EFFECT OF SUPPLEMENTING ESSENTIAL OILS CONTAINING HERBS TO TOTAL MIXED RATIONS ON THE PERFORMANCE OF GROWING MALE CROSSBRED CALVES

Thesis

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MASTER OF VETERINARY SCIENCE
in
ANIMAL NUTRITION
(Minor Subject: Veterinary Biochemistry)

By

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CERTIFICATE – I

This is to certify that the thesis entitled **"Effect of supplementing essential oils containing herbs to total mixed rations on the performance of growing male crossbred calves"** submitted for the degree of M.V.Sc in the subject of **Animal Nutrition** (Minor subject: **Veterinary Biochemistry** of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Vivek Sharma (L-2015-V-08-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

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ABSTRACT

This study was conducted to assess the effect of herbal feed additives (HFAs) containing oils supplemented at 1-3% of total mixed rations (TMR) on DM basis on the methane production potential and nutrient utilization in male crossbred calves. TMR with different roughage to concentrate ratio of 80:20, 75:25, 70:30 and 65:35 on DM basis were formulated. The roughage portion was made up of wheat straw and maize green fodder in 70:30 ratio. *In vitro* gas production studies conducted in a 4x3x3 factorial design conclusively revealed that the best response with respect to net gas production, digestibility of nutrients (OMD, NDFD and DMD), methane production, VFA production, ME availability and other fermentation parameters from TMRs with different roughage to concentrate ratios was observed in jaiphal supplemented at the rate of 1% of TMR with R: C ratio of 65:35 on DM basis. This was followed by 4x4 latin square design to assess the impact of supplementing jaiphal suva and haldi at the rate of 1% of DM basis in TMR (R:C::65:35) on the nutrient utilization of male crossbred calves. The herb supplementation did not have any significant effect on digestibility of various nutrients and percent nitrogen retention in male cross bred calves. The blood biochemical profile for various parameters did not show any significant effect on herb supplementation i.e. herbs have no deleterious effect on animal health except supplementation of herb suva in male cross bred calves significantly increased ($P<0.05$) the alkaline phosphatase activity(AKP). Herbs supplementation has stimulatory effect on rumen fermentation parameters (TN, TVFA, NPN NH_3-N) which were ($P<0.05$) higher than control group. The results conclusively revealed that supplementing TMR (R:C::65:35) with jaiphal @ 1% mitigated the methane production without affecting digestibility of nutrients.

Keywords: Herbal feed additives, Crossbred calves, *In vitro*/*In vivo* evaluation, Methane, essential oils

Signature of Major Advisor **Signature of the Student**

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LIST OF ABBREVIATIONS USED

A	:	*Acetate*
A:P	:	Acetate : Propionate
ADF	:	Acid Detergent Fibre
ADL	:	Acid Detergent Lignin
ALL	:	Allantoin
B	:	Butyrate
BCFA	:	Branched Chain Fatty Acids
CP	:	Crude Protein
CRT	:	Creatinine
CT	:	Condensed Tannins
DCP	:	Digestible Crude Protein
DM	:	Dry Matter
DMI	:	Dry Matter Intake
EE	:	Ether Extract
FE	:	Fermentation Efficiency
GFR	:	Glomerular Filtration Rate
HR	:	Hydrogen Recovery
HR-LC	:	High Roughage-Low Roughage
IB	:	Isobutyrate
IV	:	Isovalerate
IVY	:	Ivy Fruit Saponins
K	:	Rate Of Degradation
LR-HC	:	Low Roughage-High Concentrate
MCS	:	Momordica Charantia
ME	:	Metabolisable Energy
NDF	:	Neutral Detergent Fibre
NDFD	:	Neutral Detergent Fibre Digestibility
NDS	:	Neutral Detergent Solution
NGP	:	Net Gas Production
NH_3-N	:	Ammonical Nitrogen
NPN	:	Non Protein Nitrogen
OM	:	Organic Matter
PD	:	Purine Derivative
PF	:	Partition Factor

PG	:	Panax Ginseng
PO	:	Palm Oil
PSM	:	Plant Secondary Metabolites
RPM	:	Rape Seed Meal
SO	:	Saponaria Officinalis
SRL	:	Strained Rumen Liquor
TCA-N	:	Trichloroacetic Acid Nitrogen
TMR	:	Total Mixed Ration
TOMD	:	True Organic Matter Digestibility
V	:	Valerate
VFA	:	Volatile Fatty Acid
VFA UI	:	Volatile Fatty Acid Utilization Efficiency
WS	:	Wheat Straw
YS	:	*Yucca Schinindegera*

CHAPTER I

INTRODUCTION

Recently, there have been growing concerns over greenhouse gas emissions because of their effects on global warming and climate change, and consequently on the regional as well as global ecological and socio-economic vulnerability (IPCC 2007). Methane is one of the greenhouse gasses, which is normally produced during the anaerobic enteric fermentation of feeds in many animals including ruminants. Methane has 21 times more global warming potential than carbon dioxide (IPCC 2007). It is estimated that the world's population of ruminants produces about 15% of total methane emissions (Moss *et al* 2000). Besides, methane produced in ruminants represents a substantial loss of 2–15% of gross energy intake which reduces the potential conversion of feed energy to metabolizable energy. Hence, the inhibition of methanogenesis has long been considered from nutritional aspects, and more recently from the perspectives on greenhouse gas emissions. Many chemical feed additives were tried to decrease methane production in the rumen. However, these chemical additives are either toxic to host animals or have a transient effect on methanogenesis (Moss *et al* 2000). In addition, an increasing awareness of hazards associated with chemical feed additives, i.e. presence of chemical residues in animal derived foods and development of bacterial resistance to antibiotics has diverted the research on feed additive technology towards exploiting natural products as feed additives. Antibiotics and other synthetic additives used in animal feeding have been effectively banned by the European Union since 2006 (regulation 1831/2003/EC). Plants produce a diverse array of plant secondary metabolites (PSM) to protect against microbial and insects attacks. These natural plant eco chemicals such as essential oils (EOs), saponins, tannins and organosulphur compounds have been shown to selectively modulate the rumen microbial populations (Patra and Saxena 2009) resulting in an improvement of rumen fermentation and nitrogen metabolism, and a decrease in methane production and thus improving the productivity and health of animals. Essential oils (EO) are naturally occurring secondary metabolites and volatile components extracted most commonly from herbs and spices but also from various parts of plants through distillation methods, mainly steam distillation (Patra, 2007). Essential oils have been used for many centuries as folk medicines and food

preservatives because of their antimicrobial effect (Burt, 2004). They are complex mixtures of secondary metabolites and volatile compounds (Burt, 2004). Essential oils have antimicrobial activities against both gram-negative and gram-positive bacteria; this is because of the presence of terpenoid and phenolic compounds (Yang *et al* 2007). Essential oils are chemically not true oils but rather variable mixtures made up mostly out of two chemical groups. These two chemical groups include the most common essential oils: the groups are terpenoids (monoterpenoids and sesquiterpenoids) which are synthesized through the mevalonate metabolic pathway and phenylpropanoids which are synthesized through the shikimic acid metabolic pathway (Calsamiglia *et al* 2007; Patra, 2011). Of these two classes the terpenoids are the more diversified group of plant bioactives and are found in a wide variety of spices and herbs (Patra, 2011). They are derived from a basic structure of C5 isoprene units and the number of these units contained in its skeleton determines its classification. The most important components of EO of most plants within terpenoids belong to the monoterpenoids and the sesquiterpenoids. Phenylpropanoids have a different structure containing an aromatic ring of C6 with a side chain of 3 carbons bound to it (Calsamiglia *et al* 2007). Compared with the terpenoids the phenylpropanoids are less abundant. Some plants however contain significant proportions of these compounds of EO.

The plant species, maturity, botanical fraction and environment determine the composition of the EO and different variations occur as these factors change (Yang *et al* 2007). Essential oils are seen as a potential alternative to antibiotic drugs and additives in both animal and human diets because they have antimicrobial properties against a wide variety of microorganisms (Benchaar *et al* 2008). Nutritionists and rumen microbiologists have therefore recently shown great interest in exploiting EO as natural feed additives for the purpose of improving rumen fermentation through volatile fatty acid production, inhibition of methanogenesis, improving protein metabolism and increasing the efficiency of feed utilization. A wide range of herbs and spices contain EO with the potential to manipulate the rumen and enhance animal productivity. These EO are alternatives that can very successfully replace chemical feed additives (Tager and Krause, 2011). Their effectiveness in ruminant production however has not been proven to be consistent and conclusive. Essential oils have given varying results with regard to rumen microorganisms, rumen fermentation and

ruminant performance. These results depended upon the dose, feed composition, animal physiology and chemical structures of the EO.

Various parts of plants like the flowers, fruit, bark, leaves, roots, stem, seeds and pulps contain EO. Various factors such as plant health, stage of growth and environmental factors like temperature, moisture stress and light affect the concentrations of EO (Hart *et al* 2008). Because of the strong antimicrobial properties of various EO, essential oil research has recently been accelerated due to the ban in many developed countries of certain antibiotic growth promoters as feed additives (Benchaar *et al* 2006)

Hence this study was being planned with the following objectives

- To assess the effect of herbal feed additives with known active component on the methane production potential and nutrient utilization in growing male cross bred calves fed total mixed rations (TMRs) with different roughage to concentrate ratios

CHAPTER II

REVIEW OF LITERATURE:

2.3 Essential oils

2.3.2 Mode of action

Hart *et al* (2008) found that the main effects of EO in the rumen are due to the lower starch and protein degradation and the inhibition of amino acid degradation because of selective action on certain microorganisms in the rumen, especially bacteria.

Different theories have been proposed to explain the mode of action of EO. The first theory entails EO selectively inhibiting gram-positive bacteria in a similar way as ionophores (Burt 2004). Some studies however suggest that EO also inhibit gram-negative bacteria (Busquet *et al* 2005b). This is possible because of the small molecular weight of EO which allows it to penetrate the walls of these bacteria (Benchaar *et al* 2008). Gram negative bacteria have been successfully inhibited by caravacrol and thymol (Helander *et al* 1998).

The second theory entails EO changing cells by interacting with certain processes of the cell membrane (Benchaar *et al* 2008). These processes include phosphorylation, ion gradients, ATP production, protein translocation etc. Essential oils are lipophilic and hydrophobic and these characteristics contribute to this effect (Benchaar *et al* 2008). Patra (2011) also suggested that EO have 2 different modes of action. His suggestions however differed from the above. He suggested that one mode of action is that EO have an effect on the bacterial colonisation pattern of certain starch rich substrates entering the rumen. The second mode of action he suggested is that EO inhibit hyper ammonia producing bacteria involved in amino acid deamination.

2.3.3 Effect on rumen microbial populations

2.3.3.1 Rumen bacteria

The hyper ammonia producing bacteria in the rumen which cause reduced amino acid deamination is inhibited by certain EO (Wallace 2004). In a study by McIntosh *et al* (2003) it was observed that an essential oil mixture inhibited the growth of certain hyper ammonia producing bacteria like *Clostridium sticklandii* and

Peptostreptococcus anaerobius and had less of an effect on other hyper ammonia producing bacteria like *Clostridium aminophilus*.

These inhibitory effects of EO on bacteria can be diet dependant as is shown in a study by Wallace (2004). His study showed that sheep receiving a low protein diet supplemented with 100 mg a day of EO caused the number of hyper ammonia producing bacteria to decrease by 77%, but with sheep receiving a high protein diet the EO had only a small or no effect on hyper ammonia producing bacteria. Ruminal bacteria were also differently affected by individual EO (Patra 2011). Compared to the corresponding oxygenated compounds the monoterpene hydrocarbons are not as toxic and often stimulatory to microbial activity (Patra 2011).

Wallace *et al* (2002) observed that hyper ammonia producing bacteria have a great capability to produce ammonia from amino acids. Essential oils in low doses can selectively inhibit hyper ammonia producing bacteria whereas higher doses affect all microorganisms (Busquet *et al* 2005b).

Evans and Martin (2000) reported that the growth of *Selenomonas ruminantium* was selectively inhibited by thymol at a concentration of 90 mg/L whereas *S. bovis* was not inhibited. In comparison a thymol concentration of 400 mg/L inhibited all rumen organisms. The digestion and colonization of readily degradable substrates by amylolytic and proteolytic bacteria can be suppressed by EO without affecting fibre digestion (Wallace *et al* 2002). Patra *et al* (2010) however noted that carboxymethylcellulase and xylanase activity were reduced by clove and fennel extracts. This might be the result of the higher concentrations of EO found in the extracts.

2.3.3.2 Rumen protozoa

The effects of EO on rumen protozoa have delivered mixed reports. McIntosh *et al* (2003) reported that dairy cows supplemented with 1 g/day of mixed EO had no effect on the bacteriolytic activity of rumen ciliate protozoa. Benchaar *et al* (2007a) and Newbold *et al* (2004) found similar results showing that dairy cows and sheep fed 750 mg/day and 110 mg/day of a mixture of EO respectively had no effect on their ruminal protozoa counts.

Fraser *et al* (2007) observed that the number of ciliate protozoa was not affected when dairy cow diets were supplemented with 0.5 g of cinnamaldehyde per

litre of rumen fluid. The protozoa were not affected by the extract of fennel (Patra *et al* 2010). The results found in a study by Ando *et al* (2003) were contradictory to the above in that Holstein steers had a decrease in the total number of protozoa as well as the numbers of Isotrica, Diplodium and Entodinum when they were fed 30 g of peppermint (*Menta piperita L.*) per kg of total dietary dry matter intake. These results were attributed to the presence of EO (Ando *et al* 2003).

Patra *et al* (2010) also observed that the total numbers of protozoa, holotrichs and small entodiniomorphs were decreased and the large entodiniomorphs were not affected by clove extracts containing EO. In a study by Cardozo *et al* (2006) it was found that the numbers of holotrichs increased and the entodiniomorph numbers stayed the same with the addition of a mixture of cinnamaldehyde at 180 mg/day and eugenol at 90 mg/day to beef heifer diets.

There was however no effect on these protozoal numbers when the concentrations of both cinnamaldehyde and eugenol were higher at 600 mg/day and 300 mg/day respectively (Cardozo *et al* 2006). It was also observed by Yang *et al* (2010a) that total protozoal as well as Dasytricha, Entodinium and Dasytricha species numbers were not affected by cinnamaldehyde supplementation at 0.4 to 1.6 g/day in steers. In contrast to these findings Cardozo *et al* (2006) found that the numbers of holotrichs and entodiniomorphs decreased by feeding 2 g/day of anise extract containing 100 g/kg of anethol to beef heifers. The overall conclusion that can be made is that the numbers and activity of ruminal protozoa are not markedly affected by EO and their components.

2.3.4 Effect on digestibility and rumen fermentation

2.3.4.1 Feed digestion

Several studies found that EO didn’t affect the digestibility of feeds (Malecky *et al* 2009; Meyer *et al* 2009; Santos *et al* 2010). A study by Yang *et al* (2007) found that a control diet for Holstein cows containing 60% barley based concentrate and 40% forage had a 13% higher dry matter digestibility when supplemented with 2 g/day juniper berry essential oil. Experimental treatments did however not affect total tract digestibilities of organic matter, dry matter, starch and fibre (Yang *et al* 2007). Yang *et al* (2007) suggested that the increased ruminal digestion of dietary protein of the cows receiving the essential oil supplementation compared to cows receiving only

the control diet was the reason for the increased ruminal digestibility. A study by Malecky *et al* (2009) also showed that different nutrient digestibilities in dairy goats were not affected by a monoterpene blend. Dry matter and fibre digestibility in the rumen is reduced with increased essential oil concentrations (Beauchemin and McGinn 2006; Yang *et al* 2010a).

Khamisabadi *et al* (2016) carried out study to investigate the effect of inclusion of dietary supplementation of peppermint (*Mentha piperita*) and *Thymus vulgaris* on nutrient digestibility.. The results showed that addition of thyme or peppermint at a concentration of 3% on DM basis to diet improved the nutrient digestibility.

Hundal *et al* (2016) studied to assess the effect of the pure essential oils (EOs) viz. cinnamaldehyde, carvacrol, carvone and limonene supplemented individually at 1 to 5% levels of the substrate DM (wheat straw) on fiber degradation in a 4×7 factorial design. Supplementation of cinnamaldehyde and carvon, irrespective of their level had significantly ($P < 0.01$) digestibility of neutral detergent fiber (NDFD) and true organic matter (TOMD). TheNDFD and TOMD was depressed significantly ($P < 0.01$) beyond 1% level of supplementation of EO. It was concluded that carvacrol or limonene supplementation beyond 1% level the digestibility of nutrients from the substrate were also depressed significantly.

Benchaar (2016) conducted to evaluate the effect of dietary addition of cinnamon oil (CIN), cinnamaldehyde (CDH), or monensin (MON) on enteric methane (CH4) emission in dairy cows. Cows were fed (ad libitum) a total mixed ration ((TMR); 60 : 40 forage : concentrate ratio, on a dry matter (DM) basis) not supplemented (CTL), or supplemented with CIN (50 mg/kg DM intake), CDH (50 mg/kg DM intake), or monensin (24 mg/kg of DM intake). Adding CIN, CDH or MON to the diet had no effects on DMI and nutrient digestibility of the diet.

Khateri *et al* (2017) studied the effects of a specific blend of essential oils on apparent nutrient digestion, in sheep fed a 50:50 alfalfa hay: concentrate diet. They found that apparent total tract digestibility of dry matter, crude protein, organic matter, and neutral detergent fiber were not influenced by MEO supplementation. They concluded that supplementation of MEO may have limited effects on apparent nutrient digestibility.

2.3.4.2 Volatile fatty acids

Most studies showed that the total volatile fatty acid concentrations were not affected by EO (Chaves *et al* 2008c; Malecky *et al* 2009; Patra *et al* 2010). Other studies showed a decrease in the total volatile fatty acid concentrations especially if higher essential oil concentrations were fed (Macheboeuf *et al* 2008; Kumar *et al* 2009). Some studies however showed an increase in the total volatile fatty acid concentrations with the supplementation of 0.2 g cinnamaldehyde per kg DM intake (Chaves *et al* 2008b) and 0.25 g essential oil extract from oregano per kg DM intake (Wang *et al* 2009). An *in vitro* study by Castillejos *et al* (2005) found that the total volatile fatty acid concentrations were increased without the nitrogen metabolism being affected when a blend of EO were added at 1.5 mg/L. The type of substrates fed to the ruminant determines to a large extent what the essential oil responses on the total volatile fatty acid concentrations are going to be (Castillejos *et al* 2005). The total volatile fatty acid concentrations of cows fed on an alfalfa silage based diet were not affected, but cows fed a corn silage based diet with an addition of an essential oil mixture of 0.75 g/day had a decrease in volatile fatty acid concentrations (Benchaar *et al* 2007a). In some studies the addition of EO increased the acetate to propionate ratios (Benchaar *et al* 2007b; Macheboeuf *et al* 2008; Agarwal *et al* 2009), while in other studies these ratios remained unchanged (Kumar *et al* 2009; Wang *et al* 2009). A decrease in acetate to propionate ratio is normally associated with a methane production inhibition achieved by specifically targeting the methanogens (Patra 2011). The essential oil effects may depend on the rumen fluid pH (Cardozo *et al* 2005).

Cardozo *et al* (2005) observed that at a low pH the rumen volatile fatty acid profile was affected to a larger extent by some EO. They proposed that the essential oil molecule status of dissociated or undissociated depends on the rumen pH. Spanghero *et al* (2008) had similar observations of the end products of fermentation being shifted by a blend of EO at a low pH. They observed a decrease in the acetate proportion as well as the acetate to propionate ratio only if the essential oil fluid had a lower pH.

Hundal *et al* (2016) studied to assess the effect of the pure essential oils (EOs) viz. cinnamaldehyde, carvacrol, carvone and limonene supplemented individually at 1

to 5% levels of the substrate DM (wheat straw) on fiber degradation in a 4 × 7 factorial design. Supplementation of cinnamaldehyde and carvon, irrespective of their level had significantly ($P < 0.01$) affected the volatile fatty acids (VFAs) production from the substrate. Irrespective of the type of EO, at 1% level of supplementation was comp total and individual VFAs production was depressed significantly ($P < 0.01$) beyond 1% level of supplementation of EO. It was concluded that carvacrol or limonene supplementation beyond 1% level reduced the digestibility of nutrients, volatile fatty acid production and ME availability from the substrate.

Khateri *et al* (2017) studied the effects of a specific blend of essential oils on rumen fermentation in sheep fed a 50:50 alfalfa hay:concentrate diet The total volatile fatty acids (VFA) concentration, molar proportion of individual VFA, acetate: propionate ratio were not affected with MEO. The results of the study suggested that supplementation of MEO may have limited effects on ruminal fermentation.

2.3.4.3 Ammonia

Because the rumen hyper ammonia producing bacteria are inhibited by EO it leads to a decrease in ammonia and deaminase activities (Patra 2011). Only about 1% of the rumen bacteria population consists out of hyper ammonia producing bacteria, but these bacteria have very high deaminase activity (Wallace 2004). The rate of rumen ammonia production can be decreased by this which in turn may increase the rumen protein efficiency and thereby be nutritionally beneficial to the animal (Wallace *et al* 2002). A 25% decrease in bacterial deamination activity *in vitro* has been reported by Newbold *et al* (2004). Various studies reported an *in vitro* decrease in ammonia concentrations. Cardozo *et al* (2005) reported an ammonia concentration decrease of 30 to 300 mg/L with oregano oil and a decrease of 0.3 to 300 mg/L with cinnamon oil. Similarly Busquet *et al* (2006) found a decrease of up to 3000 mg/L with cinnamaldehyde. These effects however don’t always apply as was found in certain *in vitro* (Busquet *et al* 2006) and *in vivo* (Castillejos *et al* 2005; Benchaar *et al* 2007a) studies.

Certain EO reduced the ammonia concentrations at low doses compared to other EO in *in vitro* studies. Castillejos *et al* (2006) found that the rumen ammonia concentrations were reduced at 5 mg/L of Guaiacol while limonene and thymol only caused a reduction at 50 mg/L and eugenol and vanillin tended to reduce the rumen

ammonia concentrations at the 50 mg/L and the 500 mg/L concentrations. The importance of the optimum dose for a certain type of essential oil is clearly demonstrated by the above. The type of protein meal in the diet may also affect these effects. Wallace *et al* (2002) investigated the degradation rate of different protein meals and the colonisation of feedstuffs which were incubated in nylon bags by enzyme activity in the presence of EO. Pea meal which was the most rapidly degraded meal of the protein meals tested was the only meal significantly affected by EO (Wallace *et al* 2002). In animals receiving EO the bacterial proteinase and amylase affiliated with the plants protein supplement was likely to be lower, whereas the corresponding fishmeal activities were unaffected (Wallace *et al* 2002). Essential oils caused a reduction in the total microbial colonization affiliated with grass hay placed in the rumen, while the colonization of less degradable fibrous substrates like barley straw and grass silage was not affected (Wallace *et al* 2002). This served as an indication that the colonization and digestion of readily degradable substrates by proteolytic and amylolytic bacteria can be suppressed by EO without affecting fibre digestion (Wallace *et al* 2002). The adaptation of rumen microorganisms and the fast rumen metabolism of EO to a less active form may be the cause of the lack of effect of EO on rumen fermentation (Wallace *et al* 2002).

