0.12	69.97 ± 0.79	94.87 ± 1.09	96.12 ± 1.32
R_m (mL d^{-1})	12.63	37.01	36.74	38.77
Energy generation (kJ)	6.31 ± 0.05	92.99 ± 0.89	126.09 ± 1.24	127.74 ± 1.55
Energy recovery (%)	7.98 ± 0.04	70.15 ± 0.67	95.12 ± 0.94	96.34 ± 1.17

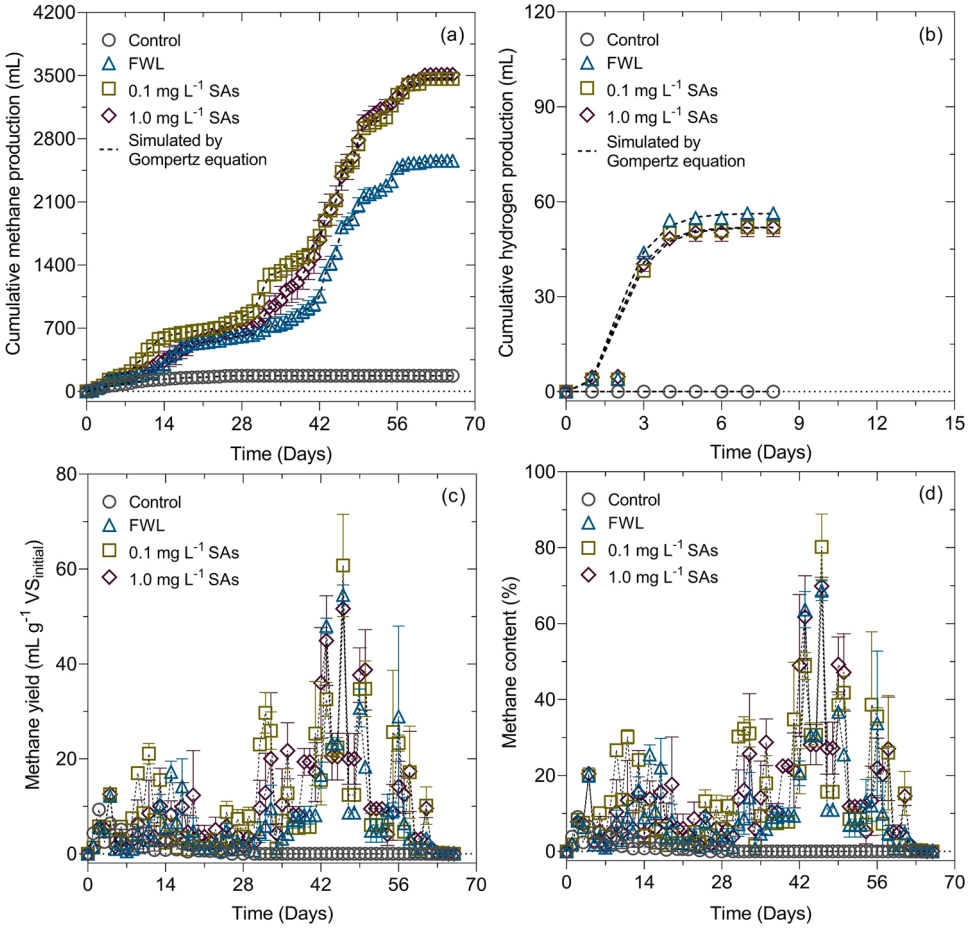

Fig. 1. Cumulative methane (a) and hydrogen (b) production with respective daily methane yields (c) and methane content (d) during co-digestion for different experimental sets. The 0.1 mg L^{-1} sulfonamide antibiotics (SAs) and 1.0 mg L^{-1} SAs indicate different concentrations of SAs in digesters with food waste leachate (FWL).

antibiotic mixtures containing sulfamethoxazole (0.5–10 mg L^{-1}), erythromycin (0.2–0.5 mg L^{-1}) and tetracycline (0.2–0.5 mg L^{-1}), indicating antibiotic tolerance of digester microbiota at comparatively high SA loads [5].

