Addition of ginkgo fruit to cattle feces and slurry suppresses methane production by altering the microbial community structure

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Abstract

The effect of ginkgo fruit addition on methane production potential of cattle feces and slurry was assessed in relation to other fermentation products and the microbial community. Holstein cattle fresh feces and slurry were left at 30°C for 0, 30, 60, 90, and 180 days with/without ginkgo fruit to monitor the effect on fermentation potential. With the addition of ginkgo fruit, methane production potential of feces was reduced on day 30 and thereafter, and that of slurry was consistently reduced over the experimental period. As a general trend, ginkgo fruit addition resulted in decreased acetate and increased propionate in feces and acetate accumulation in slurry. With ginkgo fruit addition, Miseq analyses indicated decreases in methanogen (in particular *Methanocorpusculum*), Ruminococcaceae, and Clostridiaceae populations and increases in Bacteroidaceae and Porphyloromonadaceae populations, which essentially agreed with qPCR assay results. These data indicate that direct addition of ginkgo fruit to cattle excreta is useful for reducing methane emissions by altering the microbial community structure. The application of ginkgo fruit to lower methane emissions from cattle excreta is, therefore, useful in cases in which the excreta is left without special management for a long period of time.

Key words: feces, ginkgo fruit, methane, microbiota, slurry

1 | INTRODUCTION

Although methane emissions from ruminant animals derive primarily from rumen fermentation, emissions from feces are not negligible (Husted, 1994; Khan et al., 1997). Indeed, yearly global methane emissions from cattle feces are 7,500,000 tons, which is equivalent to 10-11% of all methane of rumen origin (FAO, 2006). Feces can be used as a source for biogas production worldwide to generate methane as a fuel (USEPA, 2004). However, such biogas facilities are not widely distributed due to the high cost of construction, even for smaller and less mechanically sophisticated facilities. As a result, feces are often left on the ground or piled up without special treatment for a long period of time, a practice that is common in developing countries. Such a situation allows for continuous emission of methane under natural conditions (Rastogi et al., 2008), which could contribute to the progression of global warming. Even in biogas facilities, significant amounts of methane can be emitted from fecal slurry deposited in pre-fermentation tanks (Mer et al., 2001; Feng et al., 2018), leading to greenhouse gas release in addition to loss of usable energy. These observations suggest that a strategy for minimizing methane emission from untreated animal excreta is needed.

Methane production from feces depends on both temperature and the duration of storage (Gupta et al. 2007); longer storage and higher temperatures promote methane synthesis (Zeeman, 1994). Previous attempts to manipulate methane emissions have examined the usefulness of regulating the carbon/nitrogen ratio in animal manure by adding wheat straw (Yamulki, 2005) and the effect of storing manure under cool conditions (Monteny et al., 2006). The rumen and feces differ in terms of anaerobicity, temperature, and moisture content, which impact the respective microbial communities. After excretion, the properties of feces change from anaerobic to aerobic, with a reduced

59 moisture content, leading to the dominance of aerobic bacteria (Wong et al., 2016). With
60 regard to methanogens, fresh feces are dominated by hydrogenotrophic
61 Methanobacteriales and Methanomicrobiales populations, whereas feces left untreated
62 for 8-24 months is dominated by acetoclastic methanogens (Rastogi et al., 2008). Thus,
63 methane generation processes can change as the storage period becomes longer, leading
64 to structural changes in the microbial community.

65 Ginkgo (*Ginkgo biloba*) fruit extract was found to suppress methane production in
66 the rumen by altering the rumen microbiota (Oh et al., 2017a). This effect was consistent,
67 irrespective of dietary conditions (Oh et al., 2017b). As the preparation of ginkgo fruit
68 extract is laborious and costly, a better option could be direct application of the fruit
69 (which is considered a useless byproduct in the ginkgo nut industry) to cattle excreta.
70 However, whether addition of gingko fruit to cattle feces and slurry effectively mitigates
71 methane emissions remains to be determined.

72 The present study hypothesized that adding ginkgo fruit to cattle excreta changes the
73 fermentation pattern toward a decrease in methane generation by altering the microbial
74 community structure. The effect of ginkgo fruit addition on methane production potential
75 was therefore evaluated using in vitro cultures, monitoring changes in fermentation
76 products and microbes in feces and slurry from Holstein cattle left untended for varying
77 periods of time.

78

79 **2 | MATERIALS AND METHODS**

80 **2.1 | Samples and incubations**

81 All protocols regarding fecal sampling from cattle followed The Act on Welfare
82 and Management of Animals (2005) and were conducted according to the Animal Study

83 Guidelines of Hokkaido University (2007) with the approval by Animal Care and Use
84 Committee of Hokkaido University (no.15-0122).

85 Ginkgo fruit (cultivar name, Kyuju) was obtained from a private ginkgo farm in
86 Sobue, Aichi Prefecture, Japan, which is a major ginkgo nut producing area, and then
87 physically mashed and separated using a hand-made machine. The ginkgo fruit samples
88 were frozen at −30°C prior to shipping to the laboratory. The frozen material was thawed
89 at room temperature prior to experimental use.

90 Fresh feces was sampled just before morning feeding (0830 hours) directly from
91 the rectum of 3 Holstein milking cows (723±39 kg body weight) at the Experimental
92 Farm, Field Science Center, Hokkaido University. The cows had been given the TMR
93 consisting of corn silage and commercial formula feed (17% crude protein (CP) and 72%
94 total digestible nutrients). Collected feces were equally mixed and separated into 10
95 portions, each of which was placed in a plastic container (150 × 100 × 40 mm). The
96 containers were divided into 2 groups, one of which was supplemented with ginkgo fruit
97 (treatment), whereas the other group served as the un-supplemented control (control).
98 Ginkgo fruit was added at 6.4% (12.8 g ginkgo fruit/200 g feces), a value determined
99 from pilot study results confirming methane mitigation. All containers were covered with
100 a plastic lid and left for 0, 30, 60, 90, or 180 days in an incubator at 30°C. After incubation
101 for the above specified period, samples were taken from the treatment and control
102 containers and used for the following batch culture study to monitor fermentation
103 products and microbiota. The day zero sample consisted of feces mixed with ginkgo fruit
104 and analyzed immediately without incubation. Cattle slurry (1.5 L) was sampled from a
105 slurry tank at the same farm, thoroughly mixed, and employed for the study in the same
106 manner as the fecal samples.