2.3.4.4 Methane production

Certain essential oil components have inhibitory effects on methanogenesis (Benchaar and Greathead 2011). In a study by Evans and Martin (2000) it was found that the main component namely thymol of the essential oils derived from thymus and origanum plants inhibited *in vitro* methane production strongly and also decreased acetate and propionate concentrations. In another study by Macheoeuf *et al* (2008) a suppression of methane to the extent of 99% at a concentration of 6 mM of essential oils from origanum vulgare and its component thymol was found. Chaves *et al* (2008a) found that an inhibition of methane *in vitro* was caused by anethole at a concentration of 20 mg/L.

EO from different spices and plants have shown inhibitory effects on methane production in various studies. Strong inhibitory effects on methanogenesis have been shown by essential oils from cinnamon oil and juniper berry (Chaves *et al* 2008a) and peppermint oil (Agarwal *et al* 2009).

Macheboeuf *et al* (2008) found that cinnamaldehyde which is the active component of cinnamon oil caused a 94% methane production depression at a concentration of 5 mmol/L rumen fluid. McKay and Blumberg (2006) observed that menthol, menthone and methyl acetate containing antimicrobial properties is found in peppermint oil. Patra *et al* (2010) noted that *in vitro* methane production was inhibited by methanol and ethanol extracts of clove buds and fennel seeds.

Methane production was inhibited by eucalyptus oil by up to 58% at a concentration of 1.66 mg/L (Kumar *et al* 2009) and 90.3% at a concentration of 2 mg/L (Sallam *et al* 2009). Chaves *et al* (2008a) found that the eucalyptus oil component p-cymene caused a 29% methane reduction at a concentration of 20 mg/L. Tatsouka *et al* (2008) however found that the eucalyptus oil component α-cyclodextrin had no effect on methane production up to a concentration of 330 mg/L. Wang *et al* (2009) performed an *in vivo* study which caused a methane reduction when 0.25 g/day EO derived from oregano plants was included in the diets of sheep for 15 days. In contrast to this the *in vivo* study by Beauchemin and McGinn (2006) showed no effect on methanogenesis when an essential oil mixture was fed to beef cattle at aconcentration of 1 g/day for 21 days. The essential oil product was a commercial proprietary blend of EO and plant extracts and was added to the diet at the manufacturer's recommended level (Beauchemin and McGinn 2006). Similar to the above Soliva *et al* (2008) found that pinus mugos oil containing various essential oil components had no antimethanogenic activity.

Different responses on methanogenesis were observed for different types of EO. The dose response effects of different EO on volatile fatty acid production and methane inhibition were studied by Macheboeuf *et al* (2008). They found that methane production was linearly reduced by the essential oil mixture extracted from Anethum graveolens which contained 32% limonene. They observed a negative sigmoidal shape response with a 3 mmol/L rumen fluid threshold dose with cinnamaldehyde extracted from cinnamomum verum (Macheboeuf *et al* 2008). A concentration below this threshold dose caused methane and volatile fatty acid production not to be altered. They also found that a negative sigmoidal shape response resulted with the addition of thymol, carvacrol and EO extracted from origanum vulgare and thymus vulgare and contained threshold doses of less than 2 mmol/L

rumen fluid (Macheboeuf *et al* 2008). Concentrations higher than this caused a rapid decline in fermentation which led to decreased methane production (Macheboeuf *et al* 2008).

Cobellis *et al (*2015) evaluated the effects of increasing concentrations of oregano (*Origanum vulgare* L.) and rosemary (*Rosmarinus officinalis* L.) essentials oil (EO) on ruminal gas emissions by *in vitro gas method.* They observed reduction of methane production was 55%, 72% and 71% respectively with regard to the 1.0, 1.5 and 2.0 g/L oregano EO doses, while rosemary EO (2.0 g/L) reduced the methane production by 9%. Both EOs mitigated rumen fermentations, but oregano EO gave rise to the highest reduction in methane and ammonia production.

Ozkan *et al* (2015) determined the effect of peppermint essential oil on *in vitro* gas, methane production of barley grain in the presence (100, 200, 400, 800 and 1200 mg/L) and in the absence of peppermint essential oil. The supplementation of peppermint essential oil significantly ($P < 0.001$) reduced the methane and ammonia production. The current study had provided significant information on the anti-microbial activity of peppermint essential oil causing an inhibition of the overall fermentation process of barley grain.

Benchaar (2016) conducted a study to evaluate the effect of dietary addition of cinnamon oil (CIN), cinnamaldehyde (CDH), or monensin (MON) on enteric methane (CH4) emission in dairy cows. Eight multiparous lactating Holstein cows fitted with ruminal cannulas were used in a replicated 4×4 Latin square design (28-day periods). Cows were fed (ad libitum) a total mixed ration ((TMR); 60 : 40 forage : concentrate ratio, on a dry matter (DM) basis) not supplemented (CTL), or supplemented with CIN (50 mg/kg DM intake), CDH (50 mg/kg DM intake), or monensin (24 mg/kg of DM intake). Enteric CH4 emissions were measured over 6 consecutive days using the sulfur hexafluoride (SF6) tracer gas technique. Enteric CH4 emission and CH4 energy losses averaged 491 g/day and 6.59% of gross energy intake, respectively, and were not affected by adding CIN, CDH or MON to the diet. Results indicated that CIN, CDH and MON are not viable CH4 mitigation strategies in dairy cows

Joch *et al (*2016) studied the effects of 11 active compounds of essential oils (ACEO) on methane production. Two trials were conducted. In trial 1, ACEO (eugenol, carvacrol, citral, limonene, 1, 4-cineole, *p*-cymene, linalool, bornyl acetate,

α-pinene, and β-pinene) at a dose of 1, 000 μL/L were incubated for 24 h in diluted rumen fluid with a 70:30 forage:concentrate substrate (16.2% crude protein; 36.6% neutral detergent fiber). The reduction in methane production was observed with nine ACEO (up to 86% reduction) compared with the control ($p<0.05$). Effect of peppermint (Mentha piperita) essential oil on rumen microbial fermentation of barley grain.

Kouazounde *et al* (2016) studied the effect of selected essential oils from medicinal plants on methane production. They examined the effects of nine essential oils (EO) from *Citrus aurantifolia, Cymbopogon citratus, Eucalyptus citriodora, Laurus nobilis, Lippia multiflora, Mentha piperita, Ocimum basilicum, Ocimum gratissimum* and *Zingiber officinalis* on enteric methane production in *in vitro* batch cultures screening experiments using *Andropogon gayanus* grass. Two *in vitro* batch culture incubation runs were conducted independently on separate days at two different ranges of dosages: 0 (control), 150, 300, 600 and 1200 mg/L inoculum and 0 (control), 25, 50, 100 and 150 mg/L inoculum. The effects of EO on *in vitro* methane production was assessed relative to the control containing no additive. *O. basilicum, E. citriodora, O. gratissimum* and *C. aurantifolia*, significantly inhibited (Z' > 0 and relative decrease ≥ 15%) enteric methane production (g DM incubated) relative to control at dosages of 300-1200 mg/L and *L. nobilis, C. citratus* and *M. piperita* significantly decreased it at 600 and 1200 mg/L. A substantial decrease (Z' > 0 and relative decrease ≥ 15%) in methane production per g DM incubated was apparent for *Z. officinalis* and *L. multiflora* at dosage of 1200 mg/L. Most EO had globally negligible effects on methane production (Z' ≤ 0 and relative decrease < 15%) at dosages of 25 to 150 mg/L. Overall, screening investigation demonstrated that addition of assayed EO (except *Z. officinalis* and *L. multiflora*) at dosages close to 300 mg/L seemed to potentially decreased enteric methane production with limited negative effects on dry matter digestibility of forage grass *in vitro*.

Gunal *et al* 2017) conducted experiments on evaluating the effects of essential oils on methane production. They screeneed the effects of four different EO (clove oil (CLO), white thyme oil (WTO), citronella oil (CTO) and anise oil (ANO)) at 500 mg/L of culture fluid on methane production under *in vitro* conditions in experiment one. Treatments were a control (CON) or CON plus EO at 500mg/L. Results showed

that all EOs, except CTO, decreased ($p<0.05$) methane production. The aim of experiment two was to test the effects of three different dose levels of CLO, WTO, and ANO on methane production and fermentation in 24-h batch culture experiments. Treatments were CON or CON plus EO supplemented at 125, 250, and 500 mg/L. Relative to CON, methane production decreased (p_.05) with the three EO at the 500 mg/L dose. At the 250 mg/L dose, ANO and CLO decreased (p_.05) methane production and at the 125mg/L dose, only CLO decreased methane production. They concluded that EO effects on methane production depend on EO source and dose level. Although the addition of ANO and WTO at the high doses resulted in lower methane production, they had negatively impacted on rumen microbial fermentation. Clove oil on the other hand reduced methane production without negatively impacting rumen fermentation.

2.3.5 Effects on rumen nitrogen and energy usage

The effects of EO on rumen fermentation vary because there is a wide range in nature (Calsamiglia *et al* 2007). Some EO work similar to ionophores by affecting gram-positive bacteria and rumen volatile fatty acid proportions (Burt 2004). Other EO decrease the total volatile fatty acid production by inhibiting general rumen bacteria (Busquet *et al* 2005a).

These differences of their effects imply variations in the chemical structure of EO (Benchaar *et al* 2008). Bacterial activity is inhibited very strongly by oxygenated monoterpenes, and only slightly inhibited or stimulated by monoterpene hydrocarbons (Benchaar *et al* 2008).

Amino acid deamination is decreased by certain plant extracts and their active ingredients because of their inhibition of ammonia producing bacteria like Peptostreptococcus anaerobius and Clostridium sticklandii (McIntosh *et al* 2003). This results in decreased ammonia nitrogen (McIntosh *et al* 2003). Ionophores work in a very similar way as these EO (Calsamiglia *et al* 2007; Benchaar *et al* 2008). Other EO however have no effect on amino acid deamination (Castillejos *et al* 2005). Deamination depends not only on the type of essential oil but also on the diet and dose rate (McIntosh *et al* 2003). With the addition of EO a low protein diet resulted in deamination rather than a high protein diet (Benchaar *et al* 2008). High dosages of some EO resulted in deamination but also reduced volatile fatty acid production

which compromises the energy supply to the animal (Benchaar *et al* 2008). Some EO however result in increased volatile fatty acid production (Benchaar *et al* 2007b) and some didn't affect volatile fatty acid production (Beauchemin and McGinn 2006).

The addition of some EO resulted in increased propionate and butyrate proportions and decreased acetate proportion (Calsamiglia *et al* 2007). The essential oil eugenol however caused a decrease in propionate concentration (Adesogan 2009). Microbes have the ability to adapt to EO, which makes short term *in vitro* experiments inaccurate assessment tools (Benchaar *et al* 2008).

Certain EO, especially garlic extracts reduce methane production in the rumen (Benchaar and Greathead 2011). Garlic oil or one of its main components diallyl disulphide caused reductions of up to 70% (Benchaar and Greathead 2011). Ionophores weren't able to achieve these high reductions (Busquet *et al* 2005a). The reason why the EO affected these results was because they suppressed methanogenic bacteria directly rather than suppressing the precursors of methane (Adesogan 2009). Some EO other than garlic oils also reduce methane production, but can also decrease digestibility and propionate concentration examples include thymol, clove and fennel extracts (Patra *et al* 2006)

Some EO are pH and diet dependant only when used under certain conditions and production systems can they be a great advantage (Calsamiglia *et al* 2007). Capsaicin for example is advantageous when given in high-concentrate diets and has only small effects in high-forage diets (Santos *et al* 2010). Plant extracts act at different levels in the protein and carbohydrate degradation pathways. Carefully selecting and combining them can be a beneficial tool used for rumen microbial fermentation manipulation (Calsamiglia *et al* 2007).

2.3.6 Effects on animal performance

EO used as feed additives are still a very new concept and extra research still has to be done on this subject. Various studies done on this have shown that EO have no effect on milk production or composition. A study by Yang *et al* (2007) showed that garlic and juniper berry essential oils resulted in the same milk production and composition compared to cows receiving no feed additives. The study done by Benchaar *et al* (2006) on beef cattle showed an improved feed efficiency with the addition of EO.

2.3.6.1 Feed intake and growth

Mixed observations have been recorded on feed intake depending on essential oil type and dose. Feed intake was not influenced by EO when 250 mg/day of essential oils extracted from oregano plants were fed to sheep (Wang *et al* 2009), 2 g of juniper berry essential oils containing 35% α-pinene were fed to cows (Yang *et al* 2007), dairy cattle were fed 0.75 to 2 g of an essential oil mixture (Benchaar *et al* 2007a) and dairy goats received 0.043 g EO or 0.43 g EO per kg feed intake (Maleckey *et al* 2009). Feed intake was however adversely affected in beef cattle by an essential oil mixture of eugenol at 90 mg/day and cinnamaldehyde at 180 mg/day (Cardozo *et al* 2006) and in dairy cattle by cinnamaldehyde at high doses of 500 mg/day (Calsamiglia *et al* 2007). This feed intake reduction can be a result of palatability problems, implying that this problem can be overcome by encapsulating the product (Calsamiglia *et al* 2007; Spanghero *et al* 2008). In contrast to this Cardozo *et al* (2006) found that feed intake and rumen fermentation in beef cattle is stimulated by the addition of capsicum oil to a concentrate based diet. The effect of certain volatile compounds on the alfalfa pellet consumption by sheep was studied by Estell *et al* (1998). They found that the compounds α-pinene, borneol and camphor inhibited the alfalfa pellet consumption and the compounds limonene, β-caryophyllene and cis-jasmone had no noticeable effect on consumption. Supplementing the proper essential oil dose is very important because certain EO stimulate intake at low doses and may negatively influence intake at higher doses in ruminants (Estell *et al* 1998). A study by Yang *et al* (2010b) showed that a higher feed intake response was linked to a low dose of 0.4 g/day of cinnamaldehyde and a higher dose of 1.6 g/day had no effect on intake in steers.Limited information is available on the essential oil effects on ruminant performance.

Bampidis *et al* (2005) found that feed efficiency and average daily gain were not affected when diets supplemented with oregano leaves giving 144 mg or 288 mg of oregano oil per kilogram of diet dry matter were fed to growing lambs. Similarly Benchaar *et al* (2006b) observed no average daily gain change in beef cattle fed silage based diets supplemented with an essential oil mixture of 2 or 4 g/day consisting of eugenol, thymol, limonene and vanillin. The essential oil mixture however had a positive effect on feed conversion with the 2 g/day dose. The 2 g/day dose improved

the feed conversion compared to the 4 g/day dose (Benchaar *et al* 2006b). In the study by Chaves *et al* (2008c) it was observed that cinnamaldehyde and carvacrol had no effect on sheep growth when they were fed barley or corn based diets for 11 weeks. The barley based diet did however result in much higher growth rates. Increased average daily gain was however observed in some cases when cinnamaldehyde and juniper berry EO were added to the barley based diet at similar concentrations. The essential oil influence on growth performance thus appears to be diet dependant.

Khamisabadi *et al* (2016) carried out study to investigate the effect of inclusion of dietary supplementation of peppermint (*Mentha piperita*) and *Thymus vulgaris* on growth performance. Statistical significant differences were found in daily weight gain, dry matter intake between three groups. The results showed that addition of thyme or peppermint at a concentration of 3% on DM basis to diet or improve on nutrient digestibility and increased feed intake and average daily gain.

Valero *et al* (2016) studied the effect of feeding propolis or essential oils on bulls performance. Bulls were fed a control diet (CON) with sorghum silage (41% DM) and cracked corn, soybean meal, glycerine, limestone, and mineral salt. The propolis-supplemented group (PRO) received 3 g/animal/d in the concentrate. The essential oils-supplemented group (OIL) received 3 g/animal/d (1.5 g cashew oil + 1.5 g castor oil) added to the concentrate. They observed that final body weight, average daily gain and feed efficiency were better for bulls fed the OIL diet. Propolis or essential oils had no effect on feed intake and digestibility. They concluded that dietary addition of propolis did not affect bull performance or feed efficiency. The addition of essential oils improved the Feed intake and digestibility.

2.3.6 Combinations between different essential oils

Burt (2004) previously reported about the additive, synergistic and antagonistic effects between different combinations of EO. An *in situ* study by Newbold *et al* (2004) showed that a blend of EO containing eugenol, thymol, vanillin and limonene inhibited protein degradation. The changes they found were however small and variable depending on the feed being degraded, the type of ration fed to the animals and the adaptation period length (Newbold *et al* 2004). One or more EO have been combined in most commercial products, although there is only limited

information available on the synergies among them. Research is urgently needed on this subject to ensure proper supplementation of EO.

Various studies have been conducted in the last decade to exploit EO as feed additives for improving the ruminant production efficiency. Some essential oil components certainly affect rumen production and fermentation positively. The optimum dose, feeding system and physiological status needs to be identified to optimize these effects. Methanogens, Hyper ammonia producing bacteria and other undesirable bacteria can be specifically inhibited by EO which in turn affects rumen fermentation positively by increasing rumen VFA concentrations, inhibiting methane production, increasing CLA production and reducing ammonia concentrations. Most of the recent findings are however based on *in vitro* studies and only a few studies have been conducted *in vivo*. The *in vivo* studies that have been done are inconsistent because the essential oil constituents that have been tested differ in type and dose. Thousands of essential oil active ingredients with different range and mode of actions in the rumen have been identified. Most studies used an essential oil mixture containing different active ingredients in different proportions. To achieve more accurate results and thereby improve nutrient utilization efficiency and animal performance an optimum dose of essential oil components and their optimum combinations together with their appropriate dietary nutrient composition should be standardized. Micro-organism adaptation to EO has not been studied in detail and this aspect should be regarded for essential oil use in ruminant nutrition.

Chaturvedi *et al (*2015) studied the effect of combined herbal feed additives on methane, total gas production and rumen fermentation Different parts of the five medicinal plants were selected such as leaf and small stems of Ocimum sanctum (Tulsi), roots of Curcuma longa (Haldi), fruits of Emblica officinalis (Amla), leaves of Azadirachta indica (Neem) and leaves and small stem of Clerodendrum phlomidis (Arni) for their study. Addition of different herbal additive combinations did not influence IVDMD and total gas production however methane production (mg/g of substrate DM) was significantly ($P<0.05$) reduced in Amla: Neem and Neem: Arni combinations. Total nitrogen significantly ($P<0.01$) increased in the combinations of Tulsi: Haldi and Amla: Neem. TCA–ppt-N is significantly ($P<0.01$) increased in Tulsi: Haldi, Haldi: Amla, Amla: Neem and Neem: Arni however NH_3-N (mg/dl)

significantly decreased in all treatments. They concluded that the screening of plant combinations, Amla: Neem and Neem: Arni have potential to decrease methane production and the herbal feed supplements had no side-effects on the ruminant in small amount.

2.3.7: Blood parameters:

Effects of EO on blood metabolites in dairy cows have not been investigated widely. Devant *et al* (2007) reported that EO supplementation did not significantly affect blood glucose concentration of dairy cows. Yang *et al* (2010) reported that concentrations of some blood metabolites such as triglycerides can be influenced by EO supplementation via changing of feed intake.

Vakili *et al* (2013) observed that feeding of thyme (THY) and cinnamon (CIN) essential oils on blood metabolites in feedlot calves fed high-concentrate diets. Plasma concentrations of glucose, cholesterol, triglyceride, urea-N, beta-hydroxybutyrate, alanine aminotransferase and aspartate aminotransferase were not changed by feeding THY or CIN. Results suggested that supplementing a feedlot finishing diet with THY or CIN essential oil has minor impacts on blood metabolites.

Hashemzadeh-Cigari et al (2015) studied the effect of specific essential oil compound on blood metabolites in dairy cows. The EO treatment had no effect (P>0.05) on blood glucose, and albumin concentration. The concentration of blood total protein, cholesterol, globulin concentrations, and albumin: globulin ratio was decreased (P<0.01) by EO compared with the control, although we identified a lower greater (P<0.01) blood BHBA content in EO cows. The previous studies related to the effects of diet containing EO or blend on blood parameters in dairy cows are limit. Sahraei *et al* (2014) reported that supplementation with EO (400 mg d-1 of rosemary) had no effect on plasma concentrations of glucose, triglyceride, cholesterol, total protein and albumin in sheep.

Santos *et al* (2015) reported the effect of essential oils on blood prameters of dairy calves. Calves were assigned to one of the three treatment groups in a randomized block design. Treatments: (1) control without essential oils supplementation (C); (2) essential oils blend in the milk replacer at 400 mg/kg (MR) and (3) essential oils blend in the milk replacer (200 mg/kg) and starter feed (200

mg/kg) (MRS). They observed that blood parameters were not affected by the essential oils supplementation.

Biricik *et al* (2016) investigated the effects of increasing doses of carvacrol (C) and/or thymol (T) on the blood parameters of Merino sheep. The sheep were fed with the same concentrate mixtures including a control diet, carvacrol 100 mg/kg (C100), carvacrol 300 mg/kg (C300), thymol 100 mg/kg (T100), thymol 300 mg/kg (T300), carvacrol+thymol 100 mg/kg (C50+T50), and carvacrol+thymol 300 mg/kg (C150+T150). The serum urea and glucose in C and/or T groups were not found significant on days 0, 35, and 70 compared to the control group.

Khamisabadi *et al* (2016) conducted study to investigate the effect of inclusion of dietary supplementation of peppermint (*Mentha piperita*) and *Thymus vulgaris* on blood parameters of finishing lambs. Concentrations of cholesterol, nonesterified fatty acids (NEFA), beta-hydroxybutyric acid (BHBA), blood creatinine levels were not influenced by addition of either thyme or peppermint to the diet. While triglyceride, glucose and urea were significantly lower in lambs received these plants in their diets.

Mohamadi *et al* (2017) studied the effect of essential oils of *Mentha piperita* and *Mentha pulegium* and found no significant effects on performance and blood metabolites of Dallagh sheep

Khateri *et al* (2017) conducted study to see the effects of a specific blend of essential oils on blood parameters in sheep fed a 50:50 alfalfa hay:concentrate diet and found that relative to the control, no changes were observed in the red and white blood cells, hemoglobin, hematocrit, glucose, beta-hydroxybutyric acid, cholesterol, total protein, albumin, blood urea nitrogen and aspartate aminotransferase and alanine aminotransferase concentration. The results of the present study suggested that supplementation of MEO may have limited effects on blood cells and metabolites.

CHAPTER - III

MATERIALS AND METHODS

A brief description of experimental techniques and procedures of analysis adopted during the study are reported in this chapter. The total mixed rations (TMRs) vrying in roughage to concentrate ratio were prepared on DM basis (table 3.1). The herbs (jaiphal, suva and haldi) containing essential oils were procured from Konark herbals.

