MilkQua

Milk quality along the dairy chain for a safe and sustainable milk

Deliverable 3.4

Title: Effects of essential oils on *in vitro* ruminal fermentation parameters and methane production

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Document Abstract

This deliverable is part of WP3, and therefore is describing the in vitro effects of essential oils before being tested *in vivo*. More particularly, D3.4. is dealing with the effects of essential oils on ruminal fermentation parameters when measured in vitro in batches of ruminal microorganisms. D3.4. describes the *in vitro* trials performed in an attempt to predict the most probable effects caused when feeding these compounds to adult dairy cows. According to the results observed in vitro, no nutritional or environmental beneficial effects were observed when using EOs in the diet of ruminants.

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1. Effects of essential oils on *in vitro* ruminal fermentation parameters and methane production

1.1 Introduction

- Plant secondary compounds present in essential oils (EOs) have shown a marked antimicrobial effect, so they may be of great interest in the field of animal nutrition (e.g., ruminal microbiota modification, decrease of methane production, increased feed efficiency).
- However, the composition of EOs varies depending on many factors (phenological state of the plant, extraction protocols…).
- Therefore, a proper characterisation of the effects promoted by the different active compounds included in EOs will be required in order to obtain robust conclusions thus allowing to take advantage of the whole potential of the EOs when included in the diet of ruminants.

1.2 Objectives

- *Thymus capitatus* was selected for this task according to the phytochemical profile and bioactive effects observed in previous deliverables (D3.2, D3.3). The main objectives of this deliverable were:
  (1) testing different doses of natural and synthetic EOs on ruminal fermentation, including methane production
  (2) testing the effects of each bioactive compound on ruminal fermentation, including methane production, and
  (3) providing supernatant and microbial DNA samples obtained from in vitro incubations to feed WP5.

1.3 First trial

Evaluation of the effects of different EOs (two natural and two synthetic EOs) in a dose-response trial.

1.3.1 Material and Methods

Four different EOs [*Thymus capitatus* "natural" essential oil (**EO1**), mixture of *Thymus capitatus essential* oils (**EO2**), "Synthetic" essential oil 1 (**SEO1**), and "Synthetic" essential oil 2 (**SEO2**)] were tested in this trial.

These EOs were tested at five different doses standardised according to the carvacrol content of each essential oil (0, 25, 50, 75 and 150 mg carvacrol/L)]

EO1 and EO2 composition*

	100
Carvacrol	70.62
p-cymene	7.06
y-terpinene	7.58
β-caryophyllene	4.55
Thymol	0.24
Etanol	9.95

	100
Carvacrol	57.57
p-cymene	12.26
y-terpinene	10.43
β-caryophyllene	5.57
Thymol	0.21
Etanol	13.96

*SEO1 and SEO2 were synthesized in the lab using the same amounts of the pure bioactive compounds detected in the composition of EO1 and EO2.

Experimental design: Five different doses (0, 25, 50, 75 and 150 mg carvacrol /L)] of four different EOs [EO1, EO2, SEO1 and SEO2] were added to 120 ml glass vials. Each vial contained 0.5 g of dry matter (total mixed ration formulated for milk production). Moreover, 50 mL of medium (10 mL bovine ruminal liquid + 40 mL culture medium) were injected before being closed with rubber caps and sealed. Three replicates per treatment were used. After 24 hours of incubation at 39°C under anaerobic conditions, liquid samples were taken to measure volatile fatty acids (VFA) and gas samples were collected for methane analysis.

Data were subjected to one-way ANOVA.

1.3.2 Results and implications

The main results obtained in the **first trial** are presented below:

