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Original Research

Influence of Dietary Supplementation of Ensiled Devil Fish and *Staphylococcus saprophyticus* on Equine Fecal Greenhouse Gases Production

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A B S T R A C T

The present context was designed to investigate the efficacy of devil fish (DF; *Plecostomus* sp.) silage and *Staphylococcus saprophyticus* on fermentation characteristics as well as greenhouse gases production mitigation attributes in horses. Four levels of ensiled DF at 0 (control DF0), 6 (DF6), 12 (DF12), and 18 (DF18) % were added into the diet. Moreover, three doses of *S. saprophyticus* (0, 1, and 3 mL/g dry matter [DM]) were used for *in vitro* fecal fermentation. The use of ensiled DF resulted in increased ($P < .0001$) pH during fermentation. The asymptotic gas production was the highest ($P < .0001$) in DF6, whereas other supplementation caused lower production than that of control. *Lag* time for the asymptotic gas production decreased ($P < .05$) with increasing dietary DF doses. Inclusion of *S. saprophyticus* resulted in the lowest ($P < .05$) gas production and mL/0.5 g DM incubated and thus, the reduced gas production up to 23.17% than that of control. The interaction of DF × *S. saprophyticus* showed the lowest gas production at DF18, whereas the highest production was estimated at DF6 without *S. saprophyticus* after 48 hours. The lowest emission of CO_2 ($P < .0001$) was observed in DF18 inclusion, which was 15.25% lower than that of control at 48 hours of fermentation. In contrast, the lowest hydrogen (H_2) production was estimated in DF0, whereas DF18 exhibited the highest. Inclusion of DF12 and DF18 reduced ($P < .05$) methane (CH_4) emission by 58.24% and 59.33%, respectively. However, DF, *S. saprophyticus*, and DF × *S. saprophyticus* interaction had no significant effect ($P > .05$) on CH_4 production. In conclusion, ensiled DF and *S. saprophyticus* could be supplemented in equine diet as promising alternatives to corn for mitigating the emission of greenhouse gases effectively.

1. Introduction

Horses are monogastric, and hindgut fermenting herbivores where cecum and colon are the fermentative chambers for disparate microbiota. The microbial population present in hindgut are known for the stimulation of immunity, exclusion of pathogens, and detoxification of hazardous components [1]. The diet of horses is enriched with fibers; however, hindgut microbiota enables digesting fiber-based diets gradually due to the fact that the fiber is indigestible by secreted enzymes. However, alteration in feeding practices and activities of modern-day horses have led to an increased level of grain or starch and lowered levels of fiber in their diet [2,3]. This is done to provide quick energy release to meet the energy need of high-paced activity of equine [4]. However, such feeding practices lead to the leading causes of several disorders, namely gastric ulceration, hindgut acidosis, and endotoxemia [5]. In addition, feeding such diets may decrease the starch digestion trait in the small intestine and alter the microbial population as well as fibrolytic characteristics in the hindgut, thereby reducing the ability

Animal welfare/ethical statement: The research was performed in accordance with the ethical standard laid down in the 1996 Declaration of Helsinki and its later amendments.

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to use energy from the diets, as a result of alteration in hindgut pH [4,6]. However, pectin-rich by-product (lemon, tangerine, and pineapple) and agro-based by-product (sugar beet pulp and soybean hull) have been put forward to provide energy for horses without causing digestive disturbances or offset [4]. Still, there is an urgency to explore other resources for providing the energy demands, intestinal health, and enhance the athletic high-level performances of modern horses.

Currently, the supplementation of diversified additives into the feeds is considered auspicious strategies to enhance the energy utilization in horses. Unfortunately, the perpetual emission of greenhouse gases (GHG), particularly methane (CH_4) and carbon dioxide (CO_2), from animals due to the fermentation is the colossal burden globally. These GHGs are considered not only environmental pollutants but also hazardous to human health, resulting in global warming [7]. The quest for auspicious natural alternative resources to mitigate the emission of GHG for cleaner society and sustainable environment has gained immense interest. For instance, distinct natural feed additives such as plant extract [8], enzyme [9], yeasts [7], and lactobacilli [10] had been used. Nevertheless, the exploitation of coagulase-negative staphylococci (CNS) as feed additive in horse nutrition for mitigating the emission of GHG is not evidenced yet. *Staphylococcus equorum*, *S. hominis*, *S. cohnii*, *S. capitis*, *S. condimenti*, *S. succinus*, and *S. xylosus* belong to CNS group [11]. In recent times, CNS have emerged as the prevalent heterogeneous group of bacteria and included under Qualified Presumption of Safety status by the European Food Safety Authority Scientific Committee on a case-by-case basis within a particular taxonomic group [12].

