EFFECT OF AJWAIN SEED (*Trachyspermum ammi*) AS FEED ADDITIVE ON RUMEN ECOSYSTEM, ENTERIC METHANE PRODUCTION AND NUTRIENT UTILIZATION IN BUFFALOES

Thesis

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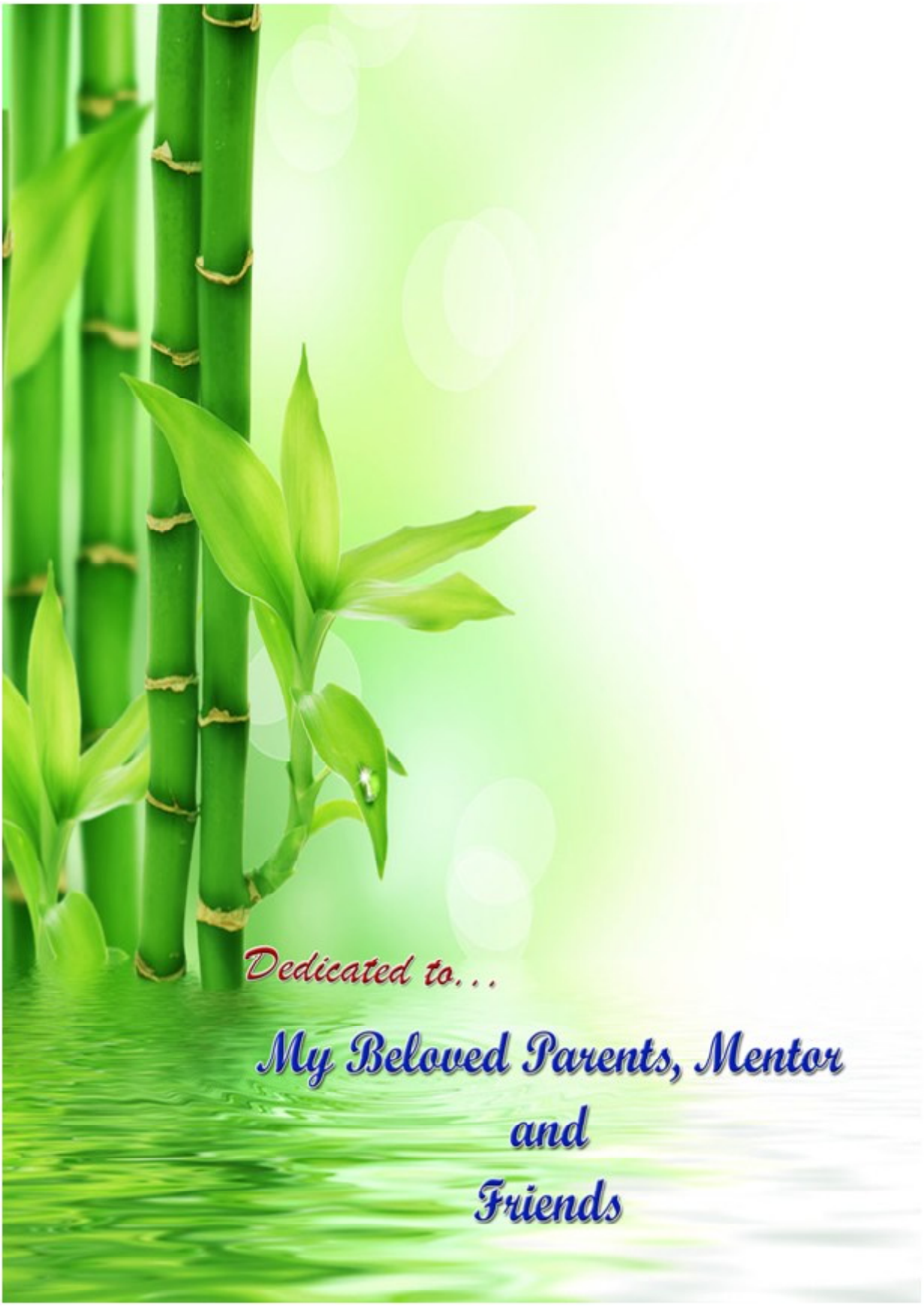
Dedicated to...
My Beloved Parents, Mentor
and
Friends

भारतीय पशु चिकित्सा अनुसंधान संस्थान
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Dated: 2018

Certificate

This is to be certified that the research work embodied in this thesis entitled "Effect of ajwain seed (*Trachyspermum ammi*) as feed additive on rumen ecosystem, enteric methane production and nutrient utilization in buffaloes" *submitted by* Dr. Kundan Kumar, Roll No. M-5776, *for the award of* Master of Veterinary Science *Degree in* Animal Nutrition *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

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We the undersigned members of Advisory Committee of **Dr. Kundan Kumar, Roll No. M-5776,** *a candidate for the degree of* **Master of Veterinary Science** *with the major discipline in* **Animal Nutrition**, *agree that the thesis entitled* "**Effect of ajwain seed (*Trachyspermum ammi*) as feed additive on rumen ecosystem, enteric methane production and nutrient utilization in buffaloes**" *may be submitted in partial fulfillment of the requirement for the degree.*

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Master of Veterinary Science** *Degree of this Institute.*

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(Kundan Kumar)

Date:
Place: *ICAR-IVRI, Izatnagar*

ABBREVIATIONS

%	:	Per cent
ADF	:	Acid detergent fibre
ADG	:	Average daily gain
ANOVA	:	Analysis of variance
AOAC	:	Association of official analytical chemist
BIS	:	Bureau of Indian Standard
CP	:	Crude protein
Df	:	Dilution factor
DF	:	Dietary fibre
DM	:	Dry matter
DMI	:	Dry matter intake
DNA	:	Deoxyribonucleic acid
DOM	:	Digestible organic matter
DOMI	:	Digestible organic matter intake
EDTA	:	Ethylene diamine tetra acetic acid
EE	:	Ether extract
g	:	Gram
g/dL	:	Gram per deciliter
GIT	:	Gastro-intestinal tract
H	:	Hours
IU/kg	:	International unit/kilogram
IVDMD	:	*In vito* dry matter digestibility
IVOMD	:	*In vito* organic matter digestibility
IVTD	:	*In vito* true digestibility
Kcal	:	Kilocalorie
Kg	:	Kilogram
$Kg^{0.75}$	:	Metabolic body size
LDL	:	Low density lipoprotein
M	:	Molarity
mg/dl	:	Milligram per deciliter
ml	:	Milliliter

mm	:	Millimeter
mM	:	Millimole
mol/L	:	Mole per liter
MPA	:	Metaphosphoric acid
MPN	:	Most probable number
N	:	Normality
NDF	:	Neutral detergent fibre
NFE	:	Nitrogen free extract
NH_3N	:	Ammonia nitrogen
nm	:	Nanometer
^oC	:	Degree centigrade
OD	:	Optical density
OM	:	Organic matter
OMD	:	Organic matter digestibility
OMI	:	Organic matter intake
PBS	:	Phosphate buffer saline
pH	:	Negative logarithum of H^+ ion
rpm	:	Revolution per minute
SCFAs	:	Short chain fatty acids
SEM	:	Standard error of mean
v/v	:	Volume by volume
w/v	:	Weight by volume
WS	:	Wheat straw
µg/ml	:	Micro gram per millilitre
µl	:	Microlitre
µm	:	Micromole

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Introduction

1 INTRODUCTION

Ruminants are one of the most important groups of herbivorous mammals on the planet, represented by 200 species consisting of approximately 75 million wild and 3.5 billion domesticated individuals (Hackman *et al*., 2010). Livestock and their products play a vital role in livelihood of millions of people in developing countries and are projected to increase by 70% by 2050 making methane emission by domesticated ruminants an issue of paramount importance for greenhouse gas (GHG) control policies. Enteric fermentation is a natural part of the digestive process of ruminants where microbes (methanogenic Archaea) decompose complex feed in the rumen. Enteric methane is formed as carbon dioxide becomes terminal hydrogen acceptor for reducing equivalents, forming methane as hydrogen sink. As all animals (and humans) obey the laws of thermodynamics thus a loss is inevitable in all energy conversions steps representing an energetic loss of 2-12% of gross energy or 8–14% of the digestible energy intake of ruminants. The methane formed is a potential environmental hazard having 23 times more global warming potential than carbon dioxide with a long environmental life span of 12 years (Samal *et al*., 2016). The level of intake of feed (more specifically organic matter) influences methane production. Dairy cows ranging in live weight from 385 to 747 kg were found to produce between 45 and 199 kg methane/head/year (14 to 31 g/kg DM intake) (Yang *et al*., 2010). With an average emission of enteric methane at 4.5 gram per 100 gram of digested carbohydrates the ruminants are responsible for 30% of global methane emission and that from Indian livestock (Naqvi and Sejian, 2011).

The rumen ecosystem is dense consortia of microbes including roughly 10^{11} bacterial cells, 10^{6} protozoal cells 10^{3} fungal cells and 10^{9} methanogen cells per ml of rumen fluid. Together

with bacteria and fungi, methanogens are the earliest colonizers of the rumen accounting for 3 to 5% of rumen microbial biomass with 113 species described in the rumen (St-Pierre and Wright, 2013). Methanogens in the rumen ecosystem of farm animals include microorganisms of the genus *Methanobrevibacter* and *Methanosaricina* especially *Methanobacterium formicicum*, *Methanobrevibacter ruminantium*, *Methanosaricina barkeri*, *Methanosaricina mazei* and *Methanomicrobium mobile* (Stewart *et al*., 1997) Analysis of the process of methanogenesis in the rumen requires understanding of interactions between hydrogen producers (bacteria, protozoa, fungi) and consumers (methanogens) in the rumen ecosystem.

The reduced emission of methane by the animals will help in protecting the environment from global warming besides enhancing the feed conversion efficiency of the animals. Hence inhibition of methanogenesis has long been considered from nutritional aspects, and more recently from the perspective of greenhouse gas emissions. Many chemical feed additives have been tried to decrease methane production in the rumen, however, these chemical additives were either toxic to host animals or have transient effects on methanogenesis. Furthermore, chemical feed additives are of concern to consumers because of the presence of chemical residues in animal derived foods and the development of bacterial resistance to antibiotics.

Recently, use of plant extracts rich in phytochemicals as natural feed additives has been of interest amongst nutritionists and rumen microbiologists to modify the rumen fermentation and to decrease methanogenesis. Unlike true bacteria, rumen methanogens belong to the class of Archaea which lack murine in their cell envelope and provides an opportunity for selective CH_4 inhibition by PSM (plant secondary metabolites). The PSMs have antimicrobial activity thus their role in modulating rumen microbial ecosystem to reduce methanogenesis has been conceptualized (Patra *et al.*, 2010). These preferential inhibition properties of different PSM against a particular group of microbes can be exploited to reduce undesirable microbes in the rumen. The benefit in animal productivity and reduction in methane production relative to the cost of using different additives is continually being assessed.

The ajwain seed used in the present study is rich source of essential oils. Ajwain (*Trachyspermum ammi*) family Apiacea is a potential traditional herb used to cure various

ailments in humans and animals. Seeds contain 2-4% brown oil having active factor thymol (50%) with germicidal, carminative and laxative property (Joshi, 2000). Several *in vitro* studies have suggested that the effect of thymol (active principle of ajwain) is diet and pH dependent (Castillejos *et al.*, 2006). *In vitro* studies on *Trachyspermum ammi* have already proved that ethanol extract of its active principle thymol reduces methane emmisions by 42% while protozoal inhibition 29% and IVTD by 0.28%. (Pawar *et al*., 2012).

There is a need for testing these effects *in vivo* as many times *in vitro* effects are not translated in the *in vivo* effects due to improper dose, effects on fermentation and effects of additives on digestibility of animals. Thus, a study is proposed to assess the effects of adding intact seeds of *Trachyspermum ammi* on rumen microbiota, fermentation, nutrient utilization and methanogenesis of adult buffaloes.

Objective:

- ☞ To study the effect of supplementation of ajwain on rumen fermentation, methanogenesis, and nutrient utilization in buffaloes.

✍✍✍

Review of Literature

2 REVIEW OF LITERATURE

Ruminants have evolved various rumen anatomies and behaviours to thrive on a range of plant species by evolving a fore stomach, the rumen that allows partial microbial digestion of feed, to form volatile fatty acids that are major nutrient sources and this flexibility has enabled them to occupy many different habitats spanning a wide range of climates (Ripple *et al.*, 2014). Ruminants themselves do not produce the enzymes needed to degrade most complex plant polysaccharides, and the rumen provides an environment for a rich and dense consortium of anaerobic microbes that fulfill this metabolic role allowing conversion of human indigestible plant material into readily-accessible animal goods, especially dairy products and meat. Animals (and humans) obey the laws of thermodynamics, making a loss inevitable in all energy conversions and the acceptability of such inefficiencies depends upon the magnitude of loss and resources used to mitigate this loss. Livestock and their products play a vital role in livelihood of millions of people in developing countries and are projected to increase by 70% by 2050 making methane emission by domesticated ruminants an issue of paramount importance for greenhouse gas (GHG) control policies. Agriculture is responsible for 47% of the total anthropogenic CH_4 emissions, of which 32% is derived from enteric fermentation in livestock (Samal *et al.*, 2016). In ruminants, approximately 95.5% of CH_4 generation is produced by fermentation of feed in the rumen. Enteric fermentation is a natural part of the digestive process of ruminants where microbes (Methanogenic Archaea) decompose complex feed in the rumen. Enteric methane is formed as carbon dioxide becomes terminal hydrogen acceptor for reducing equivalents, forming methane as hydrogen sink representing an energetic loss of 6–10% of gross energy intake (GEI), or 8–14% of the digestible energy intake of ruminants (Okine *et*

al., 2001) and is a potential environmental hazard being 23 times more global warming capacity than carbon dioxide with a long environmental life span of 12 years (Samal *et al*., 2016). Methane emission from Indian livestock ranges from 7.26 to 10.4 MT/year (Naqvi and Sejian, 2011). This contributes 42% of the total livestock methane emission in India.

2.1 Rumen microbial ecosystem

Rumen harbours a complex bionetwork, where all forms of primitive microbes starting from archaea to protozoa exist in close proximity (Sakthivel *et al*., 2012). Since strict anaerobic conditions (-150 to -350 mV) prevail in the rumen the natural rumen microflora consists of strict anaerobic microbes. Woese's classification distinguished them into three domains: Bacteria (bacteria), Archaea (methanogens), and Eucarya (protozoa and fungi). There is a continuous flow of material into and out of the rumen. It has a dry matter content of 10–13%, and temperature between 38 and 41°C. The physiological pH range is between 5.5 and 6.9, and it is one of the most variable factors in the rumen environment. The rumen ecosystem is inhabited by numerous microorganism species including roughly 10^{11} bacterial cells 10^{6} protozoal cells, 10^{3} fungal cells and 10^{9} methanogen cells/ml of rumen fluid. Methanogens are the earliest colonizers of the rumen accounting for 3 to 5% of rumen microbial biomass with 113 species described in the rumen (St-Pierre and Wright, 2013). Methanogens in the rumen ecosystem of farm animals include microorganisms of the genus *Methanobrevibacter* and *Methanosaricina* especially *Methanobacterium formicicum*, *Methanobrevibacter ruminantium*, *Methanosaricina barkeri*, *Methanosaricina mazei* and *Methanomicrobium mobile* (Stewart *et al*., 1997).

2.2 Methanogenesis

Nearly after a century of observation by the great physicist Alessandro Volta in 1776, it was confirmed that formation of "marsh gas" (now called CH_4) was a microbial process. Methanogenesis is an essential metabolic process in the rumen which acts as hydrogen sink. Hydrogen produced during feed fermentation is immediately used to produce methane which does not allow its accumulation in the gaseous phase of rumen, which otherwise might hamper feed fermentation in the rumen. It is an inescapable phenomenon of rumen fermentation and

livestock production systems contribute green house gas emissions to the atmosphere causing climate change and global warming (Gerber *et al*., 2013). Anaerobic microorganisms such as ruminal bacteria, protozoa and fungi ferment dietary organic matter (OM) components (starch and plant cell wall polysaccharides, and proteins and other materials) and release end-products that include volatile fatty acids VFA CO_2, H_2 and CH_4. Fermentation reaction uses the coenzyme NAD^+ to oxidize dietary carbohydrates and $NADH/H^+$ is formed from NAD^+. In the final stages of fermentation H_2 is used as a reducing agent and NAD^+ is regenerated. In particular methanogens oxidize the H_2 (energy content 143 MJ/kg) to reduce CO_2 to CH_4 (energy content, 55 MJ/kg), thereby gaining energy for their growth (McAllister and Newbold, 2008). This H_2 removal is extremely important because, if H_2 accumulates, reoxidation of NADH to NAD^+ is restricted, and this inhibits carbohydrate degradation, ATP production and, microbial growth (Janssen, 2010).

$CO_2+8H^++8e^- = CH_4+2H_2O$ (Methanogenesis reaction)

2.3 Methods of mitigation

2.3.1 Dietary intervention

2.3.1.1 High levels of concentrate feeding

Increase the propionate production, which decreases H_2 availability for CH_4 production. For example, Lovett *et al*. (2003) reported that increasing the ratio of concentrate in the diet of beef heifers from 35% to 90% decreased CH_4 production and increased body weight gain.

2.3.1.2 Fat addition

Irrespective of fat sources, CH_4 emissions (grams per kilogram of DM intake) reduced by 5.6% with each 1% addition of fats through combined influences on the inhibition of growth of methanogens and protozoal numbers and reduction of ruminal organic matter (OM) fermentation and hydrogenation of unsaturated fatty acids (acting as an alternative H_2 sink) in the rumen (Beauchemin *et al*., 2008).

2.3.2 Suppression of rumen methanogens:

2.3.2.1 Ionophore compounds

Ionophore antibiotics such as monensin have also been shown to depress CH_4 production in ruminants. The CH_4 production has been reported to decrease up to 76% *in*

vitro and to an average of 18% *in vivo*. Ionophores do not alter the quantity and diversity of methanogens (Hook *et al*., 2009), but they change the bacterial population from Gram-positive to Gram negative organisms with a concomitant change in the fermentation from acetate to propionate. They might also reduce ruminal protozoal numbers.

2.3.2.2 Chemical compounds

For a long time, halogenated CH_4 analogs and related compounds such as chloroform and chloral hydrate were tested for CH_4 production inhibition in ruminants. Some nitro compounds such as nitroethane, 2- nitroethanol, 2-nitro-1-propanol, and 3-nitro-1-propionic acid inhibited ruminal CH_4 production *in vitro* (Anderson *et al*., 2008).

However, there is an increasing awareness of hazards associated with chemical feed additives (Moss *et al*., 2000), i.e. many of these chemical additives are either toxic to host animals or have a transient effect on methanogenesis. Presence of chemical residues in animal derived foods and development of bacterial resistance to antibiotics has diverted the research towards exploiting natural products as feed additives this has compelled nutritionists and microbiologists to explore some antimethanogenic substances preferably through natural sources for eco-friendly animal production. Plant secondary metabolites (PSM) are potent candidate being natural components of plants having the ability to modify rumen fermentation without microbial resistance and without any residual effect on animal products (Newbold *et al*., 2002).

