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Enhancing anaerobic digestion of vegetable waste and cellulose by bioaugmentation with rumen culture

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Abstract. Anaerobic digestion (AD) has been widely used to valorize food waste (FW) because of its ability to convert organic carbon into CH_4 and CO_2. Korean FW has a high content of fruits and vegetables, and efficient hydrolysis of less biodegradable fibers is critical for its complete stabilization by AD. This study examined the digestates from different anaerobic digesters, namely Rs, Rr, and Rm, as the inocula for the AD of vegetable waste (VW) and cellulose (CL): Rs inoculated with anaerobic sludge from an AD plant, Rr inoculated with rumen fluid, and Rm inoculated with anaerobic sludge and augmented with rumen fluid. A total of six conditions (3 inocula × 2 substrates) were tested in serial subcultures. Biogas yield was higher in the runs inoculated with Rm than in the other runs for both VW (up to 1.10 L/g VS added) and CL (up to 1.05 L/g VS added), and so was biogas production rate. The inocula had different microbial community structures, and both substrate type and inoculum source had a significant effect on the formation and development of microbial community structures in the subcultures. The overall results suggest that the bioaugmentation with rumen microbial consortium has good potential to enhance the anaerobic biodegradability of VW, and thereby can help more efficiently digest high fiber-content Korean FW.

Keywords: anaerobic digestion; bioaugmentation; rumen culture; vegetable waste; cellulose

1. Introduction

Food waste (FW) management has become an increasingly challenging problem worldwide with population and economic growth. FW is rich in readily biodegradable organic compounds and causes serious environmental pollution if not properly treated. Anaerobic digestion (AD) is widely used to manage FW because of its ability to mineralize organic pollutants into CH_4 and CO_2. Korean FW is characterized by a high content of fruits and vegetables (approximately 55%) (Kafle and Kim 2013), which increases to an even higher level during "Kimjang" season, resulting from the preparation of large amounts of kimchi (a traditional Korean fermented vegetable product) in late autumn. A great quantity of vegetable waste is produced during this season, and Napa cabbage, the most common ingredient in kimchi, accounts for more than 20% of the total FW production. Napa cabbage contains a high content of dietary fiber including cellulose, lignin, and non-cellulosic polysaccharides (approximately 26–32% in dry weight and 1–2% in wet weight) (Lee and Lee 1993, Hwang *et al*. 1996). Complex fibers are not easily degraded, and therefore, efficient hydrolysis of less biodegradable fibers may be a key to enhancing the biomethanation of Korean FW.

Previous studies have tried to enhance the hydrolysis of fibrous compounds by conditioning the substrate using different methods, including thermal/thermochemical (Wang *et al*. 2006, Vavouraki *et al*. 2013), microaeration (Lim and Wang 2013), microwave (Marin *et al*. 2010), biological (Ray *et al*. 2010), and enzymatic (Moon and Song 2011) pretreatments and combinations thereof. Bioaugmentation, introducing exogenous microorganisms with desired metabolic functions into a microbial system, is also considered a viable approach to improving the biodegradability of fibers. A key factor in bioaugmentation is to choose appropriate microorganisms that are able to survive and retain the desired functions in a given environment (Sharma and Melkania 2018). Bioaugmentation can be performed by adding one or more known species or a mixed consortium of diverse species. The latter could be more advantageous in maintaining a robust function of the augmented system, given that bioaugmentation is basically a method to exogenously increase the functional diversity and redundancy of a microbial community. Natural fiber-degrading systems, such as rumen, can be a source of mixed consortium for bioaugmentation to promote the degradation of fibers. The rumen microbial consortium is a robust, naturally-formed anaerobic consortium that is versatile in hydrolyzing and fermenting various organic substances including complex fibrous compounds (Jin *et al*. 2018).

Rumen microorganisms interact with each other to degrade and convert complex macromolecules into simple molecules, e.g., organic acids and H_2/CO_2, and finally into CH_4. This allows for maintenance of a low hydrogen partial

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pressure in the rumen for efficient digestion of feed under anaerobic conditions (Zhou and Hernandez-Sanabria 2009). Rumen fluid or cultures have been employed as inoculum or to pretreat feedstocks for enhanced biomethanation of fiber-rich biomass, e.g., corn stover (Hu and Yu 2005), cattail (Zhao *et al*. 2009), rice straw (Zhang *et al*. 2016), and waste paper (Baba *et al*. 2013), because of its ability to effectively digest lignocellulosic compounds under anaerobic conditions. The potential to improve the AD of FW by using rumen fluid as inoculum has also been reported in mono-digestion (Jo *et al*.) and co-digestion (Aragaw *et al*. 2013). These suggest that bioaugmentation with rumen microbial consortia has good potential for enhancing the anaerobic degradation of vegetables in Korean FW.

