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Ruminal fermentation of mixed diets supplemented with St. John's Wort (*Hypericum perforatum*) flowers and pine (*Pinus mugo*) oil or mixtures containing these preparations

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ABSTRACT

An *in vitro* investigation using the rumen simulation technique (Rusitec) was carried out to investigate the effects of dietary supplementation of plant preparations with known antimicrobial properties on ruminal fermentation. The following preparations were added to a mixed forage-concentrate (1:1) diet (g/kg feed dry matter): *Hypericum perforatum* flowers (2.86), essential oils of *Pinus mugo* (0.57) and a plant extract mixture (5.71) containing these two plant preparations in proportions of 0.5 and 0.1 of total, respectively. None of the treatments significantly affected ruminal fermentation traits. A trend towards enhanced bacterial population and daily volatile fatty acid formation was observed with the mixture. Ruminal nitrogen turnover showed a positive trend with *Hypericum*-only supplementation without affecting protozoal population. According to the present results, it is possible to use these plant extracts as alternatives to antibiotics for the lower gut in the dosages tested without adversely affecting the ruminal ecosystem.

KEYWORDS: essential oil, hypericin, ruminal fermentation, microorganisms, supplementation

INTRODUCTION

During the first few weeks of life and especially after weaning, the calf's exposition to pathogens often leads to severe health problems, such as diarrhoea,

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pneumonia or other respiratory diseases, since its immune system is not yet fully developed. Since the ban of feed antibiotics in the European Union there is a need for efficient natural feed additives (McIntosh et al., 2003; Macheboeuf et al., 2004). Such feed additives may be found in plant preparations already applied in humans. *Hypericum perforatum* L. (St. John's Wort) is a shrubby aromatic perennial herb native to Europe, Western Asia and North Africa (Burger and Wachter, 1998). It produces several types of bioactive compounds including hypericin, pseudohypericin and hyperforin (ESCOP, 2003). Thereof, hyperforin was determined as being the agent responsible for the potent antimicrobial activity of *Hypericum perforatum* (Kirakosyan et al., 2004). Essential oils (EO) are a group of plant secondary metabolites shown to have a great potential as beneficial manipulator of ruminal fermentation (Wallace et al., 2002) and therefore might represent an alternative to antimicrobials. A product only known from application in humans so far is aetheroleum pini pumilionis. These EO are obtained by steam distillation of fresh needles and branch tips of *Pinus mugo* (Burger and Wachter, 1998), a pine tree growing in the alpine regions of Middle Europe, Italy and the Balkan.

Effective feed supplements for calves should both improve the animal's health and not impede but rather stimulate ruminal microorganisms and fermentation processes. The hypothesis tested in the present study was that, despite their known activity on microorganisms, preparations of *Hypericum perforatum*, EO of *Pinus mugo*, and a mixture mainly containing these ingredients have no adverse effects on rumen microbial populations and fermentation processes.

MATERIAL AND METHODS

Experimental design

A basal diet consisting of hay, maize silage and grass silage in mixture, and a concentrate designed for weaning calves (2:1:1:4, dry matter (DM) basis) was used in all treatments (Table 1). A dietary crude protein content of 160 g/kg DM being appropriate for weaned calves was targeted. The first ingredient to be tested were flowers of *Hypericum perforatum* L. (in the following called 'hypericum flower'). It consisted of the dried, pulverized (<0.71 mm) flower material. This material was found to contain about 80 mg of total hypericin per kg DM. Hyperforin was not analysed but is known to amount to 20 to 40 g/kg DM in *Hypericum* herbs (Clayton, 2003). The other test material consisted of the EO of *Pinus mugos* (in the following called 'pine oil'). The major ingredients found in the pine oil were made up by the monoterpene hydrocarbons α-pinen, β-pinen, limonellen, ω3-caren, and β-phellandren with proportions of 22, 13, 12, 25 and 14%, respectively, of total pine

oil. Sepiolite, a magnesium trisilicate consisting of silicon dioxide, magnesium oxide and water, was used as carrier substance for the pine oil in a ratio of 8:1. Both plant materials were also available in the form of a mixture of plant preparations.