Table 3.1. Ingredients composition of TMRs for *in-vitro* studies

Feedstuffs	80:20	75:25	70:30	65:35
Roughage				
Wheat straw	56.0	52.5	49.0	45.5
Maize	24.0	22.5	21.0	19.5
Concentrate ingredients				
Maize	3.0	3.75	4.5	5.25
Wheat	3.0	3.75	4.5	5.25
Deoiled mustard cake	3.0	3.75	4.5	5.25
Mustard cake	2.0	2.5	3.0	3.5
Soybean meal	2.0	2.5	3.0	3.5
Rice bran	3.0	3.75	4.5	5.25
DORB	3.2	4.0	4.8	5.6
Urea	0.2	0.25	0.3	0.35
Mineral mixture	0.4	0.5	0.6	0.7
Salt	0.2	0.25	0.3	0.35

The roughage portion was made up of wheat straw and maize green fodder in 70:30 ratio, while conventional concentrate mixture was made up of maize 15, wheat 15, deoiled mustard cake 15, mustard cake 10, soybean meal 10, rice bran15, deoiled rice bran 16, urea 1, salt1, mineral mixture 2%

3.1 *IN VITRO* EVALUATION OF TOTAL MIXED RATIONS

3.1.1 *In vitro* gas production

The *in vitro* gas production was assessed according to Menke *et al* (1979).

3.1.2 Preparation of samples

About 375 mg of sample was weighed in a weighing boats (with removable stem) and the sample was put at the bottom of the 100 ml calibrated glass syringe taking caution that it did not stick to the walls of syringe. Then the piston, greased with petroleum jelly (vaseline) was pushed into the cylinder. The syringes containing sample in triplicate were kept in an incubator at 39°C.

3.1.3 Collection of rumen liquor

Rumen fistulated maintained on 2 kg conventional concentrate mixture, 5 kg green and ad lib wheat straw was used as a donor for rumen liquor. The rumen contents were collected at 0 hr in double walled (thermos) flask flushed with CO2 and maintained at 39°C. The rumen contents were blended for 2-3 min. in a blender, maintained at 39°C and then strained through 4 layered muslin cloth.

3.1.4 Preparation of solutions

Following solutions were prepared well in advance

1) Micro mineral solution

$CaCl_2.2H_2O$ = 13.2 g

$MnCl_2.4H_2O$ = 10.2 g

$CoCl_2.6H_2O$ = 1 g

$FeCl_2.6H_2O$ = 8 g

Dissolved in distilled water and made the volume 100 ml.

2) Macro-mineral solution

Na_2HPO_4 = 5.7 g

KH_2PO_4 = 6.2 g

$MgSO_4.7H_2O$ = 0.6 g

Dissolved in distilled water and made the volume 1000 ml.

3) Buffer solution

$NaHCO_3$ = 35.0 g

NH_4HCO_3 = 4.0 g

Dissolved in distilled water and made volume 1000 ml.

4) **Resazurine solution:** Dissolved 100 mg of resazurine in distilled water and made volume 100 ml and kept in refrigerator.

5) Reducing solution (This solution is to be prepared fresh before each incubation)

$Na_2S.H_2O$ = 373.0 mg

1N NaOH = 2.6 ml

Distilled water = 62.0 ml

The above solutions were mixed in following ratio in a Woulff f1ask (3litre cap) mixed with magnetic stirrer in a water bath at 39°C.

1. Distilled water = 960 ml
2. Micro mineral solution = 0.16 ml
3. Buffer = 660 ml
4. Macro mineral solution = 330 ml
5. Resazurine = 1.6 ml

Add 50 ml of reducing solution.

3.1.5 Procedure

While the reducing solution was added, CO_2 was flushed through a submerged tube. The slightly bluish color first turned pinkish then became colorless. The strained rumen liquor (SRL) was added to the buffer media in 1:2 ratio only when solution was colorless. The f1ushing of CO_2 was continued till the last syringe was filled.

For fi1ling up of syringes, the tube on the capillary attachment to the syringe was firmly fixed on to the bottle top dispenser. 30 ml of SRL: buffer solution from the f1ask kept in a water bath was pumped in each syringe. The contents in syringe were mixed by gentle shaking. Air bubbles were brought to the surface and removed through the capillary by careful upward movement of the piston. The clip was closed immediately and exact volume of the contents in the syringe was noted and kept in an water bath maintained at 39°C. The contents in all the syringes were swirled at 1 hour interval for first few hours. If at 8h the gas exceeded 70 ml, the volume of gas was recorded and gas was removed. After 24 hours volume of gas produced in each syringe was recorded. Blanks and standard hay in triplicate were also run with each set of incubation. After stipulated time, the contents were taken out and centrifuged. After 24 hr the NH3-N, TVFA and NDF of residue were determined. The ME value of the substrate was calculated by using the fol1owing equation developed by (Menke *et al* 1979).

$$ME\ (kg) = 1.24 + 0.146\ G\ (ml/200\ mg\ DM) + 0.007\ CP + 0.0244\ EE$$

where,

ME = Metabolisable energy, MJ/kg DM

G =Net gas production, ml/200mg DM

CP = Crude protein, g/kg

EE = Ether extract, g/kg

At the end of 24 hr incubation period methane was estimated by GLC by sampling the gas from the silicon tube of syringes and the liquid content was further processed for the estimation of VFA and ammonia nitrogen.

3.1.6 Estimation of volatile fatty acids

Volatile fatty acids were estimated using Netchrom 9100 gas chromatograph (Netal, New Delhi, India) equipped with flame ionization detector as per method described by Cottoyn and Boucque (1968). The glass column (6 ft length and 1/8 inch diameter) packed with chromosorb 101 was used for the estimation of VFA. The gas flows for nitrogen hydrogen and zero air were 30, 30, and 320 µl/min, respectively.

Temperature of injector oven, column even and detector were 270°C, 172°C respectively. Samples were prepared by adding 0.2 ml of 25% metaphosphoric acid per ml of rumen liquor, allowing it to stand for 2 hrs followed by centrifugation at 4000 rpm for 7 min. Supernatant was used for estimation of VFA. Standard VFA mixture was prepared by mixing stock solutions (each of 25 mg/ml concentration) of standard VFAs and distilled water in the following amounts: acetic acid 1.68 ml, propionic acid 0.48 ml, butyric acid 0.24 ml, distilled acid 7.24 ml to obtain final concentration of acetic acid, 7.0, propionic acid, 1.62; Valeric acid 0.68 mm/100 ml. The mixture was stored in deep freeze until further use.

3.1.7 *In vitro* true OM digestibility of substrate

The content of syringes was transferred to spoutless beaker by repeated washing with 20 ml neutral detergent solution. The flask content were refluxed for one hour and filtered through pre weighed Gooch crucibles (grade GI). The dry matter content of the residue was weighed and *in vitro* true digestibility of feeds was calculated (Van Soest and Robertson 1988)

$$\text{True OM digestibility (TOMD\%)} = \frac{\text{Initial OM of feed taken for incubation-OM residue}}{\text{Initial OM of feed taken for incubation}} \times 100$$

3.1.8 Kinetics of gas production

Air equilibrated feed samples (200 ± 10 mg) of RH, CS and mixed diets were incubated in 100 ml calibrated glass syringes in triplicate according to Menke and Steingass, (1988) with 30 ml mixed rumen suspension with three blank incubations and standards. Cumulative gas production was recorded at 2, 4, 6, 8, 10, 12, 16, 20, 24, 30, 36, 48, 60 and 72 h of incubation. The rate and extent of gas production were calculated by non-linear regression using the model $Y = D (1 – e^{-k*t})$ where, Y is gas volume (ml) at time t, D is potential gas production (ml) and k is rate (per hour) at which gas is produced (Krishnamoorthy *et al* 1995). The time at half asymptotic gas production ($t_{1/2}$) was calculated as ln2/k. Gas production at 24 h, corrected for the blank and standards was used for determination of ME.

3.1.9. HYDROGEN BALANCE

Hydrogen recovery (%) was estimated as $(4M+2P+2B) / (2A+P+4B) \times 100$, the ratio of hydrogen consumed *via* CH_4/VFA was estimated as 4M/ (2P+2B), where acetate (A), propionate (P), butyrate (B) and methane (M) production was expressed in mmol (Demeyer 1991).

3.1.10. FERMENTATION EFFICIENCY

This was calculated on the basis of the equation worked out by Orskov (1975) and modified by Baran and Zitnan (2002).

$$FE = (0.622a + 1.092p + 1.56b) 100/ (a + p + 2b)$$

where: a, p and b express the concentrations (μmol) of acetic, propionic and butyric acids respectively in the total concentration of VFAs produced. The final result of this equation is expressed in percentage and shows an amount of energy stored in VFAs as a percentage participation of the initial energy.

3.1.11. VFAs UTILIZATION INDEX

This was expressed by non-glucogenic VFAs/glucogenic VFAs ratio (NGGR) according to Orskov (1975).

$$NGGR = (A + 2B + V) / (P+V)$$

where A, P, B and V express the concentrations (μmol) of acetic, propionic, butyric and valeric acids respectively. Valeric acid is classified as both glucogenic and non-glucogenic VFA because, its oxidation creates 1 mole of acetic acid and 1 mole of the propionic acid. Too high NGGR indicates high loss of energy in the form of gases.

3.2 CONDUCTION OF METABOLIC TRIAL

Selection of animals and experimental design

Four cross bred male calves of the age group (10 to 14 months) were selected and divided into four groups of one each, based on comparable body weight and age The feeding trial was carried out in four periods in a switch over design as shown below. Each period lasted for four weeks with an adjustment period of three weeks and collection period of one week.

	Animal-1	**Animal-2**	**Animal-3**	**Animal-4**
Period-1	TMR1	TMR2	TMR3	TMR4
Period-2	TMR2	TMR3	TMR4	TMR1
Period-3	TMR3	TMR4	TMR1	TMR2
Period-4	TMR4	TMR1	TMR2	TMR3

Male cross bred calves were divided into 4 equal groups were offered four different total mixed rations i.e TMR1 (control), TMR2 (1 % Jaiphal), TMR3 (1% Suva) and TMR4 (1% Haldi) during 120 days. During this period digestibility coefficients of various nutrients, nitrogen balance studies were conducted. Blood was collected at the end of experiment.

3.2.1 Housing

The animals were housed in a concrete shed and were stall fed individually at 9:00 am daily. The animals had free access to water twice a day and were taken out in the yard for 1-hour exercise daily.

3.2.2 Metabolism trial

A 7-day metabolic trial was conducted on all the animals after 21 day of adaptation period. During metabolic trial, the animals were kept in specially designed metabolic cages having a cemented floor converging in centre, where a metallic pipe led the excreted urine into a narrow mouth plastic container (25 liters capacity) containing 400 ml of 20% H_2SO_4. The collection of faeces and urine was done for 7 days. The faeces voided were collected manually and kept in metallic drums (30 kg capacity) for 24 h. Faeces were collected manually by trained persons, who were put on duty round the clock. However, the collection of urine was automatic. The combined residue of wheat straw, green and concentrate mixture, if any, was weighed

every morning at 9:00 am., in the morning before offering the next day's ration. On the last day of trial, blood sample was taken from jugular vein of animals 4 hrs. of post feeding to determine related blood profile of animals.

3.2.3 Sampling of feed, orts, faeces and urine

3.2.3.1 Feed

Samples of feedstuffs and orts were collected at 24 h interval and dried in duplicate at 100° C in a forced air oven. The samples were pooled for 7 days, finely ground to pass through 1mm sieve and analyzed for proximate principles and cell wall contents.

3.2.3.2 Faeces

After thoroughly mixing, the 24 h faecal material of each animal, one by two hundredth parts in duplicate was weighed in circular aluminum tray for DM determination. The samples were dried at 100° C in a forced air oven overnight and weighed by taking minimum time to prevent the absorption of moisture from atmosphere. The 7 days dried faeces were pooled, and finely ground to pass through 1mm sieve and preserved in airtight glass sampling bottles. For nitrogen estimation fifty grams total faeces was preserved in previously tarred wide mouth plastic bottles daily, containing 25 ml of 20% sulphuric acid solution added on the first day of collection. Everyday, after placing the faeces in the bottles, it was thoroughly mixed to prevent loss of ammonia and possible infestation with fungi. Faecal samples were stored in a refrigerator till analyzed.

3.2.3.3 Urine

An aliquot of urine equal to one by tenth part of the total urine voided was preserved in narrow mouth glass bottles (1000 ml capacity) daily, which were kept in a refrigerator till analyzed for nitrogen content..

3.2.3.4 Analysis of feeds, faeces and urine

Samples of different concentrate mixtures, rice straw, green fodder (bajra), feed residue and faeces were analyzed for their proximate constituents and cell wall constituents as mention in the previous section.

3.3 CHEMICAL ANALYSIS

All the feed ingredients, conventional, non-conventional, concentrate mixtures and complete feeds were analyzed for proximate and cell wall constituents.

3.3.1 Proximate principle

3.3.1.1 Dry matter

For this, a known quantity of the well mixed sample was taken in an aluminum tray, then it was dried in an oven for 24 h at 100°C. The weight of the dried sample was taken as the dry matter content of the sample.

3.3.1.2 Total ash

Finely ground sample (2 g) was taken in duplicate in tarred crucibles over a hot plate and then ignited in muffle furnace for 3 hours. After that the crucibles were taken out put in desiccator and weighed. The difference between initial weight of empty crucible and final weight of crucible with ash gave the total ash content in the sample and was expressed as percent of DM (AOAC 1990). The loss in weight after ignition in muffle furnace was taken as organic matter.

3.3.1.3 Crude protein

The N content was estimated by Macro-Kjeldahl method (AOAC 1990). Finely ground sample (0.25 g) or 2 ml of urine or 5 g of fresh feaces was digested with 15 ml (25 ml for feaces) of concentrated sulphuric acid and (5-6 g of digestion mixture potassium sulphate and copper sulphate, 9:1). The material after digestion was distilled in presence of 40 per cent sodium hydroxide. The ammonia liberated was collected in 20 ml of 4 per cent boric acid-mixed indicator solution. Mixed indicator was prepared by taking bromocresol green and methyl red in 5:1 ratio in 95% ethanol. Ammonium borate thus formed was titrated against standard (0.1 N) sulphuric acid. The reading for blank was also recorded.

$$\text{Nitrogen (\%)} = \frac{\text{(Normality of acid) (Vo1.of acid, ml) x 0.014}}{\text{Wt. of sample (g)}} \times 100$$

The CP (%) content was calculated by multiplying the nitrogen by 6.25.

3.3.1.4 Ether extract

Finely ground (2 g) sample was transferred to extraction thimble (Whatman No. l). The sample was extracted in soxhlet apparatus with petroleum ether (60-80°C) for 16 hr. After 16 hr the excess of petroleum ether was collected from the Soxhlet apparatus and small volume left in f1ask at the bottom was transferred to tarred 100 ml beaker. The beaker was dried in an oven to a constant weight. The difference in initial (weight of empty beaker) and final weight (beaker with ether extract) gave the

ether extract content in the, sample. The ether extract was expressed as per cent ether extract on DM basis (AOAC 1990).

3.3.2 Cell wall constituents

3.3.2.1 Acid detergent fibre

One gram sample was transferred in spout less beaker and 100 ml of acid detergent solution was added (20 g CTAB dissolved in one litre of lN H_2SO_4). The contents were refluxed for one hour. The contents were filtered through previously weighed sintered glass crucibles (G-l) and washed with hot water followed by one washing of acetone. The residue was dried at 80°C in a hot air oven for overnight. The difference in initial (empty crucible) and final (crucible + residue) weight of crucible gave ADF content. It was expressed as per 'cent on DM basis (Robertson and Van Soest 1981).

3.3.2.2 Neutral detergent fibre

Finely ground sample (0.5 g) was transferred in spout less beaker and 50 ml of neutral detergent solution (NDS) was added. The NDS was prepared by weighing 18.61 g disodium salt of EDTA and 6.81 g sodium borate in a beaker, added some distilled water and heated till dissolved. Then 4.56 g disodium hydrogen ortho-phosphate was taken in another beaker, added distilled water and heated till dissolved. Then, added 30 g sodium lauryl sulphate and 10 ml of ethoxy ethanol in 850 ml distilled water. Then added contents of two previous beakers to it and mixed. The volume was made to one litre.

The sample and NDS were refluxed for one hour, after the boiling had started. The contents were filtered through previously tarred sintered glass crucible (G-1) and washed with hot water, till free from NDS, followed by final washing with acetone. The residue was dried at 80°C in a hot air oven for overnight. The difference in initial weight (empty crucible) and final (crucible + residue) weight of crucible gave the NDF content. It was expressed as per cent NDF on DM basis (Robertson and VanSoest 1981).

3.3.2.3 Cellulose

Half g sample was taken and 15 ml of digestion mixture solution (650 ml glacial acetic acid, 80 ml nitric acid and 150 ml distilled water) was added in plastic

tubes. The tubes were placed in boiling water bath for 30 minutes. Then contents were filtered through sintered glass crucible (G-l), washed with hot water repeatedly and then final washing with ethanol. The residue was dried at 80°C for overnight in a hot air oven. Then crucibles were weighed and ignited at 500°C in a muffle furnace for half an hour. The loss in weight on ignition represented cellulose content which was expressed as per cent cellulose on DM basis (Crampton and Maynard 1938).

3.4 BLOOD BIOCHEMICAL PROFILE:

To study the effect of herbs feeding on nutrient (carbohydrates, fat and protein) metabolism, monitoring of blood parameters was carried out. Blood samples were drawn from all male cross bred animals at the end of experimental feeding.

3.4.1 Collection of blood, serum separation and preservation

Serum was preserved at -20°C to analyze the following parameters

1. Glucose
2. BUN
3. Cholesterol
4. GGT
5. AST
6. ALT

All these biochemical parameters were estimated by using diagnostic kits from Siemens Autopack and analyze at RA-50 blood analyzer.

3.5 RUMEN FERMENTATION

This experiment was conducted to study the rumen fermentation parameters on three rumen fistulated adult male buffaloes calves

3.5.1 Collection and sampling of rumen liquor

All the male buffalo calves were given the respective experimental diets as per the experimental design. The collection of the rumen liquor was done at 0, 2 4, 6, 8, and 10 hr of feeding. Zero hour sampling was done before feeding and watering. The feeds were offered once in a day at 9.00am and water was provided twice. Rumen liquor was collected from the various sites of the rumen with the help of a specially stomach tube. The rumen liquor was filtered through four layers of muslin cloth and

was called strained rumen liquor (SRL). For the determination of pH, no preservative was added to the SRL and pH was determined immediately after each collection. For the estimation of TVFA, total nitrogen and its fractions about 30 ml of SRL was preserved with two drops of saturated mercuric chloride. All the samples of rumen liquor were pooled according to their respective hours of sampling and kept in refrigerator until analyze. The strained rumen liquor was studied for the following parameters

1. Total volatile fatty acids
2. Total nitrogen
3. TCA-precipitable nitrogen
4. Ammonia nitrogen
5. Non protein nitrogen

3.5.2 Analytical procedures for Rumen metabolites

3.5.2.1 Total nitrogen

Strained rumen liquor (SRL, 2 ml) was taken in Kjeldhal flask and 10 ml conc. Sulfuric acid along with 2 gm digestion mixture was added. Kjeldhal flask was kept on heater for digestion. The digested material then distilled in presence of 40% NaOH. The ammonia liberated was absorbed in 25 ml of 4% boric acid solution having mixed indicator. The formed ammonium borate was titrated against N/10 H_2SO_4. The % N was calculated after subtracting the blank.

$$N\ (\%) = \frac{\text{(Vol. of acid used) (Normality of acid) X 0.014}}{\text{SRL (ml)}} \text{ x 100}$$

3.5.2.2 TCA precipitable nitrogen

Strained rumen liquor (SRL, 5 ml) was taken in centrifuge tube and 5 ml of 20% TCA was added and left for overnight. It was centrifuged at 2000 rpm for 10 minutes. Sediment was transferred by repeated washing in kjeldhal flask. The digestion, distillation and titration were done as total nitrogen estimation:-

TCA ppt. N (%) = ((Vol. of acid used X Normality of acid X 0.014)/SRL, ml) X 100

3.5.2.3 Non Protein nitrogen

NPN = Total nitrogen – TCA ppt Nitrogen

3.5.2.4 Ammonical nitrogen

The centrifuged content (5 ml) from syringes (after 24 hours of incubation) was taken in one litre boiling flask. Added 250 ml of water and distilled in presence of 2 ml of NaOH. The ammonia liberated was collected in 20 ml of 4 percent boric acid solution containing mixed indictor (same as in case of nitrogen estimation). Ammonia borate thus formed was titrated against standard (0.1 N) sulphuric acid. The percent ammonical nitrogen was calculated as follows after subtracting the blank (AOAC 1990).

NH_3-nitrogen (%) = ((Vol. of acid used X Normality of acid X 0.014)/SRL, ml) X 100

3.5.2.5 Total volatile fatty acids (TVFA's) estimation

The Volatile fatty acids were estimated using Netchrom 9100 gas chromatograph (Netal, New Delhi, India) equipped with flame ionization detector as per method described by Cottoyn and Boucque (1968).

3.6 STATISTICAL ANALYSIS

Data were analysed by 3x3x4 factorial design (Snedecor and Cochran 1994), by using SPSS Version 19. The differences in means were tested by Tukey B and Duncan. The computer program Graph Pad Prism (2004, graph pad Inc, USA) was used to calculate the rate and extent of gas production in the non-linear equation.

CHAPTER IV

RESULTS AND DISCUSSION

The results have been discussed under following sub-heads.

4.1 Potential of herbs containing essential oils on methane reduction, *in vitro*

4.1.1 Chemical composition of TMRs varying in R:C ratios

4.1.2 *In vitro* screening of herbs containing essential oils and optimum level of supplementation at 24 hr and t –half

4.1.3 Digestion kinetics parameters *in vitro* gas production

4.2 *In vivo* evaluation of herbs containing essential oils selected from section 4.1

4.2.1 Utilization of nutrients

4.2.2 Nitrogen balance

4.2.3 Rumen profile

4.2.4 Blood profile

Table 4.1. Chemical composition of TMRs varying in R:C ratios

Parameters	Roughage :concentrate ratio			
	80:20	75:25	70:30	65:35
CP	11.2	11.65	11.97	13.26
ASH	10.25	9.65	9.65	9.55
OM	89.75	90.35	90.35	90.45
NDF	70.0	69.10	68.2	64.0
ADF	45.80	43.40	42.20	38.40
HC	24.20	25.70	26.00	25.60
FAT	1.43	1.63	1.76	1.80
Cellulose	37.2	35.40	31.30	29.80

4.1.2 *In vitro* evaluation of different total mixed rations (TMR) at 24 hrs, irrespective of herb and level of supplementation

The effect of different TMRs, (R:C ratios) irrespective of different levels and herbs was studied on *in vitro* utilization of nutrients and presented in (Table 4.2). The net gas production (ml/gDM) was lower ($p<0.05$) in 80;20 TMR (70.96ml) and was highest in 65:35 TMR. (81.92ml).