This study aimed to examine whether the added rumen microorganisms can increase the anaerobic degradability of fibers and retain the enhanced AD performance in the augmented system. This is an important point to consider given the high seasonality in the production of vegetable waste (VW), which leads to large fluctuations in FW composition. Napa cabbage (a representative VW) and crystalline cellulose (a model cellulosic substrate, CL) were used as substrates for AD tests using anaerobic digestates from three different continuous FW digesters inoculated with anaerobic sludge and/or rumen fluid. Methanogenic activity of each inoculum was monitored in serial subcultures for each substrate to investigate how the different inocula responded and adapted to a new substrate over the subcultures. Denaturing gradient gel electrophoresis (DGGE) was conducted to characterize and compare the microbial community structures in the subcultures.

2. Materials and methods

2.1 Anaerobic subculture tests

VW was prepared by grinding uncooked napa cabbage waste collected from a cafeteria at UNIST with a household blender. Cellulose powder (medium fibers) was purchased from Sigma-Aldrich. Digestates from three different lab-scale continuous anaerobic digesters fed with FW, i.e., one inoculated with anaerobic sludge from a full-scale AD plant co-digesting FW and primary sewage sludge (Rs), one inoculated with rumen fluid collected through a rumen fistula from a cow (Rr), and one inoculated with anaerobic sludge and augmented with rumen fluid (Rm), were used as inocula for the AD tests. Collected digestates were sieved (mesh size, 860 μm) to remove coarse particles and starved under anaerobic conditions (14 days at 35°C) prior to inoculation to minimize the endogenous biogas production.

Six substrate-inoculum combinations (2 substrates × 3 inocula) were tested for biogas production in batch mode in parallel with five controls (2 without inoculum and 3 without substrate). Each run was triplicated, and a total of 33 bottles were prepared. All test bottles were serially subcultured for three cycles at 35°C for 30 days per cycle. The detailed experimental conditions for anaerobic subculture tests are described in Table 1. Subcultures were prepared in 120-mL serum bottles and flushed with nitrogen gas to remove oxygen in the headspace before being gas-tight sealed with rubber stoppers. The amounts of inoculum and substrate added to a test bottle were adjusted according to the available amount of inoculum, i.e., digestate from the preceding subculture cycle, over subcultures to maintain the substrate-to-inoculum (S/I) ratio (on a volatile solids (VS) basis) at similar levels. The remaining volume in the test bottle was filled with distilled water as necessary. Biogas production from each bottle was periodically measured using a gas-tight syringe and corrected to standard temperature and pressure (0°C and 1 bar).

Table 1 Experimental conditions for subculture tests

	Cycle 1		Cycle 2		Cycle 3	
Substrate	VW	CL	VW	CL	VW	CL
Inoculum[a]	600	600	203	203	84	84
CL[a]	0	346	0	106	0	42
VW[a]	510	0	138	0	60	0
Test volume[b]	80	80	50	50	50	50
S/I ratio[c]	0.85	0.58	0.68	0.52	0.71	0.5

[a] mg VS
[b] mL
[c] Substrate-to-inoculum ratio determined on a VS basis

2.2 Molecular fingerprinting and sequencing

Digestate was collected from a randomly selected bottle of each triplicate culture at the end of each subculture cycle and analyzed, along with the inocula, for microbial community structure. Total DNA was extracted from the inocula and digestate samples using an automated nucleic acid extractor (Exiprogen, Bioneer, Daejeon, Korea) as previously described (Kim and Lee 2015). The purified DNA was eluted in 200 μL of elution buffer and stored at –20°C until use. Archaeal and bacterial 16S rRNA genes were amplified by touch-down polymerase chain reaction (PCR) using ARC787F/1059R and BAC338F/805R primer sets with GC clamps attached, respectively, and analyzed by DGGE as previously described (Kim and Lee 2015). The DGGE gels were stained with SYBR Safe DNA gel stain (Molecular Probes, Eugene, OR, USA) and visualized under blue light transillumination. Selected bands were cut out of the gel and eluted in 40 μL of sterile PCR-grade water. An aliquot of each elution was amplified by PCR using the same primer sets as for DGGE but without the GC clamp. The obtained amplicons were cloned (pGEM-T Easy vector (Promega, Madison, WI, USA)) and sequenced. The retrieved 16S rRNA gene sequences were compared against the GenBank and RDP databases for phylogenetic affiliation. Taxonomic assignment of the retrieved sequences was performed using the RDP classifier at a bootstrap confidence threshold of 80%. The nucleotide sequences reported in this study have been deposited in the GenBank database: MH478173–478184.

2.3 Cluster analysis

A matrix each was generated from the archaeal and

bacterial DGGE gel images based on the relative intensity (normalized to total band intensity) and position of each band in each lane analyzed using TotalLab 1D image-processing software (TotalLab, Newcastle, UK). Non-metric multidimensional scaling (NMS) was conducted on the obtained matrices to visualize the direction and magnitude of changes in the archaeal and bacterial community structures. Calculations for ordination were performed based on the Sorensen distance measure (McCune *et al*. 2002) using PC-ORD 6 software (MjM software, Gleneden Beach, OR, USA).