In recent years, CNS have emerged as different group of fermented food-associated bacteria revealing probiotic properties [13]. In addition, CNS were reported as the predominant type of bacteria in some Korean fermented food [14]. This is an indication of the fermenting properties of CNS or its probiotic properties. Furthermore, devil fish (DF) (*Plecostomus* sp.) are included in animal's diet because of its abundance and maximum digestibility [15]. In addition, ensiling the fish could pave a way of improving its usage as feed ingredient. The proteolytic enzyme in the ensiled fish could improve feed digestibility. Besides, it is a well-established fact that fermented foods are enriched with health beneficial probiotic microbes [16]. The effect of DF has been studied in ruminant diet [17] with a better response in fermentation kinetics. However, this kind of investigation is unexplored in equine.

Considering this, a further significant attempt was undertaken in this context to fill the gap of research by determining the fermentation kinetics and GHG production mitigation attributes of *S. saprophyticus* and DF in horses as ideal alternatives to feed supplements for a cleaner and ecofriendly product.

2. Materials and Methods

2.1. Substrate and Treatments

Substrate (diets) used in this study were dried at 60°C for 48 hours before in vitro incubation. The diet level was 0, 6, 12, and 18% of ensiled DF of diet dry matter (DM) and represented as DF0, DF6, DF12, and DF18, respectively. In addition, three doses (0, 1, and 3 mL/g DM substrate) of *S. saprophyticus* (SS) at 5×10^{11} CFU/g represented as SS0, SS1, and SS3 were used for *in vitro* fermentation. Diet formulation and chemical composition of diets are shown in Table 1.

To ensile the DF, the fresh live fish was obtained from the Tuxpan lagoon municipality of the city of Iguala Gro. The fish was washed with water to remove the soil and particles stuck to the fish. After milling process, 5 kg of fish were mixed with 14 L of molasses and 1 L of natural yogurt in a bucket with a capacity of 20 L in which an airtight lid was placed to avoid leaks and air entrances, and then it was kept for 30 days. The cuvette was opened after 30 days and mixed with the ingredients as mentioned in Table 1.

Table 1
Ingredients and chemical composition of the diets with different levels of ensiled devil fish used as substrates.[a]

Ingredients	DF0	DF6	DF12	DF18
Ground corn	73.5	67.5	61.5	55.5
Pastures	15	15	15	15
Soybean meal	9	9	9	9
Devil fish	0	6	12	18
Minerals	2.5	2.5	2.5	2.5
Chemical composition (%)				
Organic matter	3.4	3.5	4.01	4.10
Crude protein	45.3	45.5	46.2	47.9
Ether extract	11.4	12.2	11.3	12.13
Acid detergent fiber	2.7	2.8	2.7	3.1
Neutral detergent fiber	4.7	4.9	4.89	5.02
Acid detergent lignin	2.1	2.3	2.1	2.3
Minerals[b]	34.7	34.7	34.7	34.7

[a] Addapted form Abrego Salgado [18].
[b] Cu: 21.18 ppm, Fe: 4971.66 ppm, Zn: 343.75 ppm, Ca: 9.96%, Mg: 0.2495%, K: 0.8895%, Na: 1.296%, Pb: 0.0029%, P: 14.395%, S: 3.125%.

2.2. In Vitro Incubation

Horses were fed the compounded diet (substrate) *ad libitum* and provided fresh water for 7 days before collection phase. Fecal content (inoculums source) collected from the rectum were obtained from four Azteca horses (aged 5–8 years, 480 ± 20.1 kg). Culture broth was added to the fecal contents in a ratio of 4:1 and kept under CO_2 environment throughout the entire *in vitro* incubation process (39°C; 48 hours). All incubations were performed in triplicate, and either rumen fluid or fecal fluid was used as a blank. Data at 2, 4, 6, 8, 10, 12, 14, 24, and 48 hours using the pressure reading technique was used to estimate total gas, CO_2, CH_4, and H_2 emissions [19]. CO_2, CH_4, and H_2 concentrations were also measured in the headspace of the bottles up to 48 hours using the gas detector (AIR QUALITY MONITOR YesAIR, Critical Environment Technologies Canada Inc, Delta, British Columbia, Canada). Furthermore, pH was measured, and DM degradability (DMD) was estimated after filtration [20].

2.3. Calculations and Statistical Analyses

Kinetic parameters of gas production (mL/g DM) were calculated according to France et al. [21] using the NLIN option of SAS [22]. The DMD was calculated according to the methodology of Menke et al. [23]. Fecal fermentation data were estimated as a completely randomized design as per PROC GLM option:

$$Y_{ij} = \mu + B_i + \varepsilon_{ij}$$

where, Y_{ij} = observation obtained with i^{th} level of LAB; B_i = level of LAB (I = 1–4); μ = general mean; ε_{ij} = experimental error. Linear and quadratic polynomial contrasts were implied to assess responses for increasing concentrations of *S. saprophyticus*. Turkey's test was used to calculate multiple comparisons among means. Significance level was estimated at $P < .05$.