Table 1. Composition and daily quantities of the diets supplied to the fermenters

Item	Control	Hypericum flower	Pine oil	Plant extract mixture
Ingredients, g dry matter/d				
meadow hay	3.5	3.5	3.5	3.5
maize silage	1.75	1.75	1.75	1.75
grass silage	1.75	1.75	1.75	1.75
concentrate[1]	7.0	7.0	7.0	7.0
Hypericum perforatum flower[2]	-	0.04	-	-
aetheroleum pini pumilionis[2]	-	-	0.008	-
plant extract mixture[3]	-	-	-	0.08
Chemical composition of the complete diets, g/kg dry matter				
organic matter	936	936	935	935
crude protein	159	159	159	160
neutral detergent fibre	426	427	426	428
Quantities, g/d[4]				
dry matter	14.00	14.04	14.01	14.08
organic matter	13.10	13.14	13.11	13.18
crude protein	2.23	2.23	2.23	2.25

[1] composed, g/kg: flaked wheat 150, flaked barley 150, flaked maize 125, soyabean meal 120, maize gluten 90, oat flakes 75, rapeseed expeller 50, wheat bran 50, maize 40, carob bean 40, dextrose 38, molasses 15, rumen protected fat 8, vitamin-mineral mix 50 (providing per kg of concentrate, g: Ca 0.57, P 0.34, Na 0.13, Mg 0.12; IU: vit. A 1000, vit. D_3 125, mg: vit. E 5)

[2] levels equivalent to that provided *via* the plant extract mixture

[3] level recommended by the manufacturer; [4] provided within one bag to each fermenter

This mixture contained hypericum flower and pine oil in proportions of 0.5 and 0.1, respectively. Hypericum flower, pine oil and the plant extract mixture were mixed with the concentrate before application in dosages of 5.7, 1.14 and 11.4 g per kg DM, respectively. This ensured that the level of the plant mixture was equivalent to the manufacturer's recommendation, and that hypericum flower and pine oil had been supplemented at the same level when added alone or in mixture. Together with an unsupplemented control this added up to four treatment diets.

Experimental and technical setup

The experiment was carried out using an eight-fermenter rumen simulation technique system (Rusitec) as described by Soliva and Hess (2007). Each treatment

was tested in six replicates in three subsequent experimental periods lasting for 10 days each. Ruminal fluid was collected from a rumen fistulated Brown Swiss cow fed a diet consisting of hay and concentrate. Prior to incubation, ruminal fluid was strained through four layers of medicinal gauze. Rusitec fermenter setup was carried out as described in the technical description of Soliva and Hess (2007). Buffer flow rate was kept at 537 ml/d. Feed ingredients were freshly prepared each day. Hay and concentrate were ground to a particle size of 3 mm and the silages were minced in a regular food mixer (Moulinette® S, GROUP Moulinex, Paris, France). Fermenter fluid redox potential, pH and ammonia were measured daily using the corresponding electrodes (Redox electrode, Pt4805-DPA-SC-S8/120, Mettler Toledo, Greifensee, Switzerland; NH_3-selective electrode, 6.0506.100, Metrohm, Herisau, Switzerland) connected to a potentiometer (model 713, Metrom, Herisau, Switzerland). For the determination of volatile fatty acids (VFA), 1.8 ml of fermenter fluid was stabilized with 0.2 ml of a 46-mM $HgCl_2$-solution and frozen until analysis by gas chromatography (GC Star 3400 CX, Varian, Sugarland, TX, USA) as outlined by Tangermann and Nagengast (1996). Counts of ciliate protozoa and bacteria (the latter counted only on days 1, 3, 7 and 10) were obtained with Bürker counting chambers (0.1 and 0.02 mm depth, respectively; Blau Brand®, Wertheim, Germany). Fermentation gases, collected in gas-tight bags (TECOBAG 81, Tesseraux Container GmbH, Bürstadt, Germany) over 24 h each, were analysed for the concentrations of methane (CH_4), carbon dioxide (CO_2) and hydrogen (H_2) by a Hewlett Packard gas chromatograph (model 5890 Series II, Avondale PA, USA) equipped with an FID and WLD detector and a Carboxen-1000 column (mesh size 60/80, Fluka Chemie AG, Buchs, Switzerland) using the corresponding purified gas standards (PanGas, Dagmersellen, Switzerland). Fermentation gas volume was quantified by water replacement. Hydrogen recovery (%) was calculated by H_2 utilized ($2H_u$)/H_2 produced ($2H_p$) × 100, where $2H_u$ = 2 × propionate + 2 × butyrate + 4 × methane + valerate, and $2H_p$ = 2 × acetate + propionate + 4 × butyrate + 2 × iso-valerate + 2 × valerate (Soliva and Hess, 2007). Feed and feed residues after 48 h of incubation were lyophilized and analysed for DM, total ash, and nitrogen (N) contents (Dumas method; Leco-Analyser Type FP-2000, Leco Corporation, St. Joseph, MI, USA), following standard procedures (Naumann and Bassler, 1997). Crude protein was calculated as 6.25 × N. Contents of neutral detergent fibre (NDF) were analysed according to the protocol of Naumann and Bassler (1997) using α-amylase (Termamyl 120L, Type S, Novo Nodirsk A/S, Bagsværd, Denmark), but no sodium sulphite, and were corrected for ash content.