On the specified days, samples were removed from the containers after mixing and dispensed into a Hungate tube with McDougal's buffer (McDougal, 1948) at a 1:1 ratio, and the headspace was flushed with N_2 gas. The tubes were fitted with a butyl rubber stopper and a plastic screwcap, and then incubated at 39°C for 24 h to monitor fermentation parameters. Quadruplicate (n=4) samples were incubated per treatment.

2.2 | Chemical analysis

Major components of experimental feeds and ginkgo fruit were analyzed according to AOAC (2016) and Van Soest et al. (1991). Alkylphenolics in ginkgo fruit (anacardic acids, cardanol, and cardol) were quantified by HPLC as described by Watanabe et al. (2010). Gases (H_2, CH_4, and CO_2) in batch cultures were analyzed using a GC-8A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with parallel Porapak Q columns (Waters, Milford, MA USA), Molecular Sieve 13X (Restek, Bellefonte, PA USA), and a thermal conductivity detector. Short-chain fatty acids (SCFAs) were analyzed as described by Oh et al. (2017a). In brief, culture fluid was mixed with 25% meta-phosphoric acid at a 5:1 ratio, incubated overnight at 4°C, and centrifuged at 10,000 × *g* at 4°C. The supernatant was mixed with crotonic acid as an internal standard and injected into a GC-14B gas chromatograph (Shimadzu, Kyoto, Japan) equipped with an ULBON HR-20M fused silica capillary column (0.53 mm i.d. × 30 m length, 3.0 μm film, Shinwa, Kyoto, Japan) and a flame ionization detector. Culture pH and ammonia nitrogen concentration were determined using an electrode (pH meter F21, Horiba, Kyoto, Japan) and spectrophotometrically using the indophenol reaction (Weatherburn, 1967), respectively.

131 **2.3 | Microbial analysis**

132 Samples for microbiological analysis were taken from each culture and
133 immediately frozen and kept at −80°C. DNA was extracted for microbial analysis using
134 the RBB+C (repeated bead beating plus column) method described by Yu and Morrison
135 (2004). The DNA was subjected to quantitative real-time PCR (qPCR) to determine the
136 abundance of representative bacterial groups, including total bacteria, total methanogens,
137 Ruminococcaceae, *Clostridium leptum* subgroup, and *Bacteroides-Prevotella-*
138 *Porphyromonas*. All qPCR details, such as primers, standards, PCR conditions, and
139 calculations, were as described by Myint et al. (2017), Watabe et al. (2018), and Yamada
140 et al. (2020). In brief, standard plasmids encoding the respective target gene sequences
141 were obtained by PCR cloning using target-specific primer sets. The copy number of each
142 standard plasmid was calculated using the molecular weight of nucleic acid and the length
143 (base pairs) of the cloned standard plasmid, as described by Koike et al. (2007). A
144 LightCycler system and a FirstStart DNA master SYBR I reaction kit (Roche, Penzberg,
145 Germany) were used with 10-fold serial dilutions of standard plasmid for the respective
146 target (16S rDNA sequence specific to each target microbe). Microbial quantity was
147 calculated using amplification curves obtained from both standards and samples. The
148 specificity of PCR amplification was confirmed by melting curve analysis of the PCR
149 products by increasing the temperature from 70°C to 95°C at a rate of 0.1°C/s. Microbial
150 abundance was shown by copy number of rDNA for total bacteria, or by relative
151 proportion of the total bacterial copy number for a specific microbial group.

152 To comprehensively analyze the microbial community, DNA samples were
153 analyzed using MiSeq (Illumina, San Diego, CA USA). The employed samples were
154 cultures from slurry left for 30 d with or without ginkgo fruit, as methane mitigation was

155 most apparent (*see* Results). Sequencing was performed by Hokkaido System Science
156 Co., Ltd. (Sapporo, Japan). The V3 to V4 regions were amplified using two primer sets,
157 S-D-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-
158 21 (5'-GACTACHVGGGTATCTAATCC-3') for bacterial rDNA (Herlemann et al.,
159 2011), and arch349F (5'-GYGCASCAGKCGMGAAW-3') and arch806R (5'-
160 GGACTACVSGGGTATCTAAT-3') for archaeal rDNA (Takai and Horikoshi, 2000).
161 PCR was carried out in 50 μL total volume: 10 μL 5× PrimeSTAR Buffer, 4 μL dNTP
162 mixture (2.5 mM each), 0.5 μL PrimeSTAR HS DNA polymerase (Takara Bio Inc.,
163 Kusatsu, Japan), 1 μL each primer (10 pmol/μL), 32.5 μL dH2O, and 1 μL template DNA
164 (10 ng/μL). The following PCR conditions were used: 30 cycles for bacteria and 40 cycles
165 for archaea, consisting of denaturation at 98°C (10 s), annealing at 55°C (15 s), and
166 extension at 72°C (30 s). Amplicon sequencing was carried out using MiSeq as described
167 by Caporaso et al. (2012). Data quality control and analyses were performed using the
168 QIIME pipeline, ver. 1.8.0 (Caporaso et al., 2010). Operational taxonomical units (OTUs)
169 were generated from sequences clustered at a 97% similarity threshold using the
170 UCLUST algorithm (Edgar, 2010). Chimeric sequences were removed from the analysis
171 using the ChimeraSlayer algorithm. Taxonomy was assigned using the Greengenes
172 database (ver. 13.8) at a 90% similarity threshold. Differences in biodiversity between
173 control and treatment were compared using alpha diversity metrics: Chao1, Ace, Shannon,
174 Simpson, and observed number of OTUs. The sequences obtained were deposited in the
175 DNA Data Bank of Japan nucleotide sequence database under accession no.
176 PRJNA684980.
177
178 **2.4 | Statistical analysis**

179 The data regarding fermentation profiles and microbiota with and without ginkgo
180 fruit addition (n=4) were analyzed using the Student t-test of SPSS (version 16.0 J, Tokyo,
181 Japan). Statistical significance and trends were declared at $P<0.05$ and $P<0.10$,
182 respectively.
183

184 **3 | RESULTS**

185 The experimental ginkgo fruit had 80.0% moisture and contained 6.6% CP, 20.0%
186 neutral detergent fiber, 33.8% non-fibrous carbohydrate, and 35.8% ether extract (all on
187 a dry matter basis). The fruit also contained 54.32 μg/g total alkyl-phenols, of which the
188 proportions of C13:1, C15:1, and C17:1 anacardic acid were 9.5, 54.1, and 21.4%,
189 respectively, whereas those of C15:1 caldol and C15:1 cardanol were 12.7 and 2.2%,
190 respectively.