2.3.3 PSM as rumen modifiers

The phytogenic feed additives (PFA) are groups of plant-derived secondary metabolites (PSM) such as tannins, saponins, essential oils (EO), alkaloids, glucosides, flavonoids, amines, non-protein amino acids, organosulphur compounds not required for the primary biochemical processes of the plant itself but used as a defence mechanism for the survival of plants (Soni *et al*., 2014). These secondary metabolites are difficult to classify because their metabolic pathways of synthesis and their properties and mechanisms of action are often overlapped, and differences are difficult to ascertain (Busquet *et al*., 2005). Earliest account of medicinal use of PSM dates back to Mesopotamia, approximately 2600 BC (Greathead, 2003) These

PSM, due to their inherent nature of protection against plant pathogens (Piasecka *et al*., 2015), may be active against undesirable microbes such as methanogens. The use of tannins, saponins and EO are gaining importance as a methane-mitigation strategy because of their natural origin. These phytogenic feed additives show health promoting functions and also affected rumen ecosystem of ruminants which resulted in reduced methanogenesis and increased feed utilization efficiency (Kamra *et al*., 2008).

Numerous studies have been made to exploit these PSM like tannins, saponins, essential oils, terpenoids, flavonoids etc as natural feed additives to improve the efficiency of rumen fermentation such as enhancing protein metabolism, decreasing methane production and improving rumen ecology (Inamdar *et al.,* 2015). The tropical plants rich in saponins have been found to suppress or eliminate protozoa from the rumen and a reduction in methane and ammonia production (Kamra *et al*., 2000). Tannins, especially condensed tannins (CT), decrease methane production in the rumen (Makkar *et al.,* 1995). Similarly, some plant extracts having high content of flavonoids decrease methane production and induces extensive stimulation of microbial metabolism which increases both degradability of crude protein and cell wall constituents (Broudiscou *et al*., 2002). Essential oils are found to be beneficial for ruminal microbial metabolism reducing the risk of rumen acidosis, decreasing intra ruminal nitrogen turnover and nitrogen excretion and also inhibit methanogenesis (Sirohi, 2012). It has been proved from *in vitro* batch culture studies provide evidence that EO and their components have the potential to improve N and energy utilization in ruminants. Effects of EO on ruminal N metabolism is more likely mediated by their impact on hyper-ammonia producing (HAP) bacteria resulting in reduced deamination of amino acids (AA) and production of ammonia N. Effects on methane production are inconsistent, but evidence to date indicates that there is potential to select EO, or active components, that selectively inhibit ruminal methanogenesis (Chaves *et al*., 2008).

Ajwain (*Trachyspermum ammi*) as source of PSM

Ajwain (*Trachyspermum ammi*) of the carrot family (Apiaceae) is native to India. India is the leading producing and exporting country in the world, with maximum production in

states of Rajasthan and Telangana. The small white flowers bloom in November and December in the plains and mid-summer in hills. The harvesting is usually done from February to May. The yield is 400–600 kg/ha under a rain-fed farming system and 1200– 2000 kg/ha under irrigated conditions (Malhotra, 2012).

The principal constituents of the essential oil, which are responsible for typical ajwain flavour are phenols: thymol (35–60 %) and carvacrol (11 %). Thymol crystallizes easily from the oil on cooling. The remainder of oil is called thymene, which contains para cymene (50–55 %), beta pinene (4–5 %), limonene (Raghvan, 2006). Ajwain has been a highly esteemed medicinal herb since early times. Traditionally, the seed is used in India as a folk remedy for arthritis, asthma, coughs, diarrohea, indigestion, intestinal gas, influenza and rheumatism. Chemical composition of ajwain ground spice per 100 g Carbohydrate (g) 24.6 Protein (g) 17.1 Fibre (g) 21.2 Water (g) 7.4 Food energy (calorie) 363 Minerals (g) 7.9 Ca (g) 1.525 P (g) 0.443 Na (mg) 56 K (mg) 1.38 Fe (mg) 27.7 Thiamine (mg) 0.21 Riboflavin (mg) 0.28 Niacin (mg) 2.1 (Agarwal *et al*., 2000).

The antimicrobial activity of EO of ajwain has been attributed to a number of terpenoid and phenolic compounds (Chao *et al*., 2000) as well as the chemical constituents and functional groups contained in the EO, the proportions in which they are present and the interactions between them (Dorman and Deans, 2000). Additive, antagonistic, and synergistic effects have been observed between components of EO (Burt, 2004). Significant research has been done on combinations of phytochemical additives while scanty work has been conducted singly on *Trachyspermum ammi for* its antimethanogenic effect, improved digestibility features and enhanced animal performance. To the best of our knowledge less number of *in vivo* studies have been conducted so far on *Trachyspermum ammi* feeding in ruminants as a methanogenesis inhibitor and as a promoter of animal performance. So, the present study will be carried with the objective of determining the effect of feeding *Trachyspermum ammi* on rumen fermentation, nutrient digestion and methanogenesis in buffaloes.

Mode of action for ajwain oil (Thymol /Cravacrol /Monoterpenes)

Essential oils have a wide variety of effects on health, including positive effects on cardiovascular diseases, some tumors, inflammatory processes, and, in general, diseases in

which the uncontrolled proliferation of free radicals is very damaging (Reddy *et al*., 2003). These properties depend on their ability to scavenge free radicals, inhibit peroxidation of membrane lipids, chelate metals, and stimulate the activity of antioxidant enzyme. The antiseptic properties of many plants have been known since antiquity however, the first scientific evidence describing their antimicrobial properties did not appear until the beginning of the 20th century (Hoffmann and Evans, 1911). Given that EO comprise a large number of components, it is most likely that their antibacterial activity is not due to one specific mode of action but involves several targets in the bacterial cell (Burt, 2004). Essential oils can interact with microbial cell membranes and inhibit the growth of some gram-positive and gram-negative bacteria (Trombetta *et al*., 2005). It is believed that most EO exert their antimicrobial activities by interacting with processes associated with the bacterial cell membrane, including electron transport, ion gradients, protein translocation, phosphorylation, and other enzyme-dependent reactions. (Dorman and Deans, 2000). This interaction causes conformational changes in the membrane structure, resulting in its fluidification and expansion (Griffin *et al*., 1999). The loss of membrane stability results in the leakage of ions across the cell membrane, which causes a decrease in the trans membrane ionic gradient. In most cases, bacteria can counterbalance these effects by using ionic pumps and cell death does not occur, but large amounts of energy are diverted to this function and bacterial growth is slowed down (Ultee *et al*., 2002; Cox *et al*., 2001). In the context of continuous flow in the rumen, a change in growth rates results in changes in the proportion of rumen bacterial populations, resulting in changes in the fermentation profile. In general, the antimicrobial activity is highest in oxygenated cyclic hydrocarbons, and particularly in phenolic structures such as thymol and carvacrol, in which the hydroxyl group and the dislocated electrons allow for the interaction with water through hydrogen bridges, ionic and hydrophobic interactions as the main active site, making them particularly active against microorganisms interactions (Prescott *et al*., 2004). Ultee *et al*. (2002) proposed an alternative mechanism in which the hydroxyl group of phenols acts as a trans membrane carrier of monovalent cations and protons, such as ionophore antibiotics

On the other hand, Helander *et al*. (1998) also reported the capacity of thymol and carvacrol to disintegrate the external membrane of gram-negative bacteria, and observed the

release of membrane lipopolysaccharides and the increased permeability of the cytoplasmic membrane.

The small molecular weight of these compounds may allow them to be active in gram-positive and gram-negative bacteria. Unfortunately, this property reduces the selectivity of these compounds against specific populations, making the modulation of rumen microbial fermentation more difficult. Although the main action of essential oils as antimicrobial seems to be centered in its activity on the cell membrane, this is not the only mechanism of action. Gustafson and Bowen (1997) reported the potential of essential oils to coagulate some cell constituents, probably by denaturation of proteins. Numerous studies have also reported the capacity of some phenolic and non-phenolic compounds of essential oils to interact with chemical groups of proteins and other biologically active molecules, such as enzymes (Juven *et al.*, 1994).

As a result of such inhibition, the addition of some plant extracts to the rumen results in an inhibition of deamination and methanogenesis, resulting in lower ammonia N, methane, and acetate, and in higher propionate and butyrate concentrations. Thymol is a monoterpene [5-methyl-2-(1-methylethyl) phenol; $C_{10}H_{14}O$] with strong antimicrobial activity against a wide range of gram-positive and negative bacteria and is one of the most active components of essential oils. (Burt, 2004) reported that the antimicrobial effect of the essential oil of thyme increased as pH decreased from 6.5 to 5.5. It is likely that at lower pH, the hydroxyl group may be non-dissociated and becoming more hydrophobic, allowing an easier integration of the molecule with the lipid bilayer of the cell membrane. Therefore, it is important to define the conditions under which these additives are used to modify rumen microbial fermentation in the desired direction.

The strong and wide spectrum activity against gram-positive and gram-negative bacteria, the narrow margin of security between an optimal and a toxic dose, and the effects reported, which were not always in the desired direction emphasize the need for study of its effect on complex microbial environment as rumen (Castilejos *et al*., 2006).

Five stages to assess the effects of phytogenic substances:

Important prerequisites to understanding the modes of action of such substances in the rumen, and to assess and repeat studies and find adequate results in *in vitro* and *in vivo*

studies, include detailed description of the plants and the plant product. In addition, scientific guidance documents exist for safety assessment of botanicals and botanical preparations for use in human food and food supplements.

1) Characterization of plant description of plants, cultivation conditions, vegetation stage at harvest, processing.

2) Analytical characterization of chemical structure of the molecule(s) and their functional group(s), characterization of impurities, analytical determination in premixes and diets.

3) *In vitro* testing of the effects of various substances on rumen fermentation under various conditions (dose-level studies, various rations etc.), screening of various substances, possible deduction of mode of actions.

4) *In vivo* studies Influence of substance(s) on feed intake, rumen fermentation, digestibility, methane emissions etc. mostly in rumen fistulated target animals.

5) Long-term feeding studies with target animal species/categories Influence of substance(s) on animal health, performance and fertility, assessment of adaptation of rumen microbes to the substance(s), transfer of phytogenic substances in food of animal origin, safety and quality of food of animal origin as well as environmental impact, reproduction of results

Effect on *in vitro* fermentation

Gupta *et al*. (2017) reported that equal proportion blend of seeds of *Trachyspermum ammi* (Ajwain) + *Phyllanthus emblica* (Amla)+ *Foeniculum vulgare* (Fennel) @ 20% of the substrate along with 5% of sodium sulphate, inhibits methane by 21.3% and increased IVTD by 10.9% without affecting TVFA but acetate was increased and propionate and butyrate were significantly reduced. Ammonia production was not affected.

Yatoo *et al.* (2014*)* reported that a blend of Ajwain (*Trachyspermum ammi*) oil, Garlic (*Allium sativum)* oil and Cinnamon (*Cinnamomum zeylanicum*) leaf oil (BEO) when included at graded levels of 0.66, 1.0, 1.33, 1.66 and 2.0 µl/ml of incubation medium resulted in a linear depression ($P<0.001$) of 28.9 in total gas and 22.1 in methane production at 1.33

µl/ml level. The results indicated that the blend of EOs comprising of low levels of individual EOs suppressed methane production, however, there was also a simultaneous reduction in *in vitro* true degradability (IVTD) of feed.

Sachan *et al*. (2014) studied the effect of addition of herbs on *in vitro* rumen fermentation and digestibility of feed. Herbs namely *Trigonella foenum graecum* (Methi), *Acacia concina* (Shikakai), *Trachyspermum ammi* (ajwain), *Cinnamomum tamla* (Tejpatta) and *Aloe barbadanis* (Aloe vera) were added at the rate of 1.5%, 2.5% and 4.0% of ration. The rumen fermentation parameters and digestibility of feed was studied with buffalo rumen liquor using *in vitro* gas production test. The addition of these herbs had no adverse effect on rumen pH. Addition of other herbs *Acacia concina* (Shikakai), *Trachyspermum ammi* (Ajwain), *Cinnamomum tamla* (Tejpatta) did not show any significant effect.

Patra *et al*. (2012) reported 87% reductions in methane by thymol component when tested *in vitro* Origanum oil (thymol) + Clove + Garlic + Eucalyptus + Peppermint at 3 different doses (0.25, 0.50, and 1.0 g/liter) for their effect on methane production, fermentation ,and select groups of ruminal microbes, including total bacteria, cellulolytic bacteria, archaea, and protozoa significantly reduced methane production with increasing doses, with reductions by 34.4%, 17.6%, 42.3%, 87%, and 25.7% for Clove, Eucalyptus, Garlic, Origanum, and Peppermint, respectively, at 1.0 g/liter compared with the control. The TVFA was not affected in other cases but altered linearly by clove and quadratically by thymol of Origanum. However, apparent degradability of dry matter and neutral detergent fiber also decreased linearly with increasing doses by all EOs except garlic

Lin *et al*. (2012) (Cravacrol + Eugenol + Cinnamaldehyde + Lemon oil) applied at levels of 0-500 mg/l in five different ratios of 1:2:3:4, 2:1:4:3, 3:4:1:2, 4:3:2:1 and 1:1:1:1 to make up five different combinations (CEO1, CEO2, CEO3, CEO4, CEO5), respectively decreased gas, methane, total volatile fatty acid (VFA) production at 24 h incubation in a dose-dependent manner. Methane tended to decrease much more than total VFA and gas at the same EO level.

Borchers (1965) was the first to report that the *in vitro* incubation of casein in rumen fluid with thymol (1,000 mg/L) resulted in an accumulation of AA and a reduction in ammonia

N concentration, suggesting that deamination was inhibited. A similar conclusion was reached by Broderick and Balthrop (1979) after incubating rumen fluid with thymol *in vitro*. More recently, McIntosh *et al*. (2003) observed a 9 % reduction in the rate of AA deamination when casein acid hydrolysate was incubated *in vitro* for 48 h in batch cultures of ruminal fluid collected from cows fed a silage-based diet supplemented with 1 g/day of a commercial mixture of EO compounds (MEO; Crina. The Crina supplement contains 100–300 g/kg of phenolic compounds including cresol, resorcinol, thymol, guaiacol and eugenol. Newbold *et al*. (2004) also reported a reduction, 24%, in the *in vitro* rate of AA deamination when casein acid hydrolysate was incubated for 24 h with ruminal fluid collected from sheep fed diets containing 110 mg of MEO.

Evans and Martin (2000) reported the effects of thymol on growth and lactate production by the ruminal bacteria *Streptococcus bovis* and *Selenomonas ruminantium*. Neither 45 nor 90 mg/ml of thymol had any significant effect on growth or lactate production by *S. bovis*, but 180 mg/ml of thymol completely inhibited growth and lactate production. In the case of *S. ruminantium* 45 mg/ml of thymol had little effect on growth and lactate production; however, 90 mg/ml of thymol completely inhibited growth of *S. ruminantium*. When mixed ruminal microorganisms were incubated in medium that contained glucose, 400 mg/ml of thymol increased final pH and the acetate to propionate ratio and decreased concentrations of methane, acetate, propionate, and lactate.

Castillejos *et al*. (2006) reported that low doses of thymol (50 mg/L) had no effects on *in vitro* rumen microbial fermentation, but at higher doses (500 mg/L) total VFA and ammonia N concentrations decreased, and the acetate to propionate ratio increased. Similar results were reported in a long-term continuous culture fermentation study, suggesting that the optimal dose of thymol is somewhere between 50 and 500 mg/L.

Macheboeuf *et al*. (2008) highlighted antimicrobial rumen fermentation modulator effect of tymol/cravacrol based (EO) from ajwain (thymol). However, dose–response effects on the rumen ecosystem shown by *in vitro* response of the rumen microbial ecosystem evaluated through production curves of volatile fatty acids (VFA), ammonia, and gas produced a profile,

characterized by a virtual stop of fermentation when doses were higher than the threshold level, for thymol/carvacrol, highlighting the toxic nature of EO upon rumen microbes, and that there is a narrow dose window for the successful adoption of this technology

Effect on *in vivo* fermentation

Samal *et al.* (2016) reported that ammonia-N concentration and ciliate protozoa population were reduced significantly while rumen pH, lactic acid, volatile fatty acids and enzyme activities were not affected ($P > 0.05$) by feeding of any of these additives when Four fistulated adult Murrah buffaloes were used in 4 x 4 Latin square design to study the effects of feeding plants containing secondary metabolites on rumen metabolites and methanogen diversity. The four groups were Control (no additive), Mix-1 (ajwain oil and lemon grass oil in a 1 : 1 ratio@ 0.05% of dry matter intake), Mix-2 (Garlic and Soapnut in a 2 : 1 ratio @ 2% of dry matter intake) and Mix-3 (Garlic, Soapnut, Harad and ajwain in a 2 : 1 : 1 : 1 ratio @ 1% of dry matter intake.

Pawar *et al*. (2014) studied the effects of essential oils on *in vitro* methanogenesis and feed fermentation with buffalo rumen liquor. The data clearly indicated that among the oils tested ajwain oil was the best as the former showed 18% reduction. For the feeding trial two essential oils were selected on the basis of *in vitro* screening results Therefore, for the selection of oils, the lowest level of 0.17 µl/ml incubation medium was considered.

Pawar *et al*. (2012) reported reduced Methane emission (L/kg dry matter intake) by 13.3% Mix-1, 10.9% in Mix-2 and 5.1% in Mix-3 groups as compared with control whereas the feed conversion efficiency was higher by 9.5% in Mix-1, 7% in Mix-2 and 10.2% in Mix-3 group than in control without adversely affecting feed utilization by the animals. Mix-1 (Ajwain oil and lemon grass oil in 1:1 ratio @ 0.05% of dry matter intake), Mix-2 (Garlic and soapnut in 2 : 1 ratio @ 2% of DMI) and Mix-3 (Garlic, Soapnut, Harad and Ajwain in 2:1:1: 1 ratio @ 1% of DMI).

Five EOs i.e. Cinnamon leaf oil, Clove bud oil, Thyme oil, Origanum oil and Rosemary oil were studied for their efficacy towards methane reduction and digestibility in wheat straw based diets by Chaturvedi *et al.*, 2016. Thyme and Origanum oil showed significant CH_4

reduction 68.8 and 82.5%, respectively. IVDMD was decreased from 76.19% in case of control diet to 74.78% and 56.87% ($P<0.05$) in Origanum and Thyme oil treatments

Macheboeuf *et al*. (2008) reported suppression of CH_4 to the extent of 99% at 6 mM dose of *Origanum vulgare* EO and its component, thymol.

Pawar *et al*. (2014) studied the effects of essential oils on *in vitro* methanogenesis and feed fermentation with buffalo rumen liquor. For the feeding trial two essential oils were selected on the basis of *in vitro* screening results. The data clearly indicated that among the oils tested ajwain oil was the best. Therefore, for the selection of oils, the lowest level of 0.17 µl/ml incubation medium was considered Feeding of essential oil @ 1ml/head/day and 2ml/ head/day to growing buffalo calves resulted in10 and 16 % more body weight gain and the feed efficiency was 5.6% higher in the oil fed calves as compared to control.

