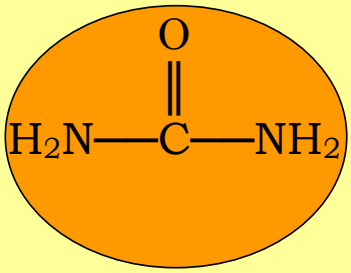
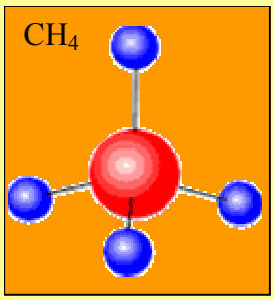
Despal

NUTRITIONAL PROPERTIES OF UREA TREATED COCOA POD FOR RUMINANT





Cuvillier Verlag Göttingen

INSTITUTE OF ANIMAL PHYSIOLOGY AND ANIMAL NUTRITION

NUTRITIONAL PROPERTIES OF UREA TREATED COCOA POD FOR RUMINANT

Doctoral Dissertation
Submitted for the degree of Doctor of Agricultural Sciences
Of the Faculty of Agricultural Sciences

By

Despal

Born in Pariaman, Indonesia

GEORG-AUGUST UNIVERSITY, GÖTTINGEN, GERMANY

Göttingen, September 2004

Bibliografische Information Der Deutschen Bibliothek
Die Deutsche Bibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über http://dnb.ddb.de abrufbar.
1. Aufl. - Göttingen : Cuvillier, 2005
Zugl.: Göttingen, Univ., Diss., 2004
ISBN 3-86537-347-X

D7

Referee: Prof. Dr. Hj. Abel

Co-referee: Prof. Dr. C. Wollny

Date of examination: 18 November 2004

Nonnenstieg 8, 37075 Göttingen
Telefon: 0551-54724-0
Telefax: 0551-54724-21
www.cuvillier.de

1. Auflage, 2005
Gedruckt auf säurefreiem Papier

ISBN 3-86537-347-X

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABREVIATIONS

^{15}N	isotope nitrogen
^{32}P	isotope phosphor
^{35}S	isotope sulphur
^{3}H	isotope hydrogen
AA	amino acids
Ac	acetate
ADF	acid detergent fibre
ADL	acid detergent lignin
AEP	aminoethylphosphonic acid
ANOVA	analysis of variance
ASH	crude ash
ATP	adenosine triphosphate
BST	bovine recombinant somatropin
Bu	butyrate
C, U0,U1,U2 and U3	treatment of cocoa pods (experiment 1)
C_2	acetate
C_3	propionate
ca.	about
CF	crude fibre
CH_4	methane
CP	crude protein
CPs	cocoa pods
cts	counts
cv.	cultivar
cwt	hundred weight (UK) = 50.8023 kg
DAPA	diaminopimelic acid
DCP	digestible crude protein
DM	dry matter

DMI	dry matter intake
DNA	deoxyribonucleic acid
EMPS	efficiency of microbial protein synthesis
FME	fermentable metabolisable energy
FOM	fermented organic matter
g	gravity
Gb	gas produced from Hohenheim gas test
GLDH	glutamate dehydrogenase
h	hour
H_2	hydrogen gas
H_2O	water
H_2-P	H_2 produced
H_2-U	H_2 utilised
HCl	hydrogen chloride
HF = $C_6H_{12}O_6$	hexose's fermented
iC_4	iso-butyrate
iC5	iso-valerate
ICCO	international cocoa organisation
iVa	iso valerate
Kg	kilo gram
KJ	kilo Joule
La	lactat
LAB	liquid associated bacteria
LCFA	long chain fatty acid
m	meter
M	mol
M/D	M is microbial fraction; D is the unfractional duodenal digesta
MC	microbial cells
MCP	microbial crude protein synthesis

Me	methane
mg	milli gram
MJ	mega Joule
ml	millilitre
mm	millimeter
mmol	millimol
MP	microbial protein
MPB	methane-producing bacteria
MPS	microbial protein synthesis
N	nitrogen
NAN	non ammonia nitrogen
NaOH	sodium hydroxide
nC4	butyrate
nC5	valerate
NDF	neutral detergent fibre
NDS	neutral detergent soluble
NFE	nitrogen free extract
NH_3	ammonia
NH_4^+	ammonium
NPN	non protein nitrogen
^oC	degree celcius
OD	optical density
OH^-	ion hydroxide
OMAD	organic matter apparently digested
OMF	organic matter fermented
P	phosphor
PPF	palm press fibre
Pr	propionate
r	infusion rate
RAC	reductive acetogens

RDN	rumen degradable nitrogen
RDP	rumen degraded protein
REP	rumen escape protein
RNA	ribonucleic acid
RUP	rumen undegraded protein
SAB	solid associated bacteria
SCFA	short chain fatty acids
s_i	^{15}N-excess in infusion
s_p	^{15}N-excess in microbial isolate or ammonia pool
SRB	sulphate-reducing bacteria
T1, T2, T3, T4, T5 and T6	ration treatments of experiment 2
TB	total bacteria
TCA	tungstic acid
TFOM	true fermented organic matter
TP	true protein
U	urea
UDP	undegradable protein
UR	Residual urea
Va	valerat
w	weight
WSN	water soluble nitrogen
XL	crude lipid

Acknowledgements

I would like to express my deepest gratitude to my supervisor, Prof. Dr. Hj. Abel, for his guidance. His compassion and honest have given me a great motivation to finish my study. Similarly, Prof. Dr. C. Wollny, the second supervisor is greatly thanked.