Calculations and statistical analysis

Means of data measured on days 5 to 10, calculated for each fermenter and experimental period, were used for data evaluation by analysis of variance with

diet and experimental period as effects. The GLM procedure of SAS (Version 8.2, SAS Institute Inc., Cary, NC, USA) was used.

RESULTS

According to the redox potential measured, fermenter fluid was clearly anaerobic in all treatments, and its average pH across all treatments amounted to 6.9 (Table 2). Fermenter fluid ammonia concentration was numerically highest

Table 2. Treatment effects on *in vitro* fermentation traits, averages of days 5-10[1]

Item	Control	Hypericum flower	Pine oil	Plant extract mixture	SEM	P-value
Properties of the fermenter fluid						
redox potential, mV	-296	-301	-300	-298	6.9	0.43
pH	6.87	6.86	6.86	6.87	0.023	0.97
ammonia, mmol/l	10.01	9.48	9.89	9.13	0.482	0.43
volatile fatty acids, mmol/l	107.1	103.9	103.3	104.7	3.03	0.83
molar proportions, %						
acetate (A)	57.4	57.6	57.0	58.2	0.47	0.34
propionate (P)	16.5	16.7	16.5	16.3	0.32	0.87
n-butyrate	17.3	17.0	17.6	16.5	0.31	0.14
iso-butyrate	0.10	0.10	0.11	0.13	0.021	0.62
n-valerate	6.75	6.71	6.89	6.76	0.136	0.80
iso-valerate	1.93	1.90	2.00	2.05	0.073	0.39
A:P ratio	3.49	3.47	3.47	3.58	0.001	0.77
Microbial counts						
bacteria, $\times 10^8$/ml	2.47	2.50	2.43	2.58	0.100	0.61
ciliate protozoa, $\times 10^3$/ml	8.44	8.22	7.80	8.44	0.877	0.95
Apparent degree of nutrient degradation						
organic matter	0.680	0.684	0.685	0.679	0.0039	0.67
crude protein	0.751	0.754	0.751	0.756	0.0055	0.88
neutral detergent fibre	0.475	0.470	0.477	0.463	0.0072	0.55

[1] SEM - standard error of mean. Means by treatment, n=6

with the control diet and with pine oil supplementation, while appearing to be slightly lower with hypericum flower and the plant extract mixture (-5.3 and -8.8%, respectively). Neither total amount nor profile of VFA was affected by the treatments. There was a weak tendency in the VFA produced per day to increase (+6.9%) with the plant extract mixture compared to the control diet. The rumen protozoal and bacterial populations were not significantly affected by any of the

dietary treatments compared with the control diet. A numerical enhancement of total bacterial counts occurred with the plant extract mixture (+4.5%) compared with the control diet. The apparent degree of nutrient degradation did not differ among treatments and amounted to 0.682, 0.753 and 0.471 on average for organic matter, crude protein and NDF degradation, respectively.