Need for *in vivo* experiments

There is a need for screening and testing these effects *in vivo* as many times *in vitro* effects are not translated in the *in vivo* effects due to improper dose selection in the ration, effects on fermentation pattern and effects of additives on digestibility of animals. Longer duration of exposure may result in shifts in microbial populations, and it is possible that some of the EO compounds are subject to degradation by ruminal bacteria. Cardozo *et al*.(2006). Busquet *et al*. (2005) observed that some of the effects of EO and their main components on rumen microbial fermentation dissipated after 6–7 days of fermentation in a dual flow continuous-culture system, suggesting that rumen microbial populations may adapt to EO. *In vitro* studies have been very useful in screening the effects of a wide variety of essential oils, and will still be useful in the screening of other extracts but doses used *in vitro*, generally reported as mg/l, are much higher than *in vivo* conditions. This is likely because the activity of these compounds depends on the probability of the active component interacting with the target (bacteria) and because the bacterial concentration *in vitro* is lower than that found *in vivo*, tests require a higher concentration of the product Chow *et al*. (1994). Therefore, results from *in vitro* batch culture studies must be interpreted with caution as they report effects over a set incubation time (e.g. 24 or 48 h) and do not account for possible shifts in microbial populates that may

occur as a result of exposure of rumen microbes to EO. Identification of EO, or their active components, that favorably alter fermentation without resulting in broad overall inhibition of rumen fermentation, continues to be a major challenge for researchers there is an urgent need to conduct *in vivo* studies to determine the optimal dose in units of the active component, the potential adaptation of rumen microflora to the action of these additives, the fate of these products in the animal and the presence of residues in meat or milk, and the effects on animal performance.

Materials and Methods

3 MATERIALS AND METHODS

The programme consisted of two animal experiments carried out at Animal Nutrition shed of A.N. Division. The laboratory work of the present study was carried out in Rumen Microbiology Laboratory and Respiration calorimetric studies was conducted in Energy Nutrition Laboratory of Centre of Advanced Faculty Training in Animal Nutrition, IVRI, Izatnagar. The proposed study was carried out to ascertain the effect of supplementation of ajwain as feed additive on rumen fermentation, methanogenesis and nutrient utilization in buffaloes. The whole study was completed in two phases, viz

3.1. Phase I

3.1.1 Selection of fistulated animals.

Three adult male Murrah buffaloes (*Bubalus bubalis*) with an average bodyweight of 470 kg with permanent fistula were used in a 3X3 Switch Over Latin Square Design.

3.1.2 Housing and management of animals

All the animals were housed in a well ventilated experimental shed having arrangement for individual feeding and watering.

3.1.3 Experimental design and feeding of animals

Animals were maintained under individual feeding on roughage (wheat straw) and concentrate based ration under stall feeding for 21 days respectively to meet out maintenance requirement as per ICAR (2013). Roughage and concentrate mixture were offered in 50:50 ratio throughout the experimental period. Composition of concentrate mixture to be fed to

experimental fistulated animals was wheat bran 59 maize 28, solvent extracted soybean meal 10, mineral mixture, 2 and salt, 1% .

The additive ajwain was mixed well with the concentrate mixture before offering it to the buffaloes. The wheat straw was offered after the concentrate mixture. To meet vitamin requirement, 5 kg chopped green maize per animal was provided twice in a week.

The experimental design was as follows:

T1 : Control diet without the phytogenic feed additive ajwain

T2 : Diet supplemented with phytogenic feed additive (ajwain @ 1% DMI)

T3 : Diet supplemented with phytogenic feed additive (ajwain @ 2% DMI)

3.1.4 Experimental animals sampling

Rumen liquor collection was done with the help of specially designed metal probe with numerous perforations wrapped with nylon cloth. The pH of the rumen liquor was recorded at every collection and samples were used for estimation of rumen metabolites, protozoa and enzyme estimation. The rumen contents were squeezed and liquid portion was used for microbial DNA isolation which was used for rumen microbial profile by Real Time PCR.

3.1.5 Parameters studied were as follows

3.1.5.1 Rumen pH

The pH of the strained rumen liquor was recorded immediately after collection, with an electronic pH meter calibrated against standard buffer solutions (Eutech instruments pH buffer solution).

3.1.5.2 Enzyme activities

The enzymes from the rumen contents were extracted as per the method described by Hristov *et al*. (1999) and Agarwal *et al*. (2000). 50 g of whole rumen content was collected from the rumen at 0 h feeding. Five g of rumen content (RC) was suspended in 25 ml phosphate buffer (0.1 M, pH 6.8) and 5.0 ml of 0.4% lysozyme solution and 5ml carbon tetrachloride were added to it. The suspension was incubated at 39^0C with continuous shaking for 3 hours

followed by freezing to terminate the reaction. The treated sample was sonicated in ice bath for 6 min at –40 mV using a sonicator (B. Braun Labsonic U model; B. Braun Biotech International). The sonicated samples were centrifuged at 27000 xg for 20 min at 4°C and clear supernatant was collected for enzyme estimation. Enzyme activity was expressed as unit per 100 g RC. The enzymes were estimated as described below.

3.1.5.2.1 Carboxymethyl cellulase (â-1, 4-D glucan glucanohydrolase, endoglucanase, EC 3.2.1.4)

Carboxymethyl cellulase activity of the samples was estimated as per the method described by Agarwal *et al*. (2000) and reducing sugars produced were estimated by following the method by Miller (1959). The details of the method followed are outlined below:

Reagents:

1. 0.1M phosphate buffer, pH 6.8
2. Carboxymethyl cellulose (1%): Weight 1.0 g carboxymethyl cellulose [(sodium carboxymethyl cellulose (Koch-Light Laboratories Ltd, Colnbrook Bucks,England)] and mixed in 100 ml distilled water. The mixture was stirred vigorously on magnetic stirrer to get homogenous viscous solution.
3. Sodium hydroxide (1%): Dissolved 10 g NaOH in 1000 ml distilled water.
4. Colour reagent: Dissolved 10 g DNS in 800 ml of 1% NaOH, added to it 2.0 g phenol and made the volume to 1000 ml. Sodium sulphite (0.05%) was added just before use.
5. Rochelle salt solution (40%): Dissolved 400 g of Rochelle salt (sodium potassium tartrate) in distilled water and made volume to 1000 ml.
6. Standard glucose (0.1%): Dissolved 100 mg of glucose (Himedia Laboratories Limited, India) in distilled water and made upto 100 ml (should be freshly prepared).

Procedure:

1. For test, took 1.0 ml phosphate buffer, 0.5 ml enzyme and 0.5 ml carboxymethyl cellulose solution in a test tube and mixed well and the tube was incubated for 1 h at 39°C.

2. For control, the enzyme was denatured by keeping it in boiling water bath for 3 min. Mixed 1.0 ml phosphate buffer, 0.5 ml denatured enzyme and 0.5 ml carboxymethyl cellulose solution in a test tube.

3. Tubes were prepared in duplicate for glucose standard as follows:

Tube No	**1**	**2**	**3**	**4**	**5**	**6**
Distill Water (ml)	2.0	1.9	1.8	1.7	1.6	1.5
Standard Glucose(ml)	0	0.1	0.2	0.3	0.4	0.5
Glucose conc.(µg)	0.00	100	200	300	400	500

*Reagent blank

4. Added 3.0 ml DNS reagent in all the tubes.

5. Kept all the tubes in boiling water bath for 10 min.

6. Added 1.0 ml Rochelle salt solution in each tube and then cool them to room temperature.

7. Read absorbance "A" at 575 nm in a spectrophotometer against reagent blank.

8. Prepared standard curve by plotting "A" against glucose concentration.

Calculations:

- Change in absorbance ΔA = "A" test - "A" control
- Read ΔA on the calibration curve to get the µg glucose released.

• Enzyme activity Units/ml = µmol glucose/min/ml = µg glucose/ T x Sx 180

Where, T = time of incubation (30 min).

S = volume of sample (0.5 ml)

180 = molecular weight of glucose.

3.1.5.2.2 Avicelase (Exo b-1, 4-D-glucanase, EC 3.2.1.91)

The assay mixture was the same as that of CMCase except for the avicel (0.1%) suspension which was used as a substrate in place of carboxymethyl cellulose at the rate of 1 ml along with 1 ml enzyme solution.

3.1.5.2.3 Xylanase (1, 4-â-xylan xylano hydrolase; Endo-1, 4-b-xylanase; EC3.2.1.8)

The procedure for estimation of xylanase activity was similar to that of endoglucanase with the following differences

1. 0.25% xylan : Suspend 0.25 g xylan (from oat spelt) (Sigma Chemical Co., St. Louis, USA) (Miller, 1959) in 100 ml distilled water and heat it at 70oC for 10 min. Centrifuge it at 3000 xg for 5 min. Used the supernatant as substrate.

2. Standard D-xylose (0.1%): Dissolved 100 mg of xylose (Sigma Chemical Company, USA) in distilled water and make up to 100 ml.

3. Assay mixture contains 1 ml phosphate buffer, 0.5 ml sample and 0.5ml xylan solution.

4. Incubation time, 30 min.

5. Tubes were prepared in duplicate for standard curve as follows.

Tube No	**1**	**2**	**3**	**4**	**5**	**6**
Distilled water (ml)	2.00	1.80	1.70	1.60	1.50	1.40
Stand. xylose soln. (ml)	0	0.2	0.3	0.4	0.5	0.6
Xylose conc. (µg)	000	200	300	400	500	600

*Reagent blank

6. Enzyme activity Units/ml = µmol xylose/min/ml = µg xylose / T x S x150

   Where,

   150 = molecular weight of xylose

3.1.5.3 Protein estimation

The protein content of enzyme samples was estimated as per the method described by Lowry *et al.* (1951). The details of the method are as follows:

Reagents:

1. Standard solution of bovine serum albumin (0.06%) in distilled water.

2. Trichloroacetic acid (TCA) 20% solution in distilled water

3. Solution A: 2.0 g sodium carbonate dissolved in 100 ml of 0.1 N NaOH.

4. Solution B: 1.0 g sodium-potassium tartrate dissolved in 100 ml distilled water. 0.5 g copper sulphate was added to it and kept overnight. Then the solution was filtered to remove the precipitate.

5. Solution C: 50 ml of solution A and 1.0 ml of solution B were mixed just before use.

6. Solution D: 1.0 ml of Folin and Ciocalteu's phenol reagent and 2.0 ml of distilled water were mixed just before use.

Procedure

Protein precipitation:

1. 1.0 ml sample and 1.0 ml TCA solution were mixed.

2. The tubes were incubated for 1 h at room temperature.

3. The tubes were centrifuged at 10,000 rpm for 10 min.

4. The precipitate was collected and dissolved in 1.0 ml of 1N NaOH and used for protein estimation.

Estimation of protein:

1. 0.1 ml sample and 0.4 ml distilled water were taken in a test tube in duplicate.

2. Standard tubes: Tubes of BSA standard were prepared in duplicate as

Tube No	**1**	**2**	**3**	**4**	**5**	**6**
Distilled water (ml)	0.50	0.40	0.30	0.20	0.10	0.00
Stand. BSA soln. (ml)	00	0.1	0.2	0.3	0.4	0.5
BSA conc. (µg)	00	60	120	180	240	300

*Reagent blank

3) 5.0 ml of solution C was added to all the tubes and left for 10 min at room temperature.

4) 0.5 ml of solution D was added and mixed immediately.

5) After 10 min, absorbance was recorded against reagent blank at 600 nm.

6) Standard curve was prepared by plotting absorbance against concentration of standard BSA solutions.

7) Protein concentration of sample was calculated by plotting the absorbance of sample on the standard graph.

Specific activity of the enzymes was calculated as follows:

Specific activity = Units/mg protein

3.1.5.4 Estimation of ammonia nitrogen

Ammonia nitrogen in the rumen liquor was estimated by the method of Weatherburn, (1967) as follows :

Reagents

a) Solution A: One g phenol was dissolved in 50 ml of distilled water. 5.0 mg of sodium nitroprusside was added and volume was made up to 100 ml with distilled water.

b) Solution B: 0.5 g of sodium hydroxide was dissolved in 50 ml of distilled water. 0.84 ml of sodium hypochlorite was added and volume was made up to 100 ml with distilled water.

c) Stock solution of standard ammonium sulphate: 0.048 g of ammonium sulphate was added in 100 ml of distilled water to get a final concentration of 10 mg of ammonia nitrogen per 100 ml solution.

d) Working standard: 10 ml of stock solution was diluted to 100 ml distilled water to get 0.01 mg ammonia nitrogen per ml.

Tubes were prepared in duplicate for standard curve as follows :

Tube	1*	2	3	4	5	6	7
Distilled water (ml)	1.0	0.90	0.80	0.60	0.40	0.20	0.00
Standard solution (ml)	-	0.10	0.20	0.40	0.60	0.80	1.00
Ammonia nitrogen (µg)	0.00	1.00	2.00	4.00	6.00	8.00	10.0

Procedure

To the rumen liquor (0.1 ml), 0.9 ml of distilled water was added, followed by addition of 5.0 ml of solution A and then immediately 5.0 ml of solution B and mixed thoroughly. The tubes were incubated at 39°C for 15 min for colour development. Samples were then read spectrophotometrically at 625 nm against a reagent blank. In a similar way standard samples (ammonia nitrogen concentration ranging from 0.5 to 10.0 µg) were processed and a calibration curve was plotted. Concentration of the unknown sample was calculated by the standard curve.

3.1.5.5 Microscopic counting of protozoa

The number of protozoa was counted as per the procedure described by Kamra *et al*. (1991). For counting protozoa 1.0 ml rumen liquor pipetted with a wide orifice pipette was mixed with 1.0 ml methyl-green formal saline solution (0.06 g methyl green, 0.85 g sodium chloride, 10 ml formaldehyde and 90 ml distilled water) and allowed to stand overnight at room temperature. If necessary, further dilution was done with 30% (v/v) glycerol. Counting was done in 20 microscopic fields in a haemocytometer counting chamber at a magnification of 100X and calculated as:

Number of protozoa / ml rumen liquor N = (n x A x D) / (a x v)

Where:

n = Average number of cells / microscopic field

A = Area on slide on which the sample is spread (area of the cavity of haemocytometer)

D = Dilution of rumen liquor

a = Area of microscopic field

V = Volume of rumen liquor in the counting chamber

Number of protozoa/ml of sample = N x D x 3919.27

3.1.5.6 Quantification of rumen microbial profile

Total bacteria, *Fibrobacter succinogenes*, methanogen, fungi, and ciliate protozoa populations were assessed by qPCR (Real time PCR) technique.

3.1.5.6.1 Genomic DNA isolation from rumen fluid (Modified Zhongtang Yu and Morrison, 2004).

Reagent:

1. TE buffer

Tris	1.21g/L (10Mm)
Di sodium EDTA	0.372g/L (1Mm)

2. Lysis buffer

NaCl	2.92g (500Mm)
Tris Hcl,	Ph-8.0 0.606g (50Mm)
EDTA	1.86g (50mm)
4% SDS	4g

3. Working Proteinase K: 20 mg/ml
4. 3M Sodium acetate PH 5.5: Dissolve 24.61g of sodium acetate in 75 ml of dH2O. Adjusted pH to 5.5 with conc.Hcl and make the volume upto 100ml with dH2O.
5. Ethanol: 100% & 70% (molecular grade)
6. RNAse: 10mg/ml or ready to use
7. QiAmp DNA stool mini kit
8. Isopropanol

Protocol

1. Cell lysis

- 1.5 ml strained rumen liquor was taken in 2 ml micro centrifuge tubes with baked zirconium bead (Biospec product, INC) and centrifuged at 14000xg for 15min at 4°C.
- Discarded the supernatant and re-suspended the pellet in 1ml TE-buffer which was then centrifuged at 14000xg for 15min at 4°C.
- Discarded the supernatant and added 1ml lysis buffer to the pellet. Fast prep for 3min at 6.5 m/s or repeat 4 step of 45 sec each was done (Kept samples in ice in between).
- Incubated tubes at 70°C for 15 min with gentle shaking by hand in every 5 min.
- Centrifuged at 16000xg for 15min at 4°C and transfered supernatant in fresh 2 ml microcentrifuge tube (in ice).
- Added 300 µl of fresh lysis buffer to the tube and repeated step 3 to 5 and then pooled the supernatant.

2. Precipitation of nucleic acid

- Added 260 µl of sodium acetate to each tube mixed well and incubated on ice for 5min, Centrifuged at 16000xg for 5 min at 4°C.
- Transfered the supernatant to two 1.5 ml microcentrifuge tube, added one volume of isopropanal, mixed well and incubated on ice for 30 min.
- Centrifuged at 16000xg for 5min at 4°C .Removed supernatant using pipette.
- Washed nucleic acid pellet with 1ml 70 % ethanol at 16000xg for 5min at 4°C
- Discarded ethanol and dry at 50°C for 15min or longer until dry.
- Dissolved nucleic acid pellet in 100 µl of TE (Tris - EDTA) buffer and pooled the two aliquots

3. Removal of RNA, Protein and Purification

- Add 3 µl of RNase and incubated at 37°C for 15min.
- Added 15 µl of proteinase K and 200 µl of buffer AL, mixed well and incubated at 70°C for 10min.
- Added 200 µl of ethanol & mixed well. Transfered to a QIAmp. column and centrifuged at 16000g for 1min.
- Discarded flow through, added 500 µl buffer AW1 and centrifuged for 1min at room temperature.
- Discarded flow through, added 500 µl buffer AW2 and centrifuge for 1 min at room temperature.
- Dried the column by centrifugation at room temperature for 1min.
- Added 200 µl of buffer AE and incubated for 2 min, centrifuged at 16000g for 1 min to elute DNA.
- Quantified DNA by Nano drop (Thermoscientific ND-1000, USA).

3.1.5.6.2 Quality and concentration of genomic DNA

Horizontal submarine agarose gel electrophoresis was performed to check the quality of genomic DNA using 0.8% gel. The gel casting tray was prepared by placing the comb. Agarose (0.8%) suspended in 1X TE buffer was boiled until the agarose was completely melted and dissolved to give a clear transparent solution. After cooling to 60°C, safeview @ 5µl/100 ml was added and mixed gently. The agarose solution was poured in the casting tray and allowed to cool down at room temperature for the polymerization of gel. After solidifying of gel, the comb was gently removed. For loading the samples, 5 µl of DNA sample was mixed with 2 µl of 6X gel loading dye and the sample was loaded in the well of agarose gel which was submerged in 1X TE buffer. Electrophoresis was performed at 100V for 30 min. Gel was visualized under UV transilluminator and documented by photography. Only DNA samples showing intact band and devoid of smearing were used for further analysis. Concentration at

260nm and 260/280 ratio of the DNA samples was recorded by Nanodrop. The samples showing ratio of 260/280 from 1.8 to 2.0 were used for further processing.

Reagents:

a) Kappa SYBR Green qPCR mastermix (2X)

b) Nuclease free water

c) Primers: Gene specific primer sets used were-

Microbe	Primer sequence	Annealing temperature	PCR product bp	Reference
Total bacteria	F5'CGGCAACGAGCGCAACCC3' R5'CCATTGTAGCACGTGTGTAGCC3'	60	130	Lane (1991)
Fibrobacter succinogens	F-5'GTTCGGAATTACTGGGCGTAAA-3' R-5'CGCCTGCCCCTGAACTATC-3'	60	121	Tajima *et al.* (2001)
Methanogens	F 5'-TTC GGT GGA TCD CAGC-3' R 5'-GBA RGT CGW AWC CGT AGA ATC C-3'	60	140	Vinh *et al.* (2001)
Fungus	F-5'CGGCAACGAGCGCAACCC-3' R-5'CCATTGTAGCACGTGTGTAGCC-3'	60	110	Lane (1991)
Ciliate Protozoa	F 5' GCT TTC GWT GGT AGT GTA TT-3'539 R 5, CTT GCC CTC YAA TCG TWC T-3'	55	223	Huws *et al.* (2009)

Reaction mixture for qPCR was prepared as follows:

Reagent	**One reaction (µl) x n**
2X Syber green mix	10
Forward primer	0.6
Reverse primer	0.6
Template	2
NFW	6.8
Total volume	20

NFW, nuclease free water; n, number of reaction

Thermal protocol was as follows

The premix was dispensed (20 µl) in duplicate in each well for each sample in 96 well PCR plate. The plate was sealed and placed in real time thermal cycler (Biorad CFX96 real time system). Cycle threshold (Ct) value was recorded for each sample. Copy number (CN) was calculated with the standard curve and the population of a microbe was expressed as Log_{10} of cells.