191 Gas production potentials assessed in batch cultures are shown in Table 1. In fecal
192 cultures, ginkgo fruit addition increased the production potentials of total gas, CO_2, and
193 CH_4 for the day 0 samples. However, production of all of these gases decreased with
194 ginkgo fruit addition in feces left for 30 d or longer. In slurry cultures, similar responses
195 to ginkgo fruit addition were observed, with the exception that the methane production
196 potential of slurry decreased with ginkgo addition even in the day 0 samples. No hydrogen
197 was detected in any sample throughout the experiment, irrespective of ginkgo fruit
198 addition.

199 Table 2 shows the SCFAs, ammonia, and pH of the cultures prepared from feces
200 and slurry left for different periods of time with or without ginkgo fruit addition. On day
201 0, the concentrations of total SCFAs and individual SCFAs increased with ginkgo fruit
202 addition in both fecal and slurry cultures. These changes were accompanied by decreases

203 in pH and ammonia. In feces left for ≥30 days with ginkgo fruit addition, acetate
204 decreased, whereas propionate and ammonia increased, even though some exceptions
205 were observed. In slurry left for ≥30 days, SCFA levels were below the quantification
206 limit for the control, but a small amount of acetate was detected in cultures with ginkgo
207 fruit addition.

208 Microbes identified in feces and slurry by qPCR analysis are listed in Table 3.
209 Similar responses to ginkgo fruit addition were observed in both feces and slurry. The
210 abundance of total bacteria, total methanogens, and Ruminococcaceae was lowered by
211 ginkgo fruit, whereas that of *Bacteroides-Prevotella-Porphyromonas* was increased
212 almost consistently in feces and slurry left for different periods of time. For the *C. leptum*
213 subgroup, the response to ginkgo fruit addition differed between feces and slurry, showing
214 that the abundance of the *C. leptum* subgroup varied with ginkgo fruit in feces, whereas
215 it decreased consistently in slurry throughout the experimental period.

216 MiSeq analysis of cultures prepared from slurry left for 30 d gave a satisfactorily
217 high number of reads: 14,657-18,779 (control) and 20,120-22,945 (ginkgo) for bacteria,
218 and 8,897-9,758 (control) and 14,377-16,670 (ginkgo) for methanogens. The diversity of
219 bacteria and methanogens in slurry is illustrated in Figure 1. All indices, including Chao
220 1, Ace, Shannon, Simpson, and OTUs, gave higher values in slurry cultures with ginkgo
221 fruit added.

222 The bacterial community structure of slurry was markedly influenced by ginkgo
223 fruit addition, as shown in Figure 2. At the family level, the detection frequency of
224 unclassified Bacteroidales, Clostridiaceae, Ruminococcaceae, and other families
225 decreased with ginkgo fruit addition, whereas that of Synergistaceae,
226 Porphyromonadaceae, Bacteroidaceae, and other families increased.

227 Figure 3 shows changes in the methanogenic archaeal community structure in slurry
228 resulting from ginkgo fruit addition. At the genus level, although *Methanocorpusculum*
229 was dominant in control cultures, *Methanoplanus* and *Methanobrevibacter* became
230 dominant in slurry cultures with ginkgo fruit added. In addition, *Methanogenium* and
231 *Methanosarcina* increased in slurry cultures with ginkgo fruit added.
232
233 **4 | DISCUSSION**
234 On day 0, all gas production potentials in feces and slurry increased with ginkgo
235 fruit addition (Table 1), which could have been caused by fermentation of the added fruit
236 itself. The increase in SCFA concentrations on day 0 (Table 2) reflects this assumption.
237 Indeed, ginkgo fruit contains carbohydrates (Oh et al., 2017a) that could be preferentially
238 fermented by fecal and slurry microbes. However, on day 30 and thereafter, the gas
239 production potentials, in particular that of methane, decreased consistently in both feces
240 and slurry (Table 1). Although changes in SCFA profiles with ginkgo fruit addition were
241 not consistent, propionate in feces increased in proportion on day 60 and after, whereas
242 acetate was dominant in slurry (Table 2). Thus, ginkgo fruit addition can modulate excreta
243 fermentation toward methane mitigation with changes in the SCFA profile, possibly by
244 influencing the microbiota (discussed below), as described for rumen fermentation (Oh
245 et al. 2017a; 2017b). Ginkgo fruit is a source of anti-bacterial alkyl-phenols, including
246 anacardic acid, which selectively inhibits gram-positive bacteria (Kubo et al., 1993).
247 Another source of alkyl-phenols is cashew nut shells, which are already in use as a feed
248 additive to modulate rumen fermentation (Kobayashi et al., 2016). With regard to gingko
249 fruit, Oh et al. (2017a) proposed the use of fruit extract to mitigate rumen-derived
250 methane in ruminant livestock. Furthermore, the present results suggest that direct

application of ginkgo fruit itself to animal excreta (rather than ginkgo fruit extract) works as a mitigating agent for methane production from feces and slurry.

Changes in the microbial community with ginkgo fruit addition were very similar between feces and slurry according to qPCR results; ginkgo fruit decreased populations of methanogens and bacteria possibly involved in hydrogen/formate production, such as Ruminococcaceae and the *C. leptum* subgroup, and increased *Bacteroides-Prevotella-Porphyromonas* (gram-negatives, including bacteria related to propionate production) (Table 3). These selective effects of ginkgo fruit can alter the excreta fermentation pattern by, for example, changing the hydrogen-utilization pathway to reduce methane production (Schink 1997).

The present qPCR results essentially agreed with the MiSeq results for slurry. Microbial populations that decreased with ginkgo fruit addition included Clostridiaceae and Ruminococcaceae, whereas populations that increased were represented by Porphyromonadaceae and Bacteroidaceae (Fig. 2). All of these changes are quite reasonable when considering the inhibitory action of alkyl-phenols against gram-positive organisms (Kubo et al. 1993; Watanabe et al. 2010; Oh et al. 2017a).