3. Results

3.1. In Vitro Gas Kinetics

Figs. 1 and 2 showed the effect of ensiled DF and *S. saprophyticus* on horse fecal total gas, CH_4, CO_2, and H_2 production. Inclusion of *S. saprophyticus* had no significant effect ($P > .05$) on total gas, CH_4, CO_2, and H_2 production. Furthermore, Table 2 showed that ensiled DF had a linear effect ($P < .0001$) on the asymptotic gas production ($P = .031$), rate of gas production, and lag time ($P < .0001$). The DF6 showed the highest asymptomatic gas production and CO_2 production, whereas the DF18 exhibited the lowest gas production and CO_2 emission. Interaction of DF × *S. saprophyticus* had no effect ($P >$ .05) on CH_4, CO_2, and H_2 except for the asymptotic gas production ($P = .0017$) and the lag time ($P = .039$).

3.2. Fecal Fermentation Parameters

The DF supplementation showed a linear effect ($P < .0001$) on the pH and gas production at 24 and 48 hours. The DF12 and DF0 showed the highest and the lowest pH values, respectively. Furthermore, DF6 and DF18 doses resulted in the highest and the lowest mL gas/0.5 g DM at 24 and 48 hours. Similarly, *S. saprophyticus* had a linear effect ($P < .05$) on gas production, but it had no effect ($P > .05$) on fecal pH. There was a linear decrease ($P <$ .05) in gas production in a dose-dependent manner at 24 and 48 hours. DF × *S. saprophyticus* estimated linear effect ($P < .002$) on gas production (mL/0.5 g DM incubated) at 48 hours of incubation. The DF, *S. saprophyticus*, and DF × *S. saprophyticus* interaction showed no significant effect on DMD. Similarly, *S. saprophyticus* exhibited no influence on the rumen fluid pH (Table 3).

3.3. Fecal Greenhouse Gas Production

Table 4 showed that DF doses, *S. saprophyticus* concentrations, and DF × *S. saprophyticus* interaction had neither linear nor quadratic effect ($P > .05$) on mL CH_4/0.5 g DM incubated, mL CH_4/0.5 g DM degraded, and proportional CH_4 production. At 8 hours of incubation period, there was complete absence of in vitro fecal CH_4 production. However, DF and *S. saprophyticus* doses revealed quantitative reduction in CH_4 production (Fig. 2).

Table 5 showed that DF doses had linear effect ($P < .05$) on mL CO_2/0.5 g DM incubated and mL CO_2/0.5 g DM degraded but showed no effect on the proportional CO_2 production. The DF6 and DF12 revealed the highest mL CO_2/0.5 g DM degraded and mL CO_2/0.5 g DM incubated, whereas DF18 exhibited the lowest mL CO_2/0.5 g DM degraded and mL CO_2/0.5 g DM incubated at 24 and 48 hours of incubation. Furthermore, DF × *S. saprophyticus* interaction had a linear effect ($P < .05$) on the mL CO_2/0.5 g DM degraded and proportional CO_2 production with DF6 exhibiting the highest, whereas DF18 revealing the lowest CO_2 production. However, *S. saprophyticus* estimated no significant ($P > .05$) impact on CO_2 emission (Fig. 2).

Table 6 showed that DF doses had a linear ($P < .02$) influence on the proportional H_2 production. The DF18 produced the highest H_2, whereas DF0 quantified the lowest H_2 production. *S. saprophyticus* doses and DF × *S. saprophyticus* interaction had no significant ($P > .05$) impact on mL H_2/0.5 DM incubated and mL H_2/0.5 g DM degraded. In contrary, *S. saprophyticus* doses and DF ×