Calculation

per ng of DNA=CN x Total Volume of DNA x Conc of DNA (ng/µl) / total volume of sample

Phase II

3.2. Effect of feed additive on methane production and nutrient utilization in buffalo calves

3.2.1. Experimental animals, feeding and sampling

To test the effect of ajwain as additive on methane production and nutrient utilization fifteen male buffaloes of about 15-20 months ,average body weight 270 kg were used. After acclimatization, the animals were randomly divided into three groups of five animals each, following Completely Randomized Design (CRD). The experiment was conducted for 30 days under similar managemental conditions in the animal shed of Animal Nutrition Division, Indian Veterinary Research Institute, Izatnagar. Prior to initiation of the experimental trial, animals were treated for ecto and endo-parasites as per the standard protocol.

The three groups were as follows:

T1 : Control diet without the phytogenic feed additive ajwain

T2 : Diet supplemented with phytogenic feed additive (ajwain @ 1% DMI)

T3 : Diet supplemented with phytogenic feed additive (ajwain @ 2% DMI)

The animals were fed concentrate mixture and wheat straw in the ratio of 50:50 as per ICAR (2013) feeding standards for 500g daily body weight gain. The concentrate mixture was composed of maize 36, solvent extracted soybean meal 22, wheat bran 39, mineral

mixture 2 and salt 1%. The feed additive was mixed well with the concentrate mixture before offering to buffaloes. The wheat straw was offered after the concentrate mixture was completely consumed by the animals. Fresh and clean drinking water was made available *ad libitum* twice a day. To meet vitamin requirement, 5 kg chopped green maize per animal was provided once in a week.

3.2.2 Parameters studied were as follows

3.2.2.1 Feed intake

The feed offered and residue left by the animals was recorded daily. The feed intake was determined by subtracting DM of residue from DM of feed offered.

3.2.2.2 Metabolism trial

A metabolism trial of 8 days duration including, 2 days adaptation in metabolic cages followed by 6 days collection was conducted after 30 d of experimental feeding to evaluate the effect of feeding ajwain as feed additive on nutrient utilization and nitrogen balance. Body weight of the animals was recorded before and after the metabolism trial.

3.2.2.2.1 Feeding and sampling of feed and residue during metabolism trial

During the metabolism trial, weighed quantity of concentrate mixture (with additive for treatment groups) was offered at 9.00 A.M. When the animals consumed concentrate mixture, weighed quantity of wheat straw was offered. Water was offered at 11.00AM, 16.00PM and 20.00 PM. Well-mixed representative samples of concentrate, wheat straw and residue left was taken daily in previously tared trays and dried at 100±2 ^{o}C for overnight for dry matter estimation. The dried material obtained during trial period was pooled animal wise, ground to pass through 1 mm sieve and stored for proximate and fibre analysis .

3.2.2.2.2 Collection and sampling of faeces

The faeces voided in 24 h by the individual animal was mixed properly and representative sample from each animal was taken separately in a labelled polythene bag. A suitable aliquot was kept (1/100 of fresh faeces) for drying at 100±2 ^{o}C in a hot air oven for dry matter estimation. The dried faeces obtained daily were pooled animal wise, ground to pass through

1 mm sieve and used for proximate analysis. A suitable aliquot (1/1000 of fresh faeces) was mixed with suitable quantity of 25% sulphuric acid and preserved for nitrogen estimation in previously weighed air-tight bottles. The weight of the faeces with acid was calculated by subtracting bottle containing faeces and acid from empty weight of the bottle. A suitable quantity (10 g) of pooled sample was taken daily for digestion after thorough mixing and analyzed for nitrogen in faeces

3.2.2.2.3 Collection and sampling of urine

Urine excreted by the animals was collected in the containers having 50 ml of 25% sulphuric acid. The total urine excreted in 24 h was measured and 1/1000 ml of representative sample was taken for digestion in duplicate in Kjeldahl flask containing 50 ml of concentrated sulphuric acid and analyzed for nitrogen in urine

3.2.2.3 Proximate analyses

The AOAC (1995) methods of analyses were followed for estimation of proximate principles as follows:

The samples of feed, faeces and urine were analyzed for proximate principles as per standard procedures of Association of Official Analytical Chemists (AOAC, 1995). A brief description of the method employed is given below

3.2.2.3.1 Dry Matter (DM)

Representative sub-samples were taken in pre-weighed moisture cups and kept in a hot air oven at 100±2°C until constant weight was obtained. Dried samples were cooled in desiccator, weighed, and DM was calculated as follows:

$$DM\ (\%) = (a/b) \times 100$$

Where,

a = dry weight of sample

b = fresh weight of sample

3.2.2.3.2 Crude Protein (CP)

Nitrogen/crude protein content of the sample was determined by the standard Kjeldahl method. One gram of the sample was taken in Kjeldahl flask having 25 ml concentrated commercial sulphuric acid and to it 3-5 g digestion mixture (sodium sulphate and copper sulphate, 9:1) was added as a catalyst. The flasks were heated on a digestion bench till the contents were free from carbon particles. The flask was then cooled to room temperature, and contents were transferred into a 250 ml volumetric flask and volume was made up to mark by repeated washings with distilled water. A suitable aliquot (10 ml) of digested sample was then distilled in a Micro-Kjeldhal distillation apparatus and sufficient amount (about 10 ml) of 40% NaOH was added to make the content alkaline. Gaseous ammonia thus released was trapped in a conical flask containing 10 ml of 2% boric acid solution having Tashiro's indicator (0.1% methyl red and 0.1% bromocresol green in the ratio of 2:1 in absolute alcohol). Approximately 60 to 80 ml of the distillate was collected which was subsequently titrated against standard N/ 100 H_2SO_4. A blank was also run, the value of which was subtracted from sample's reading. The normality of the acid was checked by titrating against sodium carbonate using methyl orange as indicator. The crude protein content was determined as follows:

$$CP\ (\%) = \frac{100 \times Y \times (B\text{-}B_1) \times 0.00014 \times 6.25}{X \times W} \times 100$$

Where,

Y = volume (ml) made out of digested sample

X = volume (ml) of aliquot taken for distillation

B = volume (ml) of N/100 H_2SO_4 consumed for titration of samples.

B_1 = volume (ml) of N/100 H_2SO_4 consumed for titration of blank distillate

W = weight (g) of oven dried sample taken for digestion

6.25 = Factor for converting nitrogen into protein of sample

3.2.2.3.3 Ether Extract (EE)/Crude Fat

The ether extract/crude fat was determined by extracting weighed quantity (about 2-3 g) of ground moisture free sample with petroleum ether (B.P. 60-80°C) in Soxhlet apparatus for 8-10 h. The extracted oil in the flask was dried to constant weight at 80°C. The increase in weight of oil flask was taken as ether extract and expressed in percent on DM basis by the formula:

Ether extract (%) = (y-x)/w×100

Where,

y = Weight of oil flask after extraction

x = Weight of oil flask before extraction

w = Weight of oven dried sample

3.2.2.3.4 Total Ash (TA)

A known quantity of sample was taken in pre-weighed silica crucible, decarbonized on heater to make it smoke free and, ignited in a muffle furnace for ignition at 550±50°C for 3h. The weight of the residue left was expressed as total ash content of the sample. The percent total ash was calculated from the following formula:

$$\text{Total Ash (\%)} = (c - a)/(b - a) \times 100$$

Where,

a = empty weight (g) of silica basin

b = weight (g) of silica basin plus oven dried sample

c = weight (g) of silica basin plus ash

3.2.2.3.5 Organic Matter (OM)

Per cent OM in feed and faeces samples were calculated by subtracting the total ash from 100 and expressed as per cent on DM basis.

$$\text{OM (\%)} = 100 - \text{TA (\%)}$$

3.2.2.3.6 Fibre fractions

Fibre fractions were determined as per the methods of Van Soest *et al*. (1988).

3.2.2.3.6.1 Neutral Detergent Fibre (NDF)

One-gram ground feed was taken in a spout less beaker and 100 ml of neutral detergent solution was added to it. The beaker along with contents was heated to boil and refluxed for 1h. The contents were filtered through a pre-weighed Gooch crucible (Grade 1) of 50 ml capacity under vacuum with 3-4 washings of hot distilled water. The crucibles were dried to a constant weight at 100°C. Cell wall contents or NDF was calculated as follows:

$$\text{NDF (\%)} = \frac{\text{(Weight of crucible + NDF) - (Weight of crucible)}}{\text{Weight of sample (DM basis)}} \times 100$$

3.2.2.3.6.2 Acid Detergent Fibre (ADF)

One-gram ground feed was taken in a spout less beaker and 100 ml of acid detergent solution was added to it. The contents were heated to boil and refluxed for 1 h and filtered through a pre-weighed Gooch crucible under vacuum with 3- 4 washings of hot distilled water. The crucibles were dried to a constant weight at 100°C. ADF was calculated as follows:

$$\text{ADF (\%)} = \frac{\text{[(Wt. of crucible + Residue) - Wt. of crucible]}}{\text{Wt. of the sample on DM basis}} \times 100$$

3.2.2.4 RESPIRATION CALORIMETRIC STUDY

3.2.2.4.1 Measurement of respiratory exchange

The animals were shifted in open circuit respiration chamber and after acclimatization, respiration exchange was measured. The flow rate, temperature of dry and wet bulb and atmospheric pressure were recorded at hourly intervals. The chamber was opened after every 22 h to offer feed and to collect residues of feed and feces. As the collection of urine and faeces separately was not possible (because of mixing), the calculated digestibility of the animal in the consecutive metabolism trial was used for the calculations.

3.2.2.4.2 Calibration of respiration apparatus

The calibration of equipment of respiration calorimeter was done as per the standard procedure given below-

3.2.2.4.2.1 Calibration of flow meter

The flow meters were periodically calibrated against a standard dry gas meter. The standard meter was attached between animal chamber and flow meters to be calibrated. The respiration chamber was run for four h and the volume of outgoing air was measured in both the meters and the factor were derived with four such observations for the differences and accordingly, a correction factor was calculated to rectify the difference in the flow rate.

3.2.2.4.2.2 Calibration of methane and CO_2 analyzer

The infrared methane analyzer was allowed to run for one hour and thereafter, nitrogen gas was passed through the analyzer and indicator was adjusted to zero. Standard gas mixture containing known concentration of methane was passed at the rate of 0.51 /min and the indicators were adjusted according to the concentrations of representative gases.

3.2.2.4.3 Respiratory exchange measurements

The concentration of methane, CO_2 and oxygen in the chamber air was recorded after running it for one hour. A known amount of methane gas was flowed into the chamber and concentration was recorded by carbon dioxide analyzer till the increased methane reached to the initial concentration. Analysis of outgoing air was done by pre-calibrated infrared methane analyzer till the recovery was completed.

The total volume of methane produced was computed as per the following formula:

3.2.2.4.3.1 Methane production

$CH4\ (L) = VSTP\ (Mf - Mi)/100$

Where,

Mf = Methane present in outgoing air from the chamber

Mi = Methane present in incoming air into the chamber

VSTP = Volume at standard temperature, and pressure

3.2.2.4.3.2 Carbon dioxide expired

CO_2 (L) = VSTP (Cf - Ci)/100

Where,

Cf = Methane present in outgoing air from the chamber

Ci = Methane present in incoming air into the chamber

VSTP = Volume at standard temperature, and pressure

3.2.2.4.3.3 Oxygen consumed

O_2 (L)= (Difference in the concentration of oxygen between ingoing and outgoing air of the chamber) VSTP

Where,

VSTP = Volume at standard temperature, and pressure

3.2.2.5 Estimation of gross energy

Gross energy (GE) content of feed offered, residue, faeces and urine, was determined by Ballistic Bomb Calorimeter (Gallenkamp, C.B.370) was used.

3.2.2.5.1 Calibration

The bomb was calibrated with thermo-chemical grade benzoic acid to establish the relationship between galvanometer reading and the heat released by the combustion of sample. Approximately 0.7 g of benzoic acid was taken in a pre-weighed nickel crucible and melted to the bottom of the crucible. Weight of crucible and benzoic acid was recorded. Then the crucible was placed in the bomb with cotton thread in the position. Bomb was then screwed down. Up to 20 atmospheric pressure of oxygen was passed and galvanometer was set to zero. The bomb was fired by pressing the ignition key and the galvanometer reading was noted. A correction for heat liberated by heating wire and cotton thread was made by repeating the above test for crucible and thread without sample in it. The correction factor thus obtained was subtracted from the reading of benzoic acid.

Calculation of calibration factor:

Caloric value of one division = $(6.322 \times X)/(Y - Z)$

Where,

X = Weight of benzoic acid

Y = Galvanometric reading for benzoic acid and cotton thread

Z = Galvanometric reading for blank

Y–Z = Actual galvanometer reading

6.322 = Calorific value of benzoic acid (kcal/g)

3.2.2.5.2 Procedure for feed and faeces

About one gram of dry powdered sample of feed, faeces was converted to pellets using pellet-press.

Calculation of gross energy

The gross energy of the sample = ac/b kcal

Where,

a = Corrected reading of galvanometer b = Weight of the sample

c = Calibration constant

3.2.2.5.3 Procedure for the urine

Approximately, 10 ml of urine sample was taken in a glass beaker and its pH was adjusted to 6.0 with concentrated sulphuric acid. Ten ml of this urine was taken into a previously weighed butter paper sheet of known energy value in a crucible and kept in incubator for drying at 50°C. Dried urine was ignited in the same way as feed and deflection in the galvanometer was recorded. Calorific value of butter paper sheet was determined separately to get exact calorific value of urine.

3.2.2.6 Heat production

It was calculated using Brouwer equation

$HP(kcal/d) = 3.866\ O_2 + 1.2\ CO_2 - 0.518\ CH_4 - 1.431\ N_2$

Where,

O_2 = Volume of oxygen (l) consumed/d

CO_2 = Volume of carbon dioxide (l) produced/d

CH_4 = Volume of methane (l) produced /d

N_2 = Amount of nitrogen (l) excreted in urine g/d

3.2.2.6 Assesment of economic aspects of ajwain feeding

Cost of ajwain per animal per day was considered and the economic return in terms of body weight gain and the cost of concentrate used to supply equivalent amount of energy was taken into account for calculating economic aspect of feeding ajwain to buffaloes.

3.3 Statistical Analyses

The statistical analyses were performed as per standard methods by using SPSS computer package (SPSS version 20.0, SPSS Inc., Chicago, USA). The phase I data obtained was done using factorial (3x3) univariate ANOVA. The data of phase II were statistically analyzed by using one way ANOVA with Duncan post hoc testing to compare experimental groups. For all statistical analyses, probability values less than 0.05 were considered as significant.

Results

4 RESULTS

The present study was undertaken to explore the possibility of using a phytogenic feed additive ajwain as a rumen modifier, fed at two different levels to reduce methane emission and improve nutrient utilization along with assessing its effect on rumen fermentation, enzyme and microbial profile in buffaloes. Accordingly, the experiment was conducted in two phases.

Phase 1: Effect of ajwain feeding on rumen fermentation, enzyme and microbial profile of adult fistulated buffaloes

Phase 2: Effect of ajwain feeding on methane emission and nutrient utilization of buffalo calves

The results obtained in both phases of experiment are detailed in this section along with pertinent discussion so as to validate the same with sound scientific basis.

PHASE -1

4.1 Effect of ajwain feeding on rumen fermentation, enzyme and microbial profile of adult fistulated buffaloes

Three adult male Murrah buffaloes average body weight 475 kg with a permanent fistula were maintained and used in a 3X3 Switch Over Latin Square Design. The animals were fed on wheat straw and concentrate (50:50) to meet the maintenance requirement as per ICAR (2013) with the switch over of feeding regime after 21 days. The three groups (T1, T2 and T3) were fed on the diet without additive, with ajwain (1% of DMI) and with ajwain (2% of DMI), respectively. The rumen liquor/content was sampled for 2 consecutive days and analyzed for various rumen fermentation parameters besides microbial and enzyme profiles.

4.1.1 Chemical composition of feed offered

The chemical composition of concentrate mixture, wheat straw and ajwain used for the feeding of the buffalo calves is given in Table 4.1.1 The concentrate mixture, wheat straw and ajwain contained 15.80, 2.53 and 14.9% CP, respectively.

Table 4.1.1: Chemical Composition (% DM) of wheat straw and concentrate mixture and ajwain fed to fistulated animals

Attributes	Concentrate 1	Wheat straw	Ajwain
Organic matter	91.3	92.4	87.25
Crude protein	15.8	2.53	14.9
Ether extract	3.43	1.24	2.65
NDF	40.37	84.45	32.2
ADF	9.54	44.15	28.5
Total ash	8.7	7.56	12.75

4.1.2 Rumen fermentation patterns

4.1.2.1. pH, Ammonia nitrogen

The pH and ammonia nitrogen in the rumen liquor as affected by addition of ajwain (T1, T2 and T3) in the diet is presented in Table 4.1.2. Rumen pH was found to be comparable ($P>0.05$) among the groups indicating that feeding of ajwain had no effect on rumen environment. The mean values of rumen pH ranged from 6.72±0.05 to 6.82±0.08 . Nitrogen balance and rumen ammonia nitrogen are the most accessible parameters to describe nitrogen available to the animals and incorporated into animal tissues. The concentration of ammonia nitrogen (mg/dl) was similar ($P>0.05$) in all the three groups and ranged from 13.91±0.57 to 14.79±0.69.

Table 4.1.2: Effect of ajwain feeding on rumen pH and ammonia nitrogen in fistulated buffaloes

Attributes	T1	T2	T3	SEM	P value
pH	6.82±0.08	6.83±0.05	6.72±0.05	0.06	0.435
NH_3-N (mg/dl)	13.91±0.57	14.79±0.69	14.32±0.57	0.61	0.609

T1: without additive; T2: with ajwain (1% of DMI);T3: with ajwain (2% of DMI); SEM: standard error of mean

4.1.2.2 Rumen enzyme activities

Results of ajwain feeding on enzyme activities in rumen content of fistulated male buffaloes are presented in Table 4.1.3. The mechanism by which PSM inhibit or stimulate any enzyme could be explained by a change in the conformation of the enzyme in the presence of PSM, so as to increase or decrease the availability of substrate at the catalytic site of the enzyme. The feeding of ajwain had no effect (P>0.05) on the activities of rumen enzymes and the (U/mg protein) values of enzymes ranged from 0.33±0.06 to 0.38±0.04; 0.14±0.02 to 0.15±0.02 and 1.22±0.08 to1.25±0.09 for carboxymethylcellulase, avicelase and, xalanase respectively.