The highest methane yield (61 mL g^{-1} $VS_{initial}$) was achieved in the FWL digester with 0.1 mg L^{-1} SAs on the 46th day of digestion, with methane comprising 80 % of the produced biogas (Fig. 1c, d). The cumulative methane yields were 37 % and 1 % higher in digesters with 1.0 mg•L^{-1} SAs after 66 days of operation, compared to FWL (497 mL•g^{-1} $VS_{initial}$) and 0.1 mg L^{-1} SAs (673 mL g^{-1} $VS_{initial}$) digesters, respectively. Maximum methane production (95–96 %) in comparison to their respective theoretical yields occurred in FWL digesters containing SAs owing to the consumption of carbohydrates (69–73 %) and lipids (64–69 %) present in digester VS (Table 2). During digestion, the maximum methane yields facilitated a total energy generation and recovery of 128 kJ and 96 %, respectively. Sulfamethazine was found to promote methanation (5–9 %) substantially during anaerobic sludge digestion [65]. The efficiency of acidogenesis and acetogenesis has been attributed to improved methanation (five times higher than that of the control) in sulfamethoxazole (5 mg L^{-1})-spiked anaerobic digesters [35]. SAs stimulated the hydrolysis of waste-activated sludge through the disruption of extracellular polymeric substances in sludge and facilitated acidogenesis and acetogenesis via anaerobes that enhance VFA production (32–173 %) during anaerobic fermentation [18,58]. SA-promoted protease, α—glucosidase, and acetate kinase activities in anaerobes accelerated the hydrolysis and acidogenesis of activated sludge, which favored hydrogenotrophic methanogenesis [18,31]. Enhancement of the syntrophic association among acidogenic *Clostridia* and Bacteroidia and toxicant-resistant hydrogenotrophic methanogens in the presence of sulfadiazine and Fe^0 improved methanation (36.9 %) and pollutant degradation during anaerobic digestion of swine manure [19]. The presence of various metal ions in wastewater sludge and FW facilitates interspecies electron transfer (IET) among acidogens/acetogens and hydrogenotrophic/acetoclastic methanogens and assists in the utilization of hydrogen and VFAs, thus leading to improved methanation [47,53]. Nevertheless, statistical analysis did not reveal any significant ($p > 0.99$) differences in cumulative methane yields among the FWL digesters with or without SAs in this study, when calculated in terms of $g_{methane}$ g^{-1} $VS_{initial}$ (Table 2). Endogenous protein-degradation compounds in the inoculum have been known to limit the bacteriostatic activity of SAs in susceptible gram-positive and gram-negative anaerobes, indicating uninterrupted digestion even at an SA concentration of 280 mg L^{-1} [39].

3.2. Microbial taxonomy during co-digestion

High-throughput amplicon sequencing of the digester microbiota revealed the dominance of eight major bacterial phyla and two archaeal phyla throughout the digestion period under all experimental conditions (Table S4). Among the major phyla, Actinobacteriota (14–23 %) and Firmicutes (49–72 %) were dominant during the phase shift of digestion in their respective digesters, followed by Chloroflexi and Proteobacteria (Fig. 2). The populations of Chloroflexi and Proteobacteria showed a declining tendency in consecutive digestion phases when compared to the microbiota of the initial ADS. However, these changes were not statistically significant ($p > 0.99$). The predominant acidogenic and acetogenic abilities of members of these phyla favored their proliferation during AD of carbohydrate- and lipid-rich FWL [48,49]. The population of pathogenic *Mycobacterium* in the phylum Actinobacteriota reached 12 % during the exponential phase in digesters with 0.1 mg•L^{-1} SAs and were the most abundant among major genera across all experimental sets (Fig. 3). The high potential of *Mycobacterium* for developing resistance to numerous antibiotics could explain its presence in all digesters with SAs [2]. *Romboutsia* in the phylum Firmicutes was the most abundant (4–6 %), followed by *Clostridium sensu stricto* 7 (2–5 %) in FWL digesters with and without SAs (Fig. 3). However, the population of *Clostridium sensu stricto* 7 decreased with increasing SA concentrations during the exponential phase compared to populations in digesters without SAs. Populations of *Clostridium sensu stricto* 1 (2–5 %) and *Terrisporobacter* (5–7 %) were high in digesters with the highest SA concentration (1.0 mg L^{-1}) throughout the digestion period (Fig. 3). The xylanolytic, amylolytic, and glycolytic activities of *Romboutsia*, *Clostridium*, and *Terrisporobacter*, respectively, probably assisted their proliferation during FWL digestion [66]. These genera are pathogenic and cause intestinal inflammation and disrupt gut homeostasis in humans and animals [17,44].

After their initial suppression, *Syntrophomonas* and *Turicibacter* in the phylum Firmicutes rapidly increased by 2–5 fold and 1.4–1.6 fold, respectively, during the late exponential phase in FWL digesters with and without SAs (Fig. 3). The members of these genera were sensitive to SAs, as indicated by the decreasing trends of their population as SA concentrations increased in the digesters. Enrichment of acetogenic