The partitioning factor (PF) is the ratio of organic matter degraded (mg) *in vitro* to the volume of gas (ml) produced. A higher partitioning factor means that proportionally more of the degraded matter is incorporated into microbial mass i.e. the efficiency of microbial protein synthesis is higher. The partition factor calculated *in vitro* provides useful information for predicting the dry matter intake, microbial mass production in the rumen and the methane emission of the whole ruminant animal. In this study PF value (ml) was lower ($p<0.05$) in 80:20 TMR (3.19) followed by 75:25TMR (3.25) and higher ($p<0.05$) PF value was observed in 65:35TMR (3.40).

The OMD % was significantly lowest ($p<0.05$) in 80:20 TMR (63.72 %) and significantly higher in 65:35 roughage to concentrate based rations (73.64%), where as NDFD % was significantly lower ($p<0.05$) in 80:20 TMR (53.48 %) and highest in 65:35 total mixed ration (65.03%). Microbial mass production (94.31mg) as well as efficiency of microbial mass production (37.79 %) was higher ($p<0.05$) in 65:35 TMR. Metabolizable energy (ME) was significantly lower (7.93) in 80 :20 R:C based TMR, however it was comparable in other TMR's (75:25 and70:30) and significantly higher in 65:35 roughage to concentrate ratio TMR. The short chain fatty acids (SCFA) was lower ($p<0.05$) in 80:20 based total mixed ration (0.84mmole) and was observed significantly higher in 65:35 TMR (0.96mmole) but it was comparable in both75:25 and 70:30 roughage to concentrate ratio based TMR. The concentration of ammonia was lower ($p<0.05$) in 65:35 total mixed ration (20.33mg/dl) and highest in 80:20 TMR (21.63 mg/dl).

The amount of fermentable methane (1.44 mmol) and fermentable carbon dioxide (2.47mmol) was lower ($p<0.05$) in 75:25 TMR where as both these were s higher ($p<0.05$) in urea based 65:35 TMR.

Table 4.2. Effect of essential oils containing herbs on *in-vitro* utilization of nutrients of different TMRs at 24h, *irrespective of herb and level*

Parameters	Roughage: Concentrate ratio				SEM
	80:20	75:25	70:30	65:35	
NGP, 375mg	70.96[a]	75.58[b]	75.38[b]	81.92[c]	0.47
NGP/gDM	179.0[a]	186.0[b]	195.00[c]	195.95[c]	1.04
OMD, mg/gDM	571.85[a]	605.28[b]	636.42[c]	666.038[d]	3.80
PF	3.19[a]	3.25[a]	3.26[b]	3.40[c]	0.013
OMD, %	63.72[a]	66.99[b]	70.44[c]	73.64[d]	0.40
NDFD, %	53.48[a]	56.78[b]	60.84[c]	65.03[d]	0.47
MMP, mg	59.73[a]	82.40[c]	77.95[b]	94.31[d]	1.43
EMMP, %	27.97a	36.50[c]	32.66[b]	37.79[d]	0.47
TD, %	65.13[a]	67.93[b]	72.43[c]	74.140[d]	0.39
SCFA, mmole	0.841[a]	0.895[b]	0.889[b]	0.967[c]	0.005
ME, MJ/kg DM	7.931[a]	8.370[b]	8.429[b]	8.993[c]	0.043
NH3-N, mg/dl	21.63[c]	20.80[b]	20.78[b]	20.33[a]	0.11
Ferm.CO_2, mmol	2.91[b]	2.47[a]	3.19[c]	3.53[d]	0.050
Ferm.CH_4, mmol	1.68[b]	1.44[a]	1.80[c]	2.01[d]	0.027

Means bearing different superscripts in a row differ significantly (P<0.05)

The effect of essential oils containing herbs on *in vitro* volatile fatty acids production of different TMRs at 24 hr, irrespective of herb and level of supplementation is presented in (Table 4.3). The TVFA was significantly lowest (p<0.05) in 75:25 TMR (4.92 mM/dl) and was significantly higher (p<0.05) in 65:35 TMR (7.35 mM/dl). The percent acetate was significantly (p<0.05) lowest (62.74%) in70:30 TMR and highest in 75:25 (64.06%) followed by 80:20 TMR (63.19%) The propionate percent was statistically higher (p<0.05) in 65:35TMR and lowest in 80:20 TMR. The percent isobutyric was significantly higher (p<0.05) in 70:30 TMR (0.54%) whereas it was significantly lower in 75:25 TMR (0.36%). The acetate to propionate ratio was significantly lowest (p<0.05) in 65:35 TMR (2.25) and statistically comparable in 75:25 and 80:20 TMRs. (Table 4.4)

Table 4.3. Effect of essential oils containing herbs on *in-vitro* volatile fatty acids production (mM/dl) of different TMRs at 24h, *irrespective of herb and level of supplementation*

Parameters	Roughage :Concentrate ratio				SEM
	70:30	65:35	75:25	80:20	
Acetic acid	3.94^{c}	4.62^{d}	3.15^{a}	3.64^{b}	0.066
Propionic acid	1.72^{c}	2.12^{d}	1.29^{a}	1.49^{b}	0.036
Iso butyric acid	0.035^{d}	0.031^{c}	0.018^{a}	0.029^{b}	0.007
Butyric acid	0.531^{d}	0.465^{b}	0.382^{a}	0.485^{c}	0.007
Isovaleric acid	0.067^{d}	0.066^{c}	0.044^{a}	0.063^{b}	0.001
Valeric acid	0.051^{c}	0.051^{c}	0.035^{a}	0.045^{b}	0.0007
TVFA	6.36^{c}	7.354^{d}	4.928^{a}	5.753^{b}	0.109
A/P ratio	2.36^{ab}	2.25^{a}	2.51^{b}	2.49^{b}	0.037

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.4. Effect of essential oils containing herbs on *in-vitro* volatile fatty acids production (% relative proportion) of different TMRs at 24h, *irrespective of herb and level of supplementation*

Parameters	Roughage :Concentrate ratio				SEM
	70:30	65:35	75:25	80:20	
Acetate	62.746^{a}	62.85^{b}	64.066^{d}	63.198^{c}	0.12
Propionate	27.08^{c}	28.79^{d}	26.192^{b}	25.989^{a}	0.13
Iso butyrate	0.547^{d}	0.425^{b}	0.368^{a}	0.508^{c}	0.007
Butyrate	8.371^{c}	6.332^{a}	7.757^{b}	8.436^{d}	0.090
Isovalerate	1.057^{b}	0.901^{a}	0.899^{a}	1.089^{c}	0.010
Valerate	0.799^{d}	0.690^{a}	0.718^{b}	0.781^{c}	0.0007
A:P ratio`	2.366^{ab}	2.258^{a}	2.510^{b}	2.497^{b}	0.037

Means bearing different superscripts in a row differ significantly (P<0.05)

This increase in gas production with increased concentrate proportion in the inoculum, might be due to increase in the nutrient availability from concentrate or might be due to lowered cell wall and lignin content in the inoculum as reduction in roughage proportion in the inoculum, which negatively affect the microbial attachment to the feed particles. The increased values might be due to gradual reduction in hemicellulose, cellulose, and lignin content in inoculum from 80R:20C to 35R:65C which act as limiting factors to lower the digestibility at excess amount or increased nutrient availability to microorganisms from increased proportion of concentrate in the ration. Similarly, Polyorach *et al* (2014) observed an increase in IVOMD in complete rations by increasing the concentrate proportion from 20% to 80%. The results of the present study were also in agreement with Blümmel *et al* (1997) and Khanum *et al* (2007) who stated that CR digestibility can be improved when CR are supplemented with concentrate which provides better nutrients to microorganisms than roughage alone. Similarly, TVFA (mEq/L) production was also increased ($p<0.01$) as the level of concentrate increased in the complete diet. This result is inconsistent with the findings of Getachew *et al* (2004) who concluded that TVFA production was positively correlated ($p<0.01$) with *in vitro* gas production. The ammonia nitrogen increased in proportion to the concentrate in complete diet might be due to active degradation of protein and hydrolysis of non-protein nitrogen substances. Kumari *et al* (*2012)* and Reddy *et al* (2015) observed increase in ammonia nitrogen concentration in complete diets having more concentrate proportion. During incubation, the liberated ammonia will be incorporated into microbial protein synthesis, but this incorporation depends on synchronization between availability of nitrogen and energy.

Similar to our results, Kumari *et al* (2012)and Reddy *et al* (2015) observed higher ($p<0.01$) MBP synthesis with 60R:40C, 50R:50C in complete diets though they noticed the highest ammonia production at 30R:70C. PF is an index for distribution of substrate truly degraded between microbial mass and fermentation products. Therefore, PF provides meaningful information for prediction of MBP and also voluntary intake in ruminants. Similarly, Thirumalesh and Krishnamoorthy (2009) noticed a positive correlation between microbial biomass flow to duodenum and PF of the total mixed ration.

With increase in the level of concentrate the methane as percent of net gas production, irrespective of herb and level of supplementation used (Table 4.5) decrease linearly ($p<0.05$). Methane production ml/ 100mg DM D and organic matter basis was significantly ($p<0.05$) lower in high concentrate based TMR (65:35) and highest in high fibre based ration i.e TMR (80:20). The feeding of high concentrate diet can modulate the rumen fermentation and can decrease the methane production. Yan *et al* (2000) reported the negative correlation between proportion of concentrate in the diet and methane production. Benchaar *et al* (2001) reported that methane production was decreased with replacement of fibrous concentrate with starchy concentrate by 22 %.Compared with the forage based diets, feeding concentrate based diets lowers the enteric methane emission (Johnson and Johnson 1995), since starch fermentation promotes the propionate production and lowers the rumen pH and inhibits the protozoa and methanogens (Williams and Coleman 1988).

Aguerre *et al* (2011) reported that increasing the R:C ratio increased the ruminal pH and methane production. They reported the effects of the level of concentrate feeding on methane emissions are not consistent.In feedlot type diets (>90%) concentrate on dry matter basis, it is clear that increased addition of starch in the diet promotes the propionate production in the rumen (Johnson and Johnson 1995). Sauvant and Giger-reverdin (2009) reported a quadratic effects of the proportion of concentrate on methane emmisions, with maximum at 35 % concentrates on DM basis.

Hydrogen balance from fermentation of TMRs at 24 hr, irrespective of herb and level of supplementation significantly influenced H recovery and ratio of H consumed via methane to H via VFA ($P<0.05$) (Table 4.6) The percent H recovery was observed to be highest ($P<0.05$) in TMR (75:25) while it was lowest in TMR (65:35) at 24 h incubation. The ratio of H consumed via methane to H via VFA followed a reverse trend being lowest ($P<0.05$) in TMR (75:25) and highest ($P<0.05$) in TMR (65:35) at 24 h. The fermentation efficiencies calculated in this study varied from 76.15.per cent (TMR75:25) to 77.09% (TMR65:35) at 24 h. It is clear that increased fermentation efficiency achieved in TMR (65:35) is actually the end result of its decremented effect on methane production and its ability to increase propionate at the expense of acetate and butyrate. The lowest value of NGGR, which indicates the best utilization of VFA, was achieved in TMR (65:35) at 24 h, irrespective of the herb and level of supplementation.

Table 4.5. Methane production from fermentation of TMRs, at 24h, *irrespective of herb and level*

Parameters	Roughage: Concentrate				
	80:20	75:25	70:30	65:35	SEM
CH4%	22.59[c]	19.61[b]	19.35[b]	18.58[a]	0.37
CH4ml/200mg	35.61[a]	36.04[a]	37.95[b]	47.40[c]	0.12
CH4ml/gDM	40.61[c]	36.47[a]	37.73[b]	36.32[a]	0.58
CH4ml/100mgDMD	6.23[c]	5.36[b]	5.25[b]	4.899[a]	0.065
CH4ml/100mgOMD	7.10[c]	6.06[b]	5.96[ab]	5.49[a]	0.071

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.6. Hydrogen balance from fermentation of TMRs at 24 hr, *irrespective of herb and level of supplementation*

Parameters	Roughage :Concentrate ratio				SEM,
	70.30	65.35	75.25	80.20	
HR%	82.01[b]	76.31[a]	94.89[d]	86.54[c]	0.86
HC via CH4/VFA	5.43[c]	6.11[d]	4.14[a]	4.88[b]	0.090
FE%	76.70[c]	77.09[d]	76.15[a]	76.23[b]	0.052
VFA utilization	2.86[b]	2.58[a]	2.98[c]	3.02[d]	0.020

Means bearing different superscripts in a row differ significantly (P<0.05)

The non-glucogenic to glucogenic VFA ratio is associated with effects on methane production, milk composition, and energy balance (MORVAY *et al* 2011). Glucogenic propionate contributes to energy deposition in body tissues, whereas nonglucogenic acetate and butyrate are sources for long-chain fatty acid synthesis. Higher NGR was related to a higher milk fat content (ABRAHAMSE 2009). Too high NGR indicates a high loss of energy in the form of gases (ØRSKOV 1975). Fermentation efficiency based on VFA production is very useful for analyzing the effect of some feed additives on ruminal fluid fermentation via microbial metabolism modulation. Since the energy in acetate, propionate and butyrate is respectively 62, 109 and 78% of that in fermented hexose, metabolically useful energy recovered in

fermentation end-products can be increased by enhancing the production of propionate and to a lesser extent butyrate at the expense of acetate production (BARAN &.ITÒAN 2002). Ruminal production of methane causes a 3 to 12% loss of feed gross energy.

The effect of supplementing herbs on digestibility of nutrients revealed that NGP (ml/24h/g DM) varied (P<0.05) from 180.42 in control and highest in suva supplemented TMR to 188.74, irrespective of R:C and level of herb supplementation (Table 4.7).

Table 4.7. Effect of different herbs containing essential oils on *in-vitro* utilization of nutrients of different TMRs at 24h, *irrespective of R:C and level of herb*

Parameters	Herbs used				SEM
	Control	Jaiphal	Suva	Haldi	
NGP, ml/gDM	180.42^{a}	186.94^{b}	188.74^{d}	186.67^{b}	1.04
OMD, mg/gDM	612.26^{a}	625.61^{b}	619.25^{ab}	618.16^{ab}	3.80
PF	3.39^{b}	3.34^{b}	3.28^{a}	3.31^{a}	0.13
OMD, %	67.85^{a}	69.33^{b}	68.62^{ab}	68.503^{ab}	0.40
NDFD, %	57.92^{a}	59.85^{b}	58.94^{ab}	58.78^{ab}	0.47
MMP, mg	81.93^{b}	80.33^{ab}	77.06^{a}	77.99^{a}	1.44
EMMP, %	35.62^{b}	34.28^{a}	33.12^{a}	33.47^{a}	0.47
TD, %	68.800^{a}	70.595^{b}	69.906^{ab}	69.6^{b}	0.39
SCFA, m mole	0.869^{a}	0.895^{b}	0.905^{b}	0.900^{b}	0.005
ME, MJ/kg DM	8.255^{a}	8.408^{b}	8.477^{b}	8.446^{b}	0.043
NH_3-N mg/dl	21.09^{d}	20.68^{a}	20.91^{b}	20.96^{c}	0.11
Ferm. $CO_{2, mmol}$	2.72^{a}	3.07^{c}	2.96^{b}	3.14^{d}	0.050
Ferm. $CH_{4, mmol}$	1.57^{a}	1.75^{c}	1.71^{b}	1.80^{d}	0.027

Means bearing different superscripts in a row differ significantly (P<0.05)

The digestibility of nutrients (DM, OM, and NDF) of varied significantly (P<0.05) amongst the herbs supplemented. Supplementation of TMR with jaiphal showed higher (P<0.05) digestibility of dry matter organic matter and neutral

detergent fibre, followed by suva and haldi. The digestibility of nutrients for DM, OM and NDF was significantly lower in control TMR (no Herb supplementation). The digestibility data points towards the fact that supplementation of herbs increased the digestibility of all nutrients but did not affect the digestibility of nutrients.

The Partitioning Factor (PF), i.e. the ratio of substrate truly degraded to the volume of gas produced was comparable in control and jaiphal supplementation in TMR and varied significantly ($P<0.05$) from 3.39 (control) to 3.34 (jaiphal) indicating proportionally more of the degraded matter will be incorporated in microbial mass. However, the PF was significantly lower in suva nad haldi supplementation TMRs. Microbial mass production was statistically comparable in control and jaiphal supplemented TMRs but the efficiency of microbial mass production was significantly lower in herbs supplemented TMRs. Metabolizable energy (ME) MJ/kgDM was significantly lower (8.25) in control TMR, but cstatistically comparable in all herbs supplemented TMRs. The short chain fatty acids (SCFA) was lower ($p<0.05$) in control total mixed ration (0.86mmole) and was comparable ill herbs supplemented TMRs. It varied from 0.89 mmole to 0.905 mmmole in herbs supplemented TMRs. The concentration of ammonia was lower ($p<0.05$) in jaiphal supplemented total mixed ration (20.68mg/dl) and highest in haldi TMR (20..96 mg/dl).

The amount of fermentable methane (1.44 mmol) and fermentable carbon dioxide (2.47mmol) was lower ($p<0.05$) in control TMR where as both these were higher ($p<0.05$) in haldi based TMR. The tendency to lower NH3-N concentrations in the fermentation fluid could be attributed either to a greater ammonia utilization by rumen microbes (Saleh 1994). Furthermore, inhibition of protozoa is often associated with reduced ruminal NH3-N concentration (Williams and Withers 1993) presumably resulting from a reduction in protozoal proteolytic and deaminative activity.

The values for total volatile fatty acid concentration obtained in this study varied ($P<0.05$) from 5.45 mM/dl (control) to 6.31 mM/dl (haldi). The acetate, propionate, iso-butyrate and butryrate levels followed the same trend as that in TVFAs (Table4.8). The A:P ratio was observed to be low in TMR supplemented with jaiphal (2.30) and highest in haldi supplemented TMR (2.55) irrespective of R:C and levels of supplementation, indicating shift towards propionate. The relative

proportions of the three main volatile fatty acids (acetate, propionate and butyrate) varied (P<0.05) amongst the herbs evaluated (Table 4.9). Acetate was significantly (P<0.01) higher in control TMR (63.44%) in comparison to the TMR supplemented with jaiphal (62.66%), whereas propionate was higher (P<0.05) in TMR supplemented with jaiphal (27.27%) closely followed by TMR supplemented with suva (27.02%) and lowest in control TMR (26.52%). The molar proportion of butyrate was observed to vary (P<0.05) from 7.65% (suva supplemented TMR) to 7.83% in control TMR, irrespective of the level of supplementation. Various research workers (Kariuki *et al* 2001 and Widiawati and Thalib 2009) have reported that if the level of acetic acid resulting from the fermentation of carbohydrate in the rumen can be reduced while the level of propionic acid is increased, the energy of the ruminant ration will be more efficiently used. This shift of the acetic to propionic acid ratio, in favour of propionic acid, may reduce the energy losses that occur in metabolism at the cellular level. The proportion of branched chain fatty acids (IB) was observed to be lowest in control TMR and highest in jaiphal supplemented TMR. The high level of these BCFAs may act as precursors for synthesis of branched chain amino acids required for synthesis of microbial protein.

Table 4.8. Effect of herbs containing essential oils on *in-vitro* volatile fatty acids production (mM/dl) of different TMRs at 24h, *irrespective of R:C and level of supplementation*

Parameters	Herbs Used				SEM
	Control	Jaiphal	Suva	Haldi	
Acetic acid	3.453^{a}	3.872^{c}	3.771^{b}	3.979^{d}	0.066
Propionic acid	1.455^{a}	1.695^{c}	1.622^{b}	1.712^{d}	0.036
Iso butyric acid	0.024^{a}	0.029^{c}	0.028^{b}	0.030^{d}	0.0007
Butyric acid	0.423^{a}	0.478^{c}	0.452^{b}	0.481^{d}	0.007
Isovaleric acid	0.055^{a}	0.062^{d}	0.058^{b}	0.062^{c}	0.001
Valeric acid	0.042^{a}	0.047^{d}	0.044^{b}	0.046^{c}	0.0007
TVFA	5.452^{a}	6.184^{c}	5.975^{b}	6.309^{d}	0.11
A/P Ratio	2.396^{ab}	2.302^{a}	2.343^{ab}	2.555^{b}	0.037

Means bearing different superscripts in a row differ significantly (P<0.05)

The methane as percent of net gas production, irrespective of R:C and level of herb supplementation is shown in the (Table 4.10) It was significantly ($p<0.05$) lower in jaiphal supplemented TMR (20.42%) and statistically comparable in all other herbs supplemented TMRs. Methane production ml/ 100mg DMD and organic matter basis was significantly ($p<0.05$) lower in jaiphal supplemented TMR (5.38 and 6.07ml) However, it was comparable in control and all other herbs supplemented TMR.

Hydrogen balance from fermentation of herbs at 24 hr, irrespective of R:C and level of supplementation significantly influenced H recovery and ratio of H consumed via methane to H via VFA ($P<0.05$) (Table 4.11). The percent H recovery was observed to be highest ($P<0.05$) in control TMR (90.50%) while it was lowest in haldi supplemented TMR (83.21%) at 24 h incubation. The ratio of H consumed via methane to H via VFA followed a reverse trend being lowest ($P<0.05$) in control TMR (4.59) and highest ($P<0.05$) in haldi supplemented TMR (5.32) at 24 h. The fermentation efficiencies calculated in this study varied from 76.34.per cent (control TMR) to 76.68% (jaiphal TMR) at 24 h. The lowest value of NGGR, which indicates the best utilization of VFA, was achieved in jaiphal supplemented TMR (2.82) at 24 h, irrespective of the herb and level of supplementation and highest in control TMR (2.93)

Table 4.9. Effect of herbs containing essential oils on *in-vitro* volatile fatty acids production (% Relative proportion) of different TMRs at 24h, *irrespective of R:C and level of supplementation*

Parameters	Herbs supplementation				SEM
	Control	Jaiphal	Suva	Haldi	
Acetate	63.445^{d}	62.663^{a}	63.153^{b}	63.188^{c}	0.12
Propionate	26.522^{a}	27.274^{d}	27.020^{c}	26.938^{b}	0.13
Iso butyrate	0.437^{a}	0.469^{c}	0.460^{b}	0.464^{b}	0.007
Butyrate	7.830^{d}	7.819^{c}	7.654^{a}	7.696^{b}	0.090
Isovalerate	1.005^{b}	1.007^{b}	0.976^{a}	0.977^{a}	0.010
Valerate	0.761^{b}	0.767^{c}	0.737^{a}	0.738^{c}	0.0007

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.10. Methane production from fermentation of TMRs, at 24h, *irrespective of R:C and level of herb supplementation*

Parameters	Herbs Used				SEM
	Control	Jaiphal	Suva	Haldi	
CH4%	21.55[b]	20.42[a]	21.14[b]	21.25[b]	0.37
CH4ml/200	7.73[c]	7.62[a]	7.96[b]	7.94[b]	0.12
CH4ml/gDM	38.68[a]	38.10[a]	39.80[b]	39.71[b]	0.58
CH4ml/100mgDMD	5.60[b]	5.38[a]	5.68[b]	5.68[b]	0.065
CH4ml/100mgOMD	6.29[b]	6.07[a]	6.42[b]	6.40[b]	0.071

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.11. Effect of different Herbs on Hydrogen balance from fermentation of TMRs at 24 hr, *irrespective of R:C and level of supplementation of herb*

Parameter	Herbs used				SEM
	Control	Jaiphal	Suva	Haldi	
HR%	90.50[d]	84.26[b]	85.78[c]	83.21[a]	0.86
HC via CH4/VFA	4.59[a]	5.24[c]	5.03[b]	5.32[d]	0.090
FE%	76.34[a]	76.68[d]	76.53[c]	76.50[b]	0.052
VFA utilization	2.93[d]	2.82[a]	2.86[b]	2.87[c]	0.020

Means bearing different superscripts in a row differ significantly (P<0.05)

Effect of different levels of herbs supplementation on *in vitro* utilization of nutrients at 24 hr, irrespective of R:C and herb is presented in the table (4.12)

The effect of different levels of supplementing herbs on digestibility of nutrients revealed that NGP (ml/24h/g DM) varied (P<0.05) from 180.42 at 0% and highest at 2 and 3% of herbs supplemented TMR (190.93), irrespective of R:C and herb supplementation (Table 4.12). The digestibility of nutrients (DM, OM, and NDF) at 1 % level of supplementation was significantly (P<0.05) higher. The digestibility data points towards the fact that supplementation of herbs at 1% level increased the digestibility of all nutrients.