I thank Mr. Rolf Jeromin and Mr. Bernardo Lenz for their help and cooperation during the laboratory work. My thanks also go to Farai for English correcting, to Mom and Elke for their collaboration and friendships.

The colleagues at the Institute of Animal Physiology and Nutrition at the University of Göttingen are greatly acknowledged for their cooperations and help.

Finally, I am very grateful to the German Academic Exchange Service (DAAD) who enabled me to study in Germany, especially to Mr. Joachim Apweiler and MS. Ruth Eberlein in Desk 424.

My success would not be possible without the support of my husband, Rudi Afnan, and my children, Insan and Ikhlas. Above all, I thank God who always guides in all of my life.

1. General introduction

Availability of quality forage is a major problem in ruminant production in tropical countries particularly during the dry season. At this season forage is expensive and out of reach to many farmers. As a result, there is over-slaughtering and selling of ruminant livestock when the production cost is elevated by the price of forage. Utilisation of other abundant agricultural by-products is one of the alternatives to substitute expensive forage to keep the ruminant body weight losses at the minimum level.

Some fibrous feed from agricultural by-products such as fresh or treated palm press fibre (PPF), rice straw, and sugarcane-bagasse have been used extensively. Cocoa pod, a by-product of cocoa plantations is a potential source of fibre for ruminants. It is easily available to the farmers. In most of tropical regions where the cocoa trees are cultivated (Figure 1.1), the availability of by-products such as cocoa pods increases with the escalation of world-wide demand of cocoa. Cocoa bean production in 2003 forecasted about 3 million tons (ICCO, 2003) which released roughly 6 million tons of cocoa pod meal (a 1 to 2 cocoa bean to cocoa pod meal ratio (DUKE, 1983)), an equivalent of about 25 million tons of fresh cocoa pods if 20% DM are assumed.

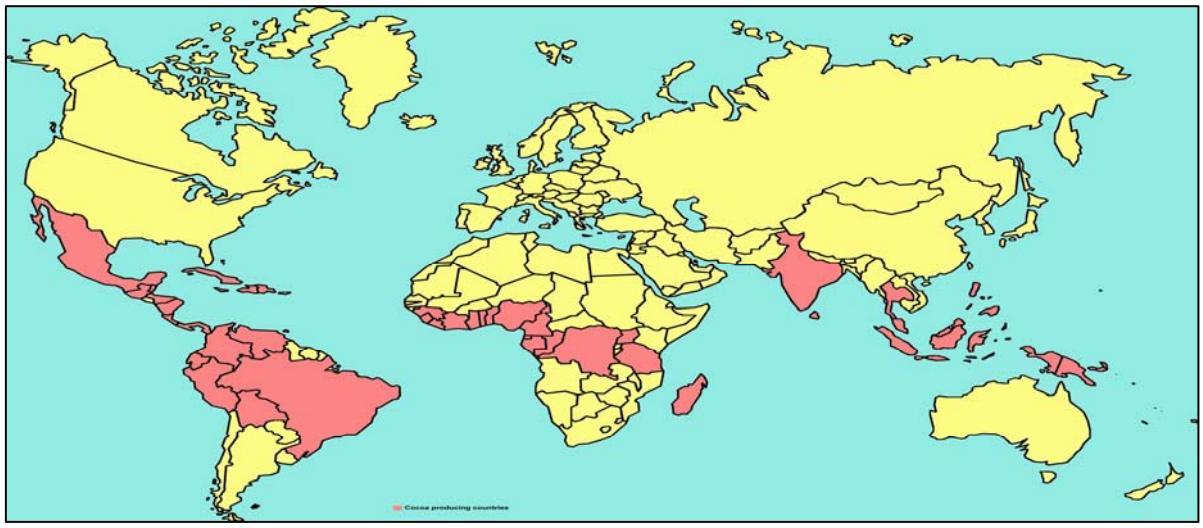


Figure 1.1: Cocoa producing countries (■ producing country)
Source: ICCO (2001)

Cocoa pods are palatable. The low protein content and high cell wall constituents, however, limit the use of cocoa pods in ruminant nutrition (reviewed by REYNOLDS, 1995). As a late-maturing plant component, the pod contains high lignocellulosic and low non-polysaccharide substances. To be used as ruminant feedstuff, cocoa pod needs quality upgrading.

The use of physical, chemical and biological treatments to improve the quality of agricultural by-products has been reviewed by FLACHOWSKY et al. (1999). Alkali agents can cleave lignocelluloses into lignin and cellulose or hemicelluloses. Ammonia and urea, a group of alkali agents, have been reported to be effective in improving fibrous feed quality (SCHIERE & IBRAHIM, 1989; SUNDSTØL et al, 1993; CHENOST & KAYOULI, 1997). In addition to improving digestibility, ammonia treatment may also supply N for microbial growths. The use of ammonia is, however, limited to industrial countries.

Urea as a source of ammonia can be applied on industrial scale as well as at farm level. Compared to other forms of chemical treatment, there are little health risks associated with the use and handling of urea. Dry urea can be easily transported compared to other sources of ammonia. Improvement of fibrous feed quality through urea treatment is due to two processes that take place simultaneously. Firstly, ureolysis splits urea into ammonia. The generated ammonia reacts with H_2O and then acts on the cell walls of the forage (CHENOST, 2001).