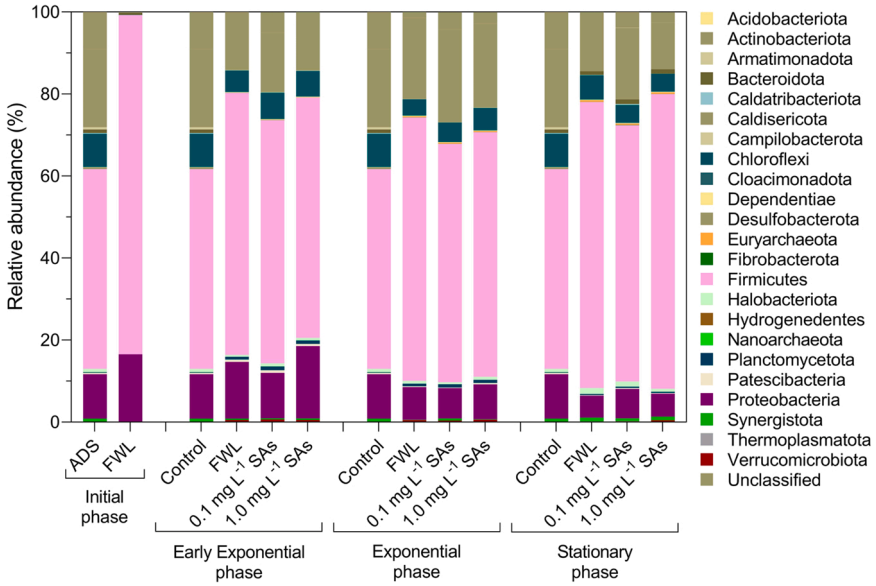

Fig. 2. Diversity in the core microbiota at the s level during co-digestion for different experimental sets. The 0.1 mg L^{-1} sulfonamide antibiotics (SAs) and 1.0 mg L^{-1} SAs indicate different concentrations of SAs in digesters with food waste leachate (FWL).

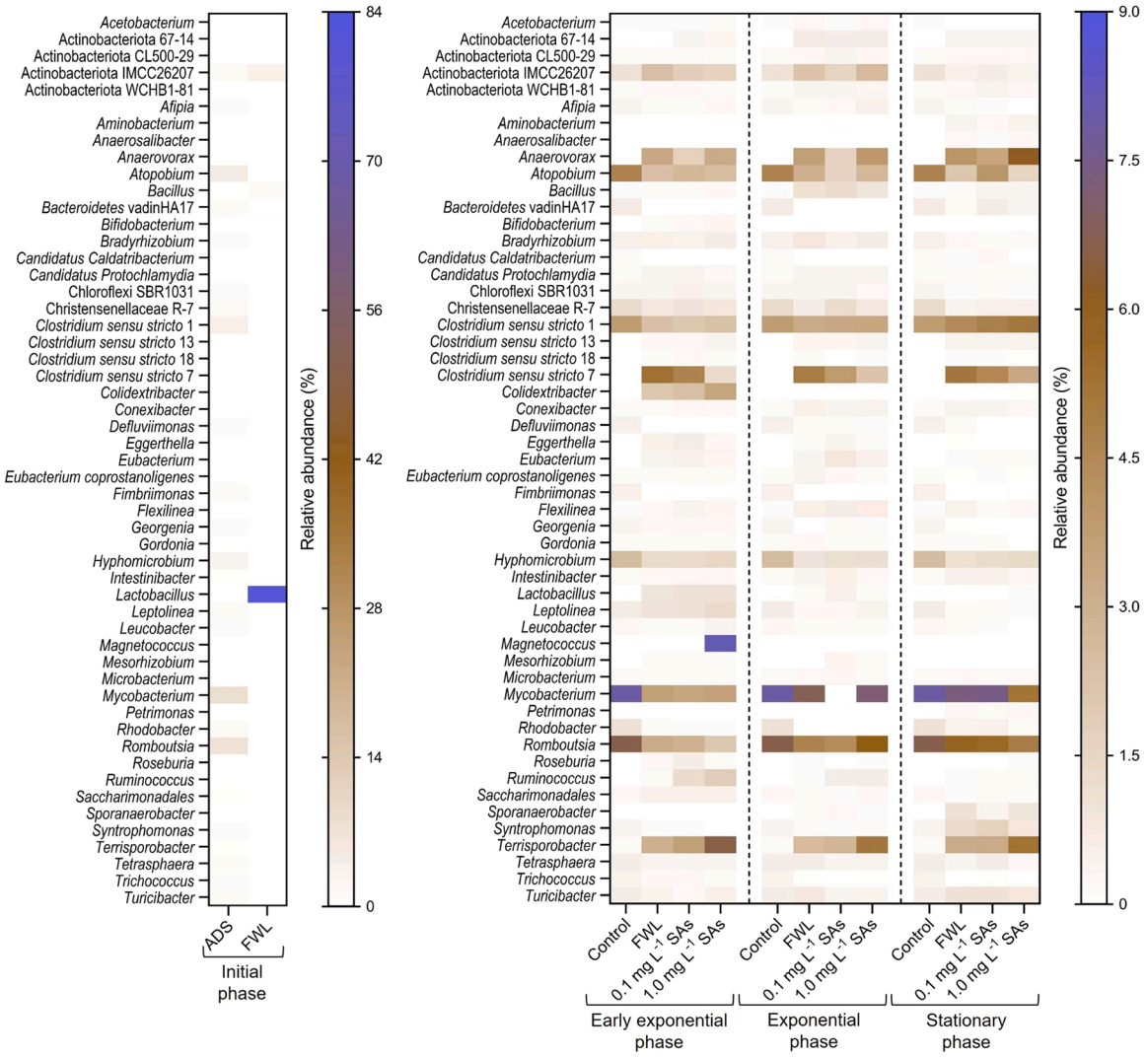

Fig. 3. Diversity in bacterial genera (abundance > 0.2) during co-digestion for different experimental sets. The 0.1 mg L^{-1} sulfonamide antibiotics (SAs) and 1.0 mg L^{-1} SAs indicate different concentrations of SAs in digesters with food waste leachate (FWL).