The Partitioning Factor (PF), i.e. the ratio of substrate truly degraded to the volume of gas produced was statistically comparable at 0% and 1% level of supplementation of herb and significantly (P<0.05) lower at 2% level of supplementation (3.24) indicating proportionally more of the degraded matter will be incorporated in microbial mass. Microbial mass production was statistically comparable at 0% and 1 % of supplementation but the efficiency of microbial mass production was significantly lower at 2and 3% level of supplementation. Metabolizable energy (ME) MJ/kgDM was significantly lower (8.25) at 0 %and highest at 2 and 3 % level of supplementation respectively. The short chain fatty acids (SCFA) was lower (p<0.05) at 0% and highest at 2 and 3 % level of supplementation. It varied from 0.86 mmole to 0.916 mmmole in all levels of herbs supplemented TMRs. The concentration of ammonia was lower (p<0.05) at 3 % level of herb supplementation (20.38mg/dl) and highest at 1 % of level (21.23 mg/dl). The amount of fermentable methane (1.57 mmol) and fermentable carbon dioxide (2.72mmol) was lower (p<0.05) at 0 % level of supplementation in control TMR where as both these were higher (p<0.05) at 2 % level of herb supplementation.

The values for total volatile fatty acid concentration obtained in this study varied (P<0.05) from 5.45 mM/dl (0 %) to 6.31 mM/dl (2%). The acetate, propionate, iso-butyrate and butryrate levels followed the same trend as that in TVFAs (Table 4.13). The A:P ratio was observed to be low at 1 % level of supplementation (2.32) and highest at 2 % level (2.61) irrespective of R:C and herb supplementation. The Acetate was significantly (P<0.01) higher at 0% (63.44%) in comparison to other levels of supplementation, whereas propionate was higher (P<0.05) at 3% level and closely followed by at 1% level of supplementation (27.16%). (Table4.14)

The methane as percent of net gas production, irrespective of R:C and level of herb supplementation is shown in the (Table 4.15) It was significantly (p<0.05) lower as the level of supplementation increased from 1 to 3 %. At 0% level it was (21.55%) whereas it was statistically comparable at all levels of herb supplementation in the TMRs. Methane production ml/ 100mg DMD and organic matter basis was significantly (p<0.05) lower at 1 % level of herb supplementation in TMR (5.35 and

6.31 ml) However, it was comparable at 0 and 2 and 3 % level of herb supplementation.

Hydrogen balance from fermentation of herbs at 24 hr, irrespective of R:C and herb supplementation significantly influenced H recovery and ratio of H consumed via methane to H via VFA ($P<0.05$). Table (4.16) The percent H recovery was observed to be highest ($P<0.05$) at 0% level of supplementation in TMR (90.50%) while it was lowest at 2 % in herb supplemented TMR (82.57%) at 24 h incubation. The ratio of H consumed via methane to H via VFA followed a reverse trend being lowest ($P<0.05$) at 0% (4.59) and highest ($P<0.05$) at 2 % level supplemented TMR (5.36) at 24 h. The fermentation efficiencies calculated in this study varied from 76.34.per cent (0%TMR) to 76.68% (3% TMR) at 24 h. The lowest value of NGGR, which indicates the best utilization of VFA, was achieved at 3% supplemented TMR (2.81) at 24 h, irrespective of the herb and level of supplementation and highest at 0% level in TMR (2.93)

Table 4.12. Effect of different levels of herbs supplementation on *in vitro* utilization of nutrients at 24 hr, *irrespective of R:C and herb*

Parameters	Levels of herbs supplementation				SEM
	Control	1%	2%	3%	
NGP/gDM	180.42^{a}	186.46^{b}	189.82^{c}	190.93^{c}	1.04
OMD, mg/gDM	612.26^{a}	626.26^{b}	616.45^{a}	624.61^{b}	3.80
PF	3.39^{c}	3.35^{c}	3.24^{a}	3.27^{b}	0.126
OMD, %	67.851^{a}	69.402^{b}	68.313^{a}	69.219^{b}	0.40
NDFD, %	57.925^{a}	59.955^{b}	58.539^{a}	59.715^{b}	0.47
MMP, mg	81.93^{c}	80.54^{c}	74.32^{a}	77.61^{b}	1.43
EMMP, %	35.62^{c}	34.24^{b}	32.00^{a}	33.07^{a}	0.47
TD, %	68.800^{a}	70.801^{b}	69.436^{a}	70.607^{b}	0.39
SCFA, mmole	0.869^{a}	0.897^{b}	0.916^{c}	0.910^{c}	0.005
ME, MJ/kg DM	8.255^{a}	8.422^{b}	8.541^{c}	8.506^{c}	0.043
NH3-N, mg/dl	21.09^{c}	21.23^{d}	20.84^{b}	20.38^{a}	0.11
Ferm.CO_{2}, mmol	2.72^{a}	3.13^{c}	3.16^{d}	3.11^{b}	0.050
Ferm.CH_{4}, mmol	1.57^{a}	1.78^{c}	1.82^{d}	1.77^{b}	0.027

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.13. Effect of different levels of herbs supplementation on *in vitro* volatile production (mM/dl) at 24 hr, *irrespective of R:C TMRs and herb*

Parameters	Levels of herbs supplementation				SEM
	Control	1%	2%	3%	
Acetic acid	3.453[a]	3.95[c]	4.026[d]	3.928[b]	0.066
Propionic acid	1.455[a]	1.718[b]	1.731[d]	1.721[c]	0.036
Iso butyric acid	0.024[a]	0.029[b]	0.030[c]	0.030[c]	0.0007
Butyric acid	0.423[a]	0.482[d]	0.481[c]	0.478[b]	0.007
Isovaleric acid	0.055[a]	0.063[d]	0.061[b]	0.061[c]	0.001
Valeric acid	0.042[a]	0.047[d]	0.046[b]	0.047[c]	0.0007
TVFA	5.452[a]	6.29[c]	6.375[d]	6.265[b]	0.11
A:P ratio	2.396[a]	2.321[a]	2.610[b]	2.304[a]	0.037

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.14. Effect of different levels of herbs supplementation on *in vitro* volatile production (% relative proportion) at 24 hr, *irrespective of R:C TMRs and herb supplementation*

Parameters	Levels of herb supplementation				SEM
	0%	1%	2%	3%	
Acetate	63.445[d]	62.874[b]	63.166[c]	62.780[a]	0.12
Propionate	26.552[a]	27.164[c]	27.015[b]	27.358[d]	0.13
Iso butyrate	0.437[a]	0.463[b]	0.472[c]	0.476[d]	0.007
Butyrate	7.830[c]	7.742[b]	7.659[a]	7.664[a]	0.090
Isovalerate	1.005[c]	1.004[c]	0.962[a]	0.975[b]	0.010
Valerate	0.761[d]	0.754[c]	0.726[a]	0.747[b]	0.0007

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.15. Methane production from fermentation of TMRs at different levels of herbs, *irrespective of R:C and Herb at 24 hr*

Parameters	Levels of herbs supplementation				SEM
	0%	1%	2%	3%	
CH4%	21.55[b]	21.10[a]	20.75[a]	20.73[a]	0.37
CH4ml/200mg	7.73[a]	7.86[b]	7.87[b]	7.91[b]	0.12
CH4ml/gDM	38.87[a]	39.34[b]	39.38[a]	39.57[b]	0.58
CH4ml/100mgDMD	5.65[b]	5.35[a]	5.67[b]	5.60[b]	0.065
CH4ml/100mgOMD	6.38[b]	6.31[a]	6.42[b]	6.37[b]	0.071

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.16. Hydrogen balance from fermentation of TMRs, *irrespective of different R:C TMRs and herb supplementation*

Parameter	Levels of herbs supplementation				SEM
	0%	1%	2%	3%	
HR%	90.50[d]	83.36[b]	82.57[a]	83.36[b]	0.86
HC via CH4/VFA	4.59[a]	5.31[c]	5.36[d]	5.29[b]	0.090
FE%	76.34[a]	76.61[c]	76.53[b]	76.68[d]	0.052
VFA utilization	2.93[d]	2.84[b]	2.86[c]	2.81[a]	0.020

Means bearing different superscripts in a row differ significantly (P<0.05)

4.1.3 Digestion kinetics parameters *in vitro* gas production

The gas production profiles presented in table 4.17 indicated that Y max (maximum potential of gas production) varied (p<0.05) amongst the herbs evaluated, irrespective of R:C and level of supplementation. The Y max (ml) was significantly lower in control TMR (45.67) and statistically comparable in jaiphal and suva supplemented herb TMR. Followed by haldi supplemented herb TMR (46.60). However, the V min (ml) followed the reversed trend. It was statistically lower in haldi supplemented TMR (15.65) and highest in control TMR (16.69). The maximum rate of degration (k) was observed in haldi supplemented TMR (6.47%, hr) and lowest in control TMR (6.07%/hr). The t ½ (time taken to reach half of asymptote) was lowest (p<0.05) for TMR supplemented with haldi (10.85h) and highest for TMR supplemented with jaiphal (11.21 h).

Table 4.17. Effect of different herbs containing essential oils on the fermentation kinetics of TMRs, ***irrespective of type of R:C and level of supplementation***

Parameters	Herbal feed additives				SEM
	Control	Jaiphal	Suva	Haldi	
2h	2.25[a]	2.81[b]	2.85[b]	2.48[a]	0.077
4h	5.58[a]	6.44[b]	6.58[b]	6.27[b]	0.12
6h	9.25[a]	10.47[b]	10.77[b]	10.52[b]	0.16
8h	13.58[a]	15.11[b]	15.40[b]	15.35[b]	0.19
10h	18.17[a]	19.50[b]	19.92[b]	19.77[b]	0.21
12h	21.92[a]	23.14[b]	23.67[b]	23.67[b]	0.22
24h	34.17[a]	35.81[b]	35.96[b]	35.81[b]	0.21
36h	39.92[a]	41.36[b]	41.54[b]	41.40[b]	0.20
48h	42.75[a]	43.92[b]	44.21[b]	43.98[b]	0.19
60h	43.83[a]	45.22[b]	45.44[b]	45.25[b]	0.21
72h	44.76[a]	46.17[b]	46.33[b]	46.13[b]	0.21
Plateu (Vmax)	45.67[a]	46.78[c]	46.88[c]	46.60[b]	0.13
Yo (lag time)	-4.87[b]	-4.42[c]	-4.41[d]	-4.88[a]	0.062
K (Degradation rate)	0.0607[a]	0.0627[b]	0.0639[c]	0.0647[d]	0.006
Tau (Vmin)	16.69[d]	16.17[c]	15.90[b]	15.65[a]	0.16
Half time	11.57[a]	11.21[c]	11.02[b]	10.85[a]	0.11
Span	50.54[a]	51.45[b]	51.30[b]	51.53[b]	0.13

Means bearing different superscripts in a row differ significantly (P<0.05)

The gas production profiles of roughage to concentrate ratio, irrespective of herb and level of supplementation are presented in the table 4.18. The Y max (ml) was significantly lower in 70:30 TMR (44.91) anantly higher in 75:25 TMR (47.17) followed by 65:35 TMR (47.29). The V min (ml) was statistically lower in 70:30 TMR (13.91) and highest in 80:20 TMR (18.28). The maximum rate of degradation (k) was observed in 70:30 TMR (7.23%, hr) and lowest in 80:20 TMR (5.48%/hr). The t½ (time taken to reach half of asymptote) was lowest (p<0.05) for 70:30TMR (9.64h) and highest for 80:20 TMR (12.67 h).

The gas production profiles of levels of herbs supplementation, irrespective of herb and R:C are presented in the table 4.19. The Y max (ml) was significantly lowerat 0 % TMR (45.67) and significantly higher at 3% level of herb supplementation (47.58) followed by 2% (47.11). The V min (ml) was statistically lower at 3 % (15.33) and highestat 0% (16.69) level of supplementation. The rate of degration (k) was observed to increased with the level of herb supplementation. The maximum rate of degradation was observed at 3% (6.63%, hr) and was lowest at)% (6.07%/hr). The t ½ (time taken to reach half of asymptote) was lowest ($p<0.05$) at 3% level of herb supplementation (10.62h) and highest at 0 % (11.57 h).

Table 4.18. Effect of roughage to concentrate ratios in TMRs on the fermentation kinetics, ***irrespective of herb and level of supplementation***

Parameter	Roughage : Concentrate ratio				SEM
	80:20	75:25	70:30	65:35	
2h	1.81[a]	3.53[d]	2.28[b]	3.06[c]	0.077
4h	4.75[a]	7.81[c]	6.50[b]	6.39[b]	0.12
6h	8.56[a]	12.58[d]	11.39[c]	9.42[b]	0.16
8h	12.81[a]	17.81[d]	15.97[c]	14.06[b]	0.19
10h	16.92[a]	22.58[d]	20.39[c]	18.58[b]	0.21
12h	20.36[a]	26.06[d]	24.53[c]	22.61[b]	0.22
24h	33.42[a]	37.78[c]	36.00[b]	35.69[b]	0.21
36h	39.61[a]	43.06[c]	41.47[b]	41.11[b]	0.20
48h	42.67[a]	45.36[c]	43.56[ab]	44.17[b]	0.19
60h	44.39[a]	47.17[c]	43.69[a]	45.50[b]	0.21
72h	45.53[b]	48.00[c]	44.53[a]	46.31[b]	0.21
Plateu (Vmax)	46.56[b]	47.90[d]	44.91[a]	47.29[c]	0.13
Yo (lag time)	-4.81[b]	-3.96[d]	-5.50[a]	-4.16[c]	0.062
K (deg rate)	0.0548[a]	0.0689[c]	0.0723[d]	0.0585[b]	0.006
Tau (Vmin)	18.28[d]	14.51[b]	13.91[a]	17.11[c]	0.16
Half time	12.67[d]	10.05[b]	9.64[a]	11.86[c]	0.11
Span	51.43[b]	51.86[c]	50.40[a]	51.71[bc]	0.13

Means bearing different superscripts in a row differ significantly ($P<0.05$)

Table 4.19. Effect of different Levels of essential oils containing herbs on fermentation kinetics of TMRs, *irrespective of R:C TMRs and herbs of herbs*

Parameter	Levels of herbs supplementation				SEM
	0%	1%	2%	3%	
2hr	2.25[a]	2.75[b]	2.67[b]	3.00[c]	0.077
4h	5.58[a]	6.47[b]	6.42[b]	6.97[c]	0.12
6h	9.25[a]	10.53[b]	10.81[b]	11.36[c]	0.16
8h	13.58[a]	15.25[b]	15.50[b]	16.31[c]	0.19
10h	18.17[a]	19.61[b]	19.89[b]	20.81[c]	0.21
12h	21.92[a]	23.36[b]	23.64[b]	24.64[c]	0.22
24h	34.17[a]	35.53[b]	36.06[b]	37.14[c]	0.21
36h	39.92[a]	41.03[b]	41.81[bc]	42.50[c]	0.20
48h	42.75[a]	43.61[ab]	44.39[bc]	45.00[c]	0.19
60h	43.83[a]	44.89[b]	45.67[bc]	46.36[c]	0.21
72h	44.75[a]	45.75[b]	46.64[bc]	47.22[c]	0.21
Plateau (Vmax)	45.67[a]	46.30[b]	47.11[c]	47.58[d]	0.13
Yo (lag time)	-4.87[a]	-4.44[d]	-4.58[b]	-4.54[c]	0.062
K (degradation rate)	0.0607[a]	0.0638[b]	0.0637[b]	0.0663[c]	0.006
Tau (Vmin)	16.69[d]	15.92[c]	15.86[b]	15.33[a]	0.16
Half time	11.57[d]	11.03[c]	11.00[b]	10.62[a]	0.11
span	50.54[a]	50.70[a]	51.69[b]	52.43[c]	0.13

Means bearing different superscripts in a row differ significantly (P<0.05)

***In vitro* utilization of nutrients at t ½ :**

Fermentation pattern at t½ revealed that supplementation of herbs on invitro utilization of nutrients of different TMRs, Irrespective of herb and level of supplementation is presented in the table 4.20. The NGP mi/g DM statistically increased with increase in the level of concentrates in the TMRs. The maximum gas production was observed in 65:35TMR (101.46) and lowest in 80:20 TMR (80:25). DM digestibility was observed to be highest (P<0.05) in 65:35 TMR and lowest in 80:20 and 75:25 TMRs. (Table 4.20). The OMD% and NDFD% was statistically

increased as the proportion of concentrates increased in the different TMRs. The microbial mass production (mg) and efficiency of microbial mass production was statistically higher in 65:35 TMR and lower in 80:20 TMR.

CH_4production per 100mg DMD and OMD was higher (P<0.05) in 80:20 TMR and and lowest in 65:35 TMR. The percent methane increased as the proportion of fibre in the TMR increased being higher in 80:20 TMR (22.64) and lowest in 65:35 TMR (17.01). (Table 4.23)

The fermentation pattern revealed that volatile fatty acid concentration at t1/2 varied (P<0.05) from 3.54 mM/dl (75:25TMR) to 5.28 mM/dl in 65:35TMR (Table 4.21). The A/P ratio was statistically higher in 65:35TMR (3.05) and lowest in 80:20 TMR (2.65). The relative proportions of acetate was significantly higher in 65:35TMR (67.20)and lowest in 80:20 TMR (65.28) The propionate percent was statistically higher in 80:20TMR and lowest in 70;30TMR (22.29) respectively at t-half.

Table 4.20. Effect of herbs containing essential oils on *in-vitro* utilization of nutrients of different TMRs at t half, *irrespective of herb and level of supplementation*

Parameters	Roughage : Concentrate ratio				SEM
	80:20	75:25	70:30	65:35)	
NGP/gDM	80.85^a	84.38^b	92.50^c	101.46^d	1.36
OMD.g/DM	415.52^a	445.16^b	477.54^c	507.70^d	3.16
PF	5.13^a	5.31^c	5.19^c	5.02^b	0.059
OMD, %	55.54^c	49.27^a	52.85^b	56.13^c	0.35
NDFD, %	43.00^d	33.57^a	37.54^b	41.47^c	0.47
MMP, mg	48.73^a	51.90^b	54.80^c	56.90^d	0.63
EMMP, %	48.77^a	58.24^c	57.27^c	55.91^b	0.54
TD, %	50.52^a	51.09^a	54.50^b	57.62^c	0.32
SCFA, mmole	0.510^d	0.370^a	0.406^b	0.446^c	0.006
ME, MJ/kg DM	5.75^d	3.85^a	5.23^b	5.56^c	0.079
NH3-N, mg/dl	22.23^c	22.07^b	22.78^d	20.39^a	0.13
Ferm.CO_2, mmol	2.15^c	1.80^a	1.87^b	2.71^d	0.040
Ferm.CH_4, mmol	1.29^c	1.12^a	1.17^b	1.69^d	0.025

Means bearing different superscripts in a row differ significantly (*P<0.05)*

Table 4.21. Effect of essential oils containing herbs on *in-vitro* volatile fatty acids production (mM/dl) of different TMRs at t half, *irrespective of herb and level of supplementation*

Parameters	Roughage :Concentrate ratio				SEM
	80.20	**75.25**	**70.30**	**65.35**	
Acetic acid	2.76^{c}	2.38^{a}	2.47^{b}	3.54^{d}	0.052
Propionic acid	1.04^{c}	0.808^{a}	0.822^{b}	1.17^{d}	0.018
Iso butyric acid	0.0198^{a}	0.0214^{b}	0.0233^{c}	0.0311^{d}	0.0009
Butyric acid	0.314^{c}	0.272^{a}	0.283^{b}	0.427^{d}	0.006
Isovaleric acid	0.0337^{a}	0.0374^{b}	0.0424^{c}	0.0498^{d}	0.001
Valeric acid	0.0265^{c}	0.0234^{a}	0.0263^{b}	0.0441^{d}	0.001
TVFA	4.22^{c}	3.54^{a}	3.67^{b}	5.28^{d}	0.078
A/P ratio	2.65^{b}	2.94^{c}	3.03^{d}	3.05^{c}	0.024

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.22. Effect of essential oils containing herbs on *in-vitro* volatile fatty acids production (%relative proportion) of different TMRs at t half, *irrespective of herb and level of supplementation*

Parameters	Roughage : Concentrate				SEM
	80.20	**75.25**	**70.30**	**65.35**	
Acetate	65.28^{a}	67.14^{b}	67.48^{d}	67.20^{c}	0.133
Propionate	24.70^{d}	22.83^{c}	22.29^{a}	22.34^{b}	0.134
Iso butyrate	0.469^{a}	0.605^{c}	0.635^{d}	0.583^{b}	0.015
Butyrate	8.10^{b}	7.69^{a}	7.72^{a}	8.09^{b}	0.035
Isovalerate	0.799^{a}	1.06^{c}	1.15^{d}	0.939^{b}	0.019
Valerate	0.629^{a}	0.663^{b}	0.713^{c}	0.831^{d}	0.016
A:P ratio	2.65^{a}	2.94^{b}	3.03^{c}	3.05^{c}	0.024

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.23. Methane production from fermentation of TMRs, at t half, *irrespective of herb and level of supplementation*

Parameters	Roughage: Concentrate				SEM
	80:20	75:25	70:30	65:35	
CH4%	22.64^{d}	19.82^{c}	18.98^{b}	17.01^{a}	0.27
CH4ml/200mg	3.66^{c}	3.34^{a}	3.51^{b}	3.45^{b}	0.034
CH4ml/gDM	18.30^{c}	16.72^{a}	17.55^{b}	17.25^{b}	0.17
CH4ml/100mgDMD	3.62^{c}	3.27^{b}	3.22^{b}	2.99^{a}	0.033
CH4ml/100mgOMD	4.20^{c}	3.78^{b}	3.70^{b}	3.42^{a}	0.038

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.24. Hydrogen balance from fermentation of TMRs, *irrespective of herb and level of supplementation* at t half

Parameter	Roughage : Concentrate ratio				SEM
	80:20	75:25	70:30	65:35	
HR%	104.08^{b}	117.99^{d}	115.54^{c}	89.75^{a}	1.32
HC via CH4/VFA	3.52^{c}	2.83^{a}	2.91^{b}	4.22^{d}	0.063
FE%	75.54^{d}	74.69^{c}	74.47^{a}	74.54^{b}	0.060
VFA utilization	3.25^{a}	3.54^{b}	3.64^{c}	3.63^{c}	0.021

Means bearing different superscripts in a row differ significantly (P<0.05)

Fermentation pattern at t½ revealed that supplementation of herbs on *invitro* utilization of nutrients of different TMRs, Irrespective of level of herb and R:C ratio is presented in Table 4.25. The NGP mi/g DM statistically increased in haldi supplementation TMR (101.25) and lowest in control TMR (93.13). The DM digestibility (%) was observed to be lowest (P<0.05) in haldi supplemented TMR (54.21) and it was statistically comparable in control, jaiphal and suva supplemented TMRs. The OMD% and NDFD% was statistically lowest in haldi supplemented TMR and comparable in jaiphal, suva and control TMRs. The microbial mass production (mg) and efficiency of microbial mass production was statistically higher in control TMR and lower in haldi supplementation TMR.