2.4 Analytical methods

Solids were measured according to the protocols in Standard Methods (APHA-AWWA-WEF 2005), and pH was determined using a pH meter (Orion 3-Star, Thermo Scientific, MA, USA). Biogas composition was determined using an Agilent 7820A gas chromatograph (Palo Alto, CA, USA) coupled with a thermal conductivity detector and a ShinCarbon ST column (Restek, Bellefonte, PA, USA). All analyses were replicated at least twice.

3. Results and discussion

3.1 Biogas production

No significant biogas production was found in the substrate-only control, and the biogas production from each run was corrected by subtracting that of its inoculum-only control. Biogas production started immediately after the initiation of incubation without a lag phase in the runs with VW, while a lag phase of up to 6 days was observed in those with CL, in the subcultures (Fig. 1). This suggests that all inocula required an adaptation time to be able to utilize CL regardless of the inoculum source, and VW contains a considerable amount of more readily utilizable organic compounds than CL. Interestingly, a stagnant phase occurred during the mid-incubation period in the VW runs, particularly in the first cycle, but not in the runs with CL. The biphasic biogas production pattern likely reflects the sequential utilization of more and less easily biodegradable organic components of VW. Acidification, i.e., accumulation of acids with fermentation of easily biodegradable matter, can inhibit methanogenesis and also be a reason for a stagnant phase (Wang *et al*. 2014). Several studies have reported the inhibition of methanogenesis by acidification in AD processes treating VW (Bouallagui *et al*. 2003, Garcia-Peña *et al*. 2011, Lü *et al*. 2012). However, given that the mixed-liquor pH was higher than 7.2 at the end of each subculture cycle for all runs and the S/I ratio was in an appropriate range for the biogas potential assay (Li *et al*. 2011, Kafle *et al*. 2014), the temporary stagnation of biogas production was unlikely to be caused by acidification. CL should first be hydrolyzed by extracellular enzymes to be used for subsequent acidogenesis and methanogenesis (Bouallagui *et al*. 2005). The results suggest that cellulolytic activity was low in the inocula but increased with subculturing, as seen by the decrease in lag time with cycles.

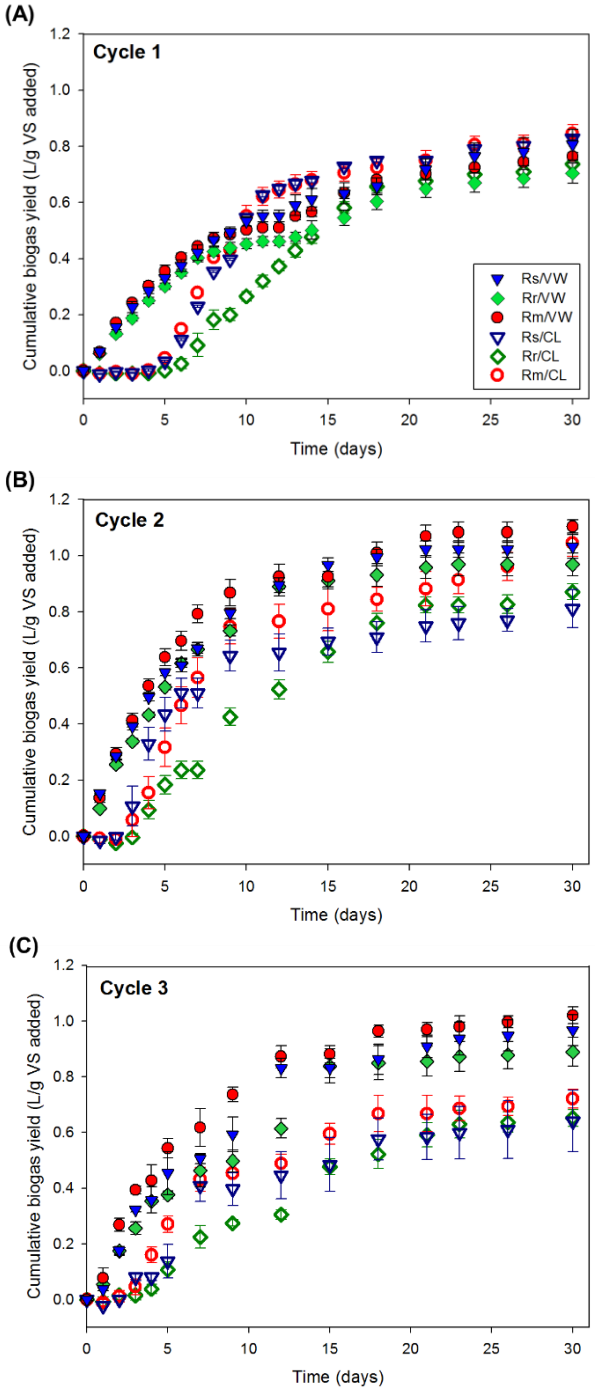

Fig. 1 Cumulative biogas yield during subcultures. Curves are labeled with the corresponding inoculum sources and substrates (CL, cellulose)