Fermentation gas volume was not affected by the dietary treatments (Table 3), and there were no differences in methane and hydrogen formation as well as

Table 3. Treatment effects on fermentation gases, averages of days 5-10[1]

Item	Control	Hypericum flower	Pine oil	Plant extract mixture	SEM	P-value
Gaseous emissions						
methane, mmol/d	8.95	9.19	8.84	9.00	0.270	0.92
methane, mmol/g aNDF[2]	1.50	1.54	1.48	1.50	0.045	0.94
hydrogen, mmol/d	0.80	0.65	0.69	0.70	0.098	0.82
carbon dioxide, mmol/d	71.6	73.0	73.0	73.2	1.33	0.74
Hydrogen balance						
produced, mmol/day	102.9	107.5	103.4	108.7	2.29	0.23
utilized, mmol/day	69.9	72.4	69.0	71.9	1.30	0.54
recovered, %	70.1	69.4	70.8	66.9	1.54	0.46

[1] SEM - standard error of mean. Means by treatment, n=6
[2] aNDF - apparently degraded neutral detergent fibre

hydrogen balance. Fermenter fluid N turnover, as calculated from measured disappearances from the nylon bag and the amounts of ammonia produced, is presented in Table 4. Three fractions were computed being the dietary N

Table 4. Calculated nitrogen (N) turnover in fermenter fluid, mg per day, averages of days 5-10[1]

Item	Control	Hypericum flower	Pine oil	Plant extract mixture	SEM	P-value
N supply	357	357	357	359	-	-
Apparently degraded N compounds	268	269	268	272	1.9	0.45
N recovered in ammonia	140	133	138	128	6.7	0.42
Degraded and not recovered N[2]	128	136	130	144	6.5	0.29
Apparently not degraded N[3]	89	88	89	88	2.0	0.96
Non-ammonia N	217	224	219	232	6.8	0.38
Microbial efficiency, mg N/g OMD[4]	14.6	15.2	14.3	16.3	0.71	0.33

[1] SEM - standard error of mean. Means by treatment, n=6
[2] N which is apparently degraded and not recovered as ammonia N (as measured in fermenter fluid) is assumed to be incorporated into microbial protein
[3] this term refers to degradation of nitrogenous compounds
[4] calculated as N incorporated into microbial protein per g of organic matter apparently degraded (OMD)

recovered in fermenter fluid as ammonia, the N incorporated in compounds apparently degraded (i.e. not present any more in the nylon bag) but not recovered as ammonia, and the dietary N compounds not degraded at all and recovered in feed residues. The last two fractions together form the non-ammonia N (NAN) which is the N amount potentially available at the small intestine as absorbable protein. With the plant extract mixture numerically less (-8.6%) dietary N was recovered in ammonia while N apparently degraded and not recovered in ammonia seemed to be higher (12.5 and 6.9%, respectively) than with the control treatment. The microbial efficiency, calculated as N incorporated into microbial protein per g of organic matter apparently degraded did not significantly differ among dietary treatments but was numerically lowest with the pine oil and the control diet and highest when the plant extract mixture was supplemented.

DISCUSSION

After weaning, calves are especially susceptible to pathogenic microorganisms affecting the health and limiting growth performance. Effective plant extracts substituting antibiotics might therefore also act as growth promoters during this phase of life, but an important condition is that they do not impede the ruminal microorganisms and, consequently, fermentation processes. In the calf's rumen, bacterial population starts to colonize already few days after birth starting with establishing amylolytic, sulphate-reducing, xylan- and pectin-fermenting bacteria, followed by cellulolytic bacteria (Minato et al., 1992). Ruminal population, including ciliate protozoa, is fully developed within 8 to 10 weeks of age when appropriate supplementary feeding has been performed (Minato et al., 1992). Therefore the usage of ruminal fluid of an adult cow seemed to be justified for the present *in vitro* study simulating ruminal fermentation processes of calves after weaning.

