

Use of essential oils in combination with fibrolytic enzymes to decrease in vitro ruminal methane production

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Abstract

Exogenous enzymes and essential oils (EOs) have both improved fibre digestion in ruminants, but the effect on ruminal fermentation and methane emission of combining these additives requires further evaluation. Various EOs were used in combination with an enzyme in an in vitro gas production system to measure organic matter digestibility and methane after 48 hours of incubation of *Eragrostis curvula*. The treatments consisted of T1) unaugmented control; T2) enzyme (cellulose-xylanase mixture, 1:1 w/w, 1.5 mL/g DM); T3) enzyme combined with Next Enhance® (cinnamaldehyde with diallyl disulfide and diallyl trisulfide, 43:3.85 w/wt); T4) enzyme and cinnamon oil; T5) enzyme and garlic oil; and T6) enzyme with cinnamon and garlic oil, 1:1 w/w). At 2, 4, 8, 12, 24, 36, and 48 hours of incubation, gas production was measured, and gas samples were analysed for methane concentration. The inclusion of additives affected cumulative gas production ($P < 0.05$), but only slightly affected methane production ($P = 0.05$) after 48 hours of incubation. The addition of enzymes increased both gas and methane production. T5 and T6 both reduced gas production compared with T2. Combining EOs with an enzyme reduced methane production compared with T2 after 48 hours of incubation. The additives tended to increase in vitro organic matter digestibility ($P = 0.09$), although they showed a tendency towards reduced methane production per unit of organic matter digested ($P = 0.07$). Further studies on these enzyme and EO combinations under different doses and substrates are required to validate their efficacy for ruminant production.

Keywords: cinnamon oil, cinnamaldehyde, garlic oil, gas production, glucanase, in vitro digestibility, xylanase

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Introduction

Efforts to improve growth by natural means have led to increased research into plant-derived compounds and their effects on ruminal fermentation (Calsamiglia *et al.*, 2007). Essential oils are volatile plant secondary metabolites that are currently gaining popularity as ruminant feed additives because they could act as alternatives to traditional antibiotic growth promoters (Wallace, 2004). The yield and composition of EOs from plants of the same species vary and this is responsible for each plant's characteristic flavour and fragrance (Benchaar & Greathead, 2011). The original role of EOs was to protect plants from abiotic and biotic stressors through their fragrance. They also attract organisms that pollinate and disperse seeds (Wink, 2010). Essential oils have promising effects through selective anti-bacterial activity, inhibition of ruminal methane emissions, enhancement of ruminal propionate proportions, and bypass of protein to the intestine (Benchaar *et al.*, 2007; Calsamiglia *et al.*, 2007). The antibacterial activity of garlic oil has been related to the presence of allicin and diallyl sulfides. Equally, cinnamaldehyde, a major component of cinnamon oil, has shown strong antimicrobial properties through the disruption of cytoplasmic membranes of microbes (Ouweland *et al.*, 2010; Benchaar & Greathead, 2011).

Mcintosh *et al.* (2003) found that EOs inhibit the energy metabolism of rumen microorganisms such as *Methanobrevibacter smithii*, a rumen archaea that contributes to methane production. The effect of EOs on methane mitigation has been evaluated in vivo and in vitro, but the reported responses in methane production were variable, which may be related to the natural diversity of these compounds (Calsamiglia *et al.*, 2007). Although in vitro screening has proved an effective procedure to identify plant compounds with

potentially positive effects (Beauchemin *et al.*, 2008), screening of multiple EO combinations and at different dose rates would improve the understanding and application of EOs in ruminant feeding.

Although fibrolytic enzymes have been used successfully to improve rumen fermentation with high forage diets (Van de Vyver & Cruywagen, 2013), there is an associated increase in methane production because of a higher proportion of acetate in the fermentation product (Gemedá *et al.*, 2014; Gemedá & Hassen, 2015). In contrast, EOs have been shown to mitigate enteric methane production *in vitro* and *in vivo* (Mutavhatsindi *et al.*, 2018). In the rumen, the antimethanogenic properties of EOs can militate against hydrogen, which is produced during acetate formation, being channelled into the methanogenic pathways. It was hypothesized that a combination of fibrolytic enzymes and various EO mixtures would provide an additive effect on rumen fermentation by improving feed digestibility while reducing methane production. The aim of this study therefore was to investigate the effects of cinnamaldehyde, garlic oils and their mixture, with or without fibrolytic enzymes, on *in vitro* gas and methane production and organic matter digestibility.

