INFLUENCE OF SECONDARY PLANT METABOLITES ON GROWTH OF *CLOSTRIDIUM PERFRINGENS* FROM CHICKENS

VLIV SEKUNDÁRNÍCH ROSTLINNÝCH METABILOTŮ NA *CLOSTRIDIUM PERFRINGENS* Z KUŘAT

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ABSTRACT

Clostridium perfringens is the causative agent of necrotic enteritis. Certain plant secondary products were investigated as potential agents to reduce the risk of *C. perfringens* colonisation in chickens. Four essential oils and 3 condensed tannins were tested. Fermentative activity of *C. perfringens* was inhibited by all essential oils tested, with the most effective being Lemon Myrtle (*Backhousia citriodora*) with a MIC of 0.05% v/v. This was at least twice as effective as Tea Tree oil. Condensed tannins were also effective as antimicrobial agents, with MICs of 0.6 – 1% w/v, where feed intake impairment *in vivo* did not occur until at least 3% w/v condensed tannin. The potential application of these secondary plant metabolites to feed formulations *in vivo* needs to be tested.

Keywords: *Clostridium perfringens*, necrotic enteritis, essential oil, tannin

ABSTRAKT

Clostridium perfringens je Gram pozitivní, anaerobní, sporulující a plynotvorná bakterie, která je původcem nekrotické enteritidy a dalších onemocnění. Jedním z možných způsobů snížení rizika infekce kuřat touto bakterií je používání určitých sekundárních rostlinných produktů. V našem experimentu byly testovány čtyři esenciální oleje a tři kondenzované taniny. Všechny vykázaly schopnost inhibice fermentační aktivity bakterie *Clostridium perfringens*. Nejúčinějším inhibitorem byl olej z citronové myrty (*Backhousia citriodora*), jehož minimální inhibiční koncentrace (MIC) 0,05% byla dvakrát nižší než MIC u oleje z čajovníku. Kondenzované taniny byly účinné v rozmezí od 0,6 do 1%. Ke snížení příjmu potravy *in vivo* vlivem taninů docházelo až při koncentraci 3%. Naše výsledky ukazují na potenciální využitelnost sekundárních rostlinných metabolitů v chovu drůbeže, a proto by jim měla být věnována zvýšená pozornost, především pak při testech *in vivo*.

Klíčové slova: *Clostridium perfringens*, nekrotická enteritida, esenciální olej, tanin

INTRODUCTION

In the UK some 1,250,000 T of poultry meat were produced in 2004 (cf 680,000 T of beef and 317,000 T of sheep meat). Economic production of this quantity of poultry meat requires intensive growing techniques however, following concerns about the increase in bacteria exhibiting multiple resistance to antibiotics, the use of in-feed antibiotic growth promoters (AGP) has been severely restricted in the EU (Ross Tech, 1999). This has resulted in an upsurge in specific (eg necrotic enteritis) and non-specific (wet litter syndrome, dysbacteriosis) enteric problems in chickens. While necrotic enteritis (NE) can result in the death of up to 40% of the birds in an affected flock, it is the sub-clinical form of the disease that may, in terms of welfare and productivity, be more important as this is often not diagnosed or treated (Kaldhusal and Hofshagen, 1992).

The causative agent of NE i*s Clostridium perfringens,* Types A or C, and the rapid proliferation of this bacterium is associated with gaseous extension of the small intestine, the production of one or more exotoxins, and enteric toxicosis (Asaoka *et al*, 2004). Ultimately, foci of gut epithelial tissue become necrotic, which may lead to death of the bird. It has been estimated that 75%–95% of birds are colonised by *C. perfringens* and there are various predisposing factors (of which pre-existing disease states and dietary factors are highly significant). In the future, management, including dietary strategies will be critical methods of control of NE and similar enteric syndromes with poorly defined aetiologies or pathologies.

Essential oils have been evaluated in an attempt to control coccidiosis (Youn and Noh 2001), and *C. perfringens* (Baratta et al, 1998). Oregano essential oil was shown to be as effective as Salinomycin in reducing the severity of coccidiosis in broilers and commercial preparations of Oregano oil are available as feed additives.