The methanogen community of slurry was also dramatically changed with ginkgo fruit addition (Fig. 3). The most dominant group in the control, *Methanocorpusculum*, was replaced by *Methanoplanus* and *Methanobrevibacter* with ginkgo fruit addition. *Methanocorpusculum* is common in feces of Holstein cattle (Liu et al., 2018), Altay sheep (Liu et al., 2012), and Korean native cattle (Daquiado et al., 2014) as a hydrogenotrophic methanogen. The susceptibility of various methanogen species to alkyl-phenols was examined to find that specific methanogens are sensitive to those compounds, particularly anacardic acid (Wakai et al. unpublished results). *Methanocorpusculum* might be one

275 such sensitive methanogen. When gingko fruit was added, *Methanoplanus* became
276 dominant (Fig. 3), which is in good agreement with results reported for the bovine rumen
277 in the presence of alkyl-phenol–containing cashew nut shell liquid (Su et al., 2021).
278 Methanomicrobiaceae, including *Methanoplanus*, express S-layer protein on the cell
279 surface (Sowers 2009), which might act as a barrier against surfactant alkyl-phenols (Su
280 et al., 2021).

281 *Methanosarcina* and *Methanosaeta* are acetoclastic methanogens that convert
282 acetate to methane (Schink et al., 1997). Ginkgo fruit addition to slurry lowered the
283 detection frequency of *Methanosaeta* and increased that of *Methanosarcina* (Fig. 3). The
284 former change could explain in part the acetate accumulation in slurry with ginkgo fruit
285 added (Table 2). In the genus *Methanosarcina*, *Methanosarcina mobile* is tolerant to
286 alkyl-phenols (Wakai et al., unpublished results), although the contribution of this
287 methanogen to acetate accumulation is unclear due to limited information regarding its
288 metabolic activity.

289 Increased microbial diversity resulting from ginkgo fruit addition (Fig.1) could be
290 caused in part by exogenous fruit-associated microbes. However, most of these organisms
291 would be aerobic, and few methanogens are likely associated with fruit because
292 methanogens are enriched only in the plant rhizosphere (Borrel et al., 2020). As the
293 bacterial and methanogenic archaeal community consisted mostly of anaerobic organisms
294 (Table 3, Figs. 2 & 3), it is reasonable to conclude that community changes resulting from
295 ginkgo fruit addition are primarily induced by the selection of indigenous microbes in
296 feces and slurry (not by fruit-associated microbes).

297 As indicated by gas and SCFA production results (Tables 1 & 2), ginkgo fruit serves
298 as an extra substrate for microbes in feces and slurry, in particular slurry in which little

fermentable substrate remains. Even in such cases, methane production was suppressed by ginkgo fruit addition, indicating that this agricultural byproduct works well for mitigating methane production originating from animal excreta left untended for a long period of time (at least 30 d). This effect is due to the selection of microbes in animal excreta. However, these possibilities must be experimentally confirmed in a practical-scale study at a facility equipped with a large manure storage area and slurry tanks.

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CONFLICT OF INTEREST

We certify that there is no conflict of interest related to the present study.

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Figure captions

Figure 1. Effect of gingko fruit addition on diversity indices of bacteria (top) and methanogenic archaea (bottom) in cattle slurry, as assessed by MiSeq analysis. Asterisk shows significant difference from control (P <0.05). Samples used were cultures from slurry left for 30 d with or without ginkgo fruit.

Figure 2. Effect of gingko fruit addition on bacteria of cattle slurry, as assessed by MiSeq analysis.
Data are shown at the phylum (top) and family (bottom) levels. Arrows in red and blue indicate significant (P <0.05) increase and decrease, respectively. Samples used were cultures from slurry left for 30 d with or without ginkgo fruit.

Figure 3. Effect of gingko fruit addition on methanogenic archaea of cattle slurry, as assessed by MiSeq analysis.
Data are shown at the phylum (top) and genus (bottom) levels. Arrows in red and blue indicate significant (P <0.05) increase and decrease, respectively. Samples used were cultures from slurry left for 30 d with or without ginkgo fruit.

TABLE 1 Effect of ginkgo fruit addition on in vitro gas production potential of cattle feces and slurry ileft for different periods

Incubation period	Treatment	Gases from feces (ml/g feces)			
		Total gas	CO_2	CH_4	H_2
0 day	Control	0.62	0.50	0.12	-
	Ginkgo	1.04 *	0.90 *	0.14 *	-
30 day	Control	0.43	0.28	0.15	-
	Ginkgo	0.29 *	0.23 *	0.06 *	-
60 day	Control	0.57	0.22	0.35	-
	Ginkgo	0.22 **	0.18 *	0.03 **	-
90 day	Control	0.37	0.27	0.1	-
	Ginkgo	0.24 **	0.22 †	0.02 **	-
180 day	Control	0.39	0.35	0.04	-
	Ginkgo	0.37	0.35	0.02 **	-

Incubation oeriod	Treatment	Gases from slurry (ml/g slurry)			
		Total gas	CO_2	CH_4	H_2
0 day	Control	0.69	0.50	0.19	-
	Ginkgo	1.09 *	0.97 *	0.12 *	-
30 day	Control	0.17	0.09	0.07	-
	Ginkgo	0.07 *	0.07 *	trace *	-
60 day	Control	0.11	0.08	0.03	-
	Ginkgo	0.06 †	0.06	trace *	-
90 day	Control	0.13	0.12	0.02	-
	Ginkgo	0.13	0.12	trace *	-
180 day	Control	0.22	0.22	0.01	-
	Ginkgo	0.27 **	0.26 **	trace *	-

Gas production potential was measured after incubating feces or slurry left for 0 - 180 days at 30℃ with or without gingko fruit.

†, *, **: Significantly different from control at $P < 0.1$, 0.05 and 0.01, respectively.