Materials and Methods

This study was conducted at the University of Pretoria's Experimental Farm following a protocol approved by the Animal Ethics Committee of the University of Pretoria (EC057-15).

Eragrostis curvula hay, which was used as substrate for *in vitro* fermentation, was obtained from a commercial feed mill in Gauteng, South Africa. Substrate samples were dried at 55 °C for 48 hours in a forced draft oven. For all *in vitro* studies and chemical composition analyses, the samples were milled through a 1-mm sieve in a Wiley mill (Arthur H. Thomas, Philadelphia, PA, USA). The samples were stored in sealable plastic bags at 4 °C prior to incubation and analysis. The six experimental treatments consisted of T1) unaugmented control; T2) enzyme (cellulose-xylanase mixture, 1:1 w/w, 1.5 mL/g DM); T3) enzyme combined with Next Enhance (cinnamaldehyde with diallyl disulfide and diallyl trisulfide (43:3.85 w/wt); T4) enzyme and cinnamon oil; T5) enzyme and garlic oil; and T6) enzyme with cinnamon and garlic oil (1:1 w/w). The enzyme compound in treatments T2–T6 was a commercial product that contained a mixture of two enzymes that consisted of concentrated liquids of acid cellulase (E.C. 3.2.1.4) and acid-neutral endo-1, 4-β-D-xylanase (E.C. 3.2.1.8), whereas the EO product in T3 was Next Enhance. The cinnamaldehyde and garlic oil were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Ten mg of each EO were dissolved in 10 mL of ethanol and a 1 mL aliquot of each solution was added to the substrate in incubation vials to give a final concentration of 30 mg/L with the addition of 33 mL mixed rumen liquor. The concentrations of garlic oil and cinnamaldehyde were based on previous research (Busquet *et al.*, 2005). All additives were stored at 5 °C in a smoked glass flask before use (Busquet *et al.*, 2005). For each treatment of enzyme or EO preparation, the required dose rates were diluted to deliver the specific concentration in 1 mL aliquots. The density of the enzyme and carrier mixture was different from that of water and this was corrected for. A fresh batch of dilutions was prepared for each *in vitro* incubation run. The amount of 400 mg *Eragrostis* hay was weighed and 1 mL of enzyme or EO treatment was added to each 125 mL serum bottle 24 hours before the start of incubation as pre-treatment of the milled substrate to allow for an enzyme-substrate interaction (Van de Vyver & Cruywagen, 2013). The control treatment received 1 mL of distilled water to standardize the volume of liquor in the vials.

Rumen buffer and macro-mineral and micro-mineral solutions were prepared according to Goering and Van Soest (1970) with modifications of Mould *et al.*, (2005) by replacing magnesium sulphate heptahydrate with magnesium chloride in the macro mineral to prevent excess sulphate in the *in vitro* media. The prepared buffer solution was placed in a water bath at 39 °C with a constant stream of CO₂ until the solution turned colourless. Rumen-cannulated Merino sheep that were used as rumen fluid donors were fed lucerne hay as basal diet *ad libitum*. Rumen fluid collections were done at 08h30 throughout the trial after the morning feeding. Approximately 500 mL of ruminal fluid was collected from at least three donor animals. Rumen contents were filtered through four layers of cheesecloth into a pre-heated thermos flask. The flask was filled with rumen fluid to maintain anaerobic conditions. In the laboratory, the measured volume of rumen fluid was added to the prepared buffer solution and continuously purged with a stream of CO₂ at 39 °C.