Ongoing studies in our laboratories are aimed at identifying a range of plant secondary products, including terpenes, flavonoids, condensed tannins and saponins that may have selective antimicrobial activity against *C. perfringens*, whilst not effecting commensal bacteria in the GIT of broilers. Plant secondary metabolites have the potential to replace antibiotics as feed additives for livestock. However, the vast number of these compounds in nature (Wink, 2004) makes their testing difficult, especially if animal trials are required. The gas production technique provides an *in vitro* method to assess the effect of plant secondary products on fermentative organisms from the gastrointestinal tract of animals.

In this paper we report on studies with some essential oils containing citral and 3 different condensed tannins as potential agents to control growth of *C. perfringens*.

MATERIAL AND METHODS

Materials

Biochemicals were obtained from Sigma-Aldrich, UK. Powdered bacterial media were obtained from Oxoid Ltd, Basingstoke, UK.

Plant secondary products

Essential oils were supplied by Essentially Oils Ltd, Oxfordshire, OX7 6NP, UK: lemon myrtle (LM, *Backhousia citriodora*), eucalyptus oil (EU, *Eucalyptus citriodora*); lemon tea tree oil (LTO, *Leptospermum petersonii*); tea tree oil (TTO, *Melaleuca altemifolia*). The composition of each essential oil tested is shown in Table 1.

Tannins were supplied as follows: grape seed extract (50%), TARAC Ltd, Adelaide, Australia; mimosa powder, Roy Wilson Dickson (UK); quebracho powder (*Schinopsis balansae*) (60%), JMG Santos (Portugal).

Bacterial strains

Isolates of *Cl. perfringens* were obtained from gut samples of chickens affected with necrotic enteritis, plate purified on TSC agar (Oxoid) and typed by Professor I. Poxton, University of Edinburgh. Isolates 72 and 100 were classified as glu^+, lac^+, $inositol^+$, $gelatinase^+$, $lecithinase^+$, $indole^-$, $lipase^-$, Gram positive rods. Both isolates were characterised phylogenetically as *Cl. perfringens* Type A.

Preparation of bacterial medium

Thioglycollate broth (Oxoid) for clostridial cultures were prepared anaerobically following manufacturers instructions. Media were left to cool in an anaerobic chamber for at least 4 hours before aliquoting into Hungate tubes and autoclaving. TSC agar plates, containing the TSC supplement (Oxoid) were prepared according to the manufacturer's instructions, and then stored under anaerobic conditions until use.

Gas production measurements

Gas pressure was measured using a modification of the *in vitro* fermentation method described by Theodorou *et al.* (1994, 1998). This is a static batch culture procedure (Gibson and Fuller 2000) in which measurement of gas volume at regular intervals is used to assess fermentation kinetics. Clostridial culture, with or without additives, was prepared in Hungate tubes containing 5 ml of medium, and preincubated in a covered water bath at 39 ^oC for 15 minutes. The tubes were almost fully immersed to maintain a constant internal temperature. At zero time, pressure in each tube was equalised using a 25G syringe needle attached to a bypass tube. Every 2 hours, gas pressure was measured in each tube using a digital pressure transducer (Theodorou *et al.* 1994), and then released through a bypass tube. Tubes were incubated for up to 20 hours and the cumulative gas production over the period of the incubation was calculated from the sum of the individual measurements.

Six replicates per inoculum were used for essential oils and tannins and three replicates for microflora isolated from the gastrointestinal tract of a chicken and for experiments to investigate bactericidal or bacteriostatic effects of EO and tannins.

A standard curve of gas volume versus pressure was established using the same procedure except the culture tube was not inoculated with bacteria, and pressure changes were generated with air, using a graduated 1 ml syringe.