Syntrophomonas has been shown to induce syntrophic methanation through β-oxidization of lipidic substrates and control electron flux among exoelectrogenic partners during AD [26,51]. In this study, the populations of Patescibacteria (0.5–0.6 %), Planctomycetota (0.7–1 %), and Verrucomicrobiota (0.5–0.7 %) increased during the early exponential phase following their initial abundance in ADS (0.1–0.3 %) and declined thereafter (Fig. 3). In contrast, the population of Synergistota exhibited a V-shaped recovery during the stationary phase (0.7–0.9 %) after a steady decline in the exponential phase (0.1–0.3 %). Incremental increases in the population of *Aminobacterium* (0.2–04 %) during the late exponential phase led to the recovery of Synergistota. The protein content of FWL supports the growth of the amino acid-utilizing *Aminobacterium* (Table 1) [51]. The acetogenic activity of members of the phylum Synergistota (especially *Aminobacterium*) probably contributes to its late recovery facilitated by syntrophic interactions with aceto-clastic/hydrogenotrophic methanogens [25]. The population of *Bacteroidetes* vadinHA17 in the phylum Bacteroidota increased during the stationary phase after an initial inhibition (Fig. 3). *Bacteroidetes* vadinHA17 can degrade 16–17 types of amino acids in proteinaceous substrates owing to their amylolytic activities, which support their growth in the stationary phase [36]. Halobacteriota was the most dominant among the archaeal phyla and reached an abundance of 0.7–1.4 % at the end of the digestion process in the FWL digesters (Fig. 2). In the FWL digesters, Halobacteriota exhibited an initial decline compared to the control digesters during the early exponential phase; however, the population recovered steadily as digestion progressed. Rapid acidogenesis and acetogenesis could account for this decline via VFA accumulation, which was not sustained due to the methanogenic potential to utilize VFAs and SAs [22,62].

Methanosaeta (phylum: Halobacteriota) was predominant (89–96 %) among the methanogens during the early exponential phase in the FWL digesters and declined to 47–53 % as digestion progressed (Fig. 4). Methanogenic dominance shifted towards *Methanosarcina* (40 %) in digesters with the highest SA concentration at the end of digestion. The tolerance of *Methanosarcina* to high acetate concentrations enables their survival during SA-mediated acidosis [26]. Selection pressure by tetracyclines and sulfonamides in anaerobic sludge digestion in continuous stirred-tank digesters shifted the microbiota towards the more versatile Methanosarcinales through VFA accumulation [60]. Nevertheless, Methanosarcinales (*Methanosarcina* and *Methanosaeta*) can create syntrophic associations with acidogens/acetogens and exoelectrogens to oxidize acetate and reduce carbon dioxide for methanation during AD [47]. Another study showed that interspecies interactions among acidogenic members of the phyla Firmicutes (*Clostridium sensu stricto*, Ruminococcaceae, *Clostridium* III: ~5 %) and Bacteroidota (Bacteroidales: 5 %) and *Methanosarcina* increased methanation (21.5 %)

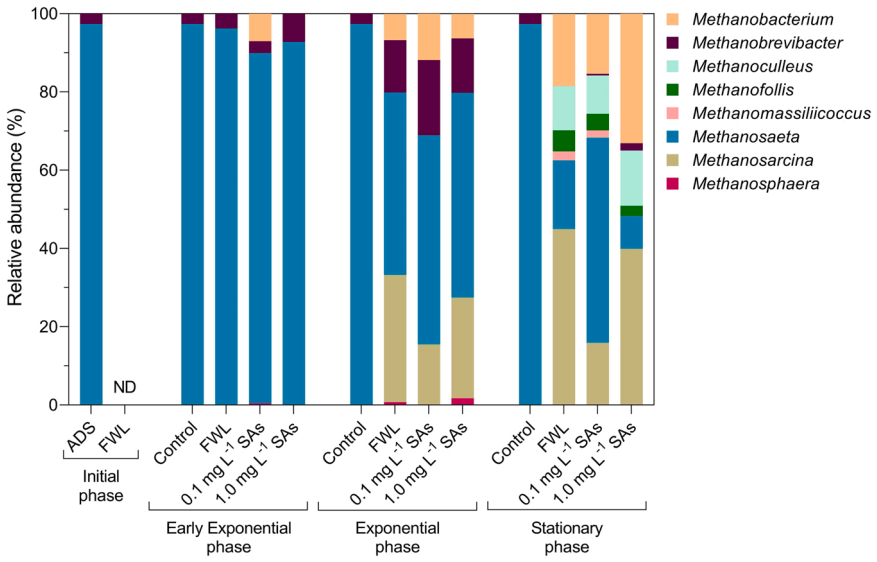

Fig. 4. Methanogenic abundance during co-digestion for different experimental sets. The 0.1 mg L^{-1} sulfonamide antibiotics (SAs) and 1.0 mg L^{-1} SAs indicate different concentrations of SAs in digesters with food waste leachate (FWL). ND: not detected.