Urea treated feed can not be evaluated based on crude protein (CP) analysis only. This is because CP analysis according to the Kjeldahl-method (N x 6.25) is unable to distinguish between true protein and non protein nitrogen and thus to evaluate the contribution of the microbial protein produced in the rumen, which in treated feed diet maybe produced from NPN as the main contribution to the intestinal protein flow (HVELPLUND, 1998). The influence of treatment on digestible crude protein (DCP) is also inadequate for analysis of urea treated feeds because non protein nitrogen (NPN), a high CP fraction in urea treated feed, is not directly related to animal performance (ORSKOV, 1982). Therefore, expressing N-fractions as true protein, non ammonia nitrogen and residual urea is needed.

Urea is a weak alkali. As a result, higher levels of urea are needed to give the same effect on fibre fraction than when using concentrated alkali. Too much urea, on the other hand, can be toxic to ruminants. Urea toxicity and ammonia poisoning occur when blood ammonia levels exceed beyond the buffering capacity of blood. Excessive levels result in a rise in pH and impairment in the capacity of blood to expel carbon dioxide (VAN SOEST, 1982).

Chemical and short in vitro evaluations of urea treated feeds are inadequate to accurately determine long time effects of urea and ammonia in ruminants. Long-term in vitro such as Rusitec or in vivo experiments to study rumen physiology are needed. In Rusitec, the effect of urea can be studied by gradually substituting a conventional ration ingredient with urea treated feed.

Since microbial protein is the major protein contributor for ruminants particularly if urea treated roughage is used in diets, information about the microbial protein synthesis (MPS) and its efficiency (EMPS) are important criteria needed for ration formulation and the estimation of animal performance.

Several methods for assessing MPS have been developed. The majority of the approaches that identify microbial protein in rumen contents (both in vitro and in vivo) and in digesta flowing at the omasum, abomasums or duodenum, have limitations (DEWHURST et al., 2000). Previous work used protein-free diets and this still can provide a useful baseline against which to asses other markers (ARAMBEL et al., 1987). Other early studies tried to distinguish feed and microbial protein on the basis of amino acid profile (ANNISON, 1975; OFFER et al., 1978). Endogenous or exogenous markers to label microbial material including ^{35}S (HUME, 1975; DURAN et al., 1975), ^{32}P (VAN NEVEL et al., 1975), ^{15}N (BRANDT, 1979; ABEL et al., 1990; HRISTOV, 2002), diaminopimelic acid (THOMAS, 1973; MILLER, 1982), RNA (BATES et al., 1985) and purine and pyrimidine bases (SHEM et al., 1999; RODRIGUEZ et al., 2000) have been used. Near infrared reflectance spectroscopy as a quick approach to distinguish the microbial content of duodenal digesta has been used successfully by LEBZIEN & PAUL (1997).

DEWHURST et al. (2000) concluded that each of these markers has its own problems including safety (radioisotopes), cost (^{15}N and amino acid profiles), difficulty of analysis (RNA and DNA) and presence of feeds, thereby lacking specificity to the microbial fraction (DAPA, nucleic acid and their bases).

1.1. Objectives

The general objective of this study was to improve the quality of cocoa pods to allow increased levels of their incorporation into ruminant rations. The first step of the study was directed to find the basic information on changes in urea treated compared to untreated cocoa pod. The in vitro gas test aimed at studying the effect of urea on the activity of rumen

microbes, at finding the optimum level to achieve maximum organic matter digestibility and metabolisable energy contents as well as to find out its toxic level.

The effect of urea treated cocoa pods on rumen microbial metabolism was then investigated by means of the rumen simulation technique Rusitec (CZERKAWSKI & BRECKENRIDGE, 1977). Barley/soybean meal-mixture was gradually substituted for urea treated cocoa pods and nutrient disappearance rates, fermentation patterns as well as microbial growth were measured in order to get more information on the optimal inclusion of urea treated cocoa pod into feed rations for the ruminant animal.

In the last experiment, two methods of determining MPS in cocoa pod-supplied Rusitec were applied and compared; the indirect continuous tracer infusion technique and the direct measurement of isolated microbes. With both methods stable isotope nitrogen (^{15}N) served as a tracer.

2. Cocoa (*Theobroma cacao,* L).

2.1. Classification

According to the PURSEGLOVE (1968), the botanical taxonomy of cocoa is as follows:

Division : Magnoliophyta
Class : Magnoliopsida
Subclass : Dilleniidae
Order : Malvales
Family : Sterculiaceae
Species : *Theobroma cacao*, L

Several subspecies and forms of cocoa have been recognised, from which a great number of cultivars have been developed. Some cultivars are named according to the place where they were found or developed. Others with elongated, ridged and pointed fruits and white cotyledons are classified as **Criollo** typed while those with short, roundish, almost smooth fruits and purplish cotyledons are categorised as **Forastero**, (DUKE, 1983). Morphology and anatomy of cocoa fruit and cocoa flower is shown in figure 2.1.