Table 4.25. Effect of different herbs containing essential oils on *in-vitro* utilization of nutrients of different TMRs at t half, *irrespective of R:C and level of herb supplementation*

Parameters	Herbs supplementation				SEM
	Control	Jaiphal	Suva	Haldi	
NGP, ml/gDM	93.13^{a}	96.25^{b}	98.91^{c}	101.25^{d}	1.36
OMD, mg/DM	493.50^{b}	487.60^{b}	484.07^{b}	473.54^{a}	3.16
PF	5.29^{d}	5.06^{c}	4.89^{b}	4.67^{a}	0.059
OMD, %	54.70^{b}	54.04^{b}	53.65^{b}	52.487^{a}	0.35
NDFD, %	40.53^{b}	39.67^{b}	39.17^{b}	37.63^{a}	0.47
MMP, mg	57.72^{c}	55.17^{b}	53.29^{b}	50.15^{a}	0.63
EMMP, %	58.65^{d}	56.62^{c}	55.10^{b}	52.92^{a}	0.54
TD, %	55.56^{b}	55.40^{b}	55.15^{ab}	54.21^{a}	0.32
SCFA, m mole	0.409^{a}	0.423^{b}	0.434^{c}	0.445^{d}	0.006
ME, MJ/kg DM	5.05^{ab}	4.99^{a}	5.11^{ab}	5.18^{b}	0.079
NH_3-N mg/dl	22.01^{c}	21.23^{a}	21.75^{b}	22.43^{d}	0.13
Ferm. CO_{2}, mmol	2.18^{c}	2.14^{b}	2.14^{b}	2.09^{a}	0.040
Ferm. CH_{4}, mmol	1.35^{d}	1.31^{b}	1.33^{c}	1.30^{a}	0.025

Means bearing different superscripts in a row differ significantly (P<0.05)

The fermentation pattern revealed that volatile fatty acid concentration at t1/2 varied (P<0.05) from 4.10 mM/dl (haldi TMR) to 4.32 mM/dl in control TMR (Table 4.26). The A/P ratio was statistically comparable lowest in control and jaiphal supplemented TMR and comparable higher in Suva and haldi TMRs. The relative proportions of acetate was significantly higher in suva and haldi supplemented TMRs and lowest in jaiphal supplemented TMR (2.84). The propionate percent was statistically higher in jaiphal supplemented TMR (23.46) and lowest in haldi TMR (22.77) respectively at t-half (Table 4.27).

CH_4production per 100mg DMD and OMD was comparable in (P<0.05) in control, suva and haldi TMRs and lowest in jaiphal supplemented TMR. The percent methane was higher in non herb supplemented TMR as compared to herbs supplemented TMRs (Table 4.28).

Table 4.26. Effect of herbs containing essential oils on *in-vitro* volatile fatty acids production (mM/dl) of different TMRs at t half, *irrespective of R:C and level of supplementation*

Parameters	Herbs supplementation				SEM
	Control	Jaiphal	Suva	Haldi	
Acetic acid	2.86^{d}	2.77^{b}	2.82^{c}	2.75^{a}	0.052
Propionic acid	1.00^{d}	0.983^{c}	0.965^{b}	0.935^{a}	0.018
Iso butyric acid	0.0309^{d}	0.0217^{a}	0.0240^{c}	0.0237^{b}	0.0009
Butyric acid	0.335^{c}	0.340^{d}	0.329^{b}	0.325^{a}	0.006
Isovaleric acid	0.0496^{d}	0.0392^{a}	0.0409^{c}	0.039^{b}	0.001
Valeric acid	0.0383^{d}	0.0286^{a}	0.0300^{c}	0.0292^{b}	0.001
TVFA	4.32^{d}	4.19^{b}	4.21^{c}	4.10^{a}	0.078
A/P Ratio	2.85^{a}	2.84^{a}	2.92^{b}	2.98^{b}	0.024

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.27. Effect of herbs containing essential oils on *in-vitro* volatile fatty acids production (% relative proportion) of different TMRs at t half, *irrespective of R:C and level of supplementation*

Parameters	Herbs supplementation				SEM
	Control	Jaiphal	Suva	Haldi	
Acetate	66.21^{a}	66.27^{a}	67.01^{b}	67.07^{b}	0.133
Propionate	23.33^{c}	23.46^{d}	22.93^{b}	22.77^{a}	0.134
Iso butyrate	0.688^{c}	0.532^{a}	0.572^{b}	0.576^{b}	0.015
Butyrate	7.77^{a}	8.09^{c}	7.79^{a}	7.90^{b}	0.035
Isovalerate	1.13^{d}	0.955^{a}	0.983^{c}	0.978^{b}	0.019
Valerate	0.849^{c}	0.683^{a}	0.703^{b}	0.699^{b}	0.016

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.28. Methane production from fermentation of TMRs, at t half, *irrespective of R:C and level of herb supplementation*

Parameters	Control	Jaiphal	Suva	Haldi	SEM
CH4%	21.327[b]	19.08[a]	19.53[a]	18.93[a]	0.27
CH4ml/200	3.91[b]	3.62[a]	3.88[b]	3.79[b]	0.034
CH4ml/gDM	19.58[b]	18.12[a]	19.00[b]	18.95[b]	0.17
CH4ml/100mg DMD	3.53[b]	3.27[a]	3.45[b]	3.49[b]	0.033
CH4ml/100mg OMD	3.97[b]	3.72[a]	3.94[b]	4.00[b]	0.038

Means bearing different superscripts in a row differ significantly (*P<0.05*)

Table 4.29: Effect of different Herbs on Hydrogen balance from fermentation of TMRs at t half, *irrespective of R:C and level of supplementation of herb*

Parameter	Herbs supplementation				SEM
	Control	Jaiphal	Suva	Haldi	
HR%	104.59[a]	106.70[c]	106.24[b]	108.12[d]	1.32
HC via CH4/VFA	3.46[d]	3.42[c]	3.38[b]	3.30[a]	0.063
FE%	74.96[c]	75.02[d]	74.74[b]	74.69[a]	0.060
VFA utilization	3.42[a]	3.46[b]	3.53[c]	3.56[d]	0.022

Means bearing different superscripts in a row differ significantly (*P<0.05*)

Hydrogen balance from fermentation of herbs at t1/2, irrespective of R:C and level of herb supplementation significantly influenced H recovery and ratio of H consumed via methane to H via VFA (P<0.05) (Table 4.29). The percent H recovery was observed to be highest (P<0.05) in haldi supplemented TMR (108.12%) while it was lowest in control TMR (104.59%). The ratio of H consumed via methane to H via VFA followed a reverse trend being lowest (P<0.05) in haldi TMR (3.30) and highest (P<0.05) in control TMR (3.46). The fermentation efficiencies calculated in this study varied from 74.69.per cent (haldi TMR) to 75.02% (jaiphal TMR) at t 1/2 The lowest value of NGGR, which indicates the best utilization of VFA, was achieved in control

TMR (3.42) at t ½, irrespective of the R:C and level of supplementation of herb and highest in haldi based TMR (3.56).

Fermentation pattern at t½ revealed that level of herbs supplementation on *in vitro* utilization of nutrients of TMRs, Irrespective of herb and R:C ratio is presented in the table 4.30. The NGP mi/g DM was statistically higher at 1 to 3 % level of herb supplementation as compared to 0 % (93.13). The DM digestibility (%) was observed to be lowest (P<0.05) at 2 and 3% level of herb supplementation but statistically higher that 0 and 1% of herb supplementation. The OMD% and NDFD% was statistically lowest at 2 and 3% level of supplementation and significantly higher at 0 and 1% level of supplementation. The microbial mass production (mg) and efficiency of microbial mass production was statistically higher at 0 % followed by 1% level of herb supplementation and significantly lower at 2 and 3% level of supplementation.

The fermentation pattern revealed that volatile fatty acid concentration at t1/2 varied (P<0.05) from 4.03 mM/dl (3%) to 4.32 mM/dl at 0 % level of supplementation. (Table 4.32). The A/P ratio was statistically comparable lowest at 0% and statistically higer at 2 % level of supplementation. The relative proportions of acetate was significantly higher at 2% (67.08%) and lowest at 0% (66.21%). The propionate percent was statistically higher at 0% (23.34) and lowest at 2 % (22.82) respectively at t-half. (Table4.33)

CH_4 production per 100mg DMD and OMD was comparable in (P<0.05) at 0, 2 and 3% level of supplementation and lowest at 1 % level of supplementation of herb. The percent methane was higher in non herb supplemented TMR (21.32) and lowest at 1 % level (18.25). (Table4.34)

The percent H recovery (%)was observed to be highest (P<0.05) at 3 % (108.52) and lowest at 0% (104.59) level of herb supplementation. The ratio of H consumed via methane to H via VFA followed a reverse trend being lowest (P<0.05) at 3 % (3.26) and highest (P<0.05) at 0% (3.46) level of supplementation. The fermentation efficiencies calculated in this study varied from 74.71.per cent (2%) to 74.96% (0%) at t 1/2. The lowest value of NGGR, which indicates the best utilization of VFA, was achieved at 0% (3.42) at t ½, irrespective of the R:C and level of supplementation of herb and highest at 2% (3.57) of supplementation (Table 4.35).

Table 4.30. Effect of different levels of herbs supplementation on *in vitro* utilization of nutrients at t half, *irrespective of R:C and herb supplementation*

Parameters	Levels of herbs supplementation				SEM
	Control	1%	2%	3%	
NGP/gDM	93.13[a]	99.58[b]	100.83[b]	100.63[b]	1.36
OMD, mg/gDM	493.50[b]	486.70[b]	474.18[a]	474.54[a]	3.16
PF	5.29[c]	4.88[b]	4.70[a]	4.71[a]	0.059
OMD, %	54.70[b]	53.94[b]	52.55[a]	52.59[a]	0.35
NDFD, %	40.53[b]	39.55[b]	37.73[a]	37.77[a]	0.47
MMP, mg	57.72[c]	53.52[b]	50.47[a]	50.63[a]	0.63
EMMP, %	58.65[c]	54.93[b]	53.19[a]	53.42[a]	0.54
TD, %	55.56[b]	55.47[b]	54.24[a]	54.46[a]	0.32
SCFA, mmole	0.409[a]	0.437[b]	0.443[b]	0.442[b]	0.006
ME, MJ/kg DM	5.05	5.09	5.13	5.12	0.079
NH3-N, mg/dl	22.01[d]	21.78[b]	21.95[c]	21.73[a]	0.13
Ferm.CO_2, mmol	2.18[c]	2.08[b]	2.20[d]	2.05[a]	0.040
Ferm.CH_4, mmol	1.35[c]	1.29[b]	1.36[d]	1.27[a]	0.025

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.31. Effect of different levels of herbs supplementation on *in vitro* volatile production (mM/dl) at t half, *irrespective of R:C TMRs and herb*

Parameters	Levels of herbs supplementation				SEM
	0%	1%	2%	3%	
Acetic acid	2.86[c]	2.72[b]	2.88[d]	2.69[a]	0.052
Propionic acid	1.00[d]	0.935[b]	0.983[c]	0.9316[a]	0.018
Iso butyric acid	0.0309[d]	0.0213[b]	0.0225[c]	0.0208[a]	0.0009
Butyric acid	0.335[c]	0.326[b]	0.343[d]	0.320[a]	0.006
Isovaleric acid	0.0496[d]	0.0378[b]	0.0389[c]	0.0371[a]	0.001
Valeric acid	0.0383[d]	0.0270[b]	0.0283[c]	0.0268[a]	0.001
TVFA	4.32[d]	4.07[b]	4.29[c]	4.03[a]	0.078
A:P ratio`	2.85[a]	2.93[bc]	2.99[c]	2.90[b]	0.024

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.32. Effect of different levels of herbs supplementation on *in vitro* volatile production (% relative proportion) at t half, *irrespective of R:C TMRs and herb supplementation*

Parameters	Levels of herb supplementation				SEM
	Control	1%	2%	3%	
Acetate	66.21[a]	66.93[b]	67.08[c]	66.87[b]	0.133
Propionate	23.34[d]	22.93[b]	22.82[a]	23.08[c]	0.134
Iso butyrate	0.688[c]	0.540[b]	0.537[b]	0.527[a]	0.015
Butyrate	7.77[a]	7.97[b]	7.96[b]	7.91[b]	0.035
Isovalerate	1.13[d]	0.955[c]	0.926[a]	0.934[b]	0.019
Valerate	0.849[c]	0.666[b]	0.657[a]	0.663[b]	0.016

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.33. Methane production from fermentation of TMRs at different levels of herbs, *irrespective of R:C and Herb at t half*

Parameters	Levels of herbs supplementation				SEM
	0%	1%	2%	3%	
CH4%	21.32[c]	18.25[a]	19.04[b]	18.85[b]	0.27
CH4ml/200mg	3.91[c]	3.63[a]	3.84[b]	3.79[b]	0.034
CH4ml/gDM	19.84[c]	18.17[a]	19.20[b]	18.97[b]	0.17
CH4ml/100mgDMD	3.57[c]	3.27[a]	3.54[c]	3.48[b]	0.033
CH4ml/100mgOMD	4.04[b]	3.75[a]	4.07[b]	4.02[b]	0.038

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.34. Hydrogen balance from fermentation of TMRs at t half, *irrespective of different R:C TMRs and herb supplementation*

Parameter	Levels of herb supplementation				SEM
	Control	1%	2%	3%	
HR%	104.59[a]	109.40[d]	104.85[b]	108.52[c]	1.32
HC via CH4/VFA	3.46[c]	3.29[b]	3.47[d]	3.26[a]	0.063
FE%	74.96[d]	74.76[b]	74.71[a]	74.81[c]	0.060
VFA utilization	3.42[a]	3.55[c]	3.57[d]	3.51[b]	0.022

Means bearing different superscripts in a row differ significantly (P<0.05)

Gas production kinetics, substrate degradation, PF, MBP and EMBS for total mixed ration, different herbs and level of herb supplementation are presented in the tables 4.35, 4.36 and 4.37 respectively. The pattern of k (h-1), gas production (ml/g) at t1/2 and 24h respectively for total mixed rations were 0.0548 0.0585, 0.0723 and 0.0680 respectively in 80:20 to 65:35 (R;C ratio) rations, The gas production at t1/2 was 80.85, 84.38, 92.50 and 101.46; at 24 hr it was 179.0, 186, 195 and 195.95 ml respectively in 80:20 to 65:35 (R:C) respectively. The TDOM (mg/g DM) and PF (mg/ml) respectively at t1/2 and 24h for TMR-1, TMR-2, TMR-3, TMR-4 were 415.52, 5.13 and 571.85, 3.19; 445.16, 5.31 and 605.28, 3.25; 477.54, 5.19 and 636.42, 3.26; 507.70, 5.02 and 666.03, 3.40.

The increased gas production at 24h over that at t1/2 in the TMRs indicated greater fermentation of truly degraded substrate, which was reflected in higher substrate degradation (TDOM). On contrary, PF at t1/2 were higher than the PF of 24h, which was partly due to incomplete fermentation of truly degraded substrate that lead to low gas production. Blummel and Orskov (1993) reported that PF estimation at 24h incubation is a distortion of PF measurement through secondary fermentation of lysed microbial cells in to SCFA and gases. Hence, Blummel *et al* (1997) suggested that as PF would depend on feed characteristics and microbial growth, the incubation time for feedstuffs should be substrate specific at half asymptotic gas production (t1/2), where active growth phase of microbes is coincided.

A different trend in gas production, substrate degradation and PF values was observed for different herbs and level of supplementation in total mixed rations (Table 4.36, 4.37).

Table 4.35. Gas production kinetics (potential gas production (*D*, ml/g DM), rate of gas production (*k* h-1)), substrate degradation (truly digested OM (TDOM, mg/g DM)), partitioning factor (PF, mg TDOM/ml gas at t1/2 and 24 h), microbial biomass production (MBP, mg) and efficiency of microbial biomass synthesis (EMBS, g/kg TDOM) for the total mixed diets used in Experiment

Kinetic Parameters	TMR1 (80:20)	TMR2 (75:25)	TMR3 (70:30)	TMR4 (65:35)	SEM
Kinetic parameters					
t1/2 (h)	12.67^{d}	10.05^{b}	9.64^{a}	11.86^{c}	0.16
k (h-1)	5.48^{a}	6.89^{c}	7.23^{d}	5.85^{b}	0.006
D (ml)	232.80	239.5	224.55	236.45	0.13
Gas at t1/2 (ml)	80.85^{a}	84.38^{b}	92.50^{c}	101.46^{d}	1.36
Gas at 24h (ml)	179.0^{a}	186.0^{b}	195.0^{c}	195.95^{c}	3.80
Substrate degradation (mg/g DM)					
TDOM at t1/2	415.52^{a}	445.16^{b}	477.54^{c}	507.70^{d}	3.16
TDOM at 24h	571.85^{a}	605.28^{b}	636.42^{c}	666.03^{d}	3.80
Microbial biomass synthesis indices					
PF at t1/2 (mg/ml)	5.13^{b}	5.27^{d}	5.16^{c}	5.00^{a}	0.059
PF at24h (mg/ml)	3.19^{a}	3.25^{a}	3.26^{b}	3.40^{c}	0.013
MBP at t1/2 (mg)	235.39	256.58	270.16	279.52	0.63
EMBS at t1/2 (g/kg)	566.63	576.37	565.73	550.56	0.54

Table 4.36. Gas production kinetics (potential gas production (*D*, ml/g DM), rate of gas production (*k* h-1)), substrate degradation (truly digested OM (TDOM, mg/g DM)), partitioning factor (PF, mg TDOM/ml gas at t1/2 and 24 h), microbial biomass production (MBP, mg) and efficiency of microbial biomass synthesis (EMBS, g/kg TDOM) for the different herbs used in Experiment

Kinetic Parameters	Control	Jaiphal	Suva	Haldi	SEM
Kinetic parameters					
t1/2 (h)	11.57^{d}	11.21^{c}	11.02^{b}	10.85^{a}	0.11
k (h-1)	6.07^{a}	6.27^{b}	6.39^{c}	6.47^{d}	0.006
D (ml)	228.35	233.90	234.40	233.00	0.13
Gas at t1/2 (ml)	93.13^{a}	96.25^{b}	98.91^{c}	101.25^{d}	1.36
Gas at 24h (ml)	180.42^{a}	186.94^{b}	188.74^{d}	186.67^{b}	1.04
Substrate degradation (mg/g DM)					
TDOM at t1/2	493.50	487.60	484.07	473.54	3.16
TDOM at 24h	612.26^{a}	625.61^{b}	619.25^{ab}	618.16^{ab}	3.80
Microbial biomass synthesis indices					
PF at t1/2 (mg/ml)	5.29^{d}	5.06^{c}	4.89^{b}	4.67^{a}	0.059
PF at24h (mg/ml)	3.39^{b}	3.34^{b}	3.28^{a}	3.31^{a}	0.13
MBP at t1/2 (mg)	288.62	275.85	266.47	250.79	0.64
EMBS at t1/2 (g/kg)	584.84	565.73	550.47	529.60	0.54

Table 4.37. Gas production kinetics (potential gas production (*D*, ml/g DM), rate of gas production (*k* h-1)), substrate degradation (truly digested OM (TDOM, mg/g DM)), partitioning factor (PF, mg TDOM/ml gas at t1/2 and 24 h), microbial biomass production (MBP, mg) and efficiency of microbial biomass synthesis (EMBS, g/kg TDOM) for the different levels of herbs used in Experiment

Kinetic Parameters	0%	1%	2%	3%	SEM
Kinetic parameters					
t1/2 (h)	11.57[d]	11.03[c]	11.00[b]	10.62[a]	0.11
k (h-1)	6.07[a]	6.38[b]	6.37[b]	6.63[c]	0.006
D (ml)	228.35	231.50	235.55	237.90	0.13
Gas at t1/2 (ml)	93.13[a]	99.58[b]	100.83[b]	100.63[b]	1.36
Gas at 24h (ml)	180.42[a]	186.46[b]	189.82[c]	190.93[c]	3.80
Substrate degradation (mg/g DM)					
TDOM at t1/2	493.50[b]	486.70[b]	474.18[a]	474.54[a]	3.16
TDOM at 24h	612.26[a]	626.26[b]	616.45[a]	624.61[b]	3.80
Microbial biomass synthesis indices					
PF at t1/2 (mg/ml)	5.29[c]	4.88[b]	4.70[a]	4.71[a]	0.059
PF at24h (mg/ml)	3.39[c]	3.35[c]	3.24[a]	3.27[b]	0.13
MBP at t1/2 (mg)	288.62	267.63	252.36	253.16	0.63
EMBS at t1/2 (g/kg)	584.84	549.88	532.20	533.48	0.54

4.2.1. DIGESTIBILITY OF NUTRIENTS

The values of different principles and fibre fractions in concentrate mixture, green fodder and wheat straw fed to the cross bred calves during experiment are presented in Table 4.38.