Table 4.1.3: Effect of ajwain feeding on enzyme activities (U/mg protein)in rumen content of fistulated buffaloes

Attributes	T1	T2	T3	SEM	P value
CMCase	0.37±0.04	0.33±0.06	0.38±0.04	0.05	0.729
Avicelase	0.15±0.02	0.14±0.02	0.15±0.02	0.02	0.837
Xylanase	1.25±0.09	1.25±0.08	1.22±0.08	0.09	0.952

Unit = µmol of glucose released/min/ml for CMCase and avicelase; µmol of xylose released/min/ml for xylanase; T1: without additive; T2: with ajwain (1% of DMI);T3: with ajwain (2% of DMI); SEM: standard error of mean

4.1.2.3 Protozoal population count

The population of ciliate protozoa in the rumen liquor of fistulated buffaloes is presented in Table 4.1.4. No significant (P>0.05) difference in protozoal numbers was observed due to ajwain feeding in treatment groups as compared to the control group. The $\log_{10}$ values varied from 4.04±0.04 to 4.08±0.04 ; 5.25±0.03 to 5.31±0.04 and 5.28±0.03 to 5.34±0.03 for holotrichous, oligotrichs and total protozoa respectively.

Table 4.1.4: Effect of ajwain feeding on Log_{10} value of ciliate protozoa population in rumen liquor of fistulated buffaloes

Attributes	T1	T2	T3	SEM	P value
Total	5.31±0.01	5.34±0.03	5.28±0.03	0.02	0.237
Oligotrichs	5.29±0.01	5.31±0.04	5.25±0.03	0.03	0.268
Holotrichs	4.08±0.04	4.04±0.04	4.04±0.04	0.04	0.716

T1: without additive; T2: with ajwain (1% of DMI);T3: with ajwain (2% of DMI); SEM: standard error of mean

4.1.2.4 Microbial profile

The horizontal agarose gel electrophoresis of DNA extracted from rumen sample showed well defined DNA bands without any shearing indicating that the DNA was well intact and was of good quality to proceed for qPCR (Figure 4.1.1). The rumen microbial population were enumerated by real time PCR. The amplification pattern and melting curves of various rumen microbial population are depicted in figures (Figure 4.1.2 to 4.1.6). The well defined sigmoid curves were there for all the microbial population during amplification indicating normal pattern of amplification. The melting curves of the microbial population had single peak which shows that the primers used in the present study to enumerate the rumen microbes were highly specific for the respective species with no non-specific binding. The changes in population density of various rumen microbes at 0 h feeding as estimated by real time PCR is presented in Table 4.1.5. Feeding of ajwain had no significant difference ($P>0.05$) on the population of total bacteria, protozoa, fungi, methanogens, Fibrobacter *succinogenes* and their $\log_{10}$ values ranged from 10.8±0.46 to 11.49±0.47; 9.6±0.32 to 9.7±0.38; 8.21±0.20 to 8.47±0.21; 8.15±0.51 to 8.33±0.2; 8.79±0.39 to 9.11±0.19 respectively.

Table 4.1.5: Effect of ajwain feeding on Log_{10} value of microbes in rumen liquor of fistulated buffaloes

Attributes	T1	T2	T3	SEM	P value
Total bacteria	11.49±0.47	11.22±0.13	10.8±0.46	0.39	0.460
Protozoa	9.7±0.38	9.66±0.18	9.6±0.32	0.31	0.972
Fungi	8.44±0.34	8.21±0.2	8.47±0.21	0.26	0.749
Methanogens	8.33±0.2	8.2±0.25	8.15±0.51	0.35	0.927
F. succinogenes	8.79±0.39	9.11±0.19	8.9±0.34	0.32	0.778

T1: without additive; T2: with ajwain (1% of DMI);T3: with ajwain (2% of DMI); SEM: standard error of mean

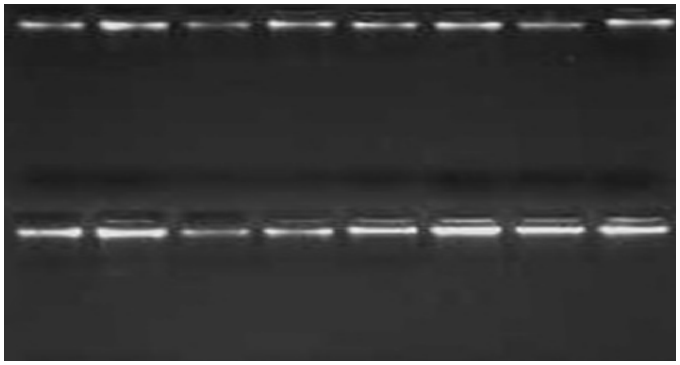
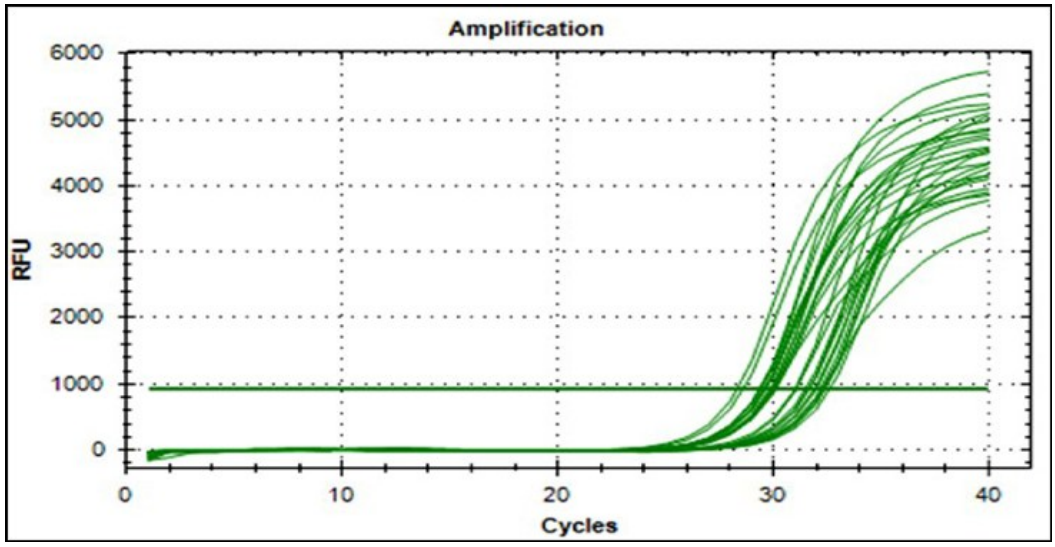


Fig. 4.1.1: Agarose gel electrophoresis of DNA of microbes



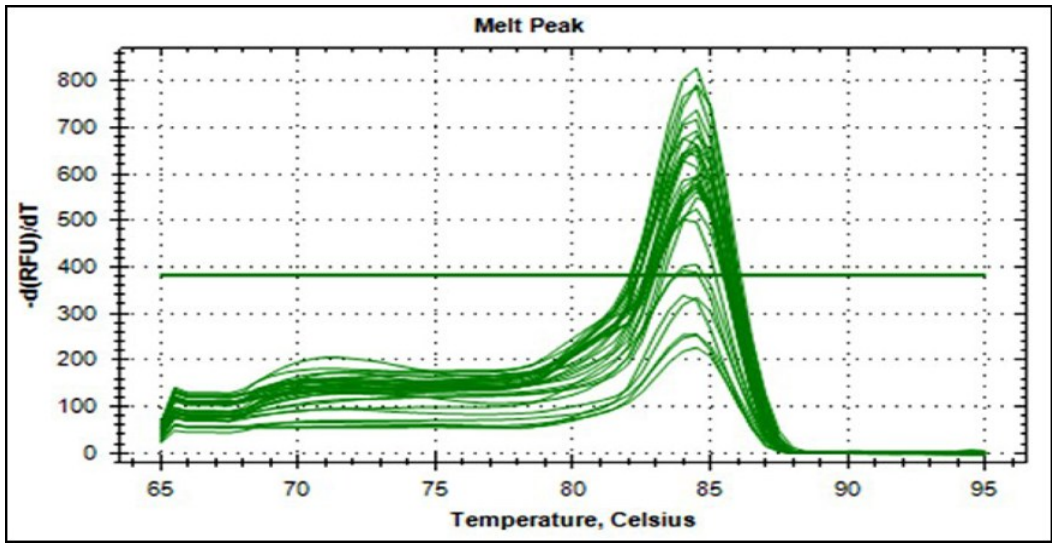


Fig. 4.1.2: Amplification curve and Melting Peak of Bacteria

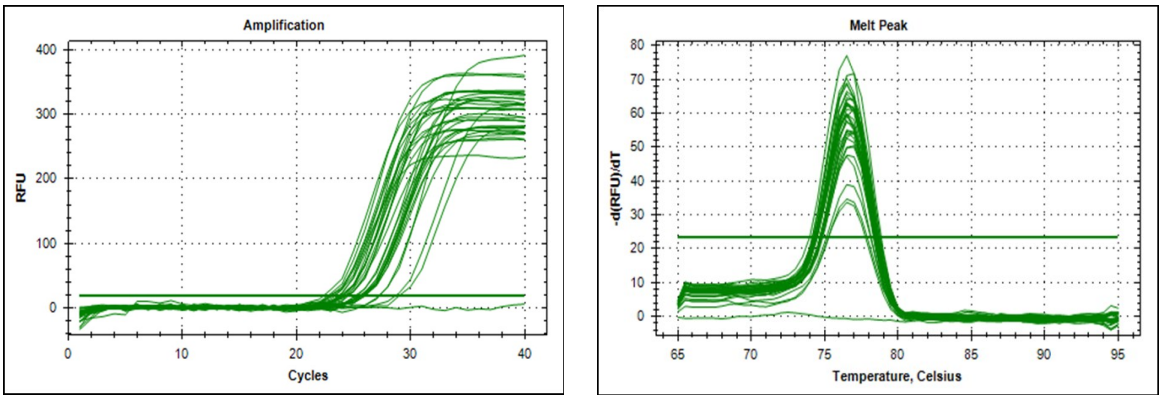


Fig. 4.1.3: Amplification curve and Melting Peak of fungus

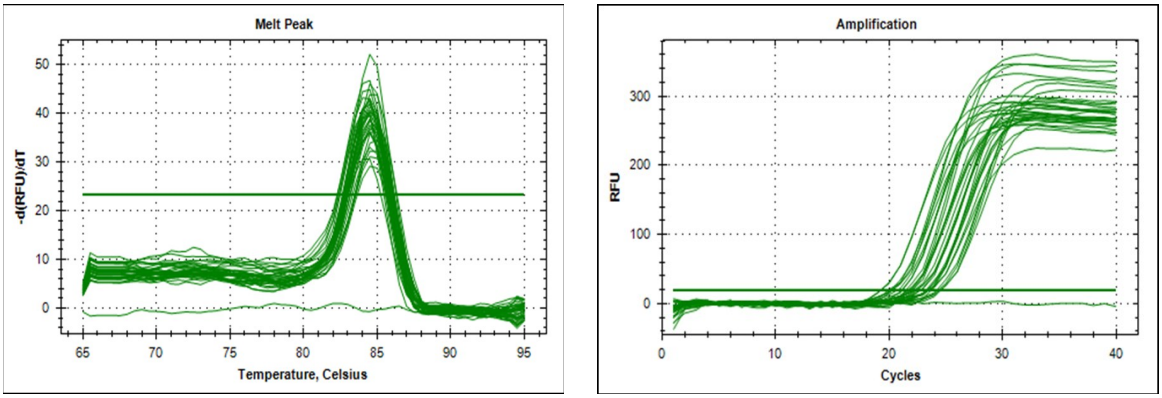


Fig. 4.1.4: Amplification curve and Melting Peak of *Fibrobacter succinogenes*

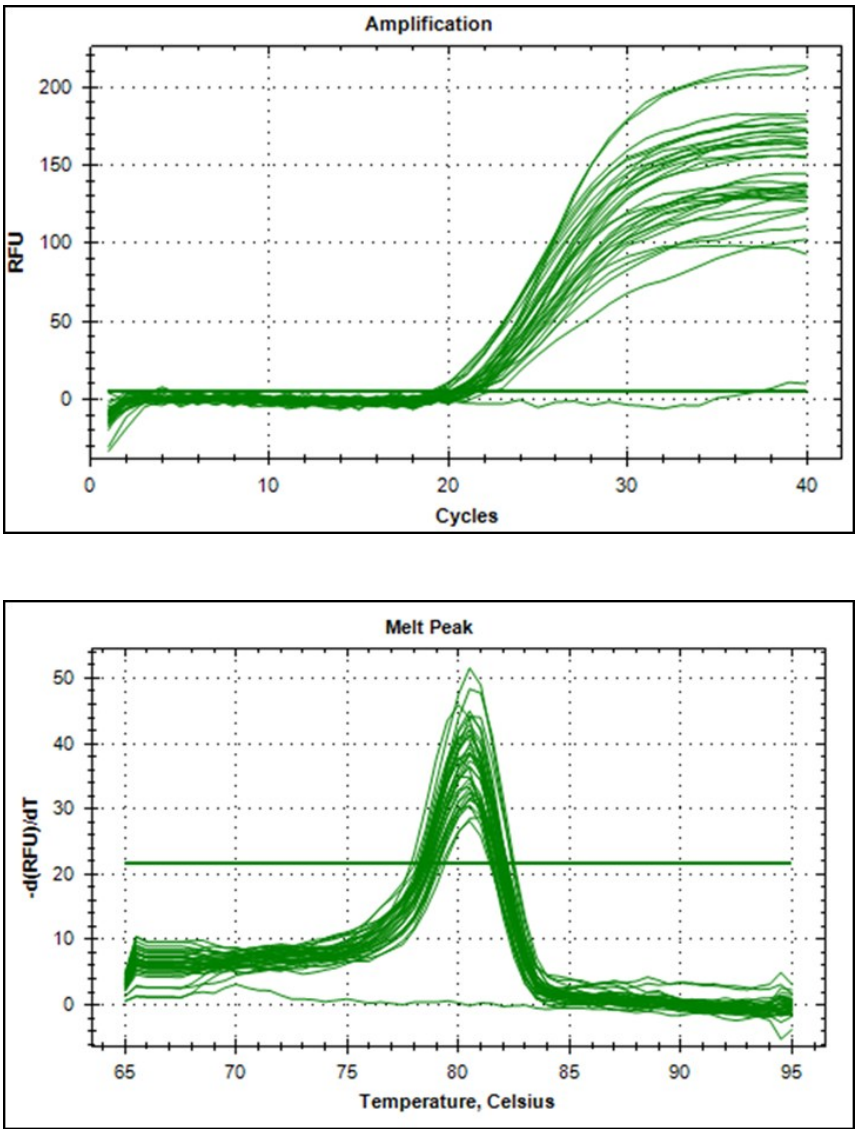


Fig. 4.1.5: Amplification curve and Melting Peak of methanogens

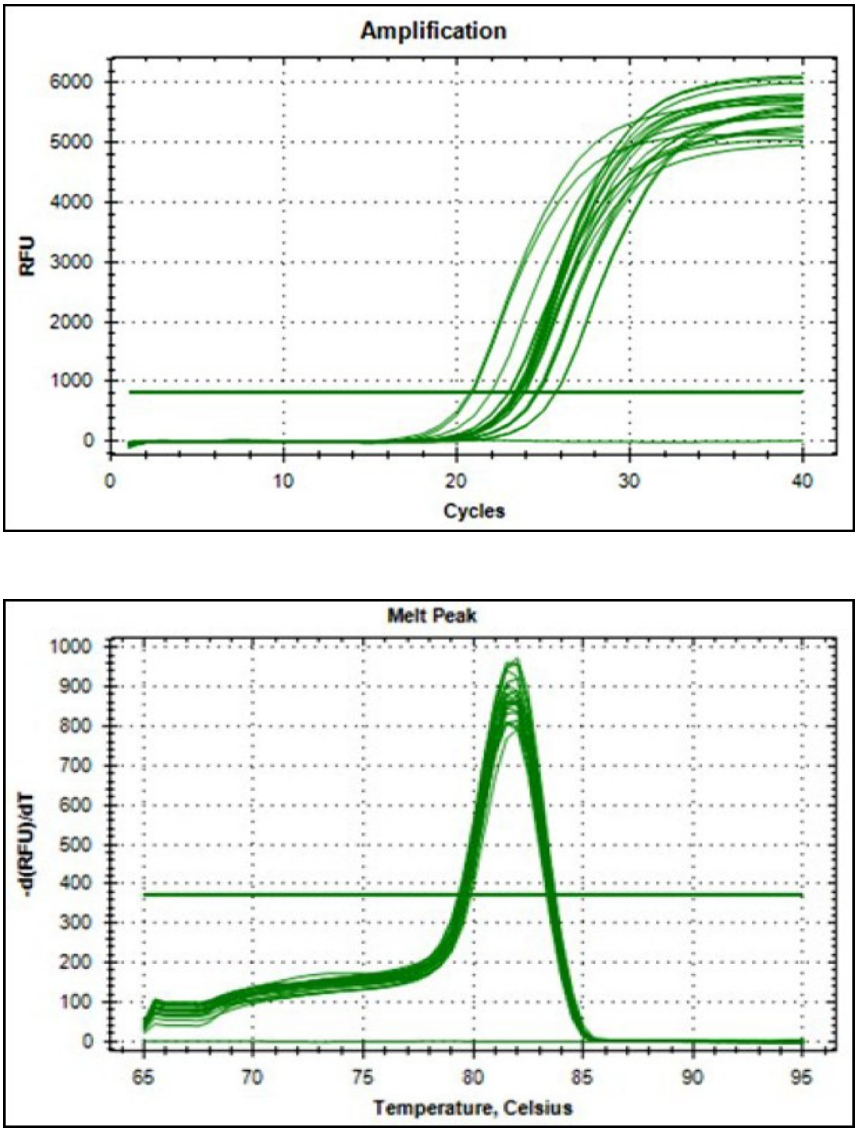


Fig. 4.1.6: Amplification curve and Melting Peak of protozoa

PHASE -2

4.2. Effect of ajwain feeding on methane emission and nutrient utilization of buffalo calves

To study the effect of ajwain on methane production and nutrient utilization, fifteen male buffalo calves were divided into three groups of five animals each in Completely Randomized Design (CRD). The three groups (T1, T2 and T3) were fed on the diet without additive, with ajwain (1% of DMI) and with ajwain (2% of DMI), respectively.

4.2.1 Chemical composition of feed offered

The chemical composition of concentrate mixture, wheat straw and ajwain used for the feeding of the buffalo calves is given in Table 4.2.1 The concentrate mixture, wheat straw and ajwain contained 18.51, 2.53 and 14.9% CP, respectively.