2.2. History and spreads

Although it is unknown exactly how or when cocoa was discovered in the wild, it is thought that the Aztec and Maya Indians, who called it the "food of the gods", domesticated and cultivated it for centuries before the discovery of the Western Hemisphere (PURSEGLOVE, 1968). It is also believed that the cocoa tree (*Theobroma cacao*) originated from the headwaters of the Amazon Basin and in early times was spread throughout the central part of Amazonia, Guinea, westward and northward to the south of Mexico (WOOD & LASS, 1985). Cocoa then has been extensively cultivated in the Old World since the Spanish conquest (COLUMBIA ENCYCLOPAEDIA, 2003).

The first European to discover cocoa was Columbus although the Spaniard Hernand Cortes was the first person to bring the cocoa seed back to Europe in 1519. In Europe, it was considered as a luxury drink by the West Europe counts (PURSEGLOVE, 1968). It was not until 1657 however, that cocoa was introduced to England, having already been used in Italy, Austria and France (ENCARTA, 1996).

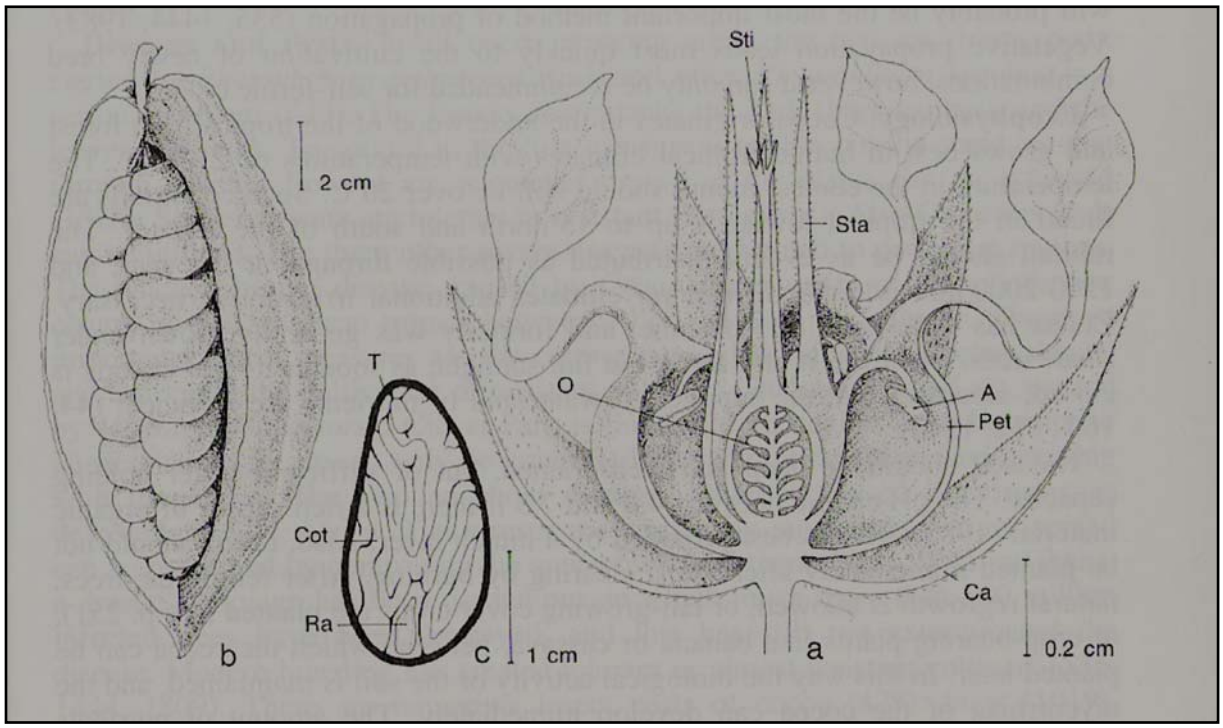


Figure 2.1: Morphology and anatomy of cocoa fruit and cocoa flower *Theobroma cacao*
(a) flower in longitudinal section, (b) opened fruit, (c) seed in longitudinal section,
A = anther, Pet = petal, Cot = cotyledon, O = ovary, Ca = calyx, Sti = stigma,
Ra = radicle, T = staminode; Source: REHM & ESPIG (1991)

In the eighteenth century chocolate started to become available to more classes of society with the establishment of chocolate houses in London, out doing the popularity of the previous centuries of coffee houses. The eighteenth century also saw the English mixing brick dust with their chocolate to thicken it, a habit that was stopped in 1727 when Nicholas Sanders mixed chocolate with milk creating the first hot chocolate. This hot chocolate was promoted by physicians as healthy for both children and adults (YOUNG, 1994).

The cocoa bean was originally roasted, ground and then mixed with maize and annatto, chilly or some other spice, this mixture made a thick drink and was known as chocolate. In this form chocolate became the first non-alcoholic stimulant drink on the European continent (YOUNG, 1994). The Spaniards also realised that the cocoa bean could be made into a delicious drink when mixed with sugar (WOOD & LASS, 1975).

TARLA (1996) summarised the chronology of the cocoa distribution to Europe as listed below:

Found	: Central America
1502, Christopher Columbus	: First European to see cocoa beans but did not show any interest
1519, Hernand Cortes	: First introduced the chocolate drink to the Spanish court.
< 1657, Antonio Carletti	: Italian, spread the chocolate in Italy
1828	: Coenraad van Houten, press 2/3 cocoa butter for better taste
1847	: Fry & Sons, England, introduced eating chocolate
1875	: Daniel Peter, Switzerland, added condensed milk to chocolate
A few years later	: Rodolphe Lindt, invented a way of refining chocolate, it was known as couching process

There have been many and varied uses of the cocoa bean since its discovery. In the early sixteenth century the beans were used for making drinks, currency and payment of tribute to Aztec overlords, and in various rituals and medicinal purposes (WOOD &LASS, 1985).