through IET during the AD of swine manure [30]. In the present study, hydrogenotrophic methanation was performed primarily by *Methanobrevibacter* (13–19 %) in FWL digesters with and without SAs during the exponential phase, which was overpowered by *Methanobacterium* (15–33 %), *Methanoculleus* (10–14 %), and *Methanofollis* (3–5 %) at the end of digestion (Fig. 4). The dominance of *Methanoculleus* and *Methanofollis* in the stationary phase suggested syntrophic associations between acetate oxidizers and hydrogenotrophic methanogens during the AD of lipidic FWL and slaughterhouse waste [7,51]. Hydrogenotrophic methanogens such as *Methanomassiliicoccus*, *Methanoculleus*, *Methanosphaera*, and *Methanobrevibacter* comprise 86 % of the archaeal population in sulfadiazine-containing digesters [19]. The presence of sulfamethoxazole in anaerobic digesters increased hydrogen partial pressure ($>10^{-4}$ atm), which triggered hydrogenotrophic methanation over the acetoclastic pathway by inhibiting the oxidation of VFAs (propionate, butyrate) to acetate [35]. Nevertheless, Methanosarcinales-mediated acetoclastic methanogenesis played a prominent role in methanation during FWL digestion with/without SAs, regardless of the SA concentrations studied.

3.3. Dissemination of antibiotic resistance through ARMs

The relative copy numbers of the sulfonamide drug resistance genes (*sul1* and *sul2*) were low during the exponential phase when compared to their respective controls (initial phase) in all digesters, but this was not the case for the class 1 integron gene (*intl1*) (Fig. 5a, b). The increase in *sul2* was inversely proportional to the SAs concentration, whereas *sul1* levels were independent of SAs concentrations in all experimental phases. The relatively low abundance of *sul1* throughout the anaerobic sludge digestion could be explained by its low sensitivity to antibiotic pressure [6]. The increase in the relative copy numbers of *sul* genes in the FWL digesters with or without SAs at various concentrations suggests that the transfer of resistance genes and proliferation of ARMs occurred during the stationary phase (Fig. 5b). Exposure to sulfamethoxazole assists the proliferation of sulfamethoxazole-resistant gram--negative bacteria in dewatered sludge [12]. Microbial exposure to sulfamethizole and sulfadiazine during AD of pig manure suggested enrichment of *sul*-genes-containing ARMs in environmental bodies after the application of digested sludge to the soil [11]. Long-term exposure to antibiotics has been shown to cause intrinsic microbial resistance to tetracycline and sulfonamide and promote the proliferation of ARMs in anaerobically digested sludge [60]. The relative abundances of pathogenic *Anaerovorax*, *Clostridium*, *Mycobacterium*, *Romboutsia*, *Terrisporobacter*, and *Turicibacter* indicate the dissemination of *sul* genes and proliferation of ARMs during the exponential and stationary phases of AD (Fig. 3). The pathogenicity of members of *Anaerovorax*, *Clostridium*, *Mycobacterium*, and *Romboutsia* and their syntrophic interactions with members of *Terrisporobacter* and *Turicibacter* lead to inflammation and antibiotic-resistant infections in humans [17,44,8].

The presence of a comparatively high copy number of *intl1* in the FWL digesters suggests that dissemination of MGEs in the experimental digesters occurred because of HGT in FWL microbiota during digestion (Fig. 5a). The abundance of ARGs in FW provides an optimum environment for the transmission of ARGs, especially *sul1* and *sul2*, across pathogenic ARMs via HGT [21,32]. Levels of the class 1 integron gene *intl1* were high in the experimental digesters throughout the digestion period (Fig. 5a, b). As an MGE, *intl1* plays a major role in the dissemination of *sul* genes (*i.e.*, *sul1* and *sul2*) among similar and diverse microbial genera through VGT and HGT in both chromosomes and plasmids [21]. The high prevalence rate of the *sul1* gene in anaerobic membrane bioreactors was associated with the abundance of *intl1*, highlighting its importance in *sul* dissemination [62]. The co-occurrence of *intl1* and *sul1* suggests that HGT-mediated transmission of ARGs occurred during AD [32]. The abundance of *sul1* and *sul2* in *Escherichia coli* plasmids carrying *intl1* suggested that HGT is the dominant route of transmission for *sul* genes [21]. The formation of biofilms in digested sludge promotes the development of spatiotemporal reserves for plasmid-carrying ARMs, which outnumber plasmid-free ARMs [45]. Additionally, the selection pressure for potential hosts and their adaptive proliferation resulted in the dissemination of various ARGs, especially *sul1*, *tetA*, *sul2*, *tetW*, and *tetM* during anaerobic sludge digestion under the influence of antibiotic mixtures containing tetracycline, oxytetracycline, chlortetracycline, and various SAs at 2 mg L^{-1} independent of *intl1*-based HGT [6]. Similarly, the maximum increase in the copy number of *sul2* observed during the stationary phase indicated *intl1*-independent transmission of ARGs and proliferation of ARMs in the FWL digesters (Fig. 5b).