RESULTS AND DISCUSSION

Effect of essential oils and condensed tannins on fermentation of C. perfringens

To Hungate tubes containing 5 ml of TGB medium were added 0.02, 0.05, 0.10, 0.20 % v/v of each essential oil (EO) or 0.05, 0.1, 0.5, 1.0 % w/v of each condensed tannin. All tubes were inoculated by 0.1 ml of a fresh overnight culture of C. perfringens (2 field isolates tested) and transferred to the water bath of 39 ^{o}C. Six fold-replicated cultures were set up to measure gas production every 2 hours over a 20 hour period. The mean volume of gas produced was calculated from the regression equation of the pressure/volume standard curve.

The results, Fig. 1a (Cp100) and 1b (Cp72), show that for the 4 essential oils tested, all inhibited fermentation of both isolates of C. perfringens at EO concentrations greater than 0.1% v/v. The minimum inhibitory concentration (MIC) for each oil and each isolate are shown in Table 2. Lemon myrtle was the most consistent of the essential oils, having an MIC of 0.05%; both grape seed and mimosa condensed tannin had MICs of 1.0% for both isolates (Fig 2a and 2b). Quebracho condensed tannin was effective against Cp100 at 0.6% w/v, but Cp72 appeared more resistant.

The data were consistent across replicates and duplicated treatments, and demonstrate that each of the EOs, as well as the condensed tannin tested, have the potential to be used as a control agent against C. perfringens. Of the oils tested, lemon myrtle was the most consistent, and had the lowest MIC. Grape seed condensed tannin was the most consistent of the tannin preparations and had the lowest MIC.

Bacteriocidal versus bacteriostatic effects

To test whether the effects of EOs and condensed tannins were bacteriocidal or bacteriostatic, cultures containing 0.1 and 0.2 % of each EO and 1, and 2 % w/v of each condensed tannin were inoculated with C. perfringens cultures and incubated at 39 ^{o}C. Gas production was measured for 20 hours, after which samples were diluted and spread on TSC agar plates. After 24 hours anaerobic incubation, colonies were counted. The results (Table 3), show that 0.2 % EO and 2 % of tannins were bacteriocidal against Cp 100, and almost no bacteria survived the treatment. In contrast, Cp 72 was more resistant and there was a low but significant survival (up to 8% with TTO) after EO treatment, although only about 1.5 % of Cp72 survived after 2 % w/v condensed tannin treatment. Grape seed condensed tannin was bacteriocidal for both isolates at the concentrations tested. The result with quebracho tannin was unusual in that at 0.6% w/v, 100% of Cp100 and 44% of Cp72 survived the treatment. In contrast, gas production of Cp100 was completely inhibited by the tannin, but for Cp72, gas production was unaffected. Higher levels of condensed tannin (2% w/v) was bacteriocidal for

both and completely inhibited gas production also. This suggests that at a critical concentration (0.6% w/v) of the quebracho condensed tannin, bacterial metabolism was inhibited, perhaps by restricting nutrient supply to the bacterium, although the bacterium itself was still viable. At the higher concentration, the tannin effect was toxic.

The data suggest that, at least for the EOs and tannins tested, at higher than critical concentrations, they were mainly bacteriocidal in action; they may therefore have potential as cleaning agents to reduce environment levels of pathogens such as C. perfringens, or as feed additives to reduce the risk of gut colonisation with this organism.

Effect of essential oils and condensed tannins on chicken gut microflora

The results above suggest that the EOs and the condensed tannins that were tested may be useful control agents against C. perfringens. However, it is important to determine whether the effective concentration of these is likely to have an impact on the commensal microflora of the gut.

A digesta sample (5 gm) from a chicken fed a standard grower diet was suspended in 5 ml of TGB medium. Hungate tubes containing 0.05, 0.10, 0.20 % of each EO were inoculated with 0.1 ml of microflora in the digesta sample and gas production was measured at 2 hourly intervals for 20 hours. The results (fig 3) show that at a concentration of 0.1% v/v, there was no significant effect on gas production with lemon myrtle, Eucalyptus citradora and lemon tea tree EOs. At 0.1% v/v, tea tree oil however inhibited gas production by 70%. At a concentration of 0.2% v/v, all EOs tested inhibited gas production by >60% (results not shown). Previous work in our laboratory has shown that grape seed tannin at up to 3% w/v had no significant effect on feed intake, FCE or live weight gain in chickens, from 7 – 42 days of age (Hughes et al, 2004, unpublished).