TABLE 2 Effect of ginkgo fruit addition on in vitro short chain fatty acid (SCFA) and ammonia production potential of cattle feces and slurry left for different periods

Incubation period	Treatment	Fermentation products from feces					pH
		Total SCFA	Acetate	Propionate	n-Butyrate	Ammonia	
		(mmol/g feces)				(mg N/g feces)	
0 day	Control	0.34	0.23	0.07	0.02	0.22	7.03
	Ginkgo	0.56 *	0.37 *	0.12 *	0.06 *	0.17 †	6.61 *
30 day	Control	0.23	0.20	0.17	-	0.26	7.54
	Ginkgo	0.1 *	0.08 *	0.16 *	0.01	0.30 *	7.68 *
60 day	Control	0.01	0.01	trace	-	0.11	7.73
	Ginkgo	0.07 *	0.05 *	0.01 *	-	0.16 *	7.78 †
90 day	Control	0.04	0.04	trace	-	0.11	7.70
	Ginkgo	0.04	0.03 **	0.01 **	-	0.17 *	7.73 †
180 day	Control	0.04	0.04	trace	-	0.11	7.83
	Ginkgo	0.04 *	0.03 **	0.01 **	-	0.15 *	7.81 *

Incubation period	Treatment	Fermentation products from slurry					pH
		Total SCFA	Acetate	Propionate	n-Butyrate	Ammonia	
		(mmol/g slurry)				(mg N./g slurry)	
0 day	Control	0.33	0.27	0.03	-	1.09	7.13
	Ginkgo	0.54 *	0.36 *	0.14 *	-	0.90 *	6.74 *
30 day	Control	trace	trace	trace	-	0.05	7.90
	Ginkgo	0.04 *	0.04 *	0.01 *	-	0.06	7.95 *
60 day	Control	trace	trace	-	-	0.02	7.96
	Ginkgo	0.01 *	0.01 *	-	-	0.02	8.04 **
90 day	Control	trace	trace	-	-	0.01	7.90
	Ginkgo	0.01 **	0.01 **	-	-	0.01	7.86 †
180 day	Control	trace	trace	-	-	0.01	7.93
	Ginkgo	0.01 **	0.01 **	-	-	0.01	7.87

Fermentation parameters were measured after incubating feces or slurry left for 0 - 180 days at 30℃ with or without gingko fruit.

†, *, **: Significantly different from control at $P < 0.1$, 0.05 and 0.01, respectively.

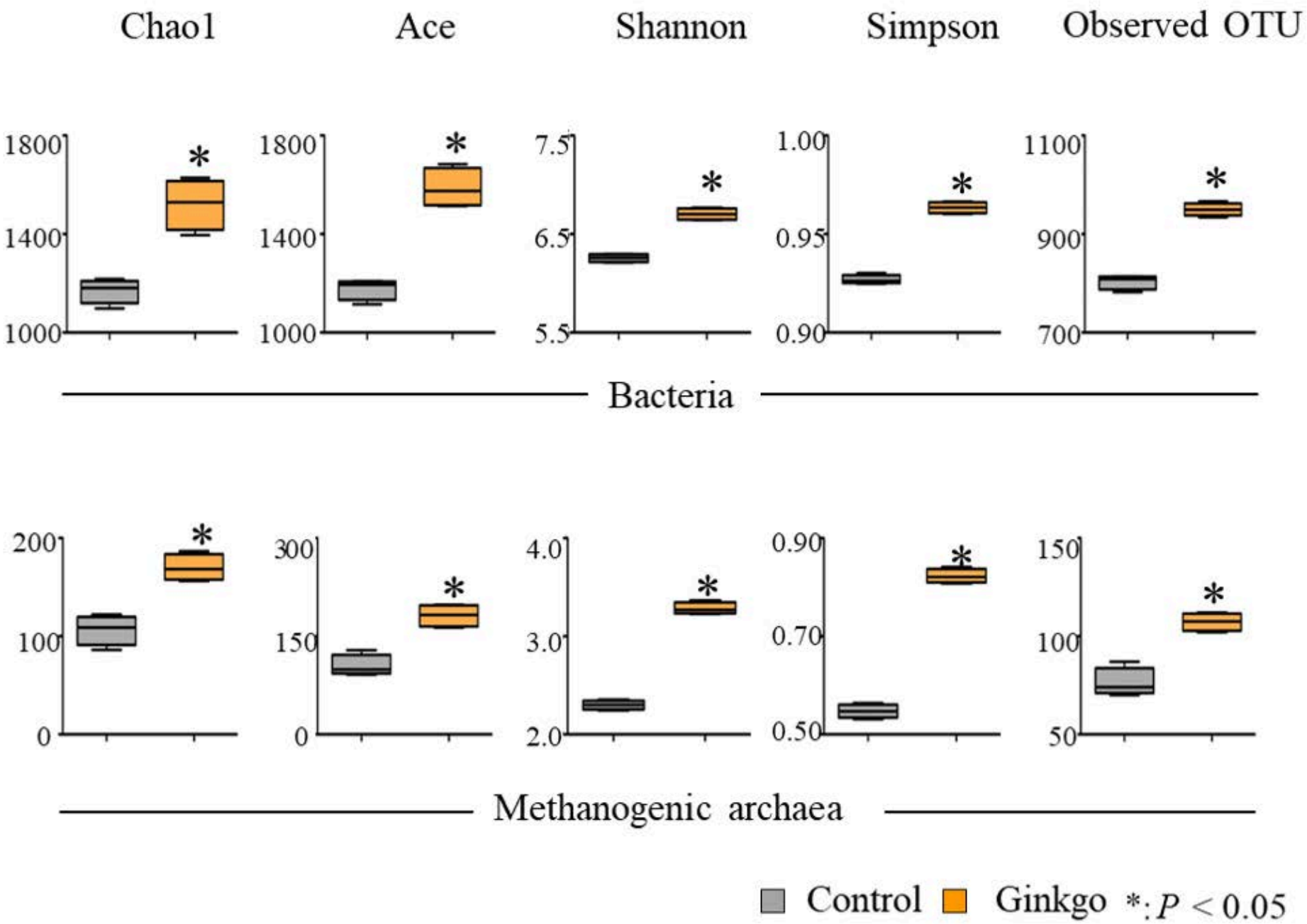
TABLE 3 Effect of ginkgo fruit addition on bacterial abundance of cattle feces and slurry left for different periods