Despite the lag phase of 5–6 days, the CL runs reached comparable or greater cumulative biogas yield than the VW runs after 30 days of incubation in Cycle 1. This may be explained in part by the need for the inocula to adapt to be able to grow on the less biodegradable fibers of VW after the depletion of readily utilizable organic matter. The VW runs were subjected to such metabolic stress after 10 days of incubation, and the time allowed for digesting less biodegradable components (approximately 15 days) might not be sufficient for their complete utilization. Another

Table 2 Modified Gompertz parameters estimated from the subculture experiments

Inoculum	Rs			Rr			Rm		
Subculture cycle	C1	C2	C3	C1	C2	C3	C1	C2	C3
VW tests									
Biogas yield[a]	0.81	1.03	0.97	0.70	0.97	0.89	0.76	1.10	1.02
B_P[b]	0.77	1.02	0.94	0.68	0.96	0.90	0.73	1.04	0.98
R_{max}[c]	0.14	0.27	0.21	0.11	0.28	0.17	0.13	0.33	0.25
λ[d]	–[e]	–	–	–	–	–	–	–	–
r^2	0.98	0.99	0.98	0.96	0.99	0.98	0.96	0.98	0.98
CL tests									
Biogas yield	0.83	0.81	0.64	0.74	0.87	0.65	0.85	1.05	0.72
B_P	0.78	0.73	0.59	0.73	0.86	0.68	0.78	0.91	0.68
R_{max}	0.30	0.35	0.17	0.17	0.18	0.11	0.28	0.36	0.19
λ	5.00	1.90	2.34	5.95	3.03	3.01	4.51	2.74	1.79
r^2	1.00	0.98	0.97	1.00	0.99	0.99	0.99	0.98	0.98

[a] The observed biogas yield (L/g VS added).
[b] B_P, the biogas yield potential (L/g VS added).
[c] R_{max}, the maximum biogas production rate (L/g VS added·d).
[d] λ, the lag phase length (day).
[e] Not detected.

possibility may be the high content of complex fibers with low biodegradability in VW (Lee and Lee 1993, Hwang *et al*. 1996), which can limit the utilization of VW. However, it appears that the former is more likely than the latter, given that the VW runs showed greater biogas yields than the CL runs in Cycles 2 and 3. The methane content was maintained fairly constant at 50–60% in the VW runs over subculture cycles, while it remained at lower levels (≤50%) in the CL runs. This may be attributed to the different characteristics of the substrates.

The cumulative biogas production profiles of the subculture tests were fitted to a modified Gompertz equation (Eq. 1) to describe the biogas production kinetics:

$$B_t = B_P \cdot \exp\left[-\exp\left\{\frac{R_{\max} \cdot e}{B_P}(\lambda - t) + 1\right\}\right] \quad (1)$$

where B_t is the cumulative biogas yield (L/g VS added) at time t, B_p is the biogas yield potential (L/g VS added), R_{max} is the maximum biogas production rate (L/g VS added·d), λ is the lag phase length (day), and t is the incubation time (day). All runs showed a good fit to the equation with a high regression coefficient ($r^2 > 0.96$). The estimated model parameters are presented in Table 2.

Consistently with the experimental observations, a lag phase was identified in all runs with VW but not in the runs with CL. The estimated lag length decreased greatly in the subsequent subcultures compared to the initial culture. This suggests that the cellulolytic activity of the inoculated microbial consortia increased while adapting to the culture conditions using CL as the sole carbon source with cycles. It is interesting to note that after the initial adaptation to new substrates (i.e., VW and CL) during Cycle 1, the runs inoculated with Rm showed superior performance, in terms of biogas yield (by 5.2–14.6% based on the observed yields) as well as production rate (by 17.9–47.1% in R_{max}), to the runs inoculated with the other inocula for both substrates. This indicates that the Rm microbial consortium responded most favorably and readily to the sudden substrate changes from FW to VW or CL. This appears to be related to the higher microbial diversity of Rm than of the other inoculum sources, given the inoculation and bioaugmentation history of the source digesters (see Subsection 2.1). More diverse microbial communities would be expected to have higher chances of being functionally more versatile and redundant, which can be beneficial in adapting to changes in the environment. The experimental results suggest that the bioaugmentation with rumen fluid had a positive effect on the metabolic capability of the Rm microbial consortium, particularly the fiber-degrading activity. It is notable that the differences in performance according to the inoculum source were more pronounced in reaction rate than biogas yield. This indicates that the beneficial effect of using the bioaugmented Rm inoculum was primarily on the hydrolysis and fermentation, which limit the overall reaction rate in the AD of complex fibers, rather than the methanogenesis.