These data suggest that at the MIC level for lemon myrtle (0.05%) and grape seed condensed tannin (1%), these agents are unlikely to have a detrimental impact on the commensal microflora in the gut. On the other hand, Tea tree oil had an MIC of 0.1-0.2% v/v, and at this level, also inhibited fermentation of the gut microflora by 60-70%. This suggests that there can be some selectivity in the organisms affected by EOs; C. perfringens is more sensitive to lemon myrtle oil than are the commensal microflora of the gut. This may be due to increased sensitivity of Gram positive organisms to EOs than Gram negative organisms, or to other factors such as membrane transport.

GC analysis of the Eos (Table 1), shows that lemon myrtle has the highest concentration of the terpene, citral. This may explain the greater inhibitory effect of this EO compared with others. However, because EOs have complex compositions, it is impossible to identify which compounds, either alone or in synergistic action with others, are actually biologically active.

CONCLUSION

Overall, these data show that the citral (neral plus geranial) containing EOs, particularly lemon myrtle, and grape seed condensed tannin, were effective at inhibiting growth of *C. perfringens*, and may have uses in the poultry industry to reduce environmental spread of this organism, and possibly to reduce gut colonisation through incorporation as feed ingredients. We have already shown that the level of condensed tannin that inhibits *C. perfringens* has no significant effect on chicken growth. Similar studies are needed with lemon myrtle oil.

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Table 1. Composition of essential oils. Composition was determined by GC and each peak is described as % of total peak area.

Plant species	EO Components	Proportional
Backhousia citriodora (LM)	neral	36.8
	geranial	50.9
Melaleuca altemifolia (TTO)	α-terpinene	11.0
	γ-terpinene	20.6
	(+)-terpinen-4-ol	39.3
Eucalyptus citriodora (EC)	citronellal	72.9
	iso-pulegol	5.0
	β-pinene	6.1
	citronellol	6.4
Leptospermum petersonii (LTT)	citronellal	23.8
	neral	20.4
	geranial	26.8
	citronellol	4.7

Table 2. MIC for each secondary plant metabolite against 2 field isolates of C. perfringens.

Secondary metabolite	MIC Cp 100	MIC Cp 72
Lemon Myrtle	0.05	0.05
Lemon Tea Tree	0.05	0.10
Eucalyptus citriadora	0.10	0.10
Tea Tree	0.20	0.10
Grape seed tannin	1.0	1.0
Mimosa tannin	1.0	1.0
Quebracho tannin	0.05	1-2

Table 3. Bacteriocidal effects of essential oils and condensed tannins

Plant secondary product	Survival (%)	Survival (%)
NIL	100	100
LM 0.1	ND	1.3
LM0.2	0.4	3.4
LTT 0.1	ND	2.1
LTT 0.2	0.0	0.3
EC 0.1	ND	7.5
EC 0.2	0.4	7.1
TTO 0.1	ND	14.4
TTO 0.2	0.2	7.9
Grape seed tannin 1%	1.2	1.5
Grape seed tannin 2%	0.0	1.4
Mimosa tannin 1%	3.8	0.7
Mimosa tannin 2%	0.5	1.4
Quebracho tannin 0.6%	104	44.4
Quebracho tannin 2 %	0.1	1.3