3.4. Microbial correlations and their metabolic activities

The correlation matrices revealed significant ($p < 0.05$) interactions among the five dominant microbial phyla (Chloroflexi, Firmicutes, Patescibacteria, Proteobacteria, and Synergistota), *sul* genes, and *intl1* (Fig. 6a and S2a, b). The significant interaction of *intl1* with *sul1*

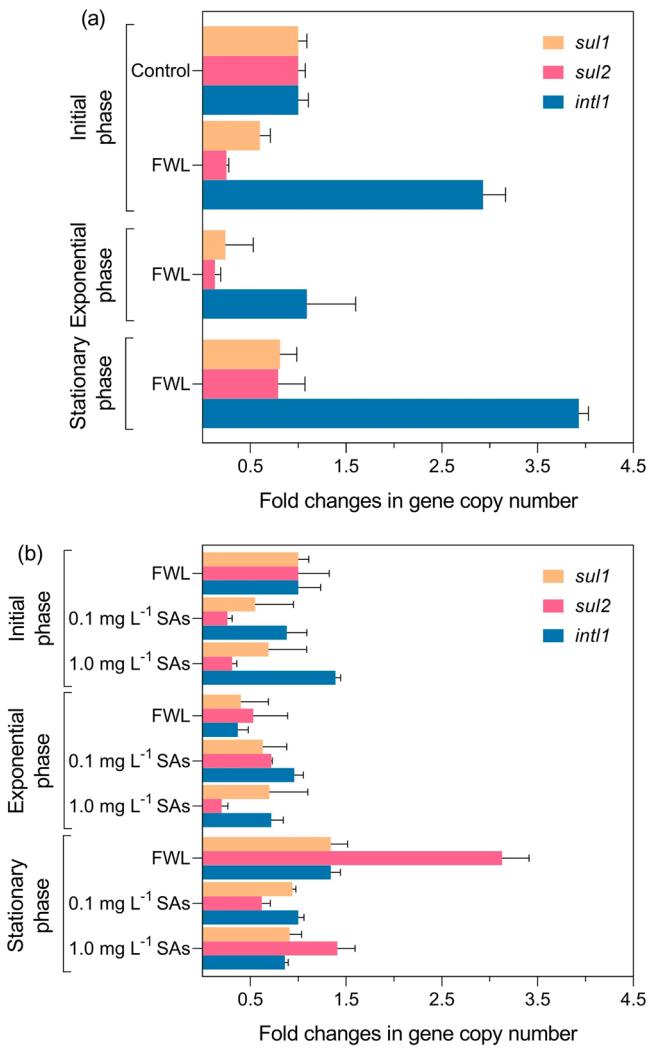

Fig. 5. Changes in the relative copy numbers of sulfonamide drug resistance genes *sul1* and *sul2*, and class 1 integron gene *intl1* in digesters with food waste leachate (FWL) compared to control (a) and other experimental sets compared to those in the digesters with FWL (b) during co-digestion. The 0.1 mg L^{-1} SAs and 1.0 mg L^{-1} SAs indicate different concentrations of SAs in digesters with FWL.

indicated that *intl1*-dependent HGT is probably responsible for the transmission of *sul1*, while the transmission of *sul2* primarily occurs via an *intl1*-independent route. This could be explained by the ability of *sul1* to be accommodated by small conjugative plasmids allowing its dissemination through *intl1*-based plasmids, whereas *sul2* is primarily carried by large plasmids with limited mobility [62]. Nevertheless, *intl1*-independent transmission of both *sul1* and *sul2* was possible in members of the phylum Synergistota (Fig. 6a). Increases in the population of Synergistota_Uncultured, copy numbers of *sul* genes, and ARM proliferation during the stationary phase confirm this interaction (Fig. 5b and S3). Bacteroidota, Firmicutes, Proteobacteria, and Synergistota are potentially responsible for the dissemination of ARGs [32]. Members of the phyla Firmicutes and Chloroflexi closely interacted with *sul1* during the exponential phase of AD, an interaction that shifted to Patescibacteria in the stationary phase along with *intl1* (Fig. S2a, b). The abundance of pathogenic genera in Firmicutes and Chloroflexi suggests the accommodation of *sul1* among the members of these phyla (Fig. 3 and S3). The shifting of the microbial community to Chloroflexi and Firmicutes was reported to be responsible for the transformation of the soil resistome through the enrichment of 108 ARGs [9]. Ultra-small parasitic bacteria *Saccharimonadales* of the phylum Patescibacteria aided *intl1*-based HGT during the stationary phase (Fig. 3). Therefore, the dissemination of ARGs during AD depends on the potential of certain bacteria to accommodate specific types of ARGs through *intl1*-dependent HGT or *intl1*-independent transmission.

Methanogenic archaea were shielded against any possible interactions with *sul* genes and *intl1* (Fig. 6a and S2a), allowing uninterrupted methanation during the AD (Fig. 1). The ability of versatile *Methanosaeta* and *Methanosarcina* in the digesters to perform both acetoclastic and hydrogenotrophic methanogenesis shielded members of the phylum Halobacteriota from the influence of ARGs (Fig. 4). Methanogenic archaea exhibit the least sensitivity to SAs during AD and can utilize SAs as a cosubstrate, which explains their low inherent risk to develop antibiotic resistance [31,62]. Notably, major acetoclastic methanogens within Halobacteriota exhibited syntrophy with members of Bacteroidota and Patescibacteria instead of with other major bacterial phyla (Fig. 6a and S2a). Euryarchaeota, which include hydrogenotrophic methanogens such as *Methanobacterium* and *Methanobrevibacter*, established positive interactions with acidogenic and acetogenic Bacteroidota, Firmicutes, and Proteobacteria. The population increases of *Methanobrevibacter* and *Methanobacterium* during the exponential and stationary phases, respectively, are indicative of their correlation (Fig. 4). The hydrogen-scavenging abilities of *Methanobacterium* and *Methanobrevibacter* during the acidogenic and acetogenic processes sustained a low hydrogen partial pressure (<2 Pa) in the digesters, which favored methanogenesis [47].