Chocolate was considered to be a cure for many illnesses and was used for provoking passion. It was, however, expensive for people belonging to the lower class society (Young, 1994).

To date the tree is also found in other tropical areas of the world, such as West Africa, notably Nigeria, Ghana and the Ivory Coast, which produce 2 million tons representing two thirds of the total world crop (ICCO, 2003).

2.3. Ecology

DUKE (1983) described the natural habitat of the cocoa tree in the lower storey of the evergreen rainforest. Climatic factors, particularly temperature and rainfall, are important for optimum growth. Across the subtropical and tropical forest zones, cocoa was reported to tolerate to soil pH of 4.3 to 8.7, annual temperature of 13.0 to 33.5^{o}C and annual precipitation of 40 to 100 inch. A constantly high temperature of about 26^{o}C is, however recommended. Cocoa is grown within 20^{o}N to 20^{o}S (the bulk of it is found within 10^{o}N and 10^{o}S) latitudes, below 300 m altitude (but in sheltered valleys of Columbia, cocoa is grown at 900 m altitude). Cocoa trees are wind-intolerant and are therefore often planted on hillsides for wind protection and good drainage. Being drought-intolerant, cocoa thrives in climates associated with high humidity and rainfall. Plants are shade-tolerant, and thrive in rich, organic, well-drained, moist and deep soils (PURSEGLOVE, 1968).

Cocoa cultivars are known to tolerate some diseases, slope and water logging (DUKE, 1978). To improve on disease resistance, hybrids have been inter-cropped with other species, such as *Th. Grandiflora* (DUKE, 1983).

Indonesia has suitable land for growing cocoa. The country produced close to one ton cocoa bean per hectare per year and was ranked first amongst the major cocoa producing countries in terms of average yields. Higher yields might be obtained from high density planting regimes. There is also possibility to achieve 2 tons of cocoa beans per hectare per year under intensive commercial conditions (ICCO, 1998[b]).

2.4. Botanical description and agronomy aspect

Cocoa tree height ranges from 6 – 8 m and rarely exceeds 14 m. At 1 – 1.5 m the terminal bud breaks into 3 – 5 meristems to give several lateral upright shoots. The fruits usually considered as drupes but referred to as pods are variable in size and shape. They could range from 10 – 32 cm long, spherical to cylindrical, pointed or blunt, smooth or warty and with or without 5 to10 furrows. The pods could be green or red before ripening and yellow after ripening. The number of seeds per pod varies from 20 – 60 and could be arranged in 5 rows that range in length from, 2 – 4 cm long and in width from 1.2 – 2 cm wide, ovoid or elliptic; cotyledons white to deep purple, convoluted, large. The roots of cocoa plants are generally

a mass of surface-feeding roots. The taproot can penetrate as deep as 2 m in friable soil or less if soils are compacted (PURSEGLOVE, 1968).

Cocoa plants can be propagated by cuttings, buds or grafts. Use of seeds however has been proven to be costs less. Seed germinate at maturities which are viable only for a short time. They may be stored for a period of 10 – 13 weeks if moisture content is kept at 50%. The pulp has to be removed from the seed soon after picking. The seeds can then be planted in shaded nursery beds or baskets. Within a few months (when approximately 0.6 m tall), the seeds are transplanted into shaded fields at 2.4 m x 2.4 m or 3.6 m x 3.6 m. Spacing can be reduced if elevated above 300 m. Cocoa fields should remain shaded for 3 years. Removing floral buds is done at 5 years old (DUKE, 1983).

Trees used for shading should be nitrogen fixing, used for firewood and be free of antagonistic amelioration effects on cacao. Cacao seedlings do best with only 25% of the sunlight radiation and 50% sapling. Energy-fixing species of *Albizia, Erythrina, Gliricidia, Inga, Leucaena, Musanga, Peltophoru*m and *Terminalia* had been recommended as shade trees or "Madre de cacao" (PURSEGLOVE, 1968). Shade trees should be pruned as the cocoa tree size increases. Such programmed (ensured the shade trees grow in the desired shape and height, allowed good air and light circulation) are less susceptible to incidences of pest and disease, and consequently produces a higher yield. Water is an important factor in plantation establishment and management. Properly constructed infield drainage ensures better performance and higher yields (DEPARTMENT OF AGRICULTURE MALAYSIA, 2001).

Weeding of cocoa is done by hand or herbicides. Optimum fertiliser recommendations in the absence of shade are 5 cwt urea, 2.5 cwt triple super phosphates, and 10 cwt potassium sulphates per hectare (DUKE, 1983).

The correct establishment of the cocoa tree into plantations is important in obtaining the desired yields. Trees could also be grown using cloned material. In this case, the density of plants per hectare should be 1241 plants per hectare for a mono-culture or 750 – 850 cocoa plants per hectare if inter-cropped with coconut (DEPARTMENT OF AGRICULTURE MALAYSIA, 2001). Cocoa is often inter-cropped with other trees of economic value, such as bananas, rubber or oil palm (DUKE, 1983).