Fig 1a

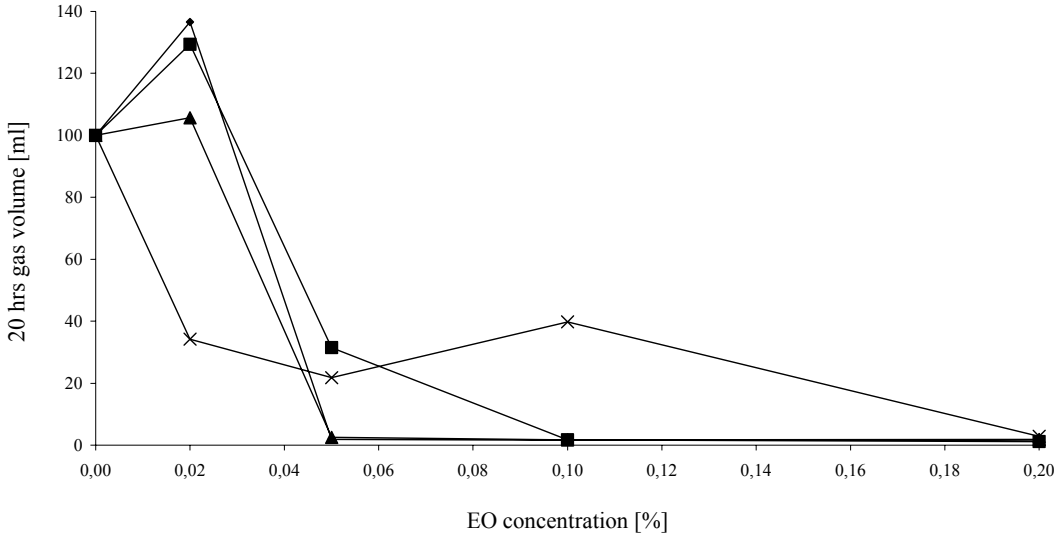

Fig 1b

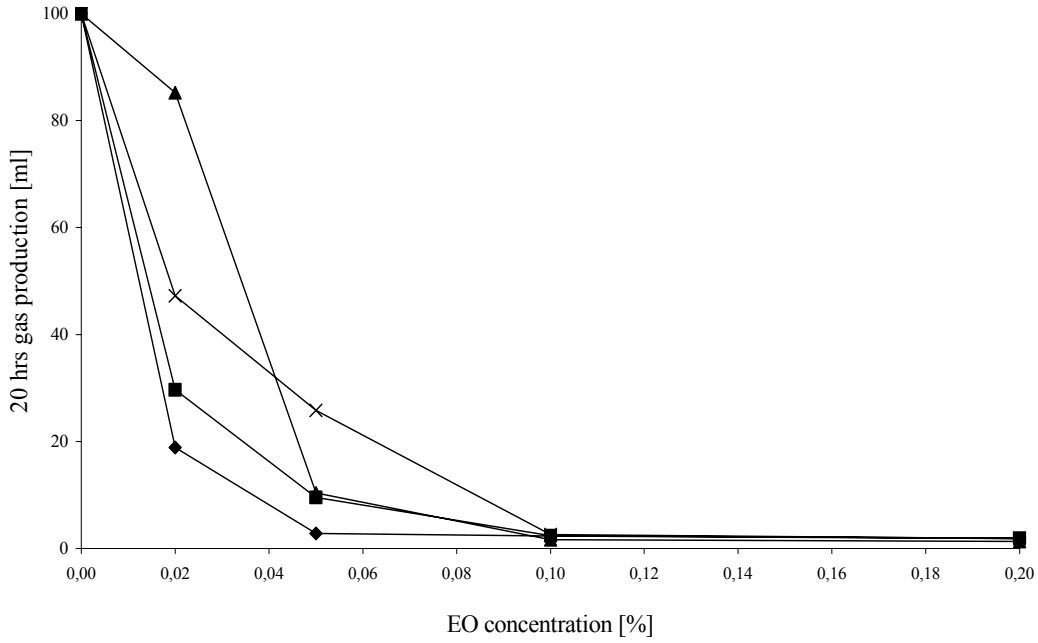

Fig 1. Effect of essential oils on gas production from* C. perfringens *Cp100 and Cp72. *Cultures of* C. perfringens *Cp100 and Cp72 were incubated in the presence of increasing EO and gas production was measured at 2 hourly intervals up to 20 hours. Results are expressed as cumulative gas production (a) Cp100, Lemon myrtle (LM), ♦;* Eucalyptus citriadora *(EC), ■; Lemon Tea Tree (LTT), ▽; and Tea Tree oil (TTO), ×. (b) Cp72, LM, ♦; EC, ■; LTT, ▽; and TTO, ×.*