KEGG ortholog analysis was used to explore the functional potency of the core microbiota during the exponential phase (Fig. 6b). The gene expression of ABC transporters (17–23 %) and genes involved in fat digestion and absorption pathways (16–34 %) were the highest among major metabolic activities in FWL digesters, followed by those involved in carbohydrate and protein digestion and absorption; butanoate, propanoate, and pyruvate metabolism; glycolysis gluconeogenesis; the citrate cycle (TCA cycle); and pentose phosphate pathway. Rapid digestion of carbohydrates, proteins, and lipids in FWL and active nutrient (e.g., acetate) transport to microbial cells for methanation accelerates the metabolic expression of these pathways [51]. Riboflavin metabolism and quorum sensing increased by 5–20 % and 2–13 %, respectively, in the FWL digesters (Fig. 6b). Induction of riboflavin metabolism and quorum sensing indicates the activity of the riboflavin cofactor during syntrophic VFA oxidation and methanation by core microbiota and their interactions through quorum sensing [46,64]. Methane metabolism did not exhibit any significant differences ($p > 0.05$) among the digesters as no inhibition was observed in any of the digesters during AD (Figs. 1 and 6b). Cationic antimicrobial peptide (CAMP) resistance emerged as the main resistance category, followed by vancomycin and β-lactam resistance during the exponential phase (Fig. 6b). Alanine, aspartate, and glutamate metabolism accounted for the increased expression of CAMP resistance, as the incorporation of D-alanine in the lipoteichoic acids of the bacterial membrane is vital for the reduction of membrane negativity, which facilitates the development of resistance in *Bacillus*, *Clostridium*, and *Lactobacillus* spp. Assoni et al., ($year$) [4]. The development of β-lactam resistance and absence of SA-mediated bacterial inhibition during AD suggest the presence of multidrug resistance gene-containing plasmids in the core microbiota, which could encode extended-spectrum β-lactamases along with other resistance genes that could confer resistance against β-lactams and several non-β-lactam antibiotics, such as aminoglycosides, fluoroquinolones, and sulfamethoxazole-trimethoprim [10]. Thus, the presence of SAs accelerated the transformation of core digester microbiota to ARMs during AD.

4. Conclusions

The addition of SAs enhanced cumulative methane yields in the FWL digesters by 37 % compared to FWL digesters without SAs by inducing the proliferation of hydrolytic, acidogenic, and acetogenic bacteria of