2.5. Harvesting and processing

The plants start to bear fruit at 18 – 30 months after planting. Pods are ready for harvest from 3 – 4 weeks after which time the beans begin to germinate (ICCO, 1998). The immature pod has a green or red colour that turns yellow or orange-like at maturity (DUKE, 1983). Harvesting should be done frequently to avoid over ripening of pods (DEPARTMENT OF AGRICULTURE MALAYSIA, 2001). The frequency of harvesting has an effect on overall yield (ICCO, 1998).

Although cocoa fruits mature throughout the year, the two main harvests are done (at the beginning and the end of rainy season) (TARLA, 1996). In Indonesia, the main harvest begins in September and extends to December, with a second smaller harvest in May-July (ICCO, 1998[a]).

During harvesting, care must be taken not to damage the flower cushion which will produce the flower and fruit of subsequent harvests. Harvesters must also avoid damage to the trees. Damaged trees are susceptible to infection by parasitic fungi (DUKE, 1983).

The harvested pods are allowed to mallow on the ground. The pods are then cracked and the beans removed (DUKE, 1983). Sometimes the pods are transported to a fermentary before splitting. If the pods are opened in the planting areas the discarded husks can be distributed throughout the fields to return nutrients to the soil (ICCO, 1998). Care should be taken during this process not to damage the bean. The wet cocoa bean should undergo fermentation for 7 days to kill the seed (at which time they change from purple to brown) and enhance the chocolate taste. After this the beans are dried under the sun or using a mechanised drier. The beans are stored for further processing (DEPARTMENT OF AGRICULTURE MALAYSIA, 2001).

2.6. Cocoa production by-product and its value

By-products that could be derived from cocoa productions are animal feed (from cocoa pod and cocoa bean husk), soft drinks, potash (from cocoa pod ash) and jam (ICCO, 2003[b]). In earlier times the cocoa pulp was also used as a beverage by South American natives (YOUNG, 1994). The gum from the pod husks was explored for uses such as binders for products like pet food, emulsifiers, and fixatives (FIGUEIRA, 1993).

Cocoa pods contain about 9% ash, 8 - 10% crude protein, 2 - 3% ether extract and 35% crude fibre. Cocoa pods contain a higher amount (3.2%) of potassium (K) compared to other roughages, but less calcium (0.3%) and phosphorus (0.1 – 0.2%). Theobromine content of cocoa pod is 0.32%. Cocoa pod has a gross energy of 20 MJ/kg DM (BARNES & AMEGA, 1984). Cell wall structure of cocoa pods after Van Soest analyses shows 11% hemicelluloses, 35% cellulose, 15% lignin and 6% pectin (SOBAMIWA & LONGE, 1994).

Nutrient composition of cocoa pods are evaluated to be relatively similar to that of king grass (*Pennisetum purpureum* cv. king grass (SUTARDI, 1988), but superior to palm press fibre and rice straw (TOHARMAT et al., 1997). An in vivo feeding trial done by LLAMOSAS et al. (1983) also showed that cocoa pod did not showed significant difference of steers live weight gain by substitution up to 100% elephant grass in the ration.

ADAMAKO (1995) recommended that dry cocoa pod husk pellets could be used as animal feed. The pelleted cocoa pod is produced by slicing the fresh cocoa husks into small flakes, partially drying the flakes followed by mincing, pelleting and drying the pellets. Dry pelleted cocoa pod husk could substitute corn and wheat bran in feed formulation for chickens, pig and sheep.

ICCO (2000) suggested to keep the inclusion rates of cocoa pods husk in the feed to a level that animals can tolerate, because of the high fibre but low protein content. In addition, theobromine, a toxic alkaloid present in cocoa pod, restricts its utilisation. Mortality due to this alkaloid has however been negligible. Research so far found that cocoa pods can replace 10% corns in diets for broiler chickens. Fifteen percent of cocoa pod is acceptable for growing pullets and 20% for laying hens. Growing and finishing pigs could use diets with up to 25% cocoa pods, while ruminants, like sheep, goats and cattle can tolerate higher levels (up to 45%).

The ratio of dry beans to pods has been found to be, 1: 2. Pruning could amount to 1 – 8 tons biomass/ha/year, depending on biological and environmental variables. During the third year, main branches might be reduced to 3 – 4, thenceforth, excess limbs and diseases tissues should be removed. One million ton production of cocoa beans therefore gives at least 2 million tons of pods and 2 million tons of prunings as residue. Yields could be increased in unshaded cocoa trees (DUKE, 1983).

Cocoa husk contains polyflavone glucoside, a pigment with a molecular weight over 1500. It is claimed that the pigment is heat and light resistant, highly stable at pH 3 – 11 and useful as food colorant. If completely isolated, this pigment constitutes about 7.9% of the total yield from cocoa beans (LEUNG, 1980).

2.7. Indonesian cocoa production

Cocoa has been known in Indonesia long time before the world war, but was exploited as a commercial plant after the second war. Criollo cocoa was introduced to Indonesia in 1560 but its production was small and limited to the island of Java. In 1888 Forastero cocoa was introduced to the Criollo stock resulting in a hardy Trintario variety producing what is known as the "Java A" bean. Cocoa plantings were cultivated by some estates in East Java and North Sumatra covering around 6,500 hectares before the Second World War, producing about 2,000 tons. Production remained small until Sulawesi, Kalimantan and Sumatra joined Java as production centres (ICCO, 2003[c]).