All subculture runs showed the highest biogas production in Cycle 2, except the CL run inoculated with Rs, where the biogas yield in Cycle 2 was slightly lower but comparable to that in Cycle 1. Interestingly, an apparent reduction in biogas yield was observed between Cycles 2 and 3 in all runs despite the serial subculture in batch mode would provide a stable environment for microbial adaptation and growth (Kim *et al*. 2013). This may be attributed to the decrease in the absolute amount of inoculum added to an assay over subculture cycles (Table 1). The inoculum size decreased by approximately 60–65% each cycle because the digestate from a test run after a cycle was used as inocula for the test subculture and the inoculum-only control bottles in the next cycle. Given that

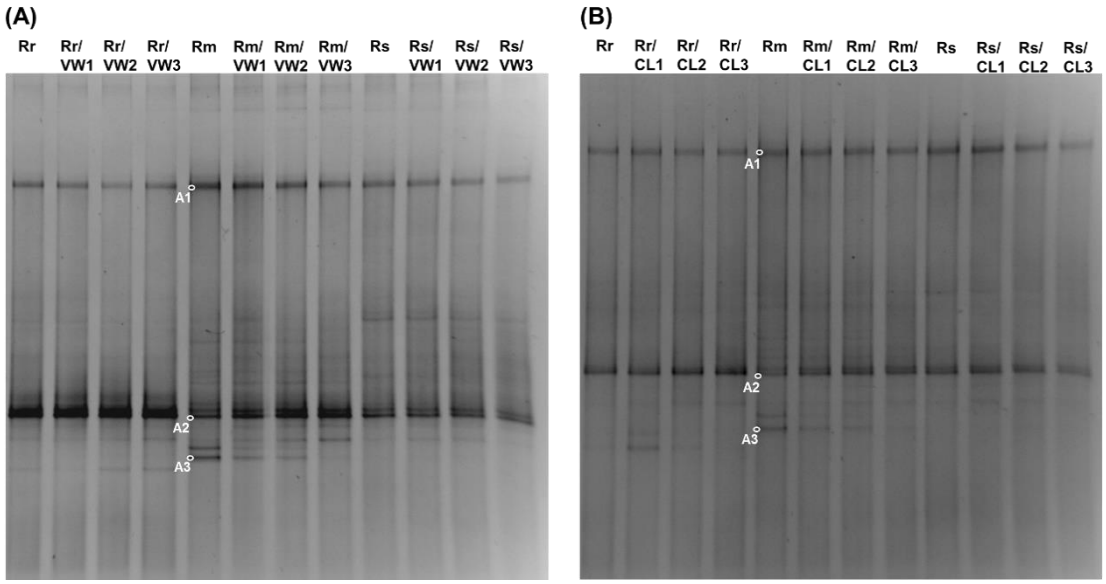

Fig. 2. Archaeal DGGE fingerprints of the anaerobic subcultures with VW (A) and CL (B). Lanes are labeled with the inocula and subculture cycles

inoculum size is a crucial factor affecting microbial growth and activity in AD processes (Divya *et al*. 2015, Ge *et al*. 2016), this may have adversely affected the methanogenic performance in Cycle 3.

3.2 DGGE results and phylogenetic affiliation

The microbial community structures in the test runs at the end of each subculture cycle and the inocula were analyzed by DGGE and sequencing. Since the DGGE gels were run separately according to the substrate used for the subculture tests, two gel images each were produced for archaea and bacteria. The images were aligned using the DNA samples of the inocula, which were loaded on both gels, as position markers to compare band patterns between the gels. Three archaeal (A1 to 3) and nine bacterial (B1 to 9) bands were selected and cut out of the gels for sequencing analysis (Figs. 2 and 3). The phylogenetic affiliations of the retrieved sequences are summarized in Table 3.