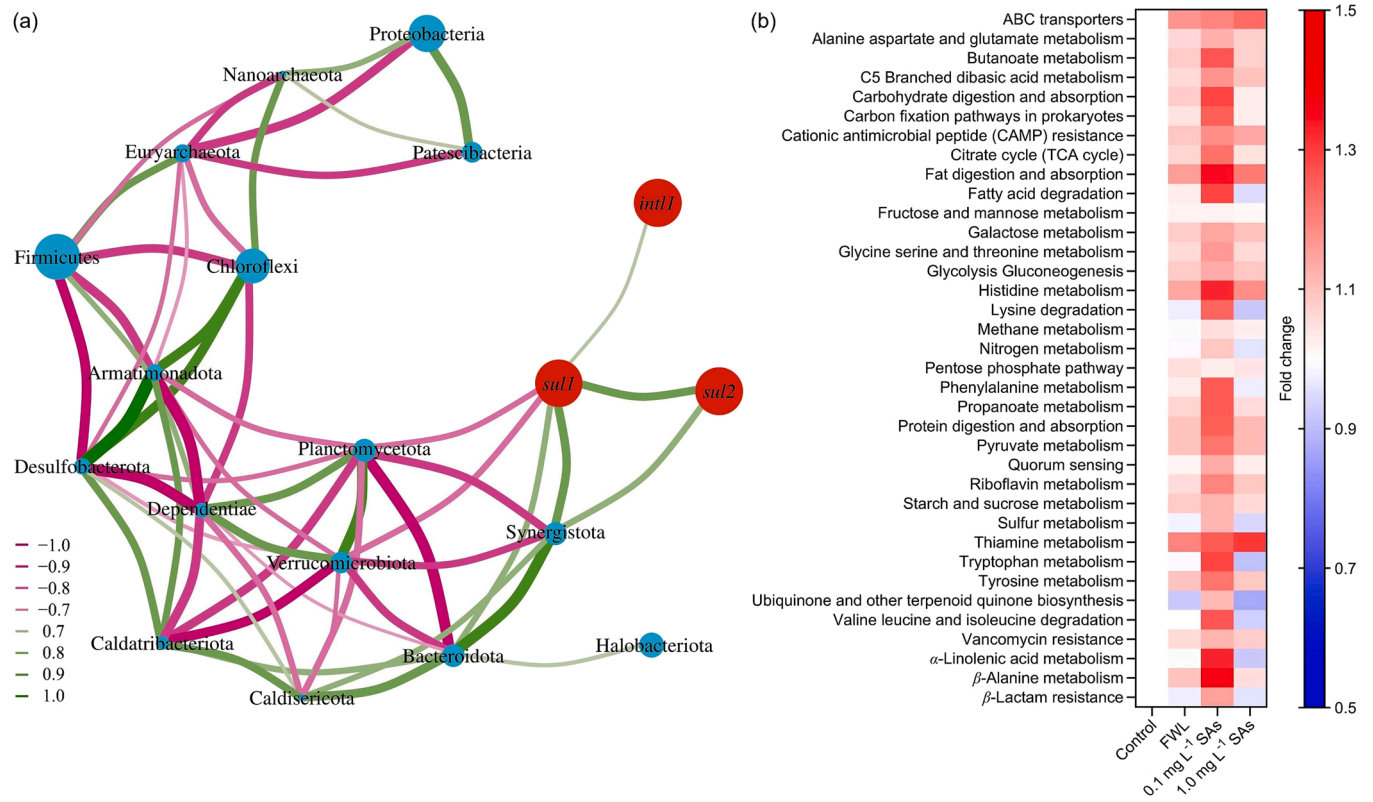

Fig. 6. Correlation matrix (Pearson's correlation coefficients > 0.6 and $p < 0.05$) among major microbial phyla, sulfonamide drug resistance genes *sul1* and *sul2*, and class 1 integron gene *intl1* over the digestion period (a) and changes in major metabolic activities during the exponential phase of different digestion sets (b). Differences in thickness represent variation in significant correlations ($r > 0.6$ or $r < -0.6$).

the phyla Actinobacteriota, Bacteroidota, Chloroflexi, Firmicutes, Proteobacteria, and Synergistota, which rapidly utilized substrates during AD. This microbial shift accelerated substrate digestion and absorption through active transport; butanoate, propanoate, and pyruvate metabolism; glycolysis gluconeogenesis; the citrate cycle (TCA cycle); and the pentose phosphate pathway. Versatile Methanosarcinales (*Methanosaeta* and *Methanosarcina*) played a predominant role in methanation through acetoclastic methanogenesis. The induction of riboflavin metabolism and quorum sensing established syntrophy among VFA oxidizers and methanogenic archaea. Syntrophic associations of hydrogenotrophic methanogens with their acidogenic and acetogenic partners in the phyla Bacteroidota, Firmicutes, and Proteobacteria stabilized the digester performance for improved methanation. The coexistence of *intl1* and *sul1* genes in the pathogenic members of the phyla Chloroflexi, Firmicutes, and Patescibacteria facilitated *intl1*-based HGT of ARGs and proliferation of ARMs during AD. The transmission of *sul2* was independent of *intl1* dissemination among the Synergistota. The performance of methanogenic archaea was not affected by SAs, regardless of the antibiotic concentrations, and methanogens were shielded against any interactions with *sul* genes or *intl1*. The emergence of CAMP, vancomycin, and β-lactam resistance in the core microbiota of the digesters suggests bacterial transformation towards multidrug resistance during SA-spiked AD of FWL. Thus, the presence of SAs in wastewater sludge could enrich environmental resistomes through the transformation of digester microbiota during AD despite improved digester performance.

Environmental Implication

Sulfonamide antibiotics are frequently detected in the influents and effluents of wastewater treatment plants worldwide because of their refractory characteristics, which exert selection pressure towards vertical and horizontal gene transfer among and across bacterial populations and intensify the emergence of antibiotic-resistant microorganisms. This study elucidates the dissemination patterns of antibiotic resistance genes (ARGs) among the members of core microbiota during anaerobic digestion of food waste leachate and the performance of core microbiota under various sulfonamide antibiotics pressure. The observed development of multidrug resistance in the digester microbiota probably occurred during digestion due to ARG transfer, although methanogens appeared unaffected from such transfer phenomena leading to antibiotic resistance.

CRediT authorship contribution statement

Shouvik Saha: Conceptualization, Methodology, Validation, Data analysis, Investigation, Data curation, Visualization, Funding acquisition, Project administration, Writing – original draft, oreview, & editing. **Jiu-Qiang Xiong:** Data analysis, Investigation, Data curation, Writing – review & editing. **Swapnil M. Patil**: High-throughput sequencing and data analysis. **Geon-Soo Ha:** Writing – review & editing. **Jeong-Kyu Hoh:** Writing – review & editing. **Hyun-Kyung Park:** Funding acquisition, Project administration. **Woojin Chung:** Writing – review & editing. **Soon Woong Chang:** Writing – review & editing. **Moonis Ali Khan:** Writing – review & editing. **Ho Bum Park:** Writing – review & editing. **Byong-Hun Jeon:** Resources, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.131200.

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