Effects on rumen microbial population. To our knowledge, no studies on the effects of hypericum flowers on ruminal fermentation have been conducted so far. However, *Hypericum perforatum* is known to affect gram positive bacteria (Gaind and Ganjoo, 1959). In the present study supplementation with hypericum flower had no obvious effect on total bacterial and protozoal numbers. One reason for the unchanged bacterial number in the present study might have been the low dosage of hypericum flower. However, an effect on specific species cannot be excluded as no quantification of the various microbial orders was conducted. Essential oils were repeatedly shown to be effective against pathogenic bacteria, while most of the ruminal bacteria tested were found to be insensitive to the addition of EO (Wallace et al., 2002). In the *in vivo* study of Benchaar et al. (2007) total viable and cellulolytic bacterial counts were not changed by the addition of EO. However,

when added to pure cultures of certain ruminal bacteria, McIntosh et al. (2003) showed an inhibited growth of some species whereas other species were able to adapt. The contradictory findings of these studies might indicate the presence of a dosage effect since Wallace et al. (2002) added only half of the amount used by the researchers of the latter study. Ineffectiveness of EO on ruminal protozoa was observed *in vivo* (Wallace et al., 2002; Newbold et al., 2004; Benchaar et al., 2007), and *in vitro* (McIntosh et al., 2003), but a reduction of protozoal counts was reported *in vitro*, too (Fraser et al., 2007). In the present investigation the pine oil had no effects on counts of total ruminal protozoa and bacteria, but again this does not exclude more specific effects. Consistent with the findings in the other treatments, the plant extract mixture, containing the same dosages of pine oil and hypericum flower, neither affected bacterial nor protozoal counts.

Effects on ruminal nitrogen turnover. While in the present study neither dietary N supply nor the amount of N apparently degraded differed among the treatments, the amount of N recovered in ammonia showed a weak trend to be reduced in the diets supplemented with hypericum flower but not with pine oil. Accordingly, the active ingredient(s) of hypericum flower might have had a slight effect on the microorganisms responsible for protein degradation. The lack of effect of the EO preparation differs from the observation made by Fernandez et al. (1997) who found ruminal ammonia concentrations being decreased when sheep received diets supplemented with EO. Also in the *in vitro* study of Fraser et al. (2007) ammonia N was decreased due to EO but only from incubation day 10 onwards. In the *in vivo* study of Benchaar et al. (2007), ammonia N concentration in ruminal fluid was not affected by the supplementation of EO to either lucerne- or maize-silage based diets. Similarly, Castillejos et al. (2006) reported no change in ammonia N concentration in a continuous culture fermenter when adding different dosages of EO. An explanation for the potential ammonia-suppressing effect of EO was given by McEwan et al. (2002) who showed that both the number and diversity of the so-called hyper-ammonia-producing bacteria in the rumen are decreased when exposed to EO. Newbold et al. (2004) found that the addition of EO to the diet of sheep decreased microbial deaminase activity but did not affect proteolytic and peptidolytic activity. Newbold et al. (2004) found microbial N leaving the rumen to be slightly increased with EO supplementation. In the present study either the low dosage of EO or the use of a high protein diet might have prevented an effect since EO only inhibited ammonia formation in sheep fed a low, but not a high, protein containing diet (McEwan et al., 2002).

The non-significantly higher amounts of dietary N apparently degraded but not recovered as ammonia N found when supplementing the plant extract mixture compared with the control treatment might have resulted from the hypericum flower addition rather than from the pine oil and is a desired effect since this fraction most likely consists of synthesized microbial protein. It can be assumed that a higher

dosage of hypericum flower and of the plant extract mixture could be clearly helpful in preventing a metabolic protein deficiency of ruminants (calves) thus improving performance and reduces losses of non-utilized N from the animal and from manure in the form of environmentally hazardous ammonia emissions (Śliwiński et al., 2004). Any action on ruminal N turnover is consistent with known effects of more commonly used plant secondary metabolites (e.g., Śliwiński et al., 2004).