Table 4.2.1: Chemical composition (% DM) of wheat straw and concentrate mixture fed to buffalo calves

Attributes	Concentrate 2	Wheat straw	ajwain
Organic matter	91.98	92.4	87.25
Crude protein	18.51	2.53	14.9
Ether extract	3.32	1.24	2.65
NDF	40.44	84.45	32.2
ADF	10.38	44.15	28.5
Total ash	8.02	7.56	12.75

4.2.2 Feed intake and body weight change

The examination of data over 1 months of experimental feeding revealed that the feeding ajwain to the animals did not impart any effect on the growth pattern of the buffaloes. The animals of all the three groups grew from their initial BW (kg) of 257.9±12.17, 259.2±16.87, 265±18 to the final BW(kg) of 270.9±11.46, 272.3±16.57 and 279.4±17.54 at the end of 1 month of feeding trial .The daily feed intake during the entire study is presented in Table 4.2.2. The initial weight, final weight and body weight gain in 1 month duration was similar among the groups. The feed intake as well as feed intake per unit gain was also similar among the groups

Table 4.2.2: Effect of ajwain feeding on body weight change and feed intake of buffalo calves

Attributes	T1	T2	T3	SEM	P value
Initial BW (kg)	257.9±12.17	259.2±16.87	265±18	8.53	0.945
Final BW (kg)	270.9±11.46	272.3±16.57	279.4±17.54	8.30	0.917
BW change (gm/day)	433.3±47.1	436.6±17.79	479.9±17.7	17.44	0.510
Concentrate (kg/d)	3.09±0.02	3.05±0.03	3.11±0.02	0.01	0.273
WS (kg/d)	2.55±0.06	2.68±0.03	2.53±0.05	0.03	0.106
TDMI (kg)	5.64±0.07	5.73±0.04	5.64±0.04	0.03	0.372
DMI (%BW)	2.2±0.1	2.25±0.14	2.17±0.16	0.07	0.919
FCR	13.17±1.47	12.76±0.57	11.39±0.46	0.55	0.410

T1: without additive; T2: with ajwain (1% of DMI);T3: with ajwain (2% of DMI); SEM: standard error of mean

4.2.3 Plane of nutrition

The plane of nutrition of the animals during metabolism trial is presented in Table 4.2.3. The body weights were comparable (P>0.05) among the groups. The intake of DM through wheat straw and concentrate mixture was maintained to 50:50 ratio in the animals of all the three groups. The CP intake for all the three groups was in the range of 627.71±5.25 to 633.77±3.38 g/d. The DCP and TDN intake ranged from 387.83±23.66 to 397.54±13.38 g/d and 3.56±0.11 to 3.7±0.08 kg/d, respectively. The data reveals that the animals of all the three groups were on the similar plane of nutrition as the intake of DM, CP and TDN were unaffected among the groups. The CP and TDN intake was as per the requirement recommended by the feeding standard of ICAR (2013).

Table 4.2.3: Effect of ajwain feeding on plane of nutrition of buffalo calves during metabolism trial

Attributes	T1	T2	T3	SEM	P value
B W (kg)	270.9±11.46	272.3±16.57	279.4±17.54	8.3	0.917
BW (kg $W^{0.75}$)	66.73±2.13	66.94±3.08	68.24±3.24	1.54	0.922
Dry matter intake					
Kg/d	5.44±0.07	5.53±0.04	5.44±0.04	0.03	0.372
g/kg $W^{0.75}$	81.79±2.53	83.29±3.43	80.43±3.99	1.83	0.837
% BW	2.02±0.08	2.06±0.12	1.98±0.13	0.06	0.877
Crude protein intake					
g/d	630.73±4.26	627.71±5.25	633.77±3.38	2.42	0.629
g/kg $W^{0.75}$	9.48±0.27	9.45±0.39	9.37±0.41	0.1	0.973
% BW	0.234±.01	0.233±0.13	0.230±0.14	0.006	0.97
DCP intake					
g/d	387.83±23.66	385.47±9.09	397.54±13.38	8.95	0.863
g/kg $W^{0.75}$	5.82±0.33	5.8±0.26	5.9±0.42	0.18	0.976
TDN intake					
Kg/d	3.56±0.11	3.7±0.08	3.58±0.07	0.05	0.515
g/kg $W^{0.75}$	53.52±2.18	55.56±2.11	52.93±3.11	1.37	0.745

T1: without additive; T2: with ajwain (1% of DMI);T3: with ajwain (2% of DMI); SEM: standard error of mean

4.2.4 Intake and digestibility of various nutrients

The daily consumption of various nutrients along with their faecal excretion and apparent digestibility, assessed through a metabolism trial are presented in Table 4.2.4 There was no significant difference (P>0.05) among the groups. Digestibility (%) of DM was similar in all the three groups being, 66.93±1.55, 68.93±1.5, 68.19±1.78 in T1, T2 and T3, respectively. Digestibility % of CP and EE varied from 61.41±1.40 to 62.7±2.22 and 76.82±0.99 to 78.86±1.46. Digestibility % of NDF and ADF varied from 57.27±2.42 to 59.75±1.77 and 47.7±2.73 to 50.48±2.59 respectively. All the parameters were in normal range.

Table 4.2.4: Effect of ajwain feeding on intake and apparent digestibility of nutrients during metabolism trial

Attributes	T1	T2	T3	SEM	P value
Dry Matter					
Intake (kg/d)	5.44±0.07	5.53±0.04	5.44±0.04	0.03	0.357
Digested (kg/d)	3.64±0.11	3.81±0.09	3.66±0.11	0.06	0.462
Digestibility (%)	66.93±1.55	68.93±1.5	68.19±1.78	0.89	0.684
Organic Matter					
Intake (kg/d)	5.00±0.06	5.09±0.03	5.00±0.04	0.03	0.355
Digested (kg/d)	3.43±0.10	3.6±0.08	3.43±0.10	0.05	0.542
Digestibility (%)	68.55±1.6	70.06±1.51	68.57±1.86	0.91	0.767
Crude Protein					
Intake (g/d)	630.74±4.27	627.71±5.25	633.77±3.38	2.42	0.629
Digested (g/d)	387.84±23.66	385.46±9.09	397.54±13.38	8.95	0.863
Digestibility (%)	61.42±3.45	61.41±1.40	62.7±2.22	1.35	0.912
Ether Extract					
Intake (g/d)	131.66±1.14	132.13±0.94	132.05±0.67	0.5	0.931
Digested (g/d)	101.14±1.66	102.68±2.09	104.12±1.79	1.04	0.543
Digestibility (%)	76.82±0.99	77.7±1.41	78.86±1.46	0.73	0.556
Neutral detergent fibre					
Intake (kg/d)	2.798±0.05	2.898±0.03	2.786±0.04	0.03	0.146
Digested (kg/d)	1.615.±0.07	1.731±0.05	1.597±0.07	0.04	0.382
Digestibility (%)	57.65±2.03	59.75±1.77	57.27±2.42	1.15	0.674
Acid detergent fibre					
Intake (kg/d)	1.347±0.02	1.400±0.01	1.340±0.02	0.01	0.131
Digested (kg/d)	0.64±0.04	0.70±0.03	0.64±0.05	0.03	0.581
Digestibility (%)	47.7±2.73	50.48±2.59	48.03±4.01	1.73	0.800

T1: without additive; T2: with ajwain (1% of DMI);T3: with ajwain (2% of DMI); SEM: standard error of mean

4.2.5 Nitrogen balance

Effect of feeding ajwain on nitrogen balance is presented in Table 4.2.5 Intake of nitrogen by buffaloes was similar among the groups. The nitrogen voided through urine was higher than nitrogen voided through faeces in all the groups being in the range of 41.75±0.8 to 42.72±0.34 g. During the experimental period, buffalo calves in all the three groups were in positive nitrogen balance. The mean group levels pertaining to nitrogen balance in T1, T2 and

T3 were 20.3±3.59, 21±1.62 and 20.89±2.38 g/d. This indicated that supplementation of the ajwain had no detrimental effect on the nitrogen balance of the animals.

Table 4.2.5: Effect of ajwain feeding on nitrogen balance in buffalo calves

Attributes	T1	T2	T3	SEM	P value
N intake (g/day)	100.92±0.68	100.43±0.84	101.41±0.54	0.39	0.627
N intake (g/kgW$^{0.75}$)	1.52±0.04	1.51±0.06	1.5±0.07	0.03	0.963
N void in faeces (g/day)	38.87±3.34	37.44±2.36	37.8±2.33	1.46	0.928
N loss in urine (g/day)	41.75±0.8	42±0.71	42.72±0.34	0.36	0.563
Total N loss (g/day)	80.62±3.19	79.44±2.05	80.52±2.62	1.43	0.942
N balance (g/day)	20.3±3.59	21±1.62	20.89±2.38	1.42	0.980
N balance (g/kgW$^{0.75}$)	0.30±0.05	0.31±0.03	0.31±0.044	0.01	0.972
N balance as % intake	20.06±3.46	20.93±1.67	20.62±2.39	1.61	0.972
N balance as % N dig.	31.79±3.98	33.15±1.66	32.45±2.91	1.40	0.951

T1: without additive; T2: with ajwain (1% of DMI);T3: with ajwain (2% of DMI); SEM: standard error of mean

4.2.6 Methane emission

Effects feeding of ajwain on methane emission during open circuit respiration calorimeter study are given in Table 4.2.6. DMI in all the three groups was found to be comparable (P>0.05). The mean values of methane emission (l/d) and (l/kg DMI) varied from127.13±6.51 to 142.06±5.43 and 24.09±1.33 to 28.15±1.07 respectively. There was no difference (P>0.05) in the average DMI (kg/d) of buffaloes. Methane emission in terms of l/d and l/kg DMI were comparable among the three groups. Methane emission in terms of l/kg DDMI was 42.54±1.20, 38.34±1.74 and 34.72±2.03 in T1, T2 and T3 groups, respectively which showed a significant reduction (P<0.05) of 18% in animals of T3 group as compared to T1 (Fig. 4.1.7). There was an improved net energy retention in the treatment groups.

Table 4.2.6: Effect of ajwain feeding on methane emission in buffalo calves

Attributes	T1	T2	T3	SEM	P value
BW(kg)	272.50±7.03	276.75±9.23	281±12.15	5.17	0.829
BW (kg $W^{0.75}$)	67.06±1.30	67.83±1.70	68.60±2.23	0.95	0.833
DMI(kg)	5.06±0.17	5.13±0.28	5.29±0.18	0.12	0.748
Methane emission					
l/d	142.06±5.43	134.00±7.25	127.13±6.51	3.83	0.308
l/kgDMI	28.15±1.07	26.20±0.82	24.09±1.33	0.76	0.078
l/kg DDMI	42.54^{a}±1.20	38.34^{ab}±1.74	34.72^{b}±2.03	1.31	0.029
l/ kg $W^{0.75}$	2.12±0.10	1.99±0.15	1.87±0.15	0.08	0.444
Kcal/ kg $W^{0.75}$	20.04±0.89	18.75±1.37	17.7±1.47	0.72	0.457

T1: without additive; T2: with ajwain (1% of DMI);T3: with ajwain (2% of DMI); SEM: standard error of mean Means bearing different superscripts (a to b) in a row differ significantly ($p<0.05$)

4.2.7 Energy metabolism

The faecal energy, urinary energy and methane energy losses were not affected due to feeding of ajwain Table 4.2.7. There was no significant difference in the GE intake, faecal and urinary loss and digestible, metabolizable and net energy among the groups. Non-significant difference in methane loss as % of GE was 6.6±0.2, 6.44±0.26, 5.85±0.23 in T1, T2 and T3 groups, respectively. The net energy retention in T1, T2 and T3 groups was 2.01±0.46, 2.35±0.51 and 2.48±0.33, respectively, indicated no influence of ajwain feeding on the energy balance.

Tab le 4.2.7: Effect of ajwain feeding on energy utilization of buffalo calves

Attributes	T1	T2	T3	SEM	P value
GE Intake (Mcal/d)	20.32±0.24	19.66±0.46	20.6±0.21	0.2	0.149
FE loss (Mcal/d)	6.47±0.22	5.93±0.81	6.56±0.55	0.32	0.717
DE (Mcal/d)	13.85±0.26	13.73±0.72	14.04±0.4	0.27	0.904
UE loss (Mcal/d)	1.81±0.11	1.82±0.12	1.64±0.11	0.06	0.454
Methane loss (Mcal/d)	1.34±0.04	1.27±0.05	1.2±0.05	0.03	0.166
Methane loss as % of GEI	6.6±0.2	6.44±0.26	5.85±0.23	0.15	0.086
ME (Mcal/d)	10.69±0.36	10.64±0.82	11.2±0.49	0.32	0.768
ME as % of GE	52.62±1.59	54.21±4.05	54.45±2.83	1.62	0.896
HP (Mcal/d)	8.63±0.27	8.56±0.5	8.25±0.33	0.20	0.762
NE (Mcal/d)	2.01±0.46	2.35±0.51	2.48±0.33	0.24	0.745

T1: without additive; T2: with ajwain (1% of DMI);T3: with ajwain (2% of DMI); SEM: standard error of mean

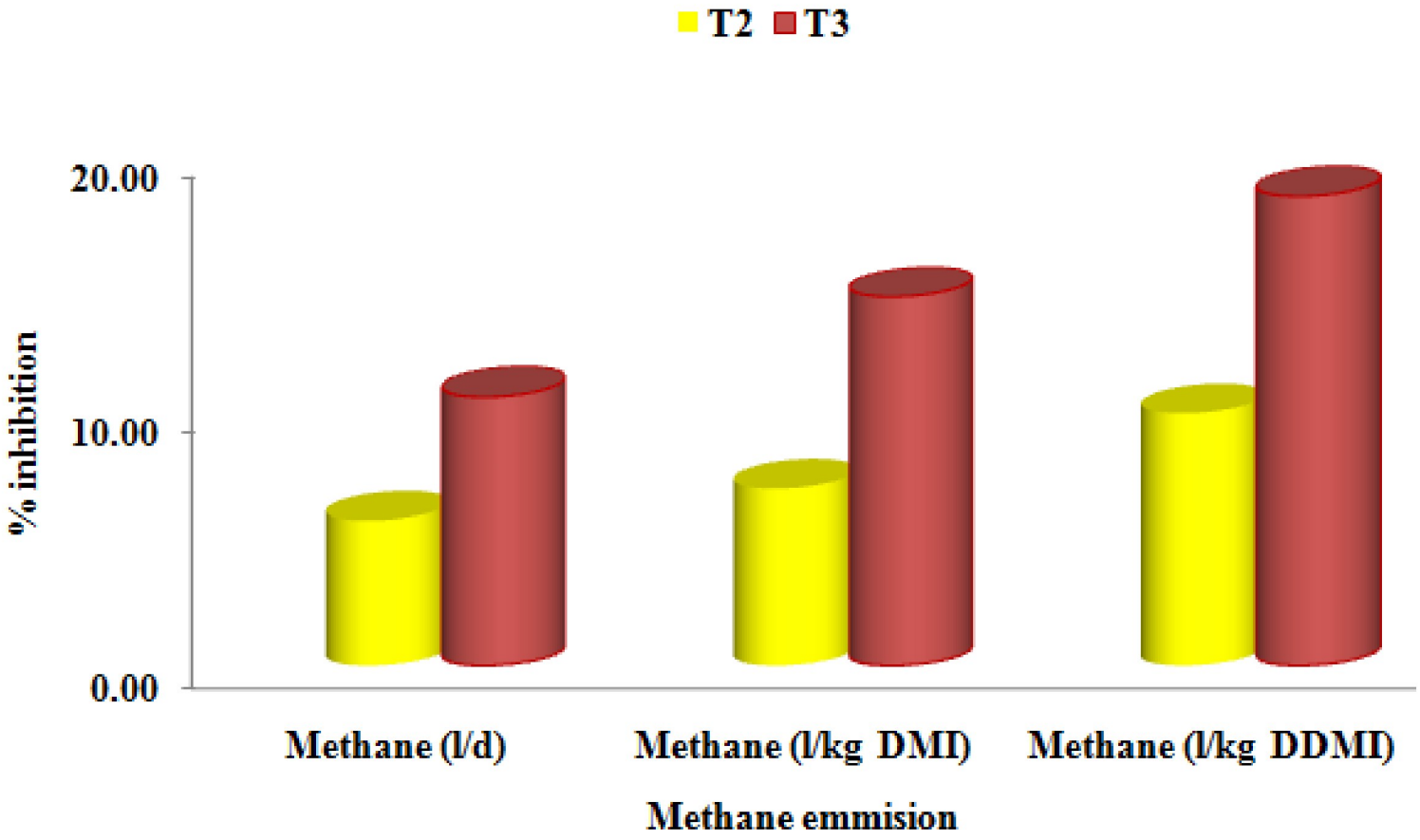


Fig. 4.1.7:Effect of ajwain on % inhibition in methane production

3.2.2.6 Assesment of economic aspects of ajwain feeding

Cost of ajwain per animal per day was considered and the economic return in terms of body weight gain and the cost of concentrate used to supply equivalent amount of energy was taken into account for calculating economic aspect of feeding ajwain to buffaloes. Since, this experiment was for short duration therefore, proper economics calculation was not possible because there was no significant change in body weight gain. Therefore, higher dose of ajwain must be tried with higher number of animals.

Discussion

5 DISCUSSION

The present study was undertaken to explore the possibility of using ajwain as a phytogenic feed additive, fed at two different levels to reduce methane emission and improve nutrient utilization in ruminants along with assessing its effect on rumen fermentation, enzyme and microbial profile of buffaloes. Accordingly, the experiment was conducted in two phases.

Phase 1: Effect of ajwain feeding on rumen fermentation enzyme and microbial profile of adult fistulated buffaloes

Phase 2: Effect of ajwain feeding on methane emission and nutrient utilization of buffalo calves

The discussion of results obtained in both phases of experiment are presented so as to validate the same with sound scientific basis.

Phase 1

5.1 Effect of ajwain feeding on rumen fermentation, enzyme and microbial profile of adult fistulated buffaloes

Three adult male Murrah buffaloes average body weight 475 kg with a permanent fistula were maintained and used in a 3X3 Switch Over Latin Square Design. The animals were fed on wheat straw and concentrate (50:50) to meet the maintenance requirement as per ICAR (2013) with the switch over of feeding regime after 21 days. The three groups (T1, T2 and T3) were fed on the diet without additive, with ajwain (1% of DMI) and with ajwain (2% of DMI), respectively.

5.1.1 Chemical composition of feed offered

The composition of feed and fodders used for feeding is given in Table 4.1.1. The chemical composition of the concentrate mixture and wheat straw used for feeding of buffaloes was within the normal range. The concentrate mixture, wheat straw and ajwain contained 15.80%, 2.53% and 14.9% CP, respectively The composition of all the nutrients matched the maintainance nutrient requirement profile of buffaloes ICAR (2013).

5.1.2 Rumen fermentation patterns

5.1.2.1 pH and ammonia nitrogen

The mean value of rumen pH and ammonia N presented in Table no.4.1.2 was within normal range in all three groups indicating that feeding of ajwain as additive had no effect on rumen environment. Pawar (2012) reported that feeding of ajwain oil (2 ml/d) as an additive did not affect ($P > 0.05$) rumen fluid pH at any post feeding time intervals. Patra (2004) also reported no change in rumen pH following supplementation of harad, garlic and a mixture of two. Hosoda *et al.* (2006) also did not report any change in rumen pH on peppermint feeding. In agreement with our finding no change in pH was also observed by Beauchemin and McGinn (2006) with feeding of essential oils (Crina Ruminants; 1g/d) in cattle, but Benchaar *et al.* (2006b) observed increase in ruminal pH (6.50 *vs.* 6.39) in lactating cows fed on diet supplemented with essential oils (Crina Ruminants; 2 g/d). Yildiz *et al.* (2005) reported that ruminal pH did not differ ($P>0.05$) between the groups of lambs receiving oak (*Quercus hartwissiana*) leaves with and without PEG. Garlic oil (GO) in different doses (0, 20, 60, 180 or 540 mg/L of incubation medium) was analyzed to observe the effects on *in vitro* fermentation of two diets comprised of medium-concentrate (MC; 500:500 alfalfa hay: concentrate) and high-concentrate (HC; 150: 850 barley straw: concentrate) using rumen fluid from eight Merino sheep (56.1±2.80 kg) (Kamel *et al*., 2009). There was no effects of GO on pH and concentration of ammonia-N. No change in pH was also observed by Kumar (2007) and Zadbuke (2009) with feeding of mixture plant parts as feed additives. Wanapat *et al.* (2008) observed no change in rumen pH by feeding garlic powder. Samal (2012) reported no affect on ruminal pH due to supplementation of feed additives of plant origin in growing male buffalo

calves. Ruminal pH and temperature were not (P>0.05) affected by eucalyptus (*E. camaldulensis*) crude oils supplementation in rice straw base diet of swamp buffaloes (Thao *et al.*, 2014). In contrast Wanapat *et al*. (2013) observed an increase in ruminal pH in all supplemented groups of rumen fistulated crossbred beef cattle (Brahman native) fed plant herb supplements in their concentrate mixture. Anantasook *et al*. (2013) reported that supplementations of rain tree pod meal (RPM) (0 and 60 g/kg of total DM intake) to basal diet comprising of two roughage to concentrate ratios (R:C, 60:40 and 40:60) to dairy steers, the ruminal pH decreased while ruminal temperature increased with high concentrate ratio (R:C40:60) ($p<0.05$). Perme (2014) reported that none of the dietary treatments in 4 groups of fistulated buffaloes, *viz.*CON (control with no additive), T1 (harad+garlic @ 2% of DMI), T2 (*S. cerevisiae,* 100g fermented feed containing 10^6 cfu/g) and T3 (combination of T1 and T2) affected (P>0.05) rumen pH, ammonia nitrogen.