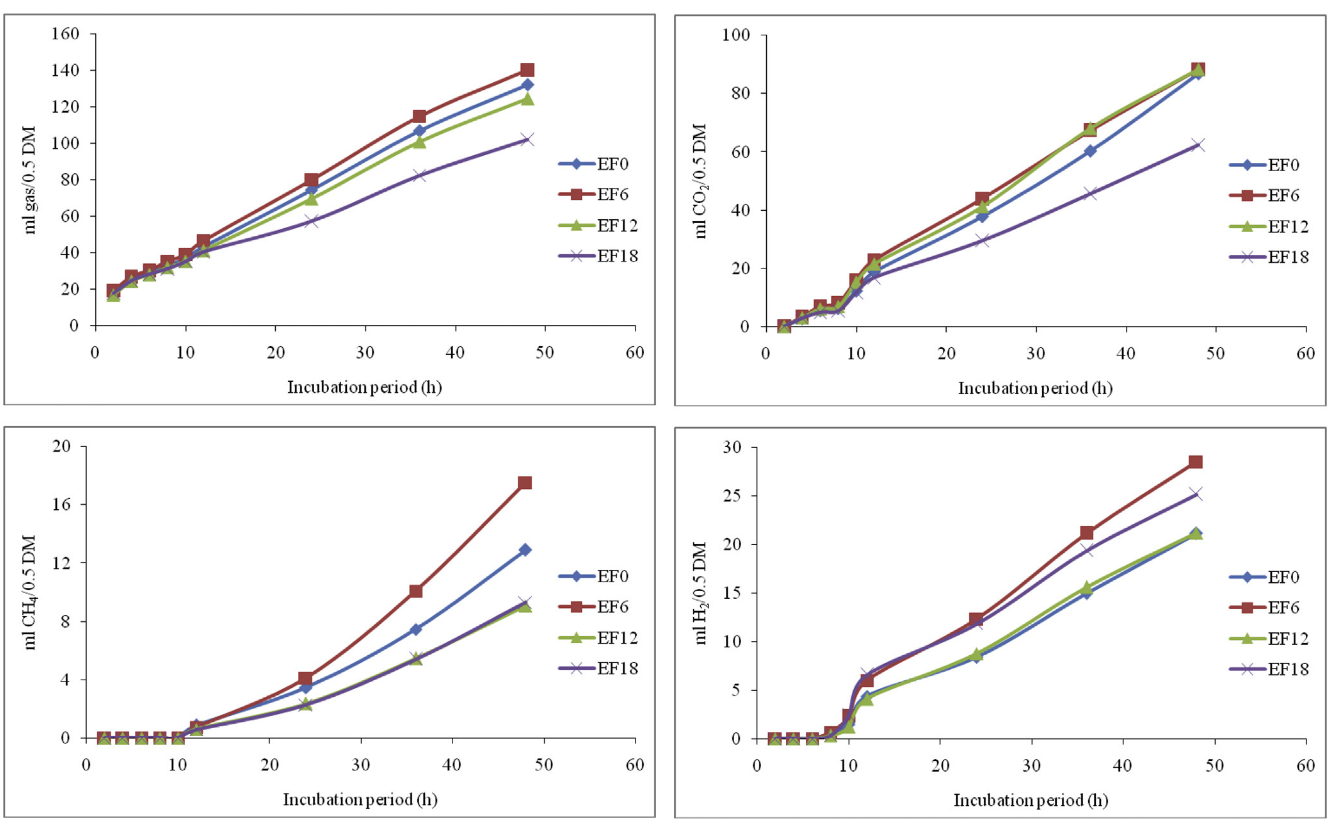

Fig. 1. Horse fecal total gas, CH_4, CO_2, and H_2 production (mL/0.5 g DM) at different incubation periods as affected by the dietary inclusion of ensiled devil fish (DF) at 0% (DF0, control), 6% (DF6), 12% (DF12), and 18% (DF18) of the diet DM. DM, dry matter.