All archaeal sequences were assigned to methanogen genera, agreeing with the general understanding that archaea in AD environments are mostly methanogens. A1 was assigned to hydrogenotrophic *Methanolinea*, while A2 and 3 were assigned to aceticlastic *Methanosaeta* and *Methanosarcina*, respectively. A1 and 2 appeared as predominant bands in all lanes, indicating that the corresponding *Methanolinea* and *Methanosaeta* populations were likely the major methanogens throughout the subculture regardless of inoculum or substrate. These suggest that methanogenesis occurred through both hydrogenotrophic and aceticlastic pathways in the subcultures and inoculum sources. *Methanolinea* and *Methanosaeta* have frequently been found in various AD processes treating different types of waste (Lee *et al.* 2010, Shin *et al*. 2010, Bialek *et al*. 2011). Given that *Methanolinea* species are hydrogenotrophic but require acetate for growth (Imachi *et al*. 2008), the organism represented by A1 likely contributed not only to the scavenging of hydrogen but also partly to the consumption of acetate. *Methanosaeta* species are strictly aceticlastic and known to play a key role in stabilizing an AD system to maintain low levels of residual acetate and other volatile fatty acids (VFAs) (Ahring 2003). The organism corresponding to A2 seems to be primarily responsible for this role in the subcultures. The *Methanosarcina* population corresponding to A3 appeared only in the subcultures inoculated with Rm. *Methanosarcina* species are metabolically versatile and able to utilize H_2/CO_2 and simple methyl compounds other than acetate, and their growth is reportedly favored at relatively high concentrations of residual VFAs (Ahring 2003). A point to note is that A3 showed the highest band intensity in the Rm inoculum and gradually disappeared with subculture cycles for both substrates. This suggests that Rm was presumably under more favorable conditions for *Methanosarcina* to grow (i.e., greater amounts of residual fermentation intermediates) compared to the other inoculum sources.

The bacterial sequences retrieved from the DGGE bands were assigned to three phyla *Bacteroidetes* (B1, 2, 3, 4, and 6), *Firmicutes* (B5, 7, and 8), and *Actinobacteria* (B9), commonly present in AD environments (Fig. 3 and Table 3). The *Bacteroidetes*-related bands appeared in all lanes although their intensities varied greatly, particularly according to the inoculum source. B1 was observed with a strong intensity in all lanes, indicating that the corresponding bacterium was commonly abundant in all inocula and able to grow well regardless of substrate. B2 and 3 appeared as more prominent bands in the subcultures inoculated with Rm or Rs, and so did B4 in the Rr-inoculated subcultures. Although their roles are unclear, the *Bacteroidetes*-related bacteria were likely involved in the hydrolysis of cellulose and other fibers given that *Bacteroidetes* species have been reported to play a key role

Table 3 Phylogenetic affiliation of the 16S rRNA gene sequences from DGGE bands

Band	Closest relatives	Accession number	Similarity (%)	Classification[a]
Archaea				
A1	*Methanolinea tarda*	NR028163	97.4	*Methanolinea*
A2	*Methanosaeta harundinacea*	NR043203	98.2	*Methanosaeta*
A3	*Methanosarcina spelaei*	NR148337	99.6	*Methanosarcina*
Bacteria				
B1	Uncultured bacterium clone 39909	MF769179	100.0	*Prolixibacteraceae*
	Prolixibacter denitrificans	NR137212	86.8	
B2	Uncultured bacterium clone CloningB5+C09	AB997663	100.0	*Bacteroidales*
	Natronoflexus pectinivorans	NR108635	87.7	
B3	Uncultured bacterium clone JKB083	LN624310	100.0	*Bacteroidales*
	Tangfeifania diversioriginum	NR134211	89.6	
B4	Uncultured bacterium clone TC(4)9	KJ734920	99.8	*Bacteroidetes*
	Solitalea canadensis	KF528160	88.4	
B5	Uncultured bacterium clone dgD-107	AB264072	98.0	*Lachnospiraceae*
	Lachnospira multipara	NR104758	96.6	
B6	*Petrimonas sulfuriphila*	LT558828	99.8	*Petrimonas*
B7	Uncultured bacterium clone CloningB3A07	AB997288	99.8	*Ruminococcaceae*
	Saccharofermentans acetigenes	NR115340	87.4	
B8	Uncultured bacterium clone QEDN5CD04	CU926267	100.0	*Firmicutes*
	Desulfotomaculum alcoholivorax	NR042970	86.6	
B9	*Atopobium* sp. canine oral taxon 418	KF030213	99.3	*Coriobacteriaceae*