The substrate, enzyme, and EO aliquots were added to serum bottles with 33 mL of mixed rumen liquor under a constant stream of CO₂ to achieve 82.5 mL/g substrate. They were then sealed with butyl rubber stoppers equipped with 1.5-inch needles and three-way stopcocks. After inoculation, the stopcock of every vial was opened for five seconds to release the small amount of gas that might have built up and create a starting point for the incubation. The vials were returned to the Incoshake incubator (Labotec (Pty) Ltd., Midrand, South Africa), which was set at 39 °C. Bottles that contained only mixed rumen liquor were used in each incubation run as blanks to correct for the gas produced as a result of rumen fluid. Four replicate bottles were incubated for the blank and for each treatment, and three repeat runs were carried out. Gas pressure was measured with a pressure transducer (PX4200-015GI, Omega Engineering, Inc., Laval,

QC, Canada) as described by Theodorou *et al.* (1994) at 2, 4, 8, 12, 24, 36, and 48 hours after incubation. Gas samples were collected and analysed using a gas chromatograph equipped with a flame ionization detector (8610C BTU Gas Analyser GC System, SRI Instruments GmbH, Bad Honnef, Germany). After 48 hours of incubation, fermentation was terminated by placing all bottles on ice. In vitro organic matter digestibility (ivOMD) was determined according to the method of Tilley & Terry (1963) as modified by Engels and Van der Merwe (1967).

Data were analysed as a randomized complete block design using the ANOVA procedure of SAS 9.4 (SAS Institute, Inc., Cary, North Carolina, USA). Blocks were defined as independent runs of the in vitro gas production system. Thus, the linear model included the effects of treatments and block. Significance was declared when $P < 0.05$ and a tendency when $P \geq 0.05$ but < 0.1 . When there were significant differences between treatments, differences among the means were assessed using Tukey's test.

Results and Discussion

The inclusion of dietary additives did not affect in vitro gas production at 2 hours of incubation ($P > 0.05$) (Table 1). However, at 4 hours of incubation and up to 48 hours, there were differences among the treatments ($P < 0.05$). T5 and T6 produced similar quantities of gas in vitro up to 48 hours incubation compared with T1. Generally, T2 had higher in vitro gas production from 8 hours to 48 hours incubation compared with T1 ($P < 0.05$). Only a slight increase in the in vitro gas production could be associated with T3 and T4 across the incubation periods compared with T1.

Table 1 In vitro gas production of *Eragrostis curvula* hay treated with various enzyme and essential oil combinations over 48 hours

Treatments	Gas production (mL/g dry matter) at various hours of incubation						
	2	4	8	12	24	36	48
T1	2.44	4.90 ^{ab}	11.7 ^{bc}	24.5 ^b	60.8 ^{bc}	93.3 ^b	118.3 ^b
T2	4.51	7.88 ^a	15.6 ^a	29.1 ^a	67.0 ^a	100.1 ^a	125.0 ^a
T3	3.46	6.31 ^{ab}	13.9 ^{ab}	27.3 ^a	64.1 ^{ab}	96.8 ^{ab}	121.0 ^{ab}
T4	5.00	8.07 ^a	14.8 ^{ab}	27.1 ^a	63.6 ^{abc}	96.0 ^{ab}	121.0 ^{ab}
T5	2.42	5.14 ^{ab}	11.8 ^{bc}	24.2 ^b	60.5 ^{bc}	93.1 ^b	116.9 ^b
T6	2.19	4.08 ^b	10.58 ^c	22.7 ^b	59.8 ^c	92.1 ^b	116.0 ^b
SE	0.84	0.76	0.64	0.48	0.76	1.07	1.09
P-value	0.16	0.02	<0.01	<0.01	<0.01	<0.01	<0.01

T1: unaugmented control, T2: enzyme (cellulose-xylanase mixture), T3: enzyme combined with Next Enhance, T4: enzyme and cinnamon, T5: enzyme and garlic oil, T6: enzyme with cinnamon and garlic oil

^{a,b,c} Within a column, means with a common superscript were not different with probability $P = 0.05$

Dietary additives affected methane production from the *Eragrostis curvula* hay ($P < 0.05$) except at 24 hours and 36 hours incubation, when there was only a tendency towards significance ($P = 0.07$). Across the incubation periods, enzyme supplementation increased methane production compared with the control diet, but this was more pronounced in the early incubation period (up to 8 hours) (Table 2). The enzyme and EO additives affected 48 hours methane ($P = 0.05$). T5 and T6 reduced ivCH₄ compared with T1, although T2 did not affect 48 hour ivCH₄ production. Nevertheless, T3 and T4 were not different from T1 or T2 in methane production. Furthermore, all treatments that contained EOs appeared to produce less ivCH₄ during the early stages of the incubation period compared with T2. T4, T5 and T6 continued to produce less ivCH₄ up to 12 hours after incubation, with T6 producing less ivCH₄ throughout the 48-hour period.