Indonesia expanded its cocoa production substantially from the early 1980s onwards. The Upper Amazon Interclonal Hybrid variety was chosen for a national expansion programme of smallholder cocoa. Millions of seedlings were distributed and by 1980 cocoa areas were covering 37,000 hectares and by 1988 more than 135,000 hectares. In 1981/82 Indonesia produced 16,000 tons of cocoa beans but by 1996/97 its production totalled 320,000 tons. In 2002/03 Indonesia planned to produce 440,000 tons of cocoa beans (ICCO, 2003[c]).

Most of Indonesian cocoa is exported and only a small portion of cocoa is retained for domestic consumption. Cocoa had become an important commodity for foreign exchange earning and its contribution is expected to increase in the future.

3. Lignocellulosic or fibrous feed

3.1. Characteristic of lignocellulosic or fibrous feed

Most of late maturing agricultural by-products have high plant cell wall content (SUNDSTØL & OWEN, 1984). They consist of three types of structural polysaccharides, namely cellulose, hemi-cellulose and pectic-polysaccharides. A major part of protein is also associated with the cell walls (THEANDER & AMAN, 1984).

Cellulose is the most abundant molecule in nature. It is a simple linear polymer composed of up to 10 000 β-1, 4 linked glucopyranosyl units but complicated by its three dimensional structure. In nature, cellulose is a largely crystalline form, organised as fibril. The cellulose chain is tightly packed together in compact aggregates surrounded by a matrix of other cell wall constituents (THEANDER & AMAN, 1984).

The major difference between cellulose and starch is the type of bonding. In starch, the dextrose units are linked by α-1, 4 and α-1, 6 bonding, whereas cellulose consists of only β-1, 4 linked dextrose. The tertiary structure of starch is therefore a wide helix disturbed by branching points, whereas the cellulose helix is very narrow and giving rise to a high degree of cristallinity. In nature, cellulose appears as a compound with lignin and hemicelluloses (DEGER, 1987).

Lignin is a family of related polymers of a three-dimensional structure, made up of phenyl propane units. It is generally agreed that p-coumaryl alcohol, coniferyl alcohol and synapyl alcohol are important precursors in the biosynthesis of lignin via complex enzymatic dehydrogenation processes. Lignin is encrusted into the cell wall and establishes covalent bonding to hemicelluloses, forming ligno-carbohydrate complexes. Lignin might also be linked to other wall constituents by covalent bonds (THEANDER & AMAN, 1984). Lignin is bound to different types of sugar units and to uronic acid residues in the hemicelluloses. The linkages to the sugar residues are probably mostly benzyl-ether bonds, while the linkages to the uronic acid residues are ester bonds or benzyl-ester bonds (ERIKSSON et al., 1980).

Silica is found in late mature plant components and is negatively correlated with degradability of the polysaccharides in the rumen (VAN SOEST & JONES, 1968). The plant cell wall is also related to the low digestibility and leads to high organic residue in faeces. These cell wall

components would become available to fermentation if the lignin-cell wall bonds are broken by pre-treatment of the dietary fibres (VAN SOEST & ROBERTSON, 1979).

3.2. Alkali treatment of fibrous feed

Treatments using concentrated alkali which break the homo-polar bonds of lignin to cellulose or hemicelluloses are extensively used to improve feed value of poor quality roughage (BODA, 1990).

Alkali treatments results in partly solubilised ligno-cellulosic, hemicelluloses and cellulose material to be more accessible for rumen microbes. In addition, saponification of the ester linkages found between acetic acid and phenolic acids, polysaccharides and/or lignin as well between the linkages in uronic acid residues in the hemicelluloses and lignin is also expected and occurs during alkaline treatment (SUNDSTØL & OWEN, 1984). The degree of polymerisation of xylans is much lower than that of cellulose. The low level of polymerisation and lower molecule weight of xylans and other hemicelluloses enables their extraction (SUNDSTØL & OWEN, 1984).

The accessibility of cellulose to hydrolysis could be increased by milling and alkali treatments to make the cellulose less crystalline and less hindered by associated components such as lignin or silica (SUNDSTØL & OWEN, 1984). The capacity of the forage to react to alkali treatment, however, depends upon the botanical family, the species and the variety to which it belongs. Unfortunately, there is limited information available to predict these differences (CHENOST, 2001).

The difference of chemical reaction between the alkali and the fibrous feed also depends on reaction-conditions such as temperature, pressure, alkali concentration, and reaction time (REXEN & KNUDSEN, 1984).

NaOH is an alkali source. Alkali treatment using sodium hydroxide (NaOH) both wet and dry methods improve the nutritive value of fibrous feed (HOMB et al., 1977). REXEN & KNUDSEN (1984); ANDERSEN & BOISEN (1989) used 4 – 5% NaOH/kg barley straw for wet treatment. While ANDERSEN et al. (1989); CHESSON & MURISON (1989) used higher level (8%). Increasing the application level (> 8%) did not release additional lignin nor result in improved digestibility.

However, NaOH is expensive for feed applications and may also have negative health and environmental aspects. The application of ammonia and urea or other ammonia releasing salts has on other hand been increased.