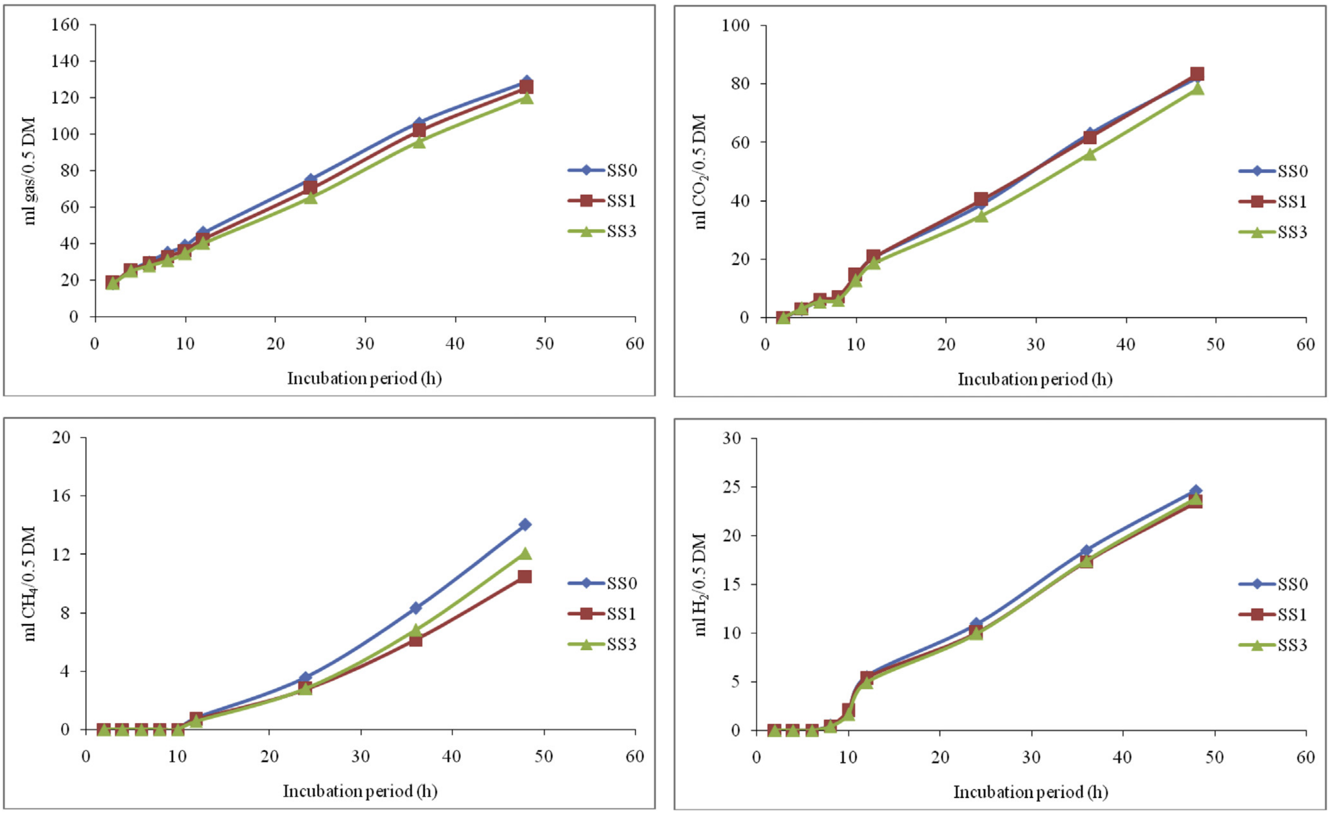

Fig. 2. Horse fecal total gas, CH_4, CO_2, and H_2 production (mL/0.5 g DM) at different incubation periods as affected by the dietary inclusion of *S. saprophyticus* (SS) at 0 (SS0, control), 1 (SS1), and 3 mL (SS3) of the diet DM. DM, dry matter.

S. saprophyticus interaction exhibited linear effect ($P < .03$) on mL H_2/0.5 DM incubated at 12 hours (Fig. 2).

4. Discussion

At present, improving the nutrition through the use of unconventional ingredients of crops or animal sources, novel additives, and microorganisms are the most common practices of researchers and farmers. In addition, reducing GHGs productions are also an important factor in equine nutritional interventions. Total gas production is an indication of feed digestibility or degradation. In this context, total gas production was estimated to be increased because of the supplementation of DF. The increment in the total gas production by DF6 with respect to the control may be attributed

Table 2
Effect of SS as feed additives on in vitro fecal total gas, CH_4, CO_2, and H_2 kinetics[a] of diets at different doses of ensiled DF.

DF Doses	SS Doses	Total gas			CH_4			CO_2			H_2		
		b	*c*	*Lag*	*b*	*c*	*Lag*	*b*	*c*	*Lag*	*b*	*c*	*Lag*
DF0	0	128.6	0.008	4.439	10.4	0.001	5.908	89.7	0.004	4.409	15.8	0.011	6.750
	1	132.8	0.003	6.042	11.9	0.001	6.941	78.1	0.001	4.844	18.2	0.007	6.695
	3	128.5	0.010	4.989	12.7	0.001	7.662	90.2	0.001	4.692	23.6	0.027	6.913
DF6	0	143.8	0.009	3.764	20.4	0.023	7.990	88.6	0.011	4.265	28.5	0.007	6.206
	1	132.8	0.005	4.370	13.7	0.001	7.761	91.8	0.012	2.957	23.1	0.020	5.815
	3	135.8	0.005	1.965	18.0	0.020	8.098	81.2	0.004	3.800	30.9	0.013	5.279
DF12	0	136.3	0.003	2.072	16.3	0.001	6.882	88.0	0.009	4.278	25.5	0.016	9.479
	1	133.3	0.004	6.276	4.8	0.001	7.560	97.1	0.006	3.643	21.0	0.013	5.712
	3	91.8	0.009	1.724	3.0	0.002	8.837	77.1	0.003	3.930	13.5	0.007	6.859
DF18	0	88.9	0.001	1.753	7.5	0.011	8.220	60.2	0.012	4.460	24.6	0.020	6.848
	1	98.9	0.004	1.632	7.6	0.001	5.079	62.8	0.010	3.988	24.1	0.018	6.070
	3	105.3	0.002	1.917	28.4	0.005	9.203	62.0	0.005	3.087	24.0	0.009	6.416
P values													
DF													
Linear		<.0001	.0307	<.0001	.8301	.446	.3764	<.0001	.0307	.2095	.1949	.8764	.6646
Quadratic		.1532	.6781	.8436	.6575	.7236	.3636	.0012	.9366	.5882	.6087	.4613	.2863
SS													
Linear		.0557	.4179	.5026	.3539	.6908	.0721	.2886	.0455	.3868	.854	.9329	.1695
Quadratic		.252	.2624	.0008	.2567	.1087	.0787	.3884	.6551	.5872	.5476	.8841	.1988
DF × SS		.0017	.3773	.0392	.3405	.6714	.2263	.1323	.9699	.8475	.4522	.2551	.5215

Abbreviations: DF, devil fish; GP, gas production; SS, *S. saprophyticus.*
[a] *b* is the asymptotic GP (mL/g DM); *c* is the rate of GP (per hour); *Lag* is the initial delay before GP begins (hour).

Table 3
Effect of SS as feed additives on in vitro fecal fermentation parameters as well as total GP at different incubation periods using different doses of ensiled DF.