Fig 2a

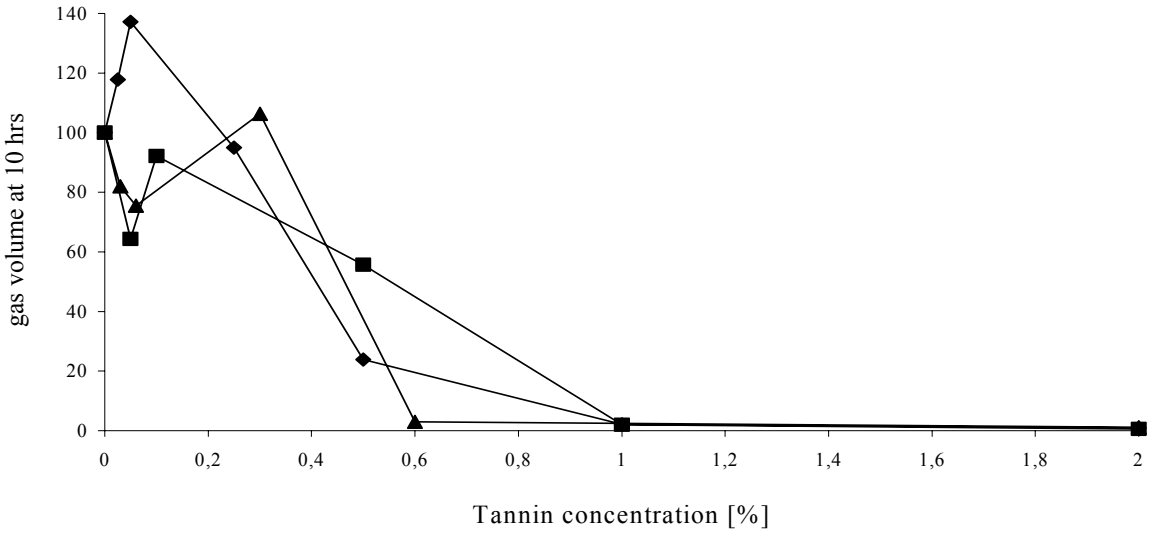

Fig 2b

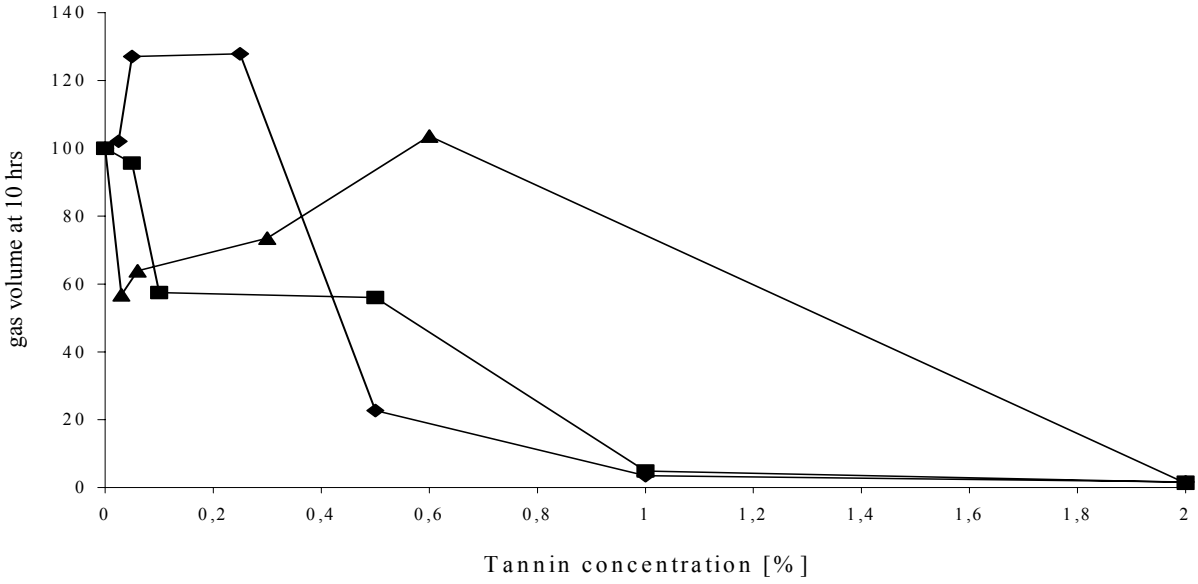

Fig 2. Effect of condensed tannins on gas production from* C. perfringens *Cp100 and Cp72. *Cultures of* C. perfringens *Cp100 and Cp72 were incubated in the presence of increasing condensed tannins and gas production was measured at 2 hourly intervals up to 10 hours. Results are expressed as cumulative gas