No differences were observed across treatments in in vitro organic matter digestibility (ivOMD), the ratio of methane to organic matter digestibility (ivCH₄/ivOMD), the ratio of methane to gas production (ivCH₄/ivGP) and the ratio of gas production to organic matter digested (ivGP/ivOMD) following the 48 hour incubation period. However, there was a tendency for the additives to affect ivOMD (g/kg DM) ($P = 0.09$), ivCH₄/ivOMD (mL/kg DM) ($P = 0.07$), and ivGP/ivOMD (mL/kg DM) ($P = 0.09$) (Table 3). In vitro organic matter digestibility (ivOMD) was highest in T2 and lowest T. Whereas ivCH₄/ivOMD was highest in T2, it was

lowest in T6. The digestion of *Eragrostis curvula* hay augmented with T5 and T3 had only slightly lower production of $\text{ivCH}_4/\text{ivOMD}$ compared with T1.

Table 2 Cumulative in vitro methane production from *Eragrostis curvula* treated with various enzyme and essential oil combinations over 48 hours

Treatments	Methane production (mL/g dry matter) at various hours of incubation						
	2	4	8	12	24	36	48
T1	0.001 ^b	0.010 ^b	0.06 ^b	0.24 ^{ab}	1.43 ^{ab}	2.89 ^{ab}	4.19 ^{ab}
T2	0.017 ^a	0.033 ^a	0.12 ^a	0.34 ^a	1.68 ^a	3.25 ^a	4.59 ^a
T3	0.003 ^b	0.010 ^b	0.06 ^b	0.25 ^{ab}	1.42 ^{ab}	2.88 ^{ab}	4.15 ^{ab}
T4	0.003 ^b	0.013 ^b	0.07 ^b	0.23 ^b	1.44 ^{ab}	2.91 ^{ab}	4.26 ^{ab}
T5	0.003 ^b	0.010 ^b	0.06 ^b	0.22 ^b	1.34 ^{ab}	2.70 ^{ab}	3.90 ^b
T6	0.001 ^b	0.003 ^b	0.05 ^b	0.20 ^b	1.26 ^b	2.59 ^b	3.89 ^b
SEM	0.01	0.01	0.01	0.02	0.08	0.13	0.14
P-value	<0.01	<0.01	<0.01	0.02	0.07	0.06	0.05

T1: unaugmented control, T2: enzyme (cellulose-xylanase mixture), T3: enzyme combined with Next Enhance, T4: enzyme and cinnamon, T5: enzyme and garlic oil, T6: enzyme with cinnamon and garlic oil
^{a,b,c} Within a column, means with a common superscript were not different with probability $P=0.05$

Table 3 In vitro organic matter digestibility and the relationship between methane, gas production and organic matter digestibility in vitro after 48 hours of incubating *Eragrostis curvula* with essential oils and fibrolytic enzymes

Treatments	ivOMD, g/kg DM	$\text{ivCH}_4/\text{ivOMD}$, mL/kg DM	$\text{ivCH}_4/\text{ivGP}$, mL/mL	ivGP/ivOMD , mL/kg DM
T1	488	8.53	3.52	241
T2	507	8.97	3.64	246
T3	495	8.32	3.41	245
T4	494	8.56	3.49	245
T5	492	7.88	3.32	237
T6	493	7.82	3.31	235
SE	0.38	0.26	0.10	2.82
P-value	0.09	0.07	0.26	0.09

T1: unaugmented control; T2: enzyme (cellulose-xylanase mixture); T3: enzyme combined with Next Enhance; T4: enzyme and cinnamon; T5: enzyme and garlic oil; T6: enzyme with cinnamon and garlic oil; ivOMD; in vitro organic matter digestibility; ivCH_4 : in vitro methane; ivGP: in vitro gas production