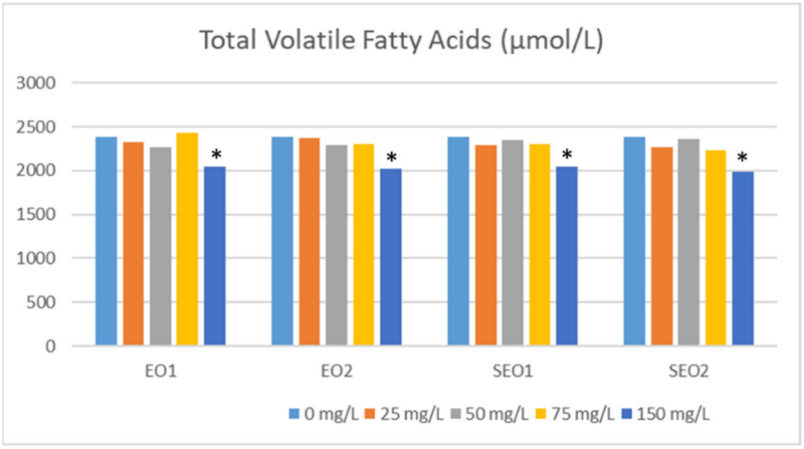

Figure 1. In vitro total volatile fatty acids production (µmol) in the dose response trial when testing four different compounds [*Thymus capitatus* "natural" essential oil (EO1), mixture of *Thymus capitatus* essential oils (EO2), "Synthetic" essential oil 1 (SEO1), and "Synthetic" essential oil 2 (SEO2)] at five different doses in batch cultures of mixed ruminal microorganisms.

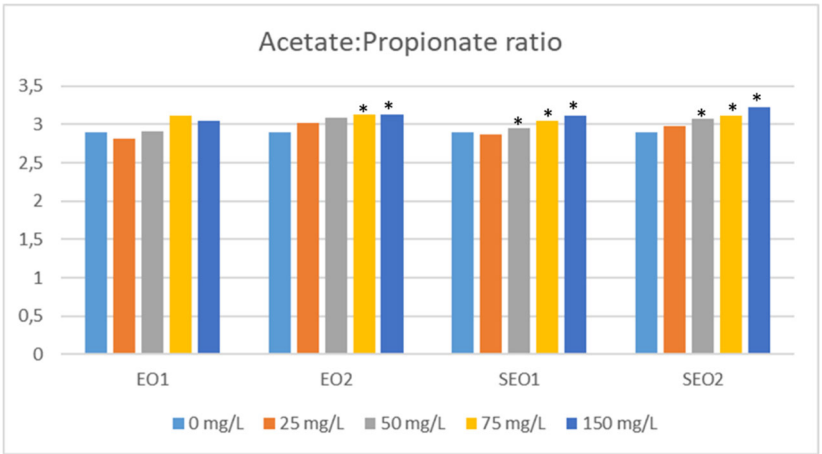

Figure 2. Acetate:Propionate ratio (mol/mol) measured in vitro in the dose response trial when testing four different compounds [*Thymus capitatus* "natural" essential oil (EO1), mixture of *Thymus capitatus* essential oils (EO2), "Synthetic" essential oil 1 (SEO1), and "Synthetic" essential oil 2 (SEO2)] at five different doses in batch cultures of mixed ruminal microorganisms.

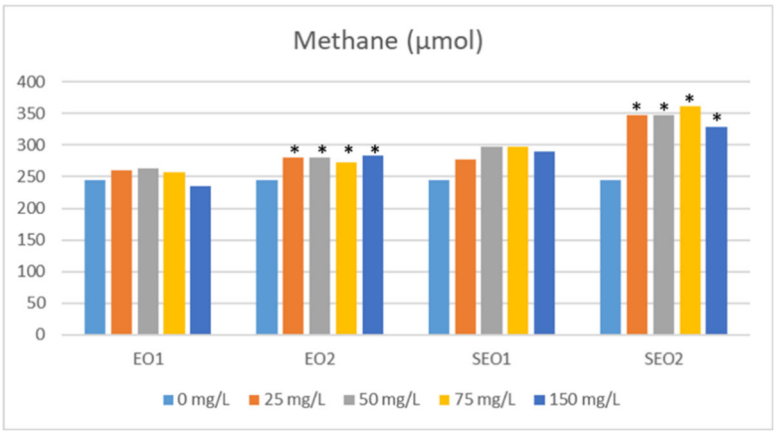

Figure 3. In vitro methane production (µmol) in the dose response trial when testing four different compounds [*Thymus capitatus* "natural" essential oil (EO1), mixture of *Thymus capitatus* essential oils (EO2), "Synthetic" essential oil 1 (SEO1), and "Synthetic" essential oil 2 (SEO2)] at five different doses in batch cultures of mixed rumen microorganisms.