production. (a) Cp100, Grape, ♦; Mimosa, ■; Quebracho, ▽. (b) Cp72, Grape, ♦; Mimosa, ■; Quebracho, ▽.*

Fig 3.

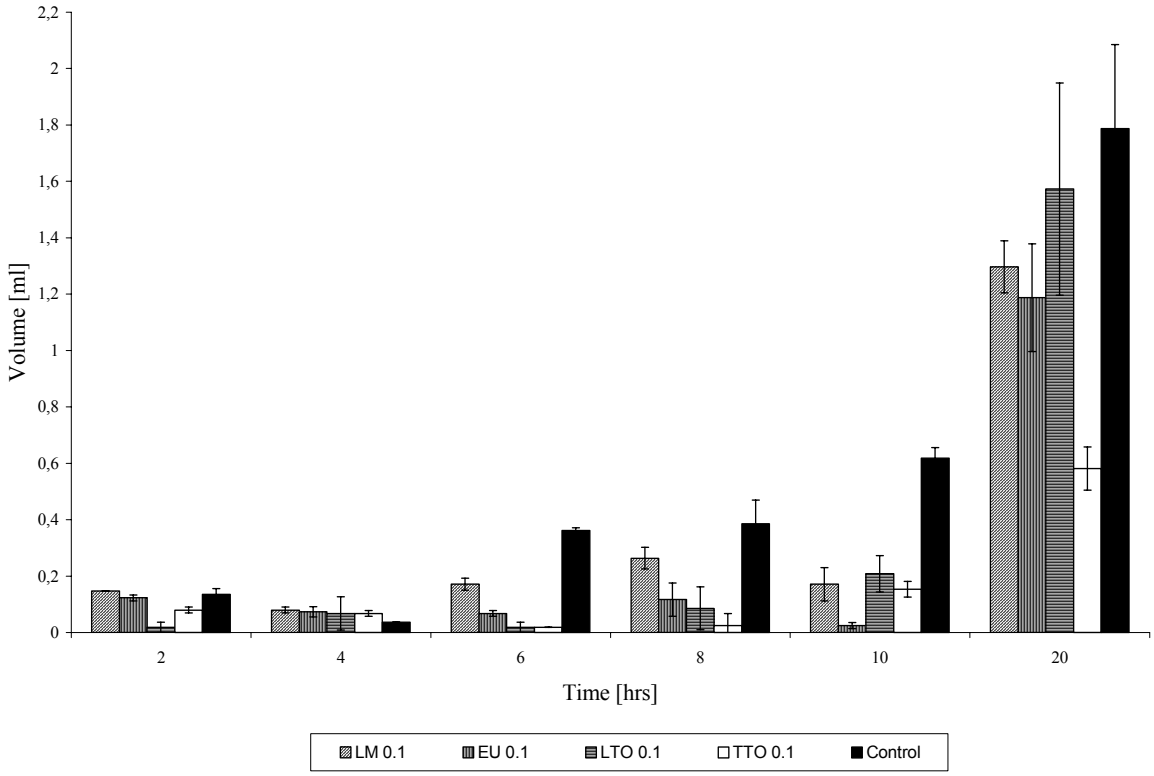

Fig 3. Effect of EO on fermentative activity of microflora from the chicken gastrointestinal tract. *Mixed microflora from samples of chicken digesta were incubated in medium for 20 hours, in the presence of 0.1% v/v EO. Results are expressed as cumulative gas production. LM, ♦; EC, ■; LTT, ▽; and TTO, ×.*