Nitrogen balance and rumen ammonia nitrogen are the most accessible parameters to describe nitrogen available to the animals and incorporated into animal tissues. The concentration of ammonia N (mg/dl) was similar (P>0.05) in all the three groups.

In the present study ammonia nitrogen was not influenced by feeding of ajwain as additive. Similarly, Yang *et al.* (2007) reported that addition of essential oils had no effect on ruminal fluid concentration of ammonia nitrogen. Patra (2010) reported a significantly ($P<0.05$) lower level of ammonia nitrogen in garlic fed fistulated buffaloes. Min *et al.* (2002) reported that action of CT of *Lotus corniculatus*, markedly reduced rumen proteolytic activity and rumen ammonia concentration in sheep. Bermingham *et al.* (2001) found decreased ammonia nitrogen concentration in sheep rumen fed sainfoin which contained 38 g CT/kg DM.

The above discussion revealed that the effect of PSM on nitrogen metabolism is governed by the type of PSM, dose of PSM and also the conditions of the experiment. In the present study, no effect was seen on rumen ammonia N concentration which might be due to reduced dose of PSM (ajwain) or due to differences of active metabolites or their interaction

5.1.3. Rumen enzyme activities

Results of ajwain feeding on enzyme activities in rumen content of fistulated male buffaloes are presented in Table 4.1.3. Fibre degrading enzymes are exclusively secreted by

the microbes which provide microbes the ability to degrade fibre and because of these microbes, ruminants can utilize fibrous diet. A rumen is an efficient fermenter and it has an active, diversified enzyme profile. Therefore, for rumen manipulation, the microbial enzymes are the ultimate targets. Changing enzyme profile by feeding any of the additive will reflect the efficiency of rumen functioning. The mechanism by which PSM inhibit or stimulate any enzyme could be explained by a change in the conformation of the enzyme in the presence of plant bioactive compound. Activity of ruminal enzymes *viz.* carboxymethyl-cellulase, avicelase, xylanase and were not affected (P>0.05) by addition of ajwain as additive to the diet of fistulated buffaloes.

Similarly, no effect on enzyme activity was observed with addition of a combination of plants containing secondary metabolites by Kumar (2007) and Zadbuke (2009). Patra (2004) supplemented harad, garlic and a mixture of the two to fistulated buffaloes and reported no change in rumen enzyme activities. In contrast to the present study, Patra *et al.* (2010) reported that activities of carboxymethyl-cellulase and xylanase were reduced by inclusion of the extracts of clove and garlic in *in vitro* gas production system which might due to the high levels of secondary metabolites present in the extracts. Paul *et al.* (2003) reported, inhibition of the activities of carboxymethyl cellulase, xylanase, acetyl esterase and proteases by phenolic monomers such as catechol. Patra *et al.* (2006a) reported the effect of seed pulp of *T. chebula* (harad) rich in tannins at the level of 10, 20, 30 mg/200 mg substrate and found no change in the activities of CMCase and xylanase whereas esterase activity was decreased significantly (P<0.05), using *in vitro* gas production system. Patra *et al.* (2006b) tested the effect of water, ethanol and methanol extracts of seed pulp of *T. chebula* on enzymatic activity and found that the specific activity of CMCase was not affected by the extract whereas specific activity of xylanase and acetylesterase was reduced significantly (P<0.05). Similarly, Samal (2012) also reported no effect on ruminal enzymes activities due to supplementation of feed additives of plant origin in growing male buffalo calves. Perme (2014) reported that in all dietary treatments in 4 groups of fistulated buffaloes , *viz.*CON (control with no additive), T1 (harad+garlic @ 2% of DMI), T2 (*S. cerevisiae,* 100g fermented feed containing 10^6 cfu/g) and T3 (combination of T1 and T2) The activities of ruminal enzymes *viz.* carboxymethylcellulase, avicelase, xylanase and acetyl esterase activity were not affected (P>0.05) by addition of any of the feed additives.

5.1.4 Protozoal population count

The population of ciliate protozoa in the rumen liquor of fistulated buffaloes is presented in Table 4.1.4. Rumen methanogens are associated with the protozoa therefore, protozoa play an important role in methanogenesis as they are the main hydrogen supplier. Any change in protozoa population can alter the extent of methane production. The additives which can reduce protozoa population can be categorized as methane inhibitors. But feeding of ajwain as additive in the present study did not have any effect on population of ciliate protozoa in the rumen liquor of fistulated buffaloes. Similar to our present finding, Hart *et al.* (2006) and Benchaar *et al.* (2007) reported that ruminal protozoa counts were not affected when sheep and dairy cows were fed 110 and 750 mg/d of a mixture of EO. Supplementation of dairy cows diet with 0.5 g of cinnamaldehyde had no effect on the number of ciliate protozoa (Fraser *et al.*, 2007). Yang *et al.* (2010) also observed that cinnamaldehyde supplemented at the rate of 0.4 to 1.6 g/d in steers did not affect total protozoa in the rumen.

5.1.5. Microbial profile by real time PCR

The changes in population density of various rumen microbes at 0 h feeding as estimated by real time PCR is presented in Table 4.1.5. In ruminants, it is the microbial ecosystem present in the rumen which enables them to extract energy from the poor quality lignocellulosic feed and convert it into utilizable energy source. The microbial community structure of rumen microenvironment dictates the performance of the animal as they are the main source of energy and protein for the host animal. The rumen microbial community is very sensitive to the type of diet taken by the animal and any change in the diet or inclusion of any additive in the diet alter the microbial populations. Therefore, shifts in various microbial population by changing the feeding pattern or an additive will reflect the efficiency of rumen. In the present study the key fibre degrading microbes and methanogens were considered as representative of rumen microbial community to study the fibre degrading capacity and methanogenesis in the rumen. Perme (2014) reported that in all dietary treatments in 4 groups of fistulated buffaloes , *viz*.CON (control with no additive), T1 (harad+garlic @ 2% of DMI), T2 (*S. cerevisiae,* 100g fermented feed containing 10^6 cfu/g) and T3 (combination of T1 and T2) The population density of

different microbes like total bacteria, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, methanogens, fungi and ciliate protozoa were assessed by qPCR (Real time PCR). None of the microbes were influenced by supplementation of any of the feed additives.

5.1.5.1. Bacteria population

The population of total bacteria and *Fibrobacter succinogenes,* was not altered due to feeding of ajwain as the additives.

Anantasook *et al*. (2013) reported that total bacterial, *R. albus* and viable proteolytic bacteria were not affected, but cellulolytic bacteria, *F. succinogenes* and *R. flavefaciens* were higher while amylolytic bacteria was lower by dietary supplementation of rain tree pod meal (RPM) containing crude tannins and saponins at 84 and 143 g/kg of DM, respectively. The population density of key fibre degrading microorganisms gave different response to the feeding of ajwain oil and cinnamon leaf oil, as EO feeding decreased ($P<0.01$) the population of *F. succinogenes* but did not affect ($P>0.05$) population of *R. flavefaciences* and *R. albus* (Pawar, 2012). Wang *et al.* (2004) observed no change in *Fibrobacter* spp. on saponin supplementation in different species of animals. Goel *et al.* (2008) observed an increased population of *F. succinogenes* by 42 and 45% with fenugreek and sesbania saponins. Thus the effects of plant secondary metabolites on fibre degrading microbes are not consistent and depend upon their source and concentration. McSweeney *et al.* (1998) reported that *Calliandra* tannin (30% in the diet) was associated with a marked decrease in the population of rumen cellulolytic bacteria including *F. succinogenes* and *Ruminococcus* spp. without significantly affecting total fungi and proteolytic bacteria. Wina *et al.* (2006) studied the effect of *Sapindus rarak* saponins in sheep and found that *Fibrobacter* sp. were not affected while *Ruminococci* and the anaerobic fungi showed an adaptation to saponin when fed over a long period. The changes in fibre degrading enzyme activity was not correlated with the changes of fibre degrading microorganism which might be due to the reason that the microbes which they tracked were not the only key microbes involved in fibre degradation. Molan *et al.* (2000) reported inhibition of ruminal bacterial strains at a CT concentration of 400 µg/ml. McNeill *et*

al. (2000) reported that *Calliandra* tannins at a level of 2-3% in diet reduced fibre degrading bacteria. Vliwiski *et al.* (2002) reported significant decrease in bacterial count by adding hydrolysable tannins in the diet of lamb @ 1 and 2 g/kg DM. Samal (2012) reported an increase in *F. succinogenes* due to supplementation of feed additives of plant origin in growing male buffalo calves. Wanapat *et al*. (2013) reported that bacteria, proteolytic bacteria were decreased, while amylolytic and cellulolytic bacteria were similar in rumen fistulated crossbred beef cattle (Brahman native) fed plant herb supplements in their concentrate mixture. Amira *et al*. (2014) reported an increase in the abundance of *F. succinogenes* in all substrates treated with secondary compounds of cactus and acacias trees; however, the tanniferous plants and Opuntia reduced the relative abundance of *R. flavefaciens*.

5.1.5.2. Methanogen population

The methanogen population density was not affected due to feeding ajwain as feed additive. In agreement with our finding, Samal (2012) also observed no change in methanogen population density due to supplementation of feed additives of plant origin in growing male buffalo calves. Supplementation of ajwain oil and cinnamon leaf oil in the diet of fistulated buffalo decreased ($P<0.05$) methanogens population Pawar (2012). Agarwal *et al.* (2009) also demonstrated *in vitro* methane inhibition associated with reduced methanogen population at higher doses by inclusion of peppermint oil. Real time PCR analysis revealed reduction in methanogen DNA indicating reduced methanogen numbers by inclusion of allicin, but total bacteria were not affected (McAllister and Newbold, 2008). Saponin fractions from Sesbania, Cardus and Knautia leaves and fenugreek seeds had inhibitory effects on methanogen population (Goel *et al.,* 2008). Anantasook *et al*. (2013) reported that methanogens was decreased ($P<0.05$) with supplementation of rain tree pod meal (RPM) and with an increasing concentrate ratio in the diet.

5.1.5.3. Fungi population

The fungi population density was not altered due to feeding ajwain as feed additive. Pawar (2012) reported that the fungi population density was not affected due to supplementation of ajwain oil and cinnamon leaf oil. Earlier McSweeny *et al.* (1998) stated that the fibre

degrading ability of rumen fungi is less sensitive to inhibitory effects of condensed tannins. McSweeney *et al.* (1994) observed no effect on ability of the cellulolytic ruminal fungus *Neocallimastix patriciarum* to degrade cellulose when exposed to condensed tannins. Paul *et al.* (2003) demonstrated that the rumen fungal isolates were resistant towards phenolic monomers and they could survive in the presence phenolic monomers upto the level of 20 g/ L.However, Samal (2012) reported an increase in fungi population density due to supplementation of feed additives of plant origin in growing male buffalo calves. Anantasook *et al*. (2013) reported that numbers of rumen fungi was higher by dietary supplementation of rain tree pod meal (RPM) containing crude tannins and saponins at 84 and 143 g/kg of DM, respectively in dairy steers.

5.1.5.4. Protozoa population

There was no effect of feeding ajwain as additive on ciliate protozoa population. Experiments with tannins (Newbold *et al.*,1997) and saponins (Sliwinski *et al.*, 2002), have shown that these secondary metabolites are not always be antiprotozoal. Wallace *et al.* (2004) reported antimicrobial properties of *Yucca* which is a rich source of saponin. Anti-protozoal effect of saponin feeding has also been demonstrated by Kamra *et al.* (2000) in buffaloes. Patra *et al.* (2006a) reported a 95 % reduction in protozoa count with methanol extract of tannin containing *T. chebula*, which was accompanied with complete inhibition of methanogenesis. Bach *et al.* (2005) reported no affect on rumen protozoa by feeding quebracho (a source of tannins). The difference in the response of rumen protozoa to tannins might be due to different chemical and physical structures of tannins in different plants as also proposed by Makkar *et al.* (1995) and Waghorn and McNabb (2003). Kamra *et al.* (2006) compiled the data of number of plants extracts screened in the laboratory for their anti-methanogenic and anti-protozoal activity and found no correlation between *in vitro* methanogenesis and protozoa because of types and levels of secondary metabolites present in different plants. The effect of tannins on protozoa count is quite variable. Patra (2004) did not find any significant antiprotozoal activity following supplementation of plant feed additives (garlic, harad and their mixture). According to McSweeney *et al.* (2001), rumen protozoa, fungi and some of the bacteria are more resistant to condensed tannin as compared to other microbial population, but Monforte-

Briceno *et al.* (2005) reported that with some plants, protozoa numbers were negatively correlated with condensed tannins, exhibiting anti-protozoal activity. Samal (2012) reported decreased protozoa population due to supplementation of feed additives of plant origin in growing male buffalo calves. Population sizes of protozoa were decreased in rumen fistulated crossbred beef cattle (Brahman native) fed plant herb supplements in their concentrate mixture (Wanapat *et al.*, 2013). Anantasook *et al*. (2013) observed a decreased ($P<0.05$) population of protozoa with supplementation of rain tree pod meal (RPM) and with an increasing concentrate ratio in the diet.

From the results of the present study and observations of other workers on plants containing secondary metabolites of any plant origin revealed that these metabolites do make shifts in rumen microbial populations and these shifts depend on the source of secondary metabolites and their levels.

Phase 2

5.2. Effect of ajwain feeding on methane emission and nutrient utilization of buffalo calves

To study the effect of ajwain on methane production and nutrient utilization, fifteen male buffalo calves were divided into three groups of five animals each in Completely Randomized Design (CRD). The three groups (T1, T2 and T3) were fed on the diet without additive, with ajwain (1% of DMI) and with ajwain (2% of DMI), respectively.

5.2.1 Chemical composition of feed offered

The chemical composition of concentrate mixture, wheat straw and ajwain used for the feeding of the buffalo calves is given in Table 4.2.1 The concentrate mixture, wheat straw and ajwain contained 18.51, 2.53 and 14.9% CP, respectively.

5.2.2 Body weight change and feed consumption

The observations on the recording of BW changes are presented in Table 4.1.2. The examination of data over 30 days of experimental feeding revealed that the ajwain supplementation did not impart any influence on the growth pattern of the buffaloes. There was no significant difference in DMI between different groups. Similar findings reported by Vakili

et al. (2013) where supplementation of essential oils, thyme and cinnamon did not improve the DMI of Holstein calves fed high concentrate diet compared to control. The DMI were comparatively higher in their studies compared to the present findings which may be due to the higher concentrate diet in the former. Pawar (2012) also found no (P>0.05) effect on DMI, ADG and FCR with ajwain oil (1 and 2 ml/h/d) supplementation. No effect on the growth and feed intake was reported by Kumar *et al*. (2011) after feeding of equal proportion of harad, seeds of fennel, fruits of amla, bulbs of garlic and rhizomes of ginger @1 and 2 % of DMI. Similarly, Wanapat *et al.* (2008) reported no effect of feeding garlic powder on DMI of ruminally fistulated Holstein Friesian crossbred steers. Benchaar *et al.* (2008) reported no change in DMI of beef cattle fed silage-based diet supplemented with 2 or 4 g/d of a commercial mixture of EO compounds consisting of thymol (main EO-ajwain seed), eugenol, vanillin and limonene.

5.2.3 Effect of ajwain on plane of nutrition during metabolism trial

The plane of nutrition of the animals during metabolism trial is presented in Table 4.2.3.The intakes of DM, OM, CP and TDN were similar (P>0.05) in all the groups. The results of this experiment suggested that the additives ajwain fed to the animals did not have any adverse effect on the plane of nutrition of the animals. In earlier studies, Zadbuke (2009) observed no change in plane of nutrition of growing buffalo calves fed plant mixture rich in plant secondary metabolites. Similar to these observations, Kumar (2007) also reported plane of nutrition was not affected by supplementation of combination of fennel seeds, harad seed pulp and leaves of jamun and guava in the diet fistulated buffaloes. Patra (2004) did not observe any change in plane of nutrition of sheep on feeding mixture of harad and garlic. Kumar *et al.* (2011) and Someshwar (2009) reported that DMI was not affected by combination of fennel seed, harad seed pulp and leaves of jamun and guava in fistulated buffaloes and soapnut, harad seed pulp, ajwain seeds and garlic bulb in equal proportions at the rate of 1% of DMI in growing buffalo calves, respectively, which was similar to the present findings. Patra *et al.* (2010) also did not observe any change in plane of nutrition of sheep on feeding mixture of harad and garlic. Inamdar *et al.* (2015) observed similar results where intake and digestibility of nutrients remained unaffected by feeding mahua and harad combination to

buffaloes. Similar results were reported by Samal *et al.* (2016) where the digestibility of nutrients was similar on feeding combinations of ajwain oil, lemongrass, garlic, soapnut, and harad to buffaloes except crude protein, which was improved ($P<0.05$) in the treatment groups. The results of the present experiment revealed that ajwain did not have any adverse effect on plane of nutrition in buffaloes.