Table 4.38. Chemical composition of total mixed ration fed to calves, %dry matter basis

Parameter	Concentrate	Green	Wheat straw
Total ash	10.3	10.2	10.35
Organic matter	89.7	89.8	89.65
Crude protein	23.45	21.10	4.41
Ether extract	4.16	2.26	0.9
Cellulose	6.8	20.70	39.10
NDF	35.4	44.0	77.6
ADF	18.30	31.10	53.70
Hemicellulose	17.1	12.9	23.9

Table 4.39. Effect of supplementing total mixed rations with essential oils containing herbs on DM intake and digestibility of nutrients in Switch over design

Parameters	Group 1 (Control)	Group 2 (Jaiphal)	Group 3 (Suva)	Group 4 (Haldi)	SEM
DM intake	5.88^{b}	5.48^{ab}	5.06^{a}	5.60^{ab}	0.097
DMD	57.46	60.15	61.03	60.60	1.23
NDFD	49.32	52.36	58.36	54.47	1.34
ADFD	44.38	41.77	46.14	42.30	1.57
EED	73.47	72.47	77.73	74.92	0.93
HCD	58.83^{a}	72.89^{b}	82.35^{c}	77.40^{bc}	1.65
OMD	57.32	62.20	67.03	62.38	1.17
CPP	70.34	72.68	75.77	72.21	0.79
TCHOD	54.32	57.11	62.38	57.45	1.31
NFCD	67.50	68.67	72.09	64.50	1.43
Cellulose D	71.43	68.87	71.08	77.43	1.29

Means bearing different superscripts in a row differ significantly ($P<0.05$)

The DM intake (Kg/d) was similar in three herbal supplemented and control groups. g (Table 4.39). This may be attributed to the comparable BW of experimental buffalo calves among the groups as no effect of niacin supplementation was observed. But in case of suva supplemented group it was statistically lower as compared to other groups. Cardozo *et al* (2006) observed no change in DMI when dietary supplementing with a mixture of 600 mg/d of cinnamaldehyde and 300 mg/d of eugenol in beef heifers fed a high concentrate diet. In a study with growing lambs, Chaves *et al* (2008b) found that addition of carvacrol or cinnamaldehyde (200 mg/kg of dietary DM) had no effect on DMI in the barley- or corn-concentrate based diets. Similar results were observed by Benchaar *et al* (2006a) who reported no change in DMI of beef cattle fed a silage based diet supplemented with 2 or 4 g/d of a commercial mixture of EO compounds consisting of thymol, eugenol, vanillin and limonene. Our results confirm these reports which showed that supplementing feedlot cattle with THY and CIN had no effect on DMI and performance. In contrast recently Yang *et al* (2010b) reported that dietary supplementation with cinnamaldehyde increased DMI of feedlot cattle in the early wk of the fattening period, although, cinnamaldehyde had no effect on DMI after 4 wk of the experiment. In some of studies, depression of DMI in cattle supplemented with EO might be related to palatability problems, proposing that the EO require to be encapsulated to overcome this problem (Calsamiglia *et al* 2007). it has been reported that DMI can be affected by a number of dietary or management factors, such as BW, animal growth stage, specific physical and chemical characteristics of diets (i.e., fiber content, particle size, amount and ruminal degradation of protein, etc.), digestion, or rumen fermentation metabolites (Allen, 2000; Yang *et al* 2007;2010c). Debashis Roy *et al* (2009 reported no statistical. variation between the groups for DM intake was not significant. Thus supplementation of EO did not make any significant impact on DM intake.

Intake and Nutrient Digestibility

There was no affect of EO supplementation on intake, fecal output, or nutrient digestibility in the current experimental trial. This is the first study to evaluate this specific blend of EO on total tract digestibility. In prior *in vitro* research decreases in nutrient digestibility have occurred at high inclusion levels which may have resulted from in antibacterial actions in the rumen.

Benchaar *et al* (2008) observed no affect on DMI or digestibility when supplementing lactating dairy cows with 1 g/d of cinnamaldehyde (one of the active compounds found in the current blend). Castillejos *et al* (2006) evaluated the addition of 5, 50, and 500 mg/L of eugenol (the other active compound found in the current blend) in a continuous-culture fermenter and observed no affect on nutrient digestibility of DM, NDF, and ADF. Digestibility of nutrients was also unaffected with the addition of CRINA EO (a blend of essential oils that contains eugenol) in several in vivo trials at various inclusion levels (Benchaar *et al* 2006, 2007). Such variability in the results could be due to many factors such as species, age, breed and body condition of the animals. There was no significant difference in DM digestibility (%) in four groups.

There was no significant difference (P<0.005) in the dry matter digestibility (DMD), Acid detergent fiber, Ether extract, Crude protein digestibility, Total carbohydrate and Total non fiber carbohydrate digestibility in control and herbs supplemented groups.

No significant difference in the digestibility (%) of OM was found among the four groups. It varied from 57.32 to 67.03 in control and herbs suva group. In herbs supplemented group it varied from 62.03 to 67.03 % respectively. This might be due to the corresponding comparable intake and digestibility (%) of DM among the groups.

The CP digestibility (%) was not modified by herbs supplementation. It varied from 70.34% to 75.77 % in all the four groups.

There were no significant differences in the mean values of EE digestibility (%) among the groups. The digestibility coefficients values for EE (%) were 73.47, 72.47, 77.73 and 74.92 respectively in control and herbs supplemented groups.

The TCHO digestibility (%) did not differ significantly among the herbs supplemented and control groups. This may be due to similar OM, CP and EE intake (g/d), digested (g/d) and digestibility (%) among the four groups. The corresponding values for digestibility (%) were 54.32, 57.11, 62.38 and 57.45 in groups I, II, III and IV respectively. The digestibility (%) of NDF was similar in four groups. The NDF digestibility (%) were 49.32, 52.36, 58.36 and 54.47 respectively in all four groups respectively.

The Non fiber carbohydrate (NFC) digestibility (%) was not significantly different. The corresponding values were 67.50, 68.67, 72.09 and 64.50 in groups I, II, II and IV respectively.

DM digestibility, CP, NDF, ADF, NFC, EE and OM digestibility. Similar results were reported by Castillejos *et al* (2005), who observed no change in DM, OM, NDF, and CP digestibility, when a Crina Ruminants EO mixture (a commercial blend of EO) was added at the dose of 3.8 mg/L of ruminal fluid in continuous-culture fermenters.

Castillejos *et al* (2006) also observed that addition of 5, 50, and 500 mg/L of eugenol in a continuous-culture fermenter did not affect DM, NDF, and ADF digestion. These results suggest that the effects of essential oil compounds on rumen microbial activity may vary depending on the dose and the type of essential oil compound used.

However, Benchaar *et al* (2006) observed that ADF digestibility was significantly increased (3 percentage points) when diets were supplemented with EO @ 2 g/ cow per day (48.9 vs. 46.0%).

4.2.2. Nitrogen balance in buffalo calves

Table 4.40. Effect of supplementation of Herbs containing essential oils on Nitrogen Retention in male crossbred calves

Parameter	Group 1 (Control)	Group2 (Jaiphal)	Group3 (Suva)	Group 4 (Haldi)	SEM
Total N intake g/day	147.16^{b}	124.49^{a}	130.70^{a}	121.45^{a}	2.37
Urinary N excretion g/d	67.74	70.45	74.17	63.90	1.65
Faecal N output g/d	44.98^{b}	35.16^{a}	32.98^{a}	34.75^{a}	1.07
Total N outgo g/d	112.73	105.62	107.15	98.65	1.99
N-retention g/day	34.43^{b}	18.86^{a}	23.54^{ab}	22.79^{ab}	2.54
%N retention	22.80	14.94	17.78	18.06	1.76

Means bearing different superscripts in a row differ significantly (P<0.05)

The intake of N (g/d) in the groups I, II, III and IV were 147.16, 124.49, 130.70 and 121.45 respectively. Nitrogen intake (g/d) was significantly lower in herbs supplemented groups as compared to control group. (Table 4.40). The mean values of total N excreted through urine (g/d) were 67.74, 70.45, 74.17 and 63.90 respectively in all the four groups. The N excreted (g/d) through feaces were 44.98, 35.16, 32.98 and 34.75 four groups respectively. Faecal N excretion was statistically higher in control group as compared to herbs supplemented groups. However, urinary and total nitrogen excreted (g/d) were statistically similar among the four groups as no significant effect was seen in herbs supplemented groups. Animals in all the four groups were in positive N balance. There were statistically lower N retention in Jaiphal supplemented group as compared to other groups. However, it was comparable in control and suva and haldi supplemented groups. The N balance (g/d) was 34.43, 18.86, 23.54 and 22.79 in all four groups.

The daily total excretion of nitrogen was highest in control non herb supplemented groups as compared to herbs supplemented groups. The urinary nitrogen excretion was highest in animals feed suva supplemented group and lowest UN excretion was observed in haldi supplemented groups. The nitrogen retention percentage was highest in control group than herbs supplemented groups though the results were non significant.

Benchaar *et al* (2006 2007) observed that there was no change in nitrogen retention and duodenal bacteria flow (estimate from urinary purine derivative) when cows were fed with EO. In addition, Tekipper *et al* (2011) reported that supplementation of 500 g of *origanum vulgare* leaves in lactating dairy cows had no effect on urinary and fecal loss as well as urinary purine derivative and microbial protein synthesis.

4.2.3. Rumen fermentation parameters

The results recorded during rumen fermentation study in crossbred calves are presented in Table 4.41. Mean ammonia-N concentration (mg/dl SRL) was significantly ($p<0.05$) lower in control group (19.87) as compared to group II (27.07) group III (28.17) and group IV (28.79) which may be due to its more incorporation into microbial protein as rumen microbes mostly prefer ammonia or peptides as nitrogen source (Bryant 1977, Horner *et al* 1988). The mean ammonia-N

concentration recorded in this study was higher than the minimum threshold of 5-8 mg/100 ml SRL, as proposed by Maynard *et al* (1979) for optimum microbial growth in all the four groups.

Table 4.41. Effect of supplementing total mixed rations with essential oils containing herbs on rumen fermentation in male cross bred calves

Parameter	TMR 1 Control	TMR2 Jaiphal	TMR 3 Suva	TMR4 Haldi	SEM
Total nitrogen, mg/dl	56.81^{a}	85.61^{d}	63.07^{b}	69.18^{c}	4.07
NPN, mg/dl	36.62^{a}	65.11^{d}	43.98^{b}	47.42^{c}	3.96
TCA-N, mg/dl	20.19	20.50	19.09	21.75	0.65
NH_3-N, mg/dl	19.87^{a}	27.07^{b}	28.17^{b}	28.79^{b}	1.37

Means bearing different superscripts in a row differ significantly (*$P<0.05$*)

In the conversion of dietary N to microbial protein, NH_3 is a prime intermediate in the rumen. Ingestion of large amount of protein can cause excessive NH_3 production in rumen. If rate of production of NH_3 is more than its utilization by rumen microbes, then concentration of NH_3 in rumen increases which is particularly evident when diet is lacking readily available carbohydrate. According to Satter and Roffler (1976), dietary CP, digestibility as well as NPN substitution for total protein, all affects ruminal NH_3 production. In group I there was significant ($p<0.05$) lower ammonia-nitrogen in comparison to herbs supplemented groups II to IV. This was probably due to better utilization of NH_3 –N by rumen microbes for the synthesis of microbial protein in the presence of supplemented herbs. But it is not evident from increased microbial protein (TCA-ppt N) in all groups as non statistically difference was observed in all the groups.

The total nitrogen in strained rumen liquor (SRL) is mainly expression of solubility of ingested protein in rumen and may also vary in relation to amount of protein intake. The total nitrogen (mg/dl)was significantly lower in control group (group I) and was statistically higher ($P<0.05$) in herbs supplemented groups (group 1I, III and IV) where it was 85.61 and 9, 63.07 and 69.18 respectively.

The TCA-ppt N mainly represents microbial N. It is evident from Table that on supplementation of herbs there were no significant ($P<0.05$) increase in the

concentration of TCA-ppt N (mg/100 ml) in all the groups as compared to control. The TCA-N values were 20.19, 20.50, 19.09 and 21.75 respectively in group I to Group IV.

The non-protein nitrogen fraction of nitrogen contains chiefly ammonia nitrogen, small quantity of amides and amino acids etc., and thus non- protein nitrogen concentration in the rumen fluid depends mainly on the production of ammonia, its uptake by microbes and absorption through rumen wall..

Non- protein nitrogen concentration (mg/100ml SRL) was also significantly ($p<0.05$) lower in control group (36.62) and highest in Jaiphal group II (65.11) as compared to other herbs supplemented groups III (43.98) and IV (47.42).

The concentration of TVFA in SRL depends upon the ingested amount of easily digestible carbohydrate, fermentable sugars and quantity and quality of CF (Phillipson, 1982). The concentration of TVFA, in this study ranged from 10.37 to 11.04 mM/dl SRL. This may be attributed to the succession of events that took place with regard to carbohydrate fermentation in the rumen (Parins and Clarke, 1979). The substrate shift in carbohydrate fermentation after feeding, involves first easily fermentable sugars followed by structural carbohydrate (cellulose) which are fermented more slowly with maximum rate of breakdown, occurring at later stages of digestion process (Leedle *et al* 1986). Results showed a significant ($P<0.05$) increase in TVFA concentration in haldi supplemented group (11.04mM/dl) and significantly lower in suva group (10.37mM/dl). This showed the stimulatory effect of herbs on rumen microorganism in the haldi supplemented groups. (Table4.42)

Mean percentage of acetate was statistically lower ($P<0.05$) in TMRgroup II (65.85) and highest in control group (69.81). The propionate was statistically higher ($P<0.05$) in all herbs supplemented groups as compared to control group. The values were 19.77, 21.40, 21.67 and 20.29 in group I, II, III and IV respectively. The butyrate percentage was statistically lower in suva group (8.31) and highest in haldi group (9.79). However, the percent isobutyrate, isovalerate and valerate in rumen liquor were statistically higher ($P<0.05$)in jaiphal supplemented groups and lowest found in control group. The A: P ratio was significantly ($P<0.05$) reduced in jaiphal supplemented group (3.07) may be due to a reduced proportion of acetic acid and an

enhanced percentage of propionic acid and found highest in non herb supplemented group i.e control group. (Table 4.43).

Table 4.42. Effect of supplementation of herbs containing essential oils on rumen volatile fatty acids production (mM/dl)

Parameter	TMR 1 Control	TMR2 Jaiphl	TMR 3 Suva	TMR4 Haldi	SEM
AA	7.54^{d}	7.06^{b}	7.02^{a}	7.45^{c}	0.086
PA	2.13^{a}	2.29^{c}	2.24^{b}	2.24^{b}	0.022
IB	0.0530^{a}	0.067^{d}	0.567^{b}	0.0615^{c}	0.002
BA	0.916^{b}	1.037^{c}	0.862^{a}	1.08^{d}	0.033
IV	0.085^{a}	0.119^{b}	0.094^{b}	0.097^{b}	0.005
V	0.072^{a}	0.139^{d}	0.87^{b}	0.110^{c}	0.011
A/P	3.53^{d}	3.07^{a}	3.12^{b}	3.32^{c}	0.083
TVFA	10.80^{c}	10.72^{b}	10.37^{a}	11.04^{d}	0.091

Means bearing different superscripts in a row differ significantly (P<0.05)

Table 4.43. Effect of supplementation of herbs containing essential oils on rumen volatile fatty acids production, % Relative Proportion

Parameter	TMR 1 Control	TMR2 Jaiphal	TMR 3 Suva	TMR4 Haldi	SEM
AA	69.81^{d}	65.85^{a}	67.67^{c}	67.48^{b}	0.53
PA	19.77^{a}	21.40^{c}	21.67^{d}	20.29^{b}	0.29
IB	0.49^{a}	0.63^{c}	0.55^{b}	0.56^{b}	0.021
BA	8.45^{b}	9.67^{c}	8.31^{a}	9.79^{d}	0.24
IV	0.79^{a}	1.11^{d}	0.91^{c}	0.88^{b}	0.043
VA	0.66^{a}	1.30^{d}	0.84^{b}	1.00^{c}	0.081

Means bearing different superscripts in a row differ significantly (P<0.05)

Castillejos *et al* (2006) using different doses (i.e., 5, 50, 500, and 5, 000 mg/L) of some EO including eugenol, thymol, guaiacol, limonene, and vanillin; and the increased pH with 500 mg/L of thymol was consisted with a depression in total VFA

concentration, reflecting a reduction in diet fermentability because of antimicrobial activity of thymol. In a subsequent research by the same authors, addition of different doses (i.e., 5, 50 and 500 mg/L) of THY in the batch culture fermentation experiment by a 10:90 forage to concentrate diet decreased pH at all used doses; whereas the decreased pH was associated with an increase in total VFA concentration (Castillejos *et al* 2008). It has been suggested that the doses of EO are need to modify rumen fermentation *in vivo* seem to be higher than those for *in vitro* experiments (Chaves *et al* 2008b). Therefore, longer term *in vivo* trials are needed to obviously establish the effects of EO supplementation at more normal feeding doses.

Essential oils had variable impacts on ruminal NH3-N concentration in the different studies. Results from different *in vitro* studies showed that the effects of EO and their main components on rumen NH3-N concentration are dose dependent and that these compounds are more effective when used at high doses compared with at low doses. F Castillejos *et al* (2006) evaluated the effects of increasing doses (0, 5, 50, 500, and 5, 000 mg/L) of thymol and eugenol on rumen fermentation in a 24-h *in vitro* batch culture fermentations; and reported that at the highest dose (i.e., 5, 000 mg/L), these compounds decreased the ruminal concentration of NH3-N, but no effects were observed at lower doses. Similarly, it has been shown that CIN and its main active component cinnamaldehyde decrease the NH3-N concentration at high (i.e., 3, 000 mg/L) level (Busquet *et al* 2006).

Most of *in vivo* studies have been shown EO often do not influence concentration of ruminal NH3-N (Benchaar *et al* 2006b;2007; Yang *et al* 2007; Yang *et al* 2010a;c) that are consistent.

Accordingly, results derived from short-term *in vitro* batch culture could be misleading and need to be interpreted with caution. Addition of EO or their main active components has caused either a reduction or no alteration in total VFA concentration in many of *in vitro* and *in vivo* studies evaluating effects of THY and CIN or their constituents on total and individual VFA concentrations. Busquet *et al* (2006) studied effects of CIN and cinnamaldehyde on rumen fermentation in an *in vitro* batch culture; and results of this study showed that high doses of CIN and cinnamaldehyde reduce total VFA and branched-chain VFA (BCVFA) concentrations. However, the proportion of propionate and acetate were only

increased by cinnamaldehyde and CIN, respectively. Although cinnamaldehyde is the main and most active component in CIN, may interact with other compounds exist within CIN, which can alter the results. Similar findings were reported by Castillejos *et al* (2006) for thymol and eugenol using doses up to 5, 000 mg/L. In a subsequent trial by this group (Castillejos *et al* 2008), all doses of THY (i.e., 5, 50 and 500 mg/L) increased total VFA concentration, but did not modify the proportions of acetate, propionate, valerate, acetate to propionate ratio and BCVFA concentration. Evans and Martin (2000) noted that 400 mg/L of thymol declined the total VFA concentration and the proportion of acetate and propionate, while acetate to propionate ratio was increased. There are very few *in vivo* studies that investigated the effects of THY and CIN or their compounds on ruminal fermentation characteristics. Yang *et al* (2010a) used cinnamaldehyde at three doses (400, 800 and 1, 600 mg/animal per d) in growing beef heifers and observed no changes in concentrations of total VFA and the molar proportions of acetate, propionate, BCVFA, and the acetate to propionate ratio. In an another study, Chaves *et al* (2008b) evaluated the effects of carvacrol and cinnamaldehyde in growing lambs and reported an increase in concentration of total VFA, but molar proportions of acetate, propionate, BCVFA, and the acetate to propionate ratio were not affected. The discrepancies among results of the present study and other studies could be attributed to the differences in type, dose or chemical composition of EO, basal diet amount and composition, and experimental conditions (e.g., *in vivo vs. in vitro*, the length of trial) as mentioned previously.

It is well documented (Wolin, 1975) that formation of methane is the main way of hydrogen elimination in the rumen, and the production of methane could be reduced by promoting a shift in fermentation toward propionate production. Propionate is an end-product of ruminal fermentation that needs hydrogen for production. Therefore, inhibition of methane production is usually accompanied with an increase in propionate production, and there is a good negative correlation between propionate production and methanogenesis activity (Wolin, 1975). This correlation could be described as competitive pathways for metabolic hydrogen use in the rumen (Moss *et al* 2000). Although the acetate:propionate ratio in the rumen has an inverse relationship with methane, but it was shown slightly lower than propionate (Russell, 1992); and whereas methane was not direct correlated with acetate and butyrate production (Moss *et al* 2000). Significant increase in propionate concentration by EO

supplementation in this experiment may be partially related to decrease in methanoghenesis activity. Therefore, changes in VFA portions may be nutritionally advantageous, because propionate is one of the main sources of metabolizable energy for ruminants and it is energetically more effective. Other *in vivo* researches showed no change in amounts of total or individual VFA by EO supplementation in dairy cattle (Benchaar *et al* 2006b;2007). These experiments and many of *in vitro* studies have suggested that the effects of EO on ruminal fermentation seem to be diet- and pH-dependent (Cardozo *et al* 2005; Castillejos *et al* 2005)

4.2.4 Blood biochemical aspects

The blood biochemical parameters estimated in cross bred calves in the present study are presented in the following Table 4.44.

Table 4.44. Effect of herbs supplementation on blood parameters of cross bred calves

Parameters	TMR1 control	TMR 2 Jaiphal	TMR 3 Suva	TMR 4 Haldi	SEM
Glucose, mg/dl	77.26	80.50	79.96	76.28	1.12
Creatinine, mg/dl	1.04	1.06	0.98	0.97	0.05
AST, (U/L)	107.70	154.31	107.14	107.86	11.15
AkP (U/L)	130.39[a]	116.35[a]	161.11[b]	121.32[a]	6.70
Total protein (g/dl)	7.10	7.72	7.97	7.68	0.19
Albumin (g/dl)	3.55	3.71	3.30	3.43	0.08
BUN, mg/dl	12.15	13.40	18.35	13.10	1.27

Means bearing different superscripts in a row differ significantly (P<0.05)

Effect of herbs supplementation on blood glucose, , creatinine, urea-nitrogen, cholesterol, ALP and AST are presented in Table 4.44. There was no significant effect of herbs supplementation on Glucose, Creatinine, Total protein, BUN and AST in all groups. The AST value (U/L) were 154.31, 107.14, 107.86 and 107.70 in groups I. II, III and IV respectively. The serum urea nitrogen concentration is closely associated

with the breakdown of protein to amino acids and their deamination in rumen and the rate of utilization of NH_3 for bacterial protein synthesis. An increase in serum urea level may reflect an accelerated rate of protein catabolism rather than decrease in urinary excretion (Kaneko 1980). The serum urea level also increases in renal tubular necrosis and decreases in hepatic insufficiency and low protein intake. Concentration of urea-N in blood serum are indicator of the adequacy or inadequacy of the nitrogen in the diet of animals (Hammond 1993) and results revealed no statistically significant difference in 4 groups. The blood urea concentration (mg/dl) were13.40, 18.35, 13.10 and 12.15 in all groups respectively. The serum creatinine concentration (mg/dl) varied from 0.97 to 1.06 in all four groups.