DF Doses	SS Doses	Fermentation Parameters		GP mL/0.5 g DM Incubated				GP mL/0.5 g DM Degraded			
		pH	DMD	8	12	24	48	8	12	24	48
DF0	0	6.3	73.4	36.7	48.1	78.3	129.9	26.9	35.2	57.5	95.5
	1	6.3	76.8	32.3	41.4	72.9	131.9	24.9	32.0	56.2	101.5
	3	6.4	74.8	30.3	40.7	72.7	135.7	22.7	30.5	54.4	101.5
DF6	0	6.6	77.5	37.0	49.1	86.6	148.7	28.6	38.0	67.1	115.3
	1	6.5	77.5	34.8	47.1	79.3	136.2	27.0	36.5	61.5	105.6
	3	6.6	76.2	33.8	43.4	73.8	136.3	25.8	33.0	56.2	103.8
DF12	0	6.6	76.8	36.4	46.8	79.3	137.5	27.9	35.9	60.8	105.5
	1	6.6	78.7	32.7	42.4	72.2	134.5	25.7	33.4	56.8	105.9
	3	6.6	75.4	27.4	35.0	58.0	102.6	20.7	26.4	43.8	77.4
DF18	0	6.6	78.2	30.1	40.2	57.7	99.8	23.5	31.4	45.1	78.0
	1	6.5	66.7	32.0	38.8	57.6	100.6	21.4	26.3	38.7	67.8
	3	6.5	72.9	31.8	42.7	56.9	106.4	23.2	31.1	41.8	77.8
P values											
DF											
Linear		<.0001	.4416	.2547	.1813	<.0001	<.0001	.1842	.1733	.0001	<.0001
Quadratic		<.0001	.2469	.9638	.7489	.1128	.0167	.477	.6607	.0798	.0333
SS											
Linear		.2386	.5432	.0046	.0041	.0002	.0061	.0127	.0135	.0037	.0507
Quadratic		.038	.7678	.9985	.6017	.9759	.6453	.9034	.6828	.9899	.8136
DF × SS		.3319	.554	.1949	.1628	.0978	.0002	.6221	.4442	.3747	.0345

Abbreviations: DF, devil fish; DMD, dry matter degradability (%); GP, gas production; SS, *S. saprophyticus.*

to the enzymatic activities such as protease and lipase from the DF, which enhanced the growth of microbes. It could also be due to the fact that the high protein content leads to more availability of ammonia nitrogen, which enhanced their growth. Makkar et al [24] reported that protein fermentation produced lesser gas compared with carbohydrate. Hence, in contrary to our study, the highest protein supplementation resulted in reduced gas production. Velazquez et al [25] stated that *Lag* time is a measure of the time required for feed digestibility by gut microbes to initiate digestibility. In this investigation, *Lag* time was reduced during ensiling due to the fermentation process. The lower *Lag* time with DF supplementation may be attributed to the ability of microbes to adapt or reveal probiotic traits [16]. Therefore, it could be stated that DF had some probiotics properties, which led to quick adherence and colonization of feed particles compared with the control.

In this study, total CO_2 emissions from horses were reduced in the presence of high doses of DF. The highest total CO_2 production in DF12 and its similarity with DF6 could be due to the fermentation process. However, the lowest total CO_2 in DF18 may be attributed to the high crude protein content of DF. The similar pattern was observed in mL CO_2/0.5 g DM incubated and degraded too (Table 5). Velázquez et al [26] demonstrated that the lower CO_2 production may be influenced by the high protein content in a diet. In addition, the ammonia–N nitrogen accumulation in the medium might have prevented the release of CO_2 in the bottle [27].

Faniyi et al [28] reported that pH is a fermentation parameter that quantifies the state of acidity and alkalinity in the gut and

Table 4
Effect of SS as feed additives on in vitro fecal CH_4 production at different incubation periods[a] using different doses of ensiled DF.

DF Doses	SS Doses	mL CH_4/0.5 g Dry Matter Incubated			mL CH_4/0.5 g Dry Matter Degraded			Proportional CH_4 Production		
		12	24	48	12	24	48	12	24	48
DF0	0	0.8	3.2	11.8	0.6	2.4	8.7	1.7	4.1	9.1
	1	1.3	4.0	12.8	1.0	3.1	9.9	3.0	5.4	9.8
	3	0.6	3.2	14.1	0.4	2.4	10.7	1.4	4.4	10.4
DF6	0	0.8	4.5	19.0	0.6	3.5	14.8	1.5	5.2	12.5
	1	0.4	3.2	14.5	0.3	2.5	11.3	0.8	4.0	10.4
	3	1.0	4.6	19.1	0.7	3.5	14.5	2.2	6.1	13.9
DF12	0	0.7	4.2	17.2	0.6	3.2	13.2	1.6	5.2	12.6
	1	0.6	1.8	6.1	0.5	1.4	4.8	1.4	2.5	4.5
	3	0.5	1.2	3.9	0.3	0.9	2.9	1.2	2.0	3.8
DF18	0	0.9	2.4	8.1	0.7	1.9	6.3	2.1	4.2	8.0
	1	0.5	2.1	8.5	0.4	1.5	6.2	1.3	3.6	8.3
	3	0.3	2.3	11.3	0.2	1.6	8.1	0.7	4.1	10.7
P values										
DF		.53	1.39	5.25	.42	1.11	4.13	1.14	1.68	3.71
Linear		.2246	.0811	.1557	.2199	.0839	.1498	.2512	.4026	.6746
Quadratic		.5725	.3876	.3511	.6307	.4802	.4475	.5462	.1393	.1267
SS										
Linear		.3329	.1848	.3794	.3241	.1695	.3198	.4788	.4373	.5902
Quadratic		.9467	.4069	.1772	.9143	.471	.2167	.8733	.36	.1645
DF × SS		.4385	.3163	.1378	.4365	.3615	.1702	.3139	.2499	.1515