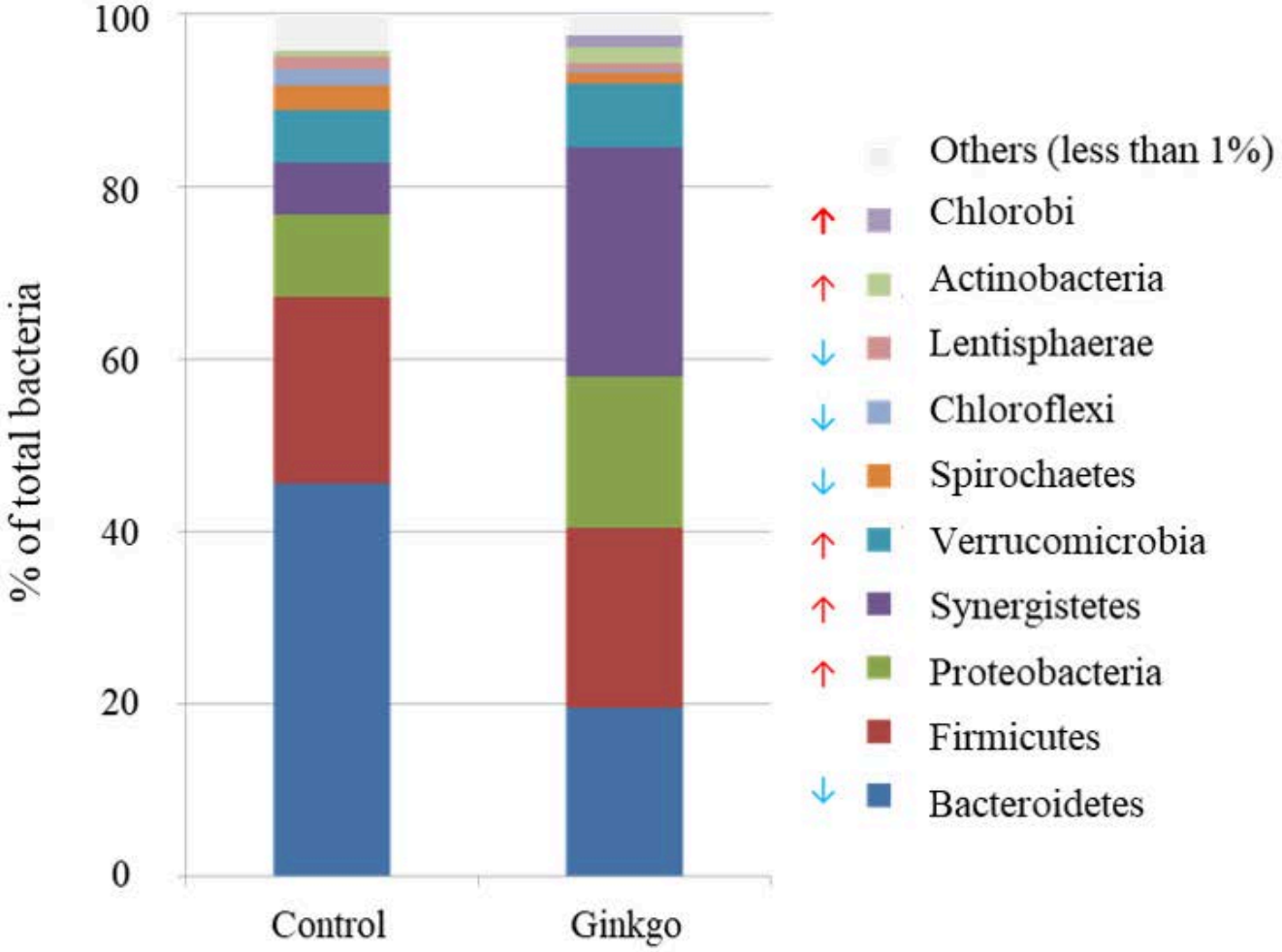
Incubation period	Treatment	Microbes in feces				
		Total bacteria	Methanogens	Rumino-coccaceae	*Clostridium leptum* subgroup	*Bacteroides-Prevotella-Porphyromonas*
		(log copy number of 16S rDNA/g feces)	(relative % of total bacteria)			
0 day	Control	11.50	2.01	17.96	8.79	19.29
	Ginkgo	11.23 **	1.04 **	3.35 **	2.25 **	19.44
30 day	Control	11.50	6.45	3.40	2.66	5.20
	Ginkgo	11.34 *	2.99 **	3.97	3.34 **	14.33 **
60 day	Control	11.55	6.00	2.01	1.71	8.63
	Ginkgo	11.37 **	1.63 **	2.06 **	1.88 †	10.11 †
90 day	Control	11.38	5.45	2.19	2.02	3.88
	Ginkgo	11.41	1.39 **	1.64 *	1.40 **	9.38 **
180 day	Control	11.28	3.65	1.98	1.43	1.35
	Ginkgo	11.42 *	2.86 **	1.90	1.56 †	3.23 **