The low nutritive value of *Eragrostis* hay was an indication that higher methane output per unit of feed consumed could be expected as a result of the associated low productivity of animals that consumed this feedstuff. Therefore, in supplementation with additives that could improve dry matter degradability and reduce methane emission, a loss of feed energy would guarantee its improved utilization for sustainable livestock production. In the early incubation period, enzyme supplementation resulted in only a slight increase in ivGP, but EO supplementation, especially cinnamon and garlic oils, reduced ivGP. Previous studies showed that higher ivGP was observed in the early incubation intervals with exogenous enzyme treatment of lucerne forage (Colombatto *et al.*, 2007), sugarcane leaves and sorghum straw (Elghandour *et al.*, 2013), and in a grass hay/concentrate substrate (Giraldo *et al.*, 2008). Higher ivGP has been used to validate the effect of fibrolytic enzyme in improving the digestibility of feedstuffs (Cantet *et al.*, 2016). Fibrolytic enzymes act on the slowly but progressively fermentable fraction of feed DM (largely the NDF and

ADF) through reduction in cell wall thickness (Van de Vyer & Cruywagen, 2013), allowing rumen microbes and fermentation enzymes an easier access to plant cell constituents (Pinos-Rodríguez *et al.*, 2008). Unlike T5 and T6, T2 resulted in a significant increase in 24 hour and 48 hour cumulative ivGP. It appears that the hemicellulose of the cell wall components was degradable more consistently in reported studies, largely because of the initial consumption of carbohydrates through the activities of endo- and exo-glucanase enzymes, with cellulose being degraded to a lesser degree, whereas lignin was undegraded (Rodrigues *et al.*, 2008). This was evident in the higher gas production observed after 12 hours incubation, unlike the early incubation period.

The various growth media in which enzymes were produced and the differences in strains usually resulted in a wide variation in response to enzyme supplementation across various trials (Castro-Montoya *et al.*, 2015). The enzyme product used in the current study was a 1:1 mixture of cellulose and xylanase enzymes. Although the potential synergistic effects of two or more enzymes in cell wall degradation was noted by De Vries *et al.*, (2000), improvements in the ivOMD, neutral detergent fibre (NDF) and crude protein (CP) degradability after the addition of these multienzyme products were variable. Whereas enzyme supplementation improved DM, NDF and CP digestibility in a dairy cow diet (Elghandour *et al.*, 2013), Jalilvand *et al.* (2008) did not observe any differences in gas production with the addition of exogenous enzyme mixtures (cellulase, xylanase, alpha-glucanase, protease and amylase) to lucerne hay, wheat straw and maize silage. Diet type and differences in enzyme activity may account for wide variations in the potency of enzyme products, even when supplemented at the same concentration. Nevertheless, Colombatto *et al.* (2007) noted that exogenous enzyme treatments tended to increase cellulose and xylan degradability *in vitro*, owing primarily to greater colonization of cell wall surfaces by enzymes. The ability of cellulases and xylanases to increase the extent of fibre digestion may be limited by the lack of enzymes that degrade the core structure of lignin-cellulose complexes in low-quality forages (Carro *et al.*, 2007).

The strong antiseptic properties of cinnamon and garlic oils exert an antimethanogenic effect on rumen fermentation, as evidenced by the tendency to reduce ivCH₄ in the current study. Cinnamaldehyde is the main active agent in the commercial EO compound and cinnamon oil treatments (Khorrami *et al.*, 2015). The antimicrobial activity of cinnamaldehyde has been linked to the reactivity of its carbonyl group, although the mechanism of antimethanogenic action is not known fully (Calsamiglia *et al.*, 2007). Equally, garlic oil is known to contain organosulfur compounds such as alliin and allicin, diallyl sulphide, diallyl disulphide (DADS) and allyl mercaptan as bioactive compounds (Patra & Yu, 2012; Pawar *et al.*, 2014). However, diallyl disulfide and allyl mercaptan were noted to be responsible for most of the effects on rumen fermentation (Busquet *et al.*, 2005). Although numerous studies have been conducted on the use of EOs in ruminant nutrition, the results have varied widely (McGinn *et al.*, 2004; Busquet *et al.*, 2005; Klevenhusen *et al.*, 2011) and the arguments against the comparison of results between studies can be justified because few studies involve identical EO compositions (Castro-Montoya *et al.*, 2015). The bioactive compounds in EOs vary in chemical structure and stereochemistry, and therefore tend to alter rumen microbial population differently (Calsamiglia *et al.*, 2007).