Abbreviations: DF, devil fish; DMD, dry matter degradability (%); GP, gas production; SS, *S. saprophyticus.*
[a] No detection of CH_4 production before 12 hr of incubation.

Table 5
Effect of SS as feed additives on in vitro fecal CO_2 production at different incubation periods using different doses of ensiled DF.

DF Doses	SS Doses	mL CO_2/0.5 g Dry Matter Incubated				mL CO_2/0.5 g Dry Matter Degraded				Proportional CO_2 Production			
		8	12	24	48	8	12	24	48	8	12	24	48
DF0	0	6.9	21.7	39.5	90.4	5.1	15.8	29.0	66.3	18.7	44.7	50.2	69.5
	1	5.3	16.1	34.5	79.1	4.0	12.4	26.4	60.4	17.0	39.7	48.2	60.3
	3	6.5	18.4	39.4	91.4	4.9	13.8	29.5	68.3	21.7	45.3	54.2	67.4
DF6	0	8.1	22.2	45.5	89.5	6.2	17.2	35.2	69.3	21.7	45.3	52.5	60.5
	1	10.1	26.1	48.8	92.9	7.8	20.2	37.8	72.0	29.0	55.3	61.6	68.4
	3	6.1	20.2	37.5	82.6	4.6	15.4	28.6	62.9	18.0	46.7	50.8	60.6
DF12	0	6.8	22.2	41.9	89.2	5.2	17.0	32.2	68.5	18.3	47.3	52.9	65.0
	1	8.1	24.4	46.6	98.1	6.3	19.2	36.8	77.4	24.7	57.7	64.6	72.9
	3	5.8	17.9	35.0	77.8	4.4	13.5	26.4	58.7	21.3	51.7	60.8	76.1
DF18	0	5.3	16.4	28.8	60.8	4.2	12.8	22.5	47.6	17.7	40.9	49.9	61.0
	1	5.3	16.1	31.7	63.7	3.6	10.9	21.4	42.1	16.7	41.9	55.5	63.7
	3	5.4	17.9	27.9	62.7	3.9	13.1	20.5	46.0	17.0	42.0	49.2	58.9
P values													
DF													
Linear		.2025	.1346	.0014	<.0001	.1500	.1055	.0029	<.0001	.2771	.5155	.8331	.2309
Quadratic		.0759	.0021	.0009	.001	.0269	.0009	.0007	.0005	.0427	.0001	.0072	.0217
SS													
Linear		.1707	.0745	.0598	.3033	.1237	.0643	.0656	.2301	.7914	.3955	.4048	.5968
Quadratic		.117	.2578	.0556	.3705	.1116	.278	.1015	.4818	.0717	.1048	.0552	.6017
DF × SS		.0799	.0162	.1032	.1218	.054	.028	.1714	.1397	.0412	.1462	.382	.3221

Abbreviations: DF, devil fish; DMD, dry matter degradability (%); GP, gas production; SS, *S. saprophyticus.*

during fermentation. Similarly, the characteristics of a feed consumed by animal influence the pH. In another words, during in vitro assay, the fluid pH is influenced by the substrate characteristics. In the present investigation, pH value was increased due to the supplementation of varied doses of DF. The increase in pH with DF supplementation may be attributed to the high protein and low carbohydrate in the substrate fermented, compared with the control, which had higher ground corn in it [29,30].

Elghandour et al [31] reported that H_2 removal stimulates bacteria during digestion. This indicates that the higher proportional of H_2 in DF18 throughout incubation period might have affected digestion. In contrast, the higher H_2 gas in DF6 could be an indication of production of more acetate and butyrate where H_2 is produced in the process [32]. The numerical reduction in CH_4 production at 24 hours of incubation by DF12 and DF18 may be attributed to the lower production of CO_2 and H_2 gas.