The four oils decreased the total VFA production at 150 mg/L, thus indicating inhibition of ruminal fermentation when added to the ruminal liquid at the highest dose. Moreover, EO1 did not affect the acetate:propionate ratio or methane production at the lowest doses (25, 50 and 75 mg/L), whereas SEO1 caused an increased acetate:propionate ratio even at 50 mg/L, with no changes on methane production for any of the doses tested.

Both, acetate:propionate ratio and methane production were increased ($P<0.05$) for EO2 at 75 mg/L and SEO2 at 50 mg/L. Due to these significant methane increments, EO2 and SEO2 were discarded for the second trial.

1.3.3 Conclusions

In conclusion, synthetic EOs have similar effects to natural EOs.

All of them inhibited ruminal fermentation above 75 mg/L.

Furthermore, according to the results obtained in this trial, EOs may increase the acetate:propionate ratio (and hence the ruminal fermentation characteristics).

Due to the lack of effects on methane production, EO1 was selected to perform a second trial with a deeper characterization on ruminal fermentation traits and microbiota, even for each particular compound.

1.4 Second trial

Second trial: Evaluation of the effects of the individual bioactive compounds present in EO1 added at a dose corresponding to 75 mg carvacrol/L (selected based on results from first trial).

1.4.1 Material and Methods

Experimental design: Two different diets (TMRs F "rich in forage" *vs*. C "rich in concentrate"), 5 compounds (EO1, SEO1, carvacrol, p-cymene, γ-terpinene) added at a dose equivalent to 75 mg carvacrol/L of the original EO1, a control treatment (no additive), 4 inoculum obtained from 4 different cows, and two replicates per treatment were used.

Batch cultures were run in 120 ml vials containing 50 ml of medium (10 mL bovine ruminal liquid + 40 mL culture medium), 0.5 g of dry matter (TMR), and the EOs or bioactive compounds added at the corresponding doses. After 24 hours of incubation at 39°C under anaerobic conditions, liquid samples were taken to measure volatile fatty acids (VFA) and gas samples were collected for methane analysis. Also, in the second

trial, supernatant and pellet were collected to measure metabolome and obtain microbial DNA samples to be shifted to WP5.

Data were subjected to one-way ANOVA and the Dunnett test was used to compare each treatment against the negative control (with no EO or compound added).

1.4.2 Results and implications

The fourth dose (75 mg/L of EO1) was selected for a **second trial**, along with SEO1 and the main individual bioactive compounds (e.g., carvacrol, cymene, terpinene) at their actual proportion in the corresponding EO1 dose. It must be considered that the diet is a key aspect in ruminal fermentation, so two different diets (F, rich in forage; C, rich in concentrate) were formulated to be used like substrate inside the 120 ml vials. The main results are presented below:

Table 1. Effects of adding 75 mg/L of EO of *Thymus capitatus* essential oil (natural –EO1– and syntethic –SEO1–) and their main pure bioactive compounds included at that dose on ruminal fermentation after 24 h in vitro fermentation of two diets (F, rich in forage; C, rich in concentrate) in batch cultures with mixed rumen microorganisms.

DIET F variable	Control	EO1	SEO1	Carvacrol	Cymene	Terpinene	SEM	P-value	Root MSE
Gas (ml)	146	140	139	139	140	141	1.92	0.222	3.847
Methane (µmol)	579	583	516	507	488	528	29.42	0.181	58.847
Total VFA (µmol/L)	2662	2579	2495*	2459*	2621	2629	40.00	0.016	79.999
Molar proportions (µmol/100 µmol)									
Acetate	66.0	66.4	65.7	66.1	66.4	66.1	0.291	0.494	0.583
Propionate	15.0	13.6*	14.2	13.8*	14.2	14.4	0.239	0.015	0.478
Butyrate	12.3	12.9	13.05*	12.9	12.8	12.6	0.170	0.081	0.340
Valerate	2.14	2.23	2.24*	2.32*	2.21	2.23	0.024	0.004	0.049
Caproate	2.22	2.78*	2.73*	2.76*	2.30	2.36	0.077	<0.001	0.153
Isoacids	2.35	2.09*	2.15	2.21	2.17	2.29	0.052	0.032	0.104
Ac:Pr (mol/mol)	4.78	5.37*	5.22*	5.30*	5.15	5.13	0.106	0.021	0.213
methane/VFA (mol/mol)	0.217	0.226	0.205	0.207	0.185	0.202	0.013	0.381	0.026
NH3-N (mg/L)	235	259*	265*	225	225	217	5.584	<0,0001	11.168