[a] The lowest rank assigned by the RDP Classifier at a bootstrap cutoff of 80%

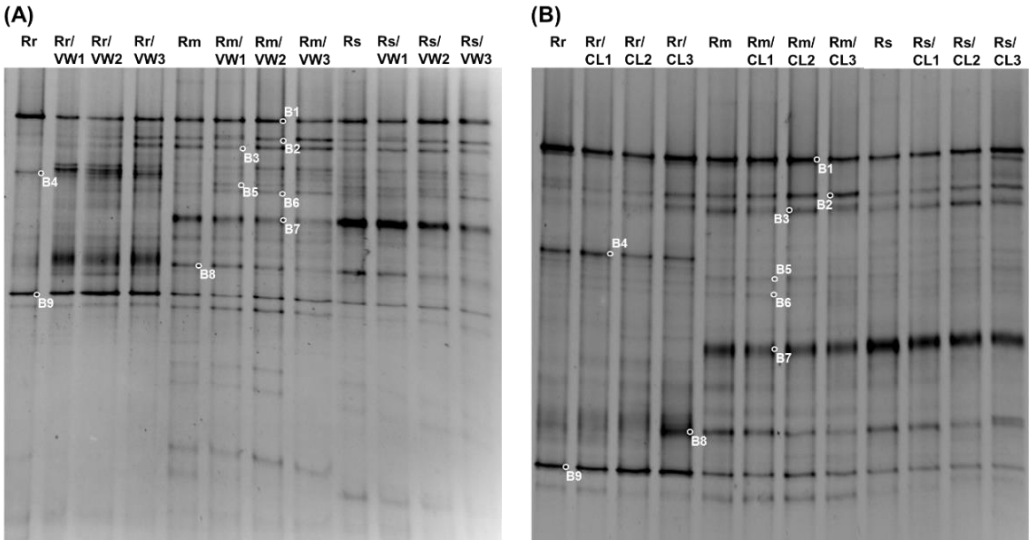

Fig. 3. Bacterial DGGE fingerprints of the anaerobic subcultures with VW (A) and CL (B). Lanes are labeled with the inocula and subculture cycles

in decomposing cellulosic matter in AD processes (Hatamoto *et al.* 2014). B6, the only one classified at the genus level, was closely related to *Petrimonas sulfuriphila* capable of utilizing glucose and cellobiose to produce acetate, H_2/CO_2, and H_2S (Grabowski *et al.* 2005). B5 and 7 were assigned to the families *Lachnospiraceae* and *Ruminococcaceae*, respectively, belonging to the order *Clostridiales*. Members of the families are present in abundance in mammalian guts and can degrade various fibrous matter including recalcitrant compounds (Biddle *et al.* 2013). Although below the cutoff, B5 showed a considerable similarity of 96.6% to a pectin-hydrolyzing rumen bacterial species *Lachnospira multipara* (Dušková and Marounek 2001). Given that B7 was observed only in

the subcultures inoculated with Rs or Rm, the bacterium represented by this band likely originated from the anaerobic sludge used to inoculate Rs and Rm (see Subsection 2.1). B9 was closely related to an *Atopobium* species whose relatives are commonly found in rumen and gut microbial consortia and able to ferment cellulose (Chassard *et al*. 2010, Mao *et al*. 2013). This band appeared with significantly higher intensity in the Rr-inoculated subcultures than in the Rm- and Rs-inoculated ones. The bacterium corresponding to B9 was likely to be a major cellulose degrader in Rr and the Rr-inoculated subcultures. Although most bacterial sequences were poorly related to known species, our results were in accordance with the finding that cellulose-degrading bacteria belong mainly to the phyla *Bacteroidetes* and *Firmicutes* in the human gut (Chassard *et al*. 2010).

3.3 Microbial community structure

Bacterial DGGE profiles were much more complex and dynamic then archaeal DGGE profiles in all subculture runs. This reflects that archaeal communities generally have less diverse structures than bacterial communities in AD processes, largely due to the very narrow substrate spectrum of methanogens (Zumstein *et al*. 2000, Kim *et al*. 2013). Fig. 4 shows the NMS plots describing the changes over subcultures in the archaeal and bacterial community structures. NMS is an ordination method which can reduce a DGGE profile generated from a microbial community (i.e., a DGGE lane) into a point in an ordination space so that communities with similar structures are closely located in the space. Both the plots showed acceptable stress (<20) and sufficiently low instability (10^{-4}) values, indicating that the ordination results provide a reliable picture of the changes in the microbial community structures in the subcultures (McCune *et al*. 2002). The cumulative r^2 for the ordination axes was 0.947 and 0.832 in the archaeal and bacterial NMS plots, respectively. This means that 94.7% and 83.2% of the total variance in the analyzed archaeal and bacterial community structures, respectively, can be explained by the obtained NMS plots. It is clearly shown in the NMS plots that both archaeal and bacterial community profiles are clustered according to the substrate and inoculum source rather than to the subculture.

This suggests that both substrate and inoculum characteristics likely had a significant influence on the development of microbial community structures in the subcultures, which agrees with previous findings in AD processes (Lee *et al*. 2009, Kim *et al*. 2013). Meanwhile, the changes in microbial community structure over subcultures were relatively minor. The archaeal and bacterial community structures of the subcultures inoculated with the same inoculum were clearly separated according to the substrate. Meanwhile, among the community profiles of the subcultures with the same substrate, those of the Rs and Rm subcultures were located close together, with those of the Rr subcultures being grouped separately. This would be expected given that Rm and Rs were initially inoculated with the same anaerobic sludge, although Rm was later augmented with rumen fluid (see Subsection 2.1). It is worth noting that the archaeal community structures