Borah et al [16] had reported the antagonistic activity of some species of *Staphylococcus*. In the present study, we observed lower gas production (mL/0.5 g DM incubated and degraded) with increasing doses of *S. saprophyticus*. This indicates that *S. saprophyticus* had some inherent antimicrobial and growth inhibitory properties. Besides, Khusro et al [11] reported that *Staphylococcus* sp. showed lack of amylolytic activity, which exhibited their inability to degrade starch. Therefore, the lower gas production encountered with *S. saprophyticus* supplementation may be attributed to the fact that bacteria are unable to degrade starch. Furthermore, Laukova' and Marekova' [33], Sung et al [34], and Khusro et al [13] had reported that CNS strains are ideal producers of bacteriocins. Thus, the bacteriocin produced by *S. saprophyticus* might have inhibited the fermentative microorganisms. The supplementation of *S. saprophyticus* at distinct doses revealed reduction in CH_4 emission. Low CH_4 production with

Table 6
Effect of SS as feed additives on in vitro fecal H_2 production at different incubation periods using different doses of ensiled DF.

DF Doses	SS Doses	mL H_2/0.5 Dry Matter Incubated				mL H_2/0.5 g Dry Matter Degraded				Proportional H_2 Production			
		8	12	24	48	8	12	24	48	8	12	24	48
DF0	0	0.11	4.22	7.77	16.89	0.09	3.14	5.78	12.55	0.33	9.00	10.0	13.00
	1	0.89	4.67	9.03	22.60	0.69	3.66	7.07	17.61	2.67	10.67	12.0	17.00
	3	0.75	4.34	8.48	23.99	0.56	3.26	6.37	17.97	2.67	10.67	11.6	17.67
DF6	0	1.24	5.91	11.85	29.48	0.96	4.58	9.19	22.87	3.33	12.00	13.6	19.67
	1	0.35	5.63	10.81	23.96	0.27	4.37	8.39	18.59	1.00	12.00	13.6	17.67
	3	0.34	6.47	14.38	32.17	0.26	4.93	10.96	24.52	1.00	15.00	19.6	23.67
DF12	0	0.36	5.56	11.79	26.69	0.28	4.25	9.02	20.45	1.00	11.67	14.6	19.33
	1	0.33	3.96	8.20	22.02	0.26	3.10	6.44	17.35	1.00	9.33	11.3	16.33
	3	0.27	2.78	6.30	14.86	0.21	2.10	4.76	11.21	1.00	8.00	10.6	14.33
DF18	0	0.30	6.56	12.49	25.68	0.24	5.12	9.76	20.08	1.00	16.33	21.6	25.67
	1	0.32	7.17	12.29	25.41	0.21	5.01	8.50	17.43	1.00	18.00	21.0	25.00
	3	0.32	6.06	10.76	24.52	0.23	4.34	7.88	17.89	1.00	14.00	18.6	23.00
P values													
DF													
Linear		.1937	.0279	.089	.3036	.1737	.0702	.1574	.4507	.1701	.0197	.0045	.0018
Quadratic		.4763	.0956	.4194	.5556	.5171	.1751	.5543	.7401	.4222	.02	.0485	.1252
SS													
Linear		.6329	.429	.5578	.8118	.5744	.3693	.4958	.6949	.895	.965	.8423	.9422
Quadratic		.9496	.8646	.7878	.7869	.9532	.9041	.7604	.7697	1.000	.2672	.774	.7695
DF × SS		.0589	.787	.6738	.4525	.0599	.8652	.7372	.5049	.099	.236	.6734	.6997

Abbreviations: DF, devil fish; DMD, dry matter degradability (%); GP, gas production; SS, *S. saprophyticus.*

S. saprophyticus supplementation may be explained by their ability to reduce nitrate to nitrite [35,36]. Furthermore, the present study showed that *S. saprophyticus* supplementation had no significant impact on CO_2 and H_2 emission. The presence of staphylococci might have enhanced formation, which would serve as terminal electron acceptors during fermentation of feeds [37].

Over the past few years, a significant attempt had been undertaken not only to improve the nutritional quality but also to reduce the emission of GHGs from livestock through the synergistic role of additives. In this regard, synergistic role of fibrous forages along with live yeasts and fibrolytic enzymes have been successfully investigated toward the improved fermentation as well as mitigation of CH_4 and CO_2 emission from equine [18,38,39]. The present investigation further filled the gap of equine research by evaluating the pivotal synergistic role of DF and *S. saprophyticus* as promising feed additives in the reduction of GHGs emission from horses.

5. Conclusions

Inclusion of DF at 6% of diet can improve feed gas production, without disrupting the gut pH. The supplementation of DF12 and DF18 reduced CH_4 production by 58.24% and 59.33%, respectively. DF18 reduced total CO_2 production by 15.25%, whereas SS1 and SS3 mitigated CH_4 production by 50.54% and 58.24%, respectively. Similarly, the low gas production pattern with *S. saprophyticus* supplementation indicates its antimicrobial properties, suggesting good prospect in livestock nutrition. The DF and *S. saprophyticus* could be potential feed additives as alternatives to conventional antibiotics.

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