5.2.4. Intake and digestibility of various nutrients

The daily consumption of various nutrients along with their faecal excretion and apparent digestibility, assessed through a metabolism trial are presented in Table 4.2.4. There was no difference in total DM, OM intake in all the three groups. The CP intake and digestibility was comparable ($P>0.05$) in all the three groups. The intake of other nutrients such as EE, NDF and ADF and their digestibility did not differ among the three groups. Similar to the present observations, Yang *et al.* (2007) found no change in intakes of DM, OM, NDF and ADF by dietary supplementation of garlic oil or juniper berry oil (2g/cow/d) in cows. Similarly, Wanapat *et al.* (2008) reported no effect of feeding garlic powder on the apparent digestibility of ruminally fistulated Holstein Friesian crossbred steers. Kumar (2007) observed no change in intake and digestibility of nutrients by supplementation of a combination of fennel seed, harad seed pulp and leaves of jamun and guava to the fistulated buffaloes. Benchaar *et al.* (2006a) observed no change in digestibility of DM, OM, CP and NDF by essential oils (2 and 4 g/ head/d) supplementation, but digestibility of ADF was significantly increased (48.9 *vs.* 46.0%). Gautam *et al*. (2015) reported no adverse effect on DM, OM and CP digestibility on feeding deoiled ajwain meal fed at 10% of the concentrate .Similarly Benchaar *et al.* (2008) reported no change in DMI of beef cattle after supplementing plant secondary metabolites (EO-thymol, eugenol, vanillin and limonene) containing commercial mixture @ 2 or 4 g/d. Arambel *et al*. (1990) reported no significant ($P>0.05$) change in digestibility on feeding neem kernel cake to7-8 months growing goats. Improved dry matter digestibility was observed by Patra (2004) with the feeding of harad and garlic as feed supplement but their mixture did not have any significant effect as compared to control and the digestibility of NDF and ADF was improved with feeding of a mixture of harad and garlic. Similarly, Lakhani (2017) reported no change in the nutrient intake and digestibility of various nutrients in buffalo calves fed a blend of seven

herbal additives. While Beauchemin and McGinn (2006) reported a lowered feed intake and digestibility of all nutrients by feeding essential oils in beef cattle. In an *in vivo* feeding experiment, Hosoda *et al.* (2006) reported that digestibility of nutrients in cows fed a diet containing 5% peppermint was significantly reduced. Like the present study findings, Benchaar *et al.* (2003) also did not observe any change in digestibility of ADF by essential oils addition in the diet of lactating dairy cows. However, Yang *et al.*(2007) observed increased ruminal digestibility of nitrogen by dietary supplementation of garlic oil (5g).

5.2.5 Nitrogen balance

Effect of feeding ajwain on nitrogen balance is presented in Table 4.2.5. The intake, excretion and balance of nitrogen were similar among the groups. The nitrogen voided through urine was higher than nitrogen voided through faeces in all the groups. The buffalo calves in all the three groups were in positive nitrogen balance. Yildiz *et al.* (2005) reported similar pattern of nitrogen excretion in lambs receiving oak (*Quercus hartwissiana*) leaves with and without PEG supplementation. Vaithiyanathan *et al.* (2007) fed graded levels of *Prosopis cineraria* leaves to kids and found urinary N excretion was higher than faecal N outgo. Nitrogen balance (g/d) was highest in T2 (24.88) followed by T3 (24.6) and T1group (23.13). The nitrogen retention, both as % of nitrogen intake and as % of nitrogen absorbed did not differ among the groups The retention of N was not affected in lactating dairy cows (Benchaar *et al.,* 2003) or in beef cattle (Benchaar *et al.,* 2006a) fed a mixture of essential oil compounds. Zadbuke (2009) observed no effects on nitrogen intake, balance and retention by feeding a plant mixture rich in plant secondary metabolites as an additive to suppress methane production. Similarly, Patra (2004) did not find any effects on nitrogen intake and balance by supplementation of a mixture of garlic and harad in sheep. Kamra *et al*. (2008) reported no significant change ($P>0.05$) in nitrogen balance on feeding harad, garlic and their mixtures @1% DMI. Rao *et al.* (2016) reported no significant ($P>0.05$) difference in nitrogen balance on feeding 34 g and 68 g of neem seed cake per kg of diet to growing kids. Samal *et al*. (2016) reported a positive nitrogen balance among all buffalo calves fed garlic, soapnut, ajwain and harad mixture. Similar type of results were found by Someshear (2009) in buffalo calves fed a mixture of soapnut, harad seed pulp, ajwain and garlic bulb in equal proportions @ 1% of DMI. Similarly, Yatoo *et al*. (2014) and Perme (2014) found no difference in nitrogen metabolism in buffalo calves. In

contrast, Pawar (2012) found significantly (P<0.05) higher N balance (g/d) on supplementation of ajwain seed oil (2 ml/d) to male buffalo calves. This might be due to inhibition of hyperammonia producing bacteria (*Clostridium sticklandii* and *Peptostreptococcus anerobiosus*) in the rumen by essential oil of ajwain which suppresses the colonization and digestion of readily degradable substrate, resulting in decreased amino acid deamination.

5.2.6 Methane emission

Effect of supplementation of ajwain on DM intake, its apparent digestibility and methane emission during open circuit respiration calorimetry studies are given in Table 4.2.6. The DM intake (kg/d) and apparent dry matter digestibility was unaffected by addition of ajwain as feed additive. Supplementation of ajwain significantly reduced methane by 18% in terms of l/ kg DDMI (P <0.005) in group T3 fed ajwain @ 2% of DMI in comparison to the control group (T1). Methane production in terms of l/d was 142.06±5.43, 134.00±7.25 and 127.13±6.51, respectively in T1, T2 and T3. The inhibition in Methane emission in terms of l/ d, l/kg DMI did not differ significantly between groups. Methane reduction might be due to the presence of array of active components in ajwain as thymol, carvacrol, and non thymol portions which might affect the microbial activity in the animals. Corroborating with our findings Malik *et al*. (2017) reported 20–26% reduction in enteric methane emission through the supplementation of three selected tanniniferous tropical tree leaves of *Ficus benghalensis, Artocarpus heterophyllus* and *Azadirachta indica* in straw based diet. Similarly, Pawar (2012) also reported significantly (P<0.05) less methane production (l/kg DMI and l/kg DDMI) by ajwain oil (1 ml/h/d) supplementation. Reduction in methanogenesis might be due to direct inhibitory effect of sulphur on methanogenic archaea and change in archeal community or reduced activity of methanogens by essential oils [thymol (ajwain seed) and anethol (fennel)]. (Calsamiglia *et al.,* 2007; Benchaar *et al.,* 2008; Ohene-Adjei *et al.,* 2008; Benchaar and Greathead, 2011). Samal *et al*. (2016) reported that methane emission (in terms of l/kg DMI and l/kg DDMI) was reduced by 13.3% and 17.8% on feeding combinations of garlic, harad, ajwain and soapnut to buffalo calves as compared with control. Similarly, Van Zijderveld *et al.* (2010) reported 16% less methane production by 2.6% sulfate supplementation in crossbred lambs. Silivong *et al*. (2011) also found less rumen methane emissions by sulphur (0.8%)

supplementation in goats. Gautam *et al*. (2016) reported lower methane production on feeding deoiled ajwain meal at 10% with no adverse effect on DM, OM and CP digestibility on feeding in the concentrate. Similarly, feeding of Japanese horseradish oil at 2% of DM intake decreased ($P<0.05$) methane production by 19% in steers (Mohammed *et al.*, 2004). In a study with different PSM's, Garcia-Gonzalez *et al.* (2008) observed methane suppressing effect in Rhubarb roots (rich in anthraquinones and other substances) and Frangula bark (rich in anthraquinones, saponins and alkaloids) .Yatoo *et al.* (2014) reported 14.1% reduction in the methane produced in terms of L/kg DMI. Similarly, Wang *et al.* (2004) observed lowered methane emission by inclusion of 0.25 g day-1 of EO mixture from oregano plants in the diet of sheep for 15 days. Contrary to this, Inamdar *et al.* (2015) found no effect ($P>0.05$) on methane emission after 0.4% of sulphur supplementation. Supplementation of EO mixture to beef cattle (1 g/d) for 21 days did not ($P>0.05$) reveal any effect on methanogenesis (Beauchemin and McGinn, 2006). Perme (2014) also reported no significant change in the methane production in buffalo calves fed harad and garlic herbal feed additive. In line with the results of the present study, Klevenhusen *et al.* (2011a) reported a decrease in methane production when sheep were fed a 50:50 forage:concentrate diet supplemented with diallyl disulfide (4 g per day), but no effects were shown with lower doses of diallyl disulfide (2 g) or 5 g of GO per day (Klevenhusen *et al.,* 2011b). No effects on methane production were found when supplementing diallyl disulfide to lactating cows @ 3.3 g per day; Van Zijderveld *et al*. (2010) or garlic bulbs @ 1% of DM intake to sheep (Patra *et al.*, 2011) and fattening bulls (Sauvant *et al.,* 2004). Kongmun *et al.* (2011) observed that supplementing swamp buffalo with a mixture of 7% coconut oil and 100 g of garlic powder reduced methane production by 9.1%, but a similar reduction was observed only with 7% coconut oil, which would indicate that the depressing effect on methane production was due to coconut oil rather than to garlic powder.

Probable mode of action of essential oil in ajwain

Thymol [5-methyl-2-(1-methylethyl) phenol; $C_{10}H_{14}O$] is the main active component in ajwain seed oil and has strong antimicrobial activity against a wide range of Gram-positive and negative bacteria. At least a part of this activity is due to the hydrophobic nature of the cyclic hydrocarbons, which allows them to interact with the cell membrane and accumulate in the lipidic bilayer of bacteria, occupying a space between the chains of fatty acids (Sikkema *et*

al., 1994; Ultee *et al*., 2002). This interaction causes conformational changes in the membrane structure, resulting in its fluidification and expansion (Griffin *et al*., 1999). The loss of membrane stability results in the leakage of ions across the cell membrane, which causes a decrease in the trans-membrane ionic gradient. In most cases, bacteria can counterbalance these effects by using ionic pumps and cell death does not occur, but large amounts of energy are diverted to this function and bacterial growth is slowed down (Griffin *et al*., 1999; Ultee *et al*., 2002; Cox *et al*., 2001). Other mechanisms of the action of essential oils are (Gustafson and Bowen, 1997) coagulation of some cell constituents, probably by denaturation of proteins, interaction of some phenolic and non phenolic compounds of essential oils with chemical groups of proteins and other biologically active molecules, such as enzymes (Juven *et al*., 1994) and interaction of some aldehyde compounds with nucleic acids and proteins, resulting in their inactivation, probably due to formation of crossed bridges (Prescott *et al*., 2004).

5.2.7 Energy metabolism

The faecal energy, urinary energy and methane energy losses were not affected due to feeding of the ajwain (Table 4.2.7). No significant effect on the energy balance was found among the three groups. Similar results were reported by Samal *et al.* (2016) that faecal (mixture of garlic and ajwain) energy, urinary energy and methane energy losses were not affected ($P>0.05$) due to feeding of harad, ajwain, soapnut and garlic to buffaloes. Lakhani (2017) also reported similar results with no effect of feeding a mixture of seven herbal additives on the energy balance of buffalo calves. Perme (2014) reported no significant effect of garlic and harad supplementation on the energy balance of buffalo calves. In the present study, the losses in the form of methane were similar in all the groups. In contrast, Patra *et al.* (2010) observed about 24% reduction in methane production (l/kg DDMI) in sheep fed on the diet supplemented with harad, whereas, this reduction was 23.6% in the sheep fed on diet supplemented with the mixture of harad and garlic resulting in decrease in methane energy loss as per cent of digestible energy intake by 20% in harad fed group and 24.8% in mix fed sheep. Yuan *et al.* (2007) reported that the addition of tea saponin @ 5 g/ kg DM and tea saponin plus disodium fumarate @ 20 g/kg DM resulted in decreased methane emissions by 8.5 and 9.6%, respectively in sheep. *Lotus pedunculatus* having condensed tannins, when fed to lamb, showed 16% reduction in methane production (Waghorn *et al.,* 2003). Samal (2016)

also reported faecal energy, urinary energy and methane energy loss were not affected by feeding feed additives containing PSM in male buffalo calves.

Summary and Conclusions

6 SUMMARY AND CONCLUSIONS

Ruminants harbour complex microbial ecosystem in the rumen which is responsible for fermentation of complex lignocellulosic feeds and non protein nitrogen to supply nutrients to the host animals but during this process, a significant amount of dietary energy is wasted in the form of methane thus reducing the feed efficiency of the animals.

In ruminants, methanogenesis is an essential metabolic process to maintain steady state feed fermentation in the rumen. But methane, being a potent green house gas is also responsible for global warming as well as loss of dietary energy. Therefore, by putting a check on methane production, the animal can be made more feed efficient and the environmental pollution due to increasing levels of green house gases can be reduced. For the past several decades, researchers have been trying to manipulate the rumen microbial ecosystem to improve the animal performance by chemical feed additives but the uses of chemicals have been criticized by researchers and consumers. The plant parts containing secondary metabolites, due to their antimicrobial activity, are considered as potent rumen modifiers. The plant secondary metabolites, due to their inherent nature of protection against plant pathogens, may be active against undesirable microbes of rumen such as methanogens.

The present study was, therefore, conducted to explore the possibility of using ajwain seed as feed additives for reducing methane emission, improving rumen fermentation, nutrient utilization and growth performance of buffaloes.

PHASE 1

Effect of ajwain feeding on rumen fermentation, enzyme and microbial profile of fistulated buffaloes

Three fistulated adult buffaloes were fed on basal diet consisting of wheat straw and concentrate mixture in 50:50 ratio in3X3 Switch Over Latin Square Design. The three groups were, (T1, T2 and T3) fed on the diet without additive, with ajwain (1% of DMI) and with ajwain (2% of DMI), respectively. The mean body weight of the animals in terms of kg and kg $W^{0.75}$ were similar in all the four groups The total DMI was similar (P>0.05) in all the three groups. None of the dietary treatments affected (P>0.05) rumen pH and ammonia nitrogen. The pH and ammonia nitrogen concentration (mg/dl) in T1, T2 and T3 groups were 6.82±0.08, 6.83±0.05, 6.72±0.05 and 13.91±0.57, 14.79±0.69, 14.32±0.57 respectively. The activities of ruminal enzymes *viz*. carboxymethylcellulase, avicelase, xylanase and were not affected (P>0.05) by addition of ajwain . The population density of different microbes like Total bacteria, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, methanogens, fungi and ciliate protozoa were assessed by qPCR (Real time PCR). None of the microbes were influenced by supplementation of ajwain. The results indicated that feeding of ajwain did not influence rumen fermentation, enzyme and microbial profile in the fistulated buffaloes.

PHASE -2

Effect of ajwain feeding on methane emission and nutrient utilization of buffalo calves

To study the effect of ajwain on, methane production and nutrient utilization, fifteen male buffalo calves were divided into three groups of five animals each. The three groups (T1, T2 and T3) were fed on the diet without ajwain, with ajwain (1% of DMI) and with ajwain (2% of DMI), respectively.The data revealed that ajwain feeding at levels of 1 and 2% of DMI did not impart any influence on the intake and growth pattern of the buffaloes. The intakes and digestibilities of nutrients i.e DM, OM, EE, NDF, ADF were also similar (P>0.05) among the groups. Animals of all the three groups were in positive nitrogen balance. Methane production in terms of l/kg DDMI was reduced by 18% in T3 as compared to T1.The gross energy intake (Mcal/d), fecal energy loss (Mcal/d), digestible energy (Mcal/d), urinary energy loss (Mcal/d), metabolizable energy (Mcal/d), heat production, net energy for production and metabolizable energy (% GE) were not affected (P>0.05) by feeding of ajwain.

The results of the study can be summarized as:

- Supplementation of ajwain as feed additives to fistulated buffaloes did not affect feed intake, rumen fermentation pattern, rumen enzyme and microbial profile.
- The supplementation of ajwain as feed additive did not affect nutrient digestibility, plane of nutrition and nitrogen balance among all three groups .
- The percent reduction in methane emission l/kg DDMI was 18% in T3 group fed ajwain @ 2% of DMI compared to control.

CONCLUSIONS

- Dietary supplementation of ajwain @ 2% of DMI resulted in 18.3% inhibition in methane production (l/kg DDMI) in buffaloes without affecting rumen fermentation, enzyme and microbial profile along with nutrient utilization. Similar feeding trials on larger number of animals with higher doses of the additive should be conducted to get better response of the additives

Mini Abstract

7 MINI ABSTRACT

The present study was conducted with the objective evaluate ajwain as feed additive for improving livestock production through improved feed utilization and inhibition of methanogenesis. In the first phase, three fistulated adult buffaloes were fed on a basal diet consisting of wheat straw and concentrate mixture in 50:50 ratio in 3x3 Latin square design.The three groups were, (T1, T2 and T3) fed on the diet without additive, with ajwain (1% of DMI) and with ajwain (2% of DMI), respectively. Daily dry matter intake was similar (P>0.05) in all the three groups. Feeding of any of the dietary treatments did not affect (P>0.05) rumen fluid pH, concentration of ammonia N activities of carboxymethylcellulase, xylanase and avicelase. The microscopic count of protozoa as well as the population density of total bacteria, *Fibrobacter succinogenes,* methanogens, fungi and protozoa assessed by real time PCR was similar in all the groups.In the second phase,1 month duration feeding trial was conducted on 15 growing buffaloes divided into 3 groups to assess the effects of ajwain as feed additives on growth, nutrient utilization and methane production. The dietary treatments were similar to that of phase I .The data revealed that adding ajwain did not impart any influence on DMI and digestibilities of nutrients i.e DM, OM, EE, NDF, ADF which were similar (P>0.05) among the groups. Animals of all the three groups were in positive nitrogen balance. Methane emission in terms of l/d and l/kg DMI were comparable in all groups where as l/kg DDMI of methane was reduced by 18% in T3 as compared to T1. However, similar feeding trials on larger number of animals using higher doses should be conducted to get better response of the additive.

लघु सारांश

8 लघु सारांश

वर्तमान अध्ययन अजवाइन खाद्य योजक को रूमेन के अपरिवर्तक के रूप में उपयोग करने की संभावना हेतु किया गया है जिससे गुगाली करने वाले पशुओं द्वारा उत्पन्न होने वाली मीथेन गैस को कम किया जा सके तथा खाद्य पाचकता को सुधारा जा सके। पहले चरण में 3 x 3 लैटिन स्क्वायर डिजाइन के अन्तर्गत परीक्षण किया गया। सभी जानवरों को 50:50 के अनुपात में भूसा और दाना खिलाया गया। 3 समूहों का निर्माण किया गया जो इस प्रकार है :– टी1 नियंत्रित रोगज के, टी2 आजवाइन शुष्क आहार का 1 प्रतिशत एवं टी3 आजवाइन शुष्क आहार का 2 प्रतिशत शुष्क आहार की दैनिक खपत सभी समूहों में समान थी। किसी भी समूह में रूमेन पीएच मान, अमोनिया नत्रजन कार्बोक्सी मेथाइल सेल्यूलेज, जाइलनेज, एवीसेलेज पर कोई प्रभाव नहीं दिखा ($P>0.05$) सभी समूहों में कुल बैक्टीरिया, फाइब्रोबैक्टर सक्सीनोजीनेज, रूमीनोकोकस फ्लेवीपोशीयन्स, मीथेनोजेन कवक व प्रोटोजोआ की संख्या समान थी दूसरे चरण में एक महीने का फीडिग्र परीक्षण 15 भैंसों के कटड़ो को तीन समूहों में बाँटकर किया गया। इसमें आजवाइन का असर पोषक तत्वों की उपयोगिता एवं मीथेन उत्सर्जन पर देखा गया। प्रयोगात्मक डिजाइन पहले चरण के समान ही रखा गया। पोषक तत्वों का अन्तर्ग्रहण एवं पाचनशीलता सभी समूहों में समान थी। टी3 समूह में ली/किलोग्रा. शुष्क पदार्थ ग्रहण के संबंध में मीथेन उत्सर्जन पर कोई प्रभाव नहीं था। समूह टी3 में ली/किलोग्रा. पाच्य शुष्क पदार्थ ग्रहण के संबंध में 18 प्रतिशत की कमी हुई। बेहतर प्रभाव प्राप्त करने के लिये इसी परीक्षण को अधिक खुराकों व अधिक पशुओं पर करने की आवश्यकता है।

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Achievements

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