There was a no significant (P<0.05) increase in blood glucose (mg/dl) level after herbs supplementation. The serum glucose (mg/dl) values varied from 80.50, 79.96, 76.28 and 77.26 in group I, II, III and IV respectively.

Table 4.45. Effect of supplementation of herbs containing essential oils on Blood Hematology parameters

Parameters	TMR1 control	TMR 2 Jaiphal	TMR 3 Suva	TMR 4 Haldi	SEM
WBC*10^3	12.39	13.57	14.03	14.92	0.68
RBC*10^6	6.94	6.90	7.15	6.90	0.32
HB	9.55	9.61	9.90	9.67	0.41
HCT	29.22	28.12	29.22	28.80	1.14
MCV	41.32	40.55	41.12	40.80	0.49
MCH	14.00	13.92	13.52	13.80	0.19
MCHC	33.92	34.75	33.20	34.17	0.32
CHCM	34.02	34.57	34.02	34.85	0.27
CH	14.02	13.90	14.00	14.12	0.16
RDW	17.20	18.85	17.75	17.87	0.47
HDW	2.03	2.12	2.06	2.22	0.033
PLT *10^3	728.75	651.50	687.75	651.50	57.5
MPV	7.15	6.82	6.75	6.65	0.20

Means bearing different superscripts in a row differ significantly (P<0.05)

The effect of herb supplementation on blood haemotology shows no significant effect on different parameters like WBC, RBC, Hb, MCH, and MCV (table 4.45). Vakili *et al* (2013)reported that no significant effect *of* essential oils effects on blood metabolites in beef cattle have not been investigated widely. In accordance to previous researches (Devant *et al* 2007; Chaves *et al* 2008a; Yang *et al* 2010b), EO supplementation did not significantly affect blood glucose concentration.

CHAPTER - V

SUMMARY AND CONCLUSIONS

The present study was conducted to assess the effect of supplementing herbs containing essential oils on the methane production potential and nutrient utilization in male crossbred calve fed total mixed rations (TMRs) with different roughage to concentrate ratios.

The TMRs were formulated with roughage to concentrate ratio of 80:20, 75:25, 70:30 and 65:35 on DM basis. The roughage portion was made up of wheat straw and maize green fodder in 70:30 ratio, while concentrate mixture was prepared of of maize 15, wheat 15, deoiled mustard cake 15, mustard cake 10, soybean meal 10, rice bran15, deoiled rice bran 16, urea 1, salt1, mineral mixture 2%. The different ratios TMRs were supplemented with herbs (Jaiphal, Suva and Haldi) @1to 3% in order to find out the optimum level by using invitro gas production technique.

1. Effect of herbs containing essential oils on *in vitro* utilization of nutrients of different TMRs at 24 hr and t ½, irrespective of herb and level of herb supplementation

The net gas production (ml/gDM) was lower ($p<0.05$) in 80;20 TMR (70.96ml) and was highest in 65:35 TMR. (81.92. In this study PF value (ml) was lower ($p<0.05$) in 80:20 TMR (3.19) followed by 75:25TMR (3.25) and higher ($p<0.05$) PF value was observed in 65:35TMR (3.40). The OMD % was significantly lowest ($p<0.05$) in 80:20 TMR (63.72 %) and significantly higher in 65:35 roughage to concentrate based rations (73.64%), where as NDFD % was significantly lower ($p<0.05$) in 80:20 TMR (53.48 %) and highest in 65:35 total mixed ration (65.03%). Microbial mass production (94.31mg) as well as efficiency of microbial mass production (37.79 %) was higher ($p<0.05$) in 65:35 TMR. Metabolizable energy (ME) was significantly lower (7.93) in 80 :20 R:C based TMR, however it was comparable in other TMR's (75:25 and70:30) and significantly higher in 65:35 roughage to concentrate ratio TMR. The short chain fatty acids (SCFA) was lower ($p<0.05$) in 80:20 based total mixed ration (0.84mmole) and was observed significantly higher in 65:35 TMR (0.96mmole) but it was comparable in both75:25 and 70:30 roughage to concentrate ratio based TMR. The concentration of ammonia was lower ($p<0.05$) in

65:35 total mixed ration (20.33mg/dl) and highest in 80:20 TMR (21.63 mg/dl). The amount of fermentable methane (1.44 mmol) and fermentable carbon dioxide (2.47mmol) was lower ($p<0.05$) in 75:25 TMR where as both these were s higher ($p<0.05$) in urea based 65:35 TMR. The TVFA was significantly lowest ($p<0.05$) in 75:25 TMR (4.92 mM/dl) and was significantly higher ($p<0.05$) in 65:35 TMR (7.35 mM/dl). The percent acetate was significantly ($p<0.05$) lowest (62.74%) in70:30 TMR and highest in 75:25 (64.06%) followed by 80:20 TMR (63.19%) The propionate percent was statistically higher ($p<0.05$) in 65:35TMR and lowest in 80:20 TMR. The percent isobutyric was significantly higher ($p<0.05$) in 70:30 TMR (0.54%) whereas it was significantly lower in 75:25 TMR (0.36%). The acetate to propionate ratio was significantly lowest ($p<0.05$) in 65:35 TMR (2.25) and statistically comparable in 75:25 and 80:20 TMRs.

Methane production ml/ 100mg DMD and organic matter basis was significantly ($p<0.05$) lower in high contrite based TMR (65:35) and highest in high fibre based ration i.e TMR (80:20). The percent H recovery was observed to be highest ($P<0.05$) in TMR (75:25) while it was lowest in TMR (65:35) at 24 h incubation. The ratio of H consumed via methane to H via VFA followed a reverse trend being lowest ($P<0.05$) in TMR (75:25) and highest ($P<0.05$) in TMR (65:35) at 24 h. The fermentation efficiencies calculated in this study varied from 76.15.per cent (TMR75:25) to 77.09% (TMR65:35) at 24 h. The lowest value of NGGR, which indicates the best utilization of VFA, was achieved in TMR (65:35) at 24 h, irrespective of the herb and level of supplementation.

At t ½

The NGP ml/g DM statistically increased with increase in the level of concentrates in the TMRs. The maximum gas production was observed in 65:35TMR (101.46) and lowest in 80:20 TMR (80:25). DM digestibility was observed to be highest ($P<0.05$) in 65:35 TMR and lowest in 80:20 and 75:25 TMRs. (Table). The OMD% and NDFD% was statistically increased as the proportion of concentrates increased in the different TMRs. The microbial mass production (mg) and efficiency of microbial mass production was statistically higher in 65:35 TMR and lower in 80:20 TMR.

CH_4production per 100mg DMD and OMD was higher ($P<0.05$) in 80:20 TMR and lowest in 65:35 TMR. The percent methane increased as the proportion of fibre in the TMR increased being higher in 80:20 TMR (22.64) and lowest in 65:35 TMR (17.01).

The fermentation pattern revealed that volatile fatty acid concentration at t1/2 varied ($P<0.05$) from 3.54 mM/dl (75:25TMR) to 5.28 mM/dl in 65:35TMR (Table -). The A/P ratio was statistically higher in 65:35TMR (3.05) and lowest in 80:20 TMR (2.65). The relative proportions of acetate was significantly higher in 65:35TMR (67.20) and lowest in 80:20 TMR (65.28). The propionate percent was statistically higher in 80:20TMR and lowest in 70: 30 TMR (22.29) respectively at t-half.

2. **Effect of different herbs containing essential oils on *in vitro* utilization of nutrients of different TMRs at 24hr and t ½, irrespective of R:C and level of herb supplementation**

The effect of supplementing herbs on digestibility of nutrients revealed that NGP (ml/24h/g DM) varied ($P<0.05$) from 180.42 in control and highest in suva supplemented TMR to 188.74, irrespective of R:C and level of herb supplementation.

The digestibility of nutrients (DM, OM, and NDF) of varied significantly ($P<0.05$) amongst the herbs supplemented. Supplementation of TMR with jaiphal showed higher ($P<0.05$) digestibility of dry matter organic matter and neutral detergent fibre, followed by suva and haldi. The digestibility of nutrients for DM, OM and NDF was significantly lower in control TMR (no Herb supplementation). The digestibility data points towards the fact that supplementation of herbs increased the digestibility of all nutrients but did not affect the digestibility of nutrients. The Partitioning Factor (PF), i.e. the ratio of substrate truly degraded to the volume of gas produced was comparable in control and jaiphal supplementation in TMR and varied significantly ($P<0.05$) from 3.39 (control) to 3.34 (jaiphal) indicating proportionally more of the degraded matter will be incorporated in microbial mass. However, the PF was significantly lower in suva and haldi supplementation TMRs. Microbial mass production was statistically comparable in control and jaiphal supplemented TMRs but the efficiency of microbial mass production was significantly lower in herbs supplemented TMRs. Metabolizable energy (ME) MJ/kgDM was significantly lower (8.25) in control TMR, but statistically comparable in all herbs supplemented TMRs.

The concentration of ammonia was lower ($p<0.05$) in jaiphal supplemented total mixed ration (20.68mg/dl) and highest in haldi TMR (20..96 mg/dl).

The values for total volatile fatty acid concentration obtained in this study varied ($P<0.05$) from 5.45 mM/dl (control) to 6.31 mM/dl (haldi). The acetate, propionate, iso-butyrate and butryrate levels followed the same trend as that in TVFAs (Table). The A:P ratio was observed to be low in TMR supplemented with jaiphal (2.30) and highest in haldi supplemented TMR (2.55) irrespective of R:C and levels of supplementation, indicating shift towards propionate. The relative proportions of the three main volatile fatty acids (acetate, propionate and butyrate) varied ($P<0.05$) amongst the herbs evaluated (Table -). Acetate was significantly ($P<0.01$) higher in control TMR (63.44%) in comparison to the TMR supplemented with jaiphal (62.66%), whereas propionate was higher ($P<0.05$) in TMR supplemented with jaiphal (27.27%) closely followed by TMR supplemented with suva (27.02%) and lowest in control TMR (26.52%). The molar proportion of butyrate was observed to vary ($P<0.05$) from 7.65% (suva supplemented TMR) to 7.83% in control TMR, irrespective of the level of supplementation.

The methane as percent of net gas production, irrespective of R:C and level of herb supplementation was significantly ($p<0.05$) lower in jaiphal supplemented TMR (20.42%) and statistically comparable in all other herbs supplemented TMRs. Methane production ml/ 100mg DMD and organic matter basis was significantly ($p<0.05$) lower in jaiphal supplemented TMR (5.38 and 6.07ml) However, it was comparable in control and all other herbs supplemented TMR.

The percent H recovery was observed to be highest ($P<0.05$) in control TMR (90.50%) while it was lowest in haldi supplemented TMR (83.21%) at 24 h incubation. The ratio of H consumed via methane to H via VFA followed a reverse trend being lowest ($P<0.05$) in control TMR (4.59) and highest ($P<0.05$) in haldi supplemented TMR (5.32) at 24 h. The fermentation efficiencies calculated in this study varied from 76.34.per cent (control TMR) to 76.68% (jaiphal TMR) at 24 h. The lowest value of NGGR, which indicates the best utilization of VFA, was achieved in jaiphal supplemented TMR (2.82) at 24 h, irrespective of the herb and level of supplementation and highest in control TMR (2.93)

At t ½

The NGP ml/g DM statistically increased in haldi supplementation TMR (101.25) and lowest in control TMR (93.13). The DM digestibility (%) was observed to be lowest (P<0.05) in haldi supplemented TMR (54.21) and it was statistically comparable in control, jaiphal and suva supplemented TMRs. The OMD% and NDFD% was statistically lowest in haldi supplemented TMR and comparable in jaiphal, suva and control TMRs. The microbial mass production (mg) and efficiency of microbial mass production was statistically higher in control TMR and lower in haldi supplementation TMR.

CH_4production per 100mg DMD and OMD was comparable in (P<0.05) in control, suva and haldi TMRs and lowest in jaiphal supplemented TMR. The percent methane was higher in non herb supplemented TMR as compared to herbs supplemented TMRs.

The fermentation pattern revealed that volatile fatty acid concentration at t1/2 varied (P<0.05) from 4.10 mM/dl (haldi TMR) to 4.32 mM/dl in control TMR. The A/P ratio was statistically comparable lowest in control and jaiphal supplemented TMR and comparable higher in Suva and haldi TMRs.

The percent H recovery was observed to be highest (P<0.05) in haldi supplemented TMR (108.12%) while it was lowest in control TMR (104.59%). The ratio of H consumed via methane to H via VFA followed a reverse trend being lowest (P<0.05) in haldi TMR (3.30) and highest (P<0.05) in control TMR (3.46). The fermentation efficiencies calculated in this study varied from 74.69.per cent (haldi TMR) to 75.02% (jaiphal TMR) at t 1/2. The lowest value of NGGR, which indicates the best utilization of VFA, was achieved in control TMR (3.42) at t ½, irrespective of the R:C and level of supplementation of herb and highest in haldi based TMR (3.56).

3. **Effect of different levels of herbs supplementation on the *in vitro* utilization of nutrients at 24 h and at t ½, irrespective of R:C and herb supplementation**

The effect of different levels of supplementing herbs on digestibility of nutrients revealed that NGP (ml/24h/g DM) varied (P<0.05) from 180.42 at 0% and highest at 2 and 3% of herbs supplemented TMR (190.93), irrespective of R:C and herb supplementation. The digestibility of nutrients (DM, OM, and NDF) at 1 % level of supplementation was significantly (P<0.05) higher.

The Partitioning Factor (PF), i.e. the ratio of substrate truly degraded to the volume of gas produced was statistically comparable at 0% and 1% level of supplementation of herb and significantly ($P<0.05$) lower at 2% level of supplementation (3.24). Microbial mass production was statistically comparable at 0% and 1 % of supplementation but the efficiency of microbial mass production was significantly lower at 2and 3% level of supplementation. Metabolizable energy (ME) MJ/kgDM was significantly lower (8.25) at 0 % and highest at 2 and 3 % level of supplementation respectively. The concentration of ammonia was lower ($p<0.05$) at 3 % level of herb supplementation (20.38mg/dl) and highest at 1 % of level (21.23 mg/dl).

The values for total volatile fatty acid concentration obtained in this study varied ($P<0.05$) from 5.45 mM/dl (0 %) to 6.31 mM/dl (2%). The acetate, propionate, iso-butyrate and butryrate levels followed the same trend as that in TVFAs. The A:P ratio was observed to be low at 1 % level of supplementation (2.32) and highest at 2 % level (2.61) irrespective of R:C and herb supplementation.

The methane as percent of net gas production, irrespective of R:C and level of herb supplementation was significantly ($p<0.05$) lower as the level of supplementation increased from 1 to 3 %. At 0% level it was (21.55 %) whereas it was statistically comparable at all levels of herb supplementation in the TMRs. Methane production ml/ 100mg DMD and organic matter basis was significantly ($p<0.05$) lower at 1 % level of herb supplementation in TMR (5.35 and 6.31 ml) However, it was comparable at 0 and 2 and 3 % level of herb supplementation.

The percent H recovery was observed to be highest ($P<0.05$) at 0% level of supplementation in TMR (90.50%) while it was lowest at 2 % in herb supplemented TMR (82.57%) at 24 h incubation. The fermentation efficiencies calculated in this study varied from 76.34.per cent (0%TMR) to 76.68% (3% TMR) at 24 h. The lowest value of NGGR, which indicates the best utilization of VFA, was achieved at 3% supplemented TMR (2.81) at 24 h, irrespective of the herb and level of supplementation and highest at 0% level in TMR (2.93).

At t ½

The NGP ml/g DM was statistically higher at 1 to 3 % level of herb supplementation as compared to 0 % (93.13). The DM digestibility (%) was observed

to be lowest ($P<0.05$) at 2 and 3% level of herb supplementation but statistically higher at 0 and 1% of herb supplementation. The OMD% and NDFD% was statistically lower at 2 and 3% level of supplementation and significantly higher at 0 and 1% level of supplementation. The microbial mass production (mg) and efficiency of microbial mass production was statistically higher at 0 %, followed by 1% level of herb supplementation and significantly lower at 2 and 3% level of supplementation.

CH_4production per 100mg DMD and OMD was comparable in ($P<0.05$) at 0, 2 and 3% level of supplementation and lowest at 1 % level of supplementation of herb. The percent methane was higher in non herb (control) supplemented TMR (21.32) and lower et at 1 % level (18.25).

The fermentation pattern revealed that volatile fatty acid concentration at t1/2 varied ($P<0.05$) from 4.03 mM/dl (3%) to 4.32 mM/dl at 0 % level of supplementation. The A/P ratio was statistically comparable lowest at 0% and statistically higher at 2 % level of supplementation. The percent H recovery (%)was observed to be highest ($P<0.05$) at 3 % (108.52) and lowest at 0% (104.59) level of herb supplementation. The ratio of H consumed via methane to H via VFA followed a reverse trend being lowest ($P<0.05$) at 3 % (3.26) and higher ($P<0.05$) at 0% (3.46) level of supplementation. The lowest value of NGGR, which indicates the best utilization of VFA, was achieved at 0% (3.42) at t ½, irrespective of the R:C and level of supplementation of herb and highest at 2% (3.57) of supplementation.

4. Digestion kinetics parameters *in vitro* gas production

The gas production profiles indicated that Y max (maximum potential of gas production) varied ($p<0.05$) amongst the herbs evaluated, irrespective of R:C and level of supplementation. The Y max (ml) was significantly lower in control TMR (45.67) and statistically comparable in jaiphal and suva supplemented herb TMR followed by haldi supplemented herb TMR (46.60). However, the V min (ml) followed the reversed trend. It was statistically lower in haldi supplemented TMR (15.65) and highest in control TMR (16.69). The maximum rate of degradation (k) was observed in haldi supplemented TMR (6.47%, hr) and lowest in control TMR (6.07%/hr). The t ½ (time taken to reach half of asymptote) was lowest ($p<0.05$) for TMR supplemented with haldi (10.85h) and highest for TMR supplemented with jaiphal (11.21 h).

The gas production profiles of roughage to concentrate ratio, irrespective of herb and level of supplementation indicated that Y max (ml) was significantly lower in 70:30 TMR (44.91) and significantly higher in 75:25 TMR (47.17) followed by 65:35 TMR (47.29). The V min (ml) was statistically lower in 70:30 TMR (13.91) and highest in 80:20 TMR (18.28). The maximum rate of degradation (k) was observed in 70:30 TMR (7.23%, hr) and lowest in 80:20 TMR (5.48%/hr). The t ½ (time taken to reach half of asymptote) was lowest (p<0.05) for 70:30TMR (9.64h) and highest for 80:20 TMR (12.67 h).

The gas production profiles of levels of herbs supplementation, irrespective of herb and R:C indicated that the Y max (ml) was significantly lower at 0 % TMR (45.67) and significantly higher at 3% level of herb supplementation (47.58) followed by 2% (47.11). The V min (ml) was statistically lower at 3 % (15.33) and highest at 0% (16.69) level of supplementation. The rate of degradation (k) was observed to increased with the level of herb supplementation. The maximum rate of degradation was observed at 3% (6.63%, hr) and was lowest at)% (6.07%/hr). The t ½ (time taken to reach half of asymptote) was lowest (p<0.05) at 3% level of herb supplementation (10.62h) and highest at 0 % (11.57 h).

5. DM intake and Digestibility of nutrients

The DM intake (kg/d) was statistically similar in jaiphal, haldi and control groups but it was statistically lower in suva herb supplemented group. Results revealed no significant difference in digestibility of dry matter (DM), neutral detergent fibre (NDF), cellulose, crude protein digestibility (CPD), ether extract digestibility (EED), Total carbohydrate digestibility (TCHOD), nonfibre carbohydrate digestibility (NFCD) and organic matter (OM). There was significant difference (P<0.005) in the digestibility of hemicellulose digestibility (HCD) as it was significantly lower in control group and highest in suva supplemented group.

6. Nitrogen balance in buffalo calves

The intake of Nitrogen intake (g/d) was statistically higher in control group but it was comparable between all the herbs supplemented groups. The urinary and total nitrogen excreted (g/d) were statistically similar among all the four groups as no significant effect was seen in herbs supplemented groups The faecal N out go (g/d) was statistically higher in control group and it was comparable in all herbs supplemented groups. The daily total excretion of nitrogen and urinary nitrogen

excretion was statistically comparable in all groups. Animals in all the four groups were in positive N balance.. The nitrogen retention was lowest in jaiphal supplemented group and highest in control group but it was comparable amongst suva, jaiphal and haldi supplemented groups.

7. Blood biochemical aspects

There was no significant effect of herbs supplementation on glucose, creatinine, AST, Total protein, Albumin and BUN and hematology parameters in all four groups. The results revealed that there was increase in AKP in suva supplemented group but it was comparable in control, haldi and jaiphal supplemented groups.

8. Rumen fermentation parameters

On supplementation of herbs there was no significant (P<0.05) increase in the concentration of TCA-ppt N (mg/100 ml) and total nitrogen (mg/dl) in control group was significantly lower (56.81) and higher in Jaiphal supplemented TMR (85.61). Non protein nitrogen concentration (mg/100ml SRL) was also significantly (p<0.05) lower in control group (36.62) and highest in Jaiphal supplemented TMR (65.11). The concentration of ammonia nitrogen (mg/dl) was significantly lower in control group but it was statistically comparable in other herbs supplemented TMRs groups. Results showed a significant (P <0.05) increase in TVFA concentration in the haldi supplemented and lower in suva supplemented group. The Mean percentage of acetate was statistically lower in jaiphal supplemented group (65.85) and highest in control group (69.81%). The propionate was statistically higher in suva supplemented group and lowest in control group. The butyrate percentage was statistically higher in haldi group and lowest in suva group. However, the percent isobutyrate, isovalerate and valerate in rumen liquor were statistically higher (P<0.05) in jaiphal supplemented group. The A: P ratio was significantly (P<0.05) reduced in jaiphal supplemented group and observed highest in control group.

Conclusions

- From *in-vitro* studies it can be concluded that TMR65:35 (R:C) and 1% of level of jaiphal herb supplementation on DM basis has significantly higher NGP, digestibility of nutrients (OMD, NDFD and DMD), VFA production and ME availability and lower methane ml/100mg of DMD and OMD. The

herb supplementation did not have any significant effect on digestibility of various nutrients and percent nitrogen retention in male cross bred calves. The blood biochemical profile for various parameters did not show any significant effect on herb supplementation i.e. herbs have no deleterious effect on animal health except supplementation of herb suva in male cross bred calves significantly increased ($P<0.05$) the alkaline phosphatase activity (AKP). Herbs supplementation has stimulatory effect on rumen fermentation parameters (TN, TVFA, NPN NH_3-N) which were ($P<0.05$) higher than control group.

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