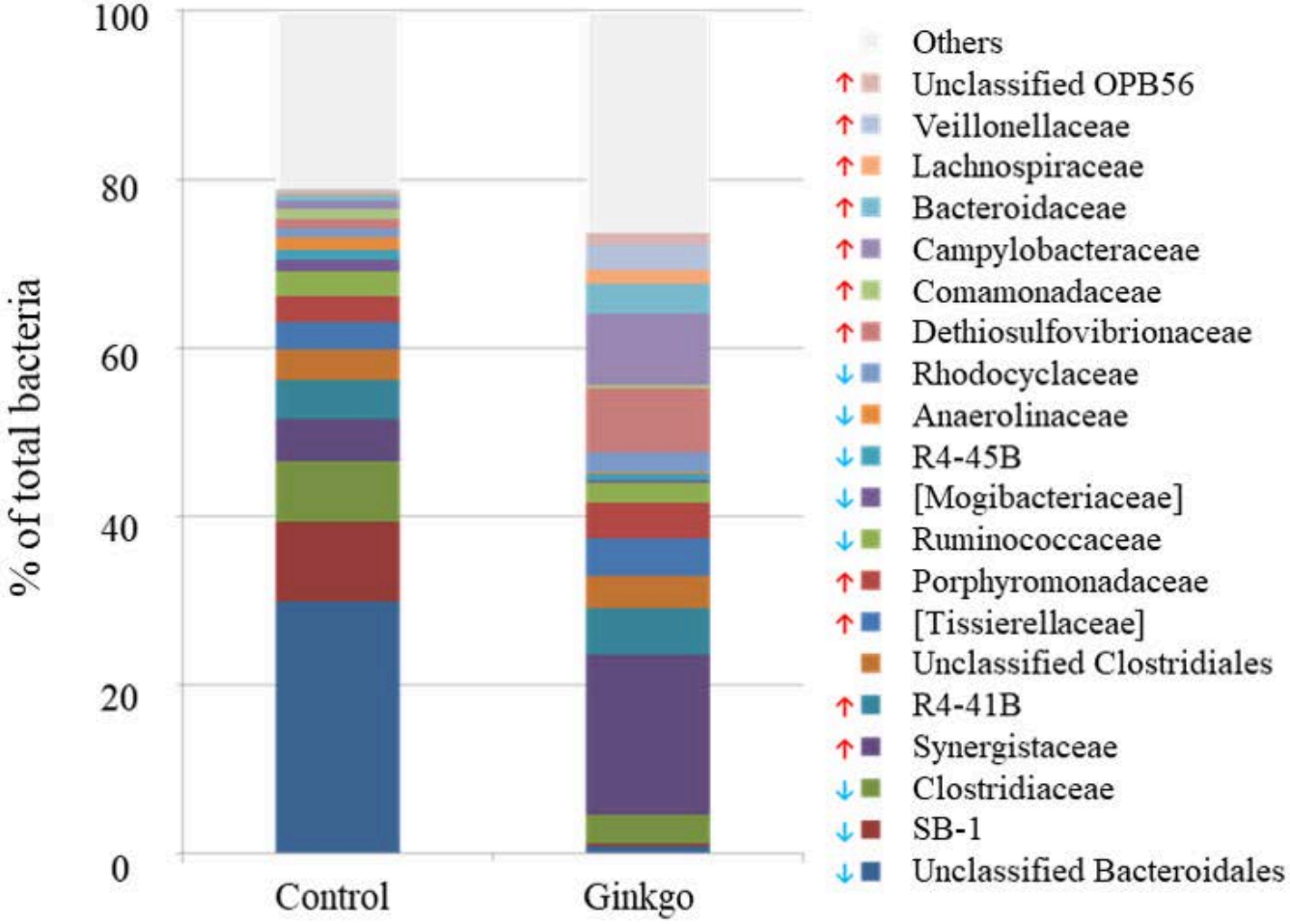
Incubation period	Treatment	Microbes in slurry				
		Total bacteria	Methanogens	Rumino-coccaceae	*Clostridium leptum* subgroup	*Bacteroides-Prevotella-Porphyromonas*
		(log copy number of 16S rDNA/g feces)	(relative % of total bacteria)			
0 day	Control	11.66	2.91	9.00	3.81	16.11
	Ginkgo	11.49 **	0.70 **	4.15 **	2.39 **	12.98 **
30 day	Control	11.62	11.44	4.72	2.23	6.80
	Ginkgo	11.51 *	2.49 **	2.75 **	1.41 **	7.35 *
60 day	Control	11.75	36.31	2.79	1.39	6.67
	Ginkgo	11.59 **	4.98 **	1.82 **	0.81 **	16.75 **
90 day	Control	11.96	32.01 **	2.35	1.05	5.76
	Ginkgo	11.69 **	3.84	1.12 **	0.67 **	13.78 **
180 day	Control	11.75	45.60	1.28	0.64	5.68
	Ginkgo	11.71	7.18 **	0.66 **	0.40 **	17.93 **

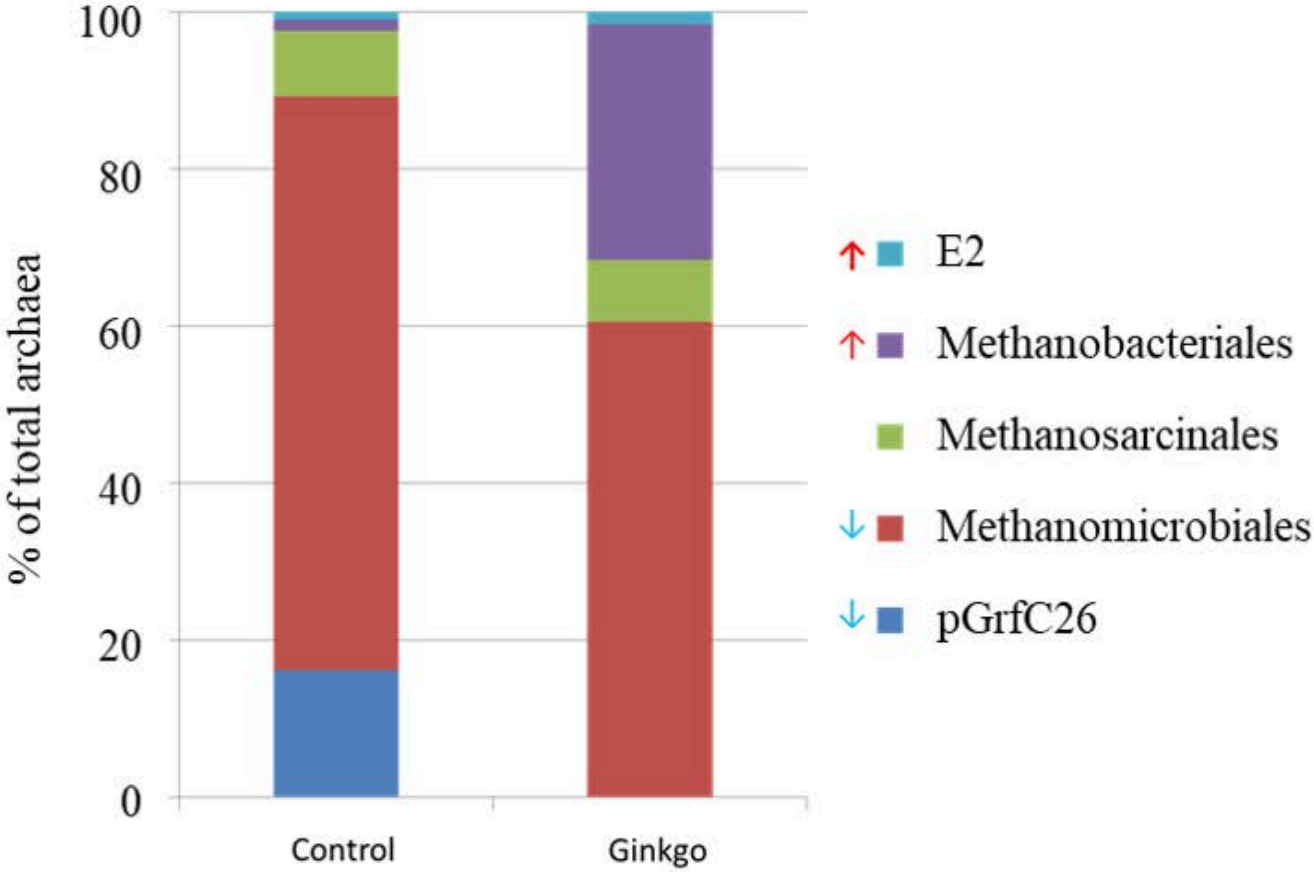
Microbes were measured by qantitative PCR after incubating feces or slurry left for 0 - 180 days at 30℃ with or without gingko fruit.