Although previous reports on the antimethanogenic effects of EOs showed some promise, the results varied widely and were inconsistent. Klevenhusen *et al.* (2011) supplemented DADS, a principal organosulfur compound, in garlic oil at 2 g/kg DM in sheep ration and did not observe any significant effect on methane emissions, although an *in vitro* study by Busquet *et al.* (2005) showed significant methane suppression with DADS at 300 mg/L inclusion level. In the study by Pawar *et al.* (2014), garlic and cinnamon oils at 167 µL/L of incubation media reduced *in vitro* methane production significantly without negative effects on the organic matter digestibility of the wheat straw and concentrate mixture. Equally, Patra and Yu (2012) observed significantly reduced methane production with increasing doses of garlic oil from 0.25 g/L, with 1.0 g/L showing a 42% decrease in the *in vitro* methane production without any significant depression in DM and NDF degradability. The effect of cinnamon oil as a rumen modifier in beef steers showed that 500 mg/kg DM cinnamon oil did not depress nutrient digestibility or alter rumen fermentation, but reduced the relative abundance of protozoa and methanogenic archaea (Khorrami *et al.*, 2015). In a similar study, horseradish oil modulated rumen fermentation in Holstein steers effectively by reducing acetate and increasing propionate of volatile fatty acids (VFAs), while decreasing methane production by 19% and showing a significant reduction in the methanogen population of the rumen (Mohammed *et al.*, 2004). The pH of rumen or *in vitro* incubation media, which is influenced by diet type, greatly affects the efficacy of EOs.

Cardozo *et al.* (2005) observed that garlic oil supplementation of a high concentrate beef ration showed that at pH 7.0, ammonia nitrogen and total VFA concentration decreased compared with the control. However, at a pH of 5.5, although ammonia nitrogen concentration decreased, total VFA concentration increased, and there was a shift in the molar proportion of VFA from acetate to propionate. This variation in response was related to the higher susceptibility of microbes to the effects of EOs at low pH (Skandamis & Nychas, 2000) with lower pH being associated more with animals that consumed high concentrate diets. In

this study, rumen fluid was obtained from donor sheep that consumed lucerne and therefore the impact of EOs on rumen fermentation might be less observable. The presence and relative position of a hydroxyl group in the phenolic structures of EOs have been noted as being capable of influencing the antimicrobial potency of EOs in the rumen (Ultee *et al.*, 2002). Garlic oil exerted a more inhibitory effect on methanogenic archaea than on rumen bacterial populations. This was associated with the effect of its organosulfur compounds in inhibiting the HMG-CoA reductase enzymes, which are responsible for the synthesis of isoprenoid side chains in the archaeal cell membrane lipids (Busquet *et al.*, 2006; Calsamiglia *et al.*, 2007; Kamel *et al.*, 2008).

Although there have been few studies on the potential interaction effect of exogenous polysaccharide degrading enzyme and plant phytochemicals on rumen fermentation (Salem *et al.*, 2015), the current study did not show a significant additive effect between enzyme and EO inclusion in reducing methane or improving digestibility. Nevertheless, the tendency to reduce methane without depressing organic digestibility justifies further studies on the optimal doses of cinnamon and garlic oils for practical application with poor-quality roughage feed such as the *Eragrostis curvula* hay.

Conclusion

The addition of an exogenous fibrolytic enzyme increased *in vitro* gas production, but a methane suppressing effect was not observed, even when augmented with an EO. However, there was a tendency for augmentation with garlic oil alone or in combination with cinnamon to reduce *in vitro* methane production after 48 hours incubation. Organic matter digestibility was not affected by the inclusion of additives. The combination of EOs with anti-methanogenic effects and that do not depress organic matter digestibility would seemingly have application in sustainable ruminant systems. A combination of an exogenous fibrolytic enzyme and garlic oil, at higher doses, is recommended for further *in vitro* and *in vivo* evaluation.

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Authors' Contributions

TCN and AH designed the study. TCN did data collection and TCN, AH and AMA did data analysis. TCN and FAA wrote the draft manuscript and AMA and AH did the editing and proofreading. AH funded and supervised the study.

Conflict of Interest Declaration

The authors declare that there was no conflict of interest in the conduct of this study.

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