Effects on apparent ruminal nutrient degradation, fermentation gases, and volatile fatty acids. Neither the apparent ruminal nutrient degradation nor the amount and composition of ruminal fermentation gases were altered by the dietary supplements tested. This indicates that overall microbial activity was not affected by any of the treatments which was also visible from the unaffected total amounts and molar proportions of VFA. This is consistent with the *in vitro* study of Castillejos et al. (2007) where different dosages of a specific blend of EO compounds did not affect ruminal nutrient degradation while only the lowest dose affected amount and composition of VFA. Also Newbold et al. (2004) reported that VFA concentrations were not affected *in vitro* by the dietary addition of EO, suggesting that no major changes in the microbial population had occurred. A change in the molar proportions of the individual VFA was noted mainly from incubation day 10 onwards in a Rusitec system but not earlier (Fraser et al., 2007). *In vivo* the addition of EO also neither affected total VFA concentration nor molar proportions of individual VFA (Castillejos et al., 2007). Taking ruminal fluid of the same sheep adapted to EO for four weeks and incubated *in vitro* resulted in an increased amount of acetate and acetate-to-propionate ratio while total concentration of VFA was not affected (Castillejos et al., 2007). A decrease of total VFA concentration, when supplementing EO, was found to be highly dose-dependent as effects were only observed with the respectively highest dosages (Macheboeuf et al., 2004; Castillejos et al., 2006). Beauchemin and McGinn (2006) found a lower total tract nutrient digestibility with the addition of EO whereas no specific effects on ruminal fermentation and methane formation was measured. Also in the study of Fraser et al. (2007) dry matter disappearance at 48 h was decreased due to EO addition. In the study of Macheboeuf et al. (2004), eight EO were compared with ruminal fermentative activity showing different responses, demonstrating the importance of the chemical composition for the ruminal activity of EO. Together with the dose dependency of EO this might be a rather likely explanation for the large variability in the effects described in the studies discussed.

Suitability of hypericum flower and pine oil as alternatives to antibiotics in calf nutrition. Subsequent to the ban of using antibiotics in ruminant nutrition in the European Union, the search for efficient feed additives has shifted to natural compounds, particularly plant secondary metabolites. In former times, EO had already been considered valuable as antimicrobial agents until interest was lost

because of the discovery of antibiotics (McIntosh et al., 2003). Nevertheless, it can be assumed that given at high dosages, plant secondary metabolites may have anti-nutritional effects such as reduced feed intake and digestibility of the diet, and toxicity may arise when supplemented at very high concentrations. In the study of Bourke (2000), Merino sheep showed a limited tolerance for *Hypericum perforatum*, eaten at flowering stage, of <10 g (plant wet weight) per day per kg of liveweight; hypericin was tolerated up to 2.65 mg/d per kg of liveweight. Since hypericin circulates in blood for several days, the safe dose will decline at continuous daily ingestion (Bourke, 2000). Furthermore, hypericin is a strong photodynamic active substance and is responsible for the phenomenon of photo toxicity of *Hypericum perforatum*, observed in grazing animals ingesting large amounts of this plant (Burger and Wachter, 1998). The dosages used in the present study, though, were far below this threshold level when hypericism could occur.

CONCLUSIONS

The two plant preparations and the plant extract mixture tested were shown not to impede ruminal microorganisms and fermentation traits. Indications of favourable effects when adding the plant extract mixture, especially on ruminal N turnover and on volatile fatty acid formation, might become more evident when increasing the level of dietary supplementation. Therefore, provided the antimicrobial action of the investigated plant preparations against pathogens can be demonstrated, they might be helpful in replacing feed antibiotics.

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