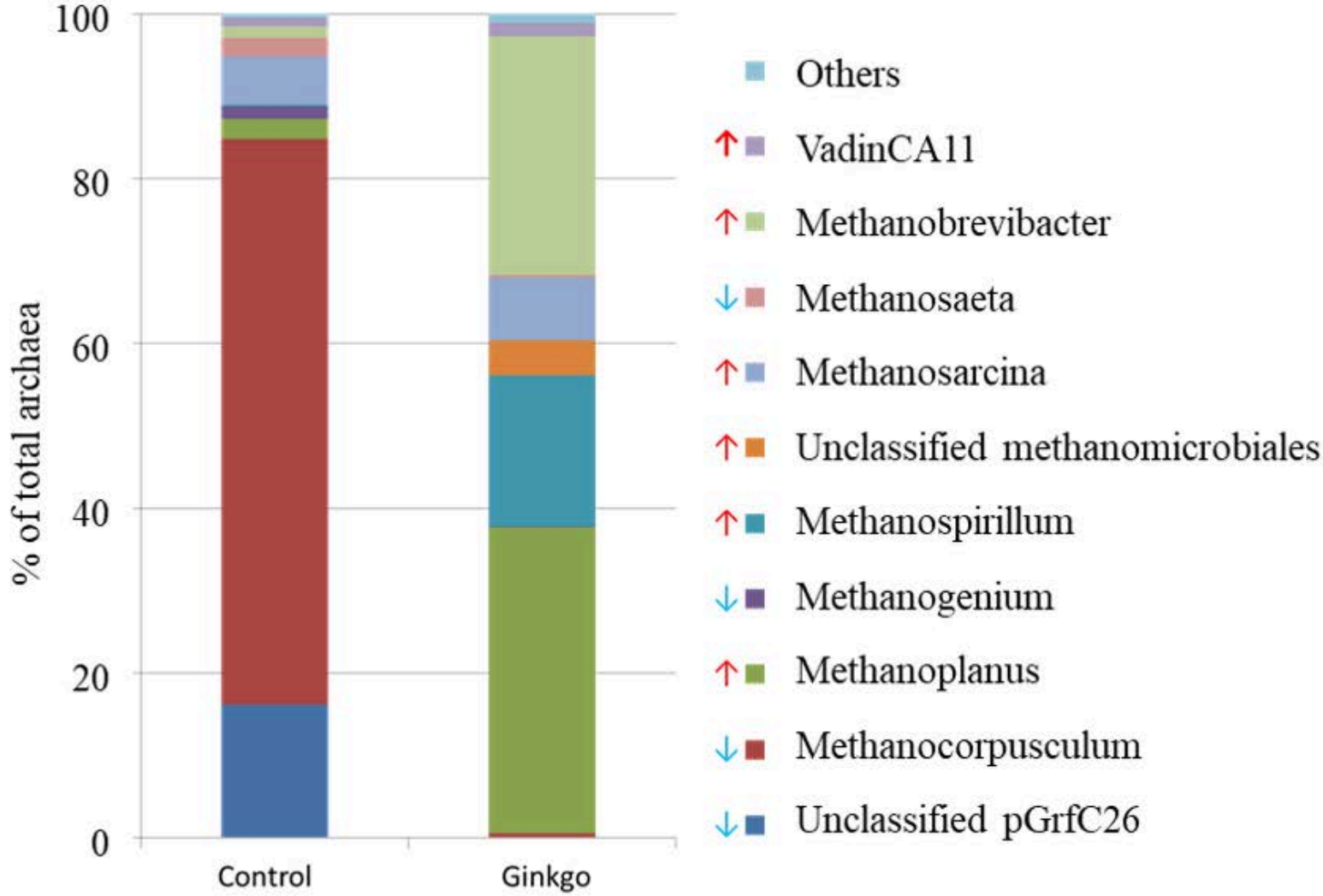
†, *, **: Significantly different from control at $P < 0.1$, 0.05 and 0.01, respectively.


Chao1
Ace
Shannon
Simpson
Observed OTU
1800
1400
1000
1800
1400
1000
7.5
6.5
5.5
1.00
0.95
0.90
1100
900
700
Bacteria
200
100
0
300
150
0
4.0
3.0
2.0
0.90
0.70
0.50
150
100
50
Methanogenic archaea
Control
Ginkgo
*: $P < 0.05$


100
80
60
40
20
0
% of total bacteria
Control
Ginkgo
Others (less than 1%)
Chlorobi
Actinobacteria
Lentisphaerae
Chloroflexi
Spirochaetes
Verrucomicrobia
Synergistetes
Proteobacteria
Firmicutes
Bacteroidetes


100
80
60
40
20
0
% of total bacteria
Control
Ginkgo
Others
Unclassified OPB56
Veillonellaceae
Lachnospiraceae
Bacteroidaceae
Campylobacteraceae
Comamonadaceae
Dethiosulfovibrionaceae
Rhodocyclaceae
Anaerolinaceae
R4-45B
[Mogibacteriaceae]
Ruminococcaceae
Porphyromonadaceae
[Tissierellaceae]
Unclassified Clostridiales
R4-41B
Synergistaceae
Clostridiaceae
SB-1
Unclassified Bacteroidales


100
80
60
40
20
0
% of total archaea
Control
Ginkgo
E2
Methanobacteriales
Methanosarcinales
Methanomicrobiales
pGrfC26


100
80
60
40
20
0
% of total archaea
Control
Ginkgo
Others
VadinCA11
Methanobrevibacter
Methanosaeta
Methanosarcina
Unclassified methanomicrobiales
Methanospirillum
Methanogenium
Methanoplanus
Methanocorpusculum
Unclassified pGrfC26