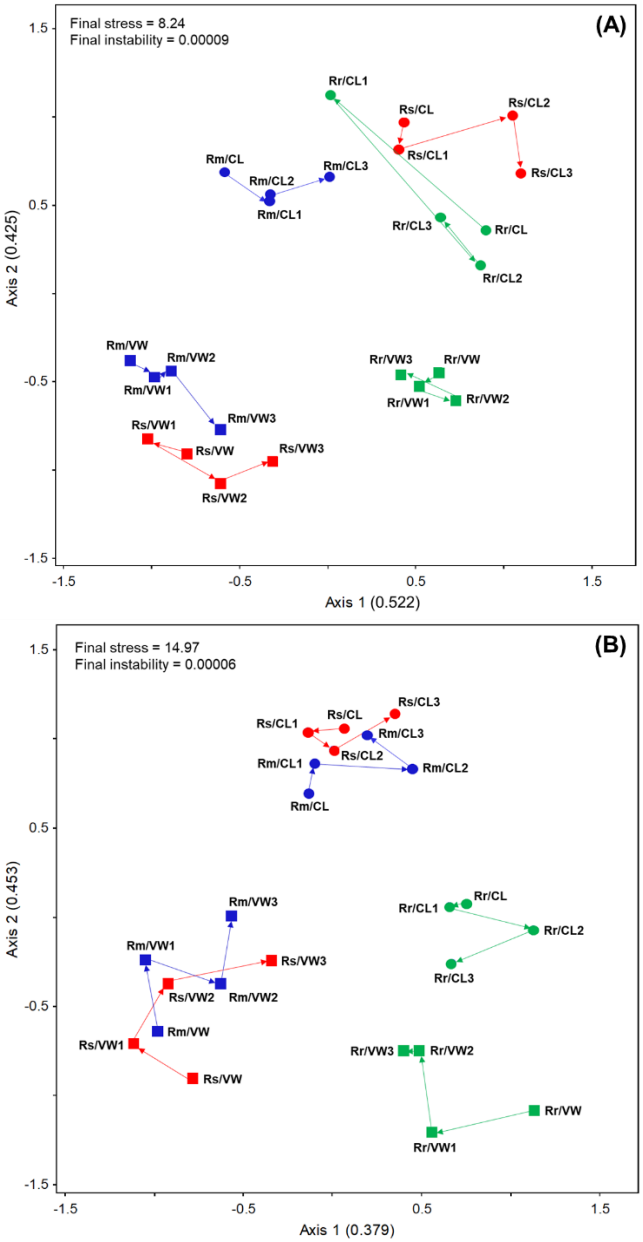

Fig. 4 NMS plots showing changes in the archaeal (A) and bacterial (B) community structures. Points are labeled with the corresponding inoculum sources followed by the substrates (CL, cellulose) and subculture cycles. Arrows indicate the shifts in community structure with subculture cycles in each run

showed a clearer separation between the subcultures inoculated with different inocula compared to the bacterial community structures. This indicates that the bioaugmentation of Rm with rumen fluid likely had a more significant effect on the archaeal community structure than the bacterial community structure in the digester. This possibility can be associated with the less diverse nature of archaeal communities than bacterial communities in AD environments (Zumstein *et al*. 2000), because a small change in band pattern (i.e., appearance or disappearance of one or a few populations) can result in a significant structural change in simple communities. It may also be

attributed in part to the distinct methanogen community structure in the rumen, often characterized by the high abundance of hydrogenotrophs, from those in typical anaerobic digesters (Zhou and Hernandez-Sanabria 2009).

The overall results suggest that the rumen microbial consortium augmented to Rm likely maintained the fiber-degrading activity and enhanced the AD of the fiber-rich substrates over repeated subcultures. Although further research is required to assess whether the beneficial effect can be maintained long term in continuous mode, it was demonstrated that the introduction of the rumen microbial consortium has promising potential as an approach to enhancing the anaerobic degradability of the fiber-rich fraction of FW, such as fruits and vegetables. Given that the inocula were sourced from three anaerobic FW digesters operated for more than ten turnovers of the working volume, the experimental results further suggest the possibility of using mixed-culture digestates from bioaugmented or co-inoculated digesters, like Rm in this study, as a microbial source for bioaugmentation. This may help avoid the difficulties in collecting large amounts of rumen fluid or culture for augmenting digesters.

4. Conclusions

This study investigated three inocula sourced from different anaerobic digesters, Rs, Rr, and Rm, for the anaerobic digestion of VW and CL in serial subcultures. For both substrates, the subcultures inoculated with Rm showed superior biogas yield and production rate to those inoculated with the other inoculum sources. Both archaeal and bacterial community structures in the subcultures were significantly influenced by the substrate characteristics and the inoculum source. The overall results suggest that the bioaugmentation with rumen microbial consortium has promising potential for the enhancement of the AD of fiber-rich substances. The outcomes of this study may help with more efficient treatment of large amounts of fruits and vegetables in the AD of Korean FW.

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