DIET C variable	Control	EO1	SEO1	Carvacrol	Cymene	Terpinene	SEM	P-value	Root MSE
Gas (ml)	146	144	144	150*	150*	149	0.82	<0,001	1.641
Methane (µmol)	586	564	538	554	601	569	45.82	0.937	91.638
Total VFA (µmol/L)	2675	2529*	2628	2679	2692	2575	33.50	0.020	67.007
Molar proportions (µmol/100 µmol)									
Acetate	59.5	57.8*	59.4	59.5	59.6	58.7	0.421	0.056	0.842
Propionate	13.7	12.2	12.0	12.4	13.0	12.9	0.575	0.374	1.149
Butyrate	18.5	19.6	18.7	18.3	18.6	19.0	0.370	0.281	0.741
Valerate	2.29	2.49	2.43	2.46	2.34	2.40	0.054	0.152	0.109
Caproate	3.98	6,02*	5,61*	5.40	4.42	4.98	0.373	0.014	0.747
Isoacids	2.03	1.88	1.90	1.87	1.99	1.97	0.048	0.153	0.096
Ac:Pr (mol/mol)	5.14	5.75	5.87	5.69	5.48	5.47	0.28	0.518	0.559
methane/VFA (mol/mol)	0.223	0.228	0.204	0.207	0.225	0.225	0.017	0.857	0.034
NH3-N (mg/L)	242	255	244	244	249	266	9.82	0.533	19.640

No positive effects on ruminal fermentation parameters were detected with any of the five compounds when using F or C diets. Compared to the control, EO1 decreased ($P<0.05$) propionate molar proportion for the F diet, and the same was observed for carvacrol. This reduction main impair the synthesis of **glucose** in the animal. EO1, SEO1 and carvacrol also showed similar effects on F diet by increasing acetate:propionate ratio, suggesting that carvacrol was the main responsible for the modification of the VFA proportions.

However, not all the effects can be attributed to carvacrol. For example, the significant increase in **NH_3-N** observed when adding EO1 to the F diet might have been caused by any other compound or combination of compounds. In fact, this parameter was significantly raised when EO1 and SEO1 (a synthetic combination of carvacrol, cymene and terpinene) were added to the vials, but not with only carvacrol, cymene or terpinene. Anyway, this would be another negative process because NH_3-N may be transformed in urea on the liver and eliminated in urine or in milk.

As far as **methane** production is concerned, carvacrol can be used like an antimicrobial against gram-positive and gram-negative bacteria. In fact, it has been shown that low doses (20 mg/L) do not affect total VFA production or the proportion of VFA, but may reduce methane production by 30% (Joch et al., 2016). However, according to our results, only numerically lower values in methane production were observed for the F diet when using p-cymene, no significant differences being detected for any of the diets assayed. Additionally, the higher acetate:propionate ratios observed when using EO1, SEO1 or carvacrol in the F diet (probably related to a higher fibre degradation) seem to corroborate the lack of potential of this EO1 (75 mg carvacrol/L) to reduce methane production.

When C diet was incubated with the additives, only subtle effects on VFA profiles were detected, but EO1 seemed to slightly inhibit ruminal fermentation, as total VFA production was lower ($P<0.05$) compared to the control.

1.4.3 Conclusions

According to the results obtained in this task, it seems that EO1 (when used at a dose of 75 mg carvacrol/L) may promote a reduction of propionate proportion, with no differences in methane production when using high forage diets. No nutritionally or environmentally beneficial effects were observed when using EO1 at a dose selected to avoid the inhibition of in vitro ruminal fermentation in batch cultures of mixed ruminal microorganisms.

1.5 References

Joch, M et al. “In vitro Screening of Essential Oil Active Compounds for Manipulation of Rumen Fermentation and Methane Mitigation.” Asian-Australasian Journal of Animal Sciences vol. 29,7 (2016): 952-9. doi:10.5713/ajas.15.0474