Ammonia is a suitable reagent for breaking hydrogen bonds. It is a gas of small molar weight, which could easily penetrate into interfibrilar space of plant cells. NH_4^+ cation are produced from cellular water ($NH_3 + H_2O \leftrightarrow NH_4^+ + OH^-$) with an equilibrium constant (K) of 1.75 x 10^{-5}. Only 1% of 0.1 M aqueous solution of ammonia is dissociated at room temperature whereas a solution of NaOH with the same concentration is completely dissociated under similar conditions (BODA, 1990).

Urea can also be used as a source of ammonia. CHENOST (2001) explained the two simultaneous processes which occur when forage is being treated with urea. The first reaction called ureolysis, splits urea into ammonia. The ammonia then acts on the cell walls of the forage. Therefore, urea treatment took longer time than treatment with anhydrous ammonia.

Ureolysis is an enzymatic reaction that requires the presence of the urease enzyme. Urease is absent in forage or roughage which are dead materials. It is produced by the telluric ureolytic bacteria under humid conditions (BESLE et al., 1990).

Although ammonia treatment with urea had been considered to be a method of choice for improving the feeding value of poor quality forage such as rice straw (SCHIERE & IBRAHIM, 1989; SUNDSTØL et al, 1993; CHENOST & KAYOULI, 1997), the specific conditions for the optimal treatments effect are not yet fully defined (TRACH et al., 1998). This may be one of several reasons why urea treatment of fibrous feed had not been widely applied in spite of expected rapid implementation in many developing countries (PRESTON, 1995).

CHENOST (2001) recommended a physio-chemical condition with 100% of humidity, 30 – 60% water content, 30 – 60^{o}C temperature, and 3 kg anhydrous ammonia per 100 kg DM straw (equal to 5.3 kg urea/100 kg straw). WILLIAMS et al. (1984); IBRAHIM & SCHIERE (1986) recommended lower urea levels (4 kg/100 kg DM). It was found that two-third of ammonia released from ureolysis was in labile form and lost; only one third of the ammonia was bound on the forage cell wall (CHENOST, 2001).

TRACH et al. (1998) found that the amount of the nitrogen that is fixed to the straw structure increases significantly over the time of treatment up to at least 30 days. When the urea level is

increased by 2% from 3% to 5% of the straw, only 17.4% of the additional urea nitrogen was fixed. This meant more nitrogen is lost when the level of urea applied is high.

It is known that a too high consumption of dietary urea results in toxicity. In the rumen, urea is converted to ammonia, which is either utilised by rumen microbes or absorbed across the rumen wall. In normal conditions, the blood levels of ammonia remains low as the liver rapidly converts ammonia back to urea. Urea toxicity and death from ammonia poisoning occurs when levels of blood ammonia are beyond the blood buffering capacity. Excessive levels results in a rise in pH and an impairment of the capacity of blood to expel carbon dioxide. Beneficially, the level of ammonia in the blood tends to be lower than that in the rumen, and the level of urea lower in the rumen than in the blood, the potential for a perpetual cycle exists, particularly under conditions of dietary nitrogen overfeeding (VAN SOEST (1982).

In an experiment, 0.5 g urea per kilogram bodyweight proved to have been toxic to half of experimental cattle. In the toxic cases, blood ammonia was elevated to 0.9 mg per 100 ml blood in 60 min while in the non-toxic cases blood ammonia remained significantly lower (0.5 mg per 100 ml in 60 min). Blood ammonia and toxicity were strongly correlated (HELMER & BARTLEY, 1971).

In toxic cases, the rumen pH was elevated to 7.41 in 60 min, significantly higher than 7.16 for the non-toxic cases. However, rumen ammonia concentration was found to be the same for toxic and non-toxic cases (ca. 80 mg per 100 ml rumen fluid) and did not correlate with toxicity. High concentrations of rumen ammonia did not necessarily indicate ammonia toxicity. High rumen ammonia concentration combined with high rumen pH, however, would indicate toxicity as the free NH_3 concentration would be much higher at high pH than at low pH. Ammonia exists as free NH_3 at high pH but as the ammonium ion (NH_4^+) at lower pH. Tissue membranes are permeable to the lipophilic NH_3 form but impermeable to the charged NH_4^+ form and as a result more ammonia is absorbed at high pH than at low pH (BARTLEY & DEYOE, 1981).

SMITH (1974) suggested that urea toxicity can be controlled by ensuring an adequate supply of available energy instead of replacing urea with other, more expensive, NPN sources.

Ensiling straws and other fibrous by-products after high doses of alkali treatment means anaerobic storage, rather than fermentation as a method of preservation. Ensiling is generally a method of conservation for grass and forage crops. It causes preservation by bacterial

fermentation of carbohydrates (usually glucose and fructose) to short-chain organic acids such as lactic and acetic acids. By contrast, straws and other by-products that had been treated with alkali have a relatively high pH (up to pH 12). In this case, "ensiling" is just as good as storage of the treated crop in a silo, though some bacteria could tolerate alkaline conditions, and if sufficient moisture is present fermentation, albeit at a very restricted level, might occur (WILKINSON, 1984).

3.3. Quality of alkali treated fibrous feed

Alkali treatment with NaOH solubilised the lignin fraction of barley straw, increased rate of protein disappearance which probably related to the higher release of protein associated with cell walls (HVELPLUND, 1998). Alkali treatment with NaOH also increased ash, fat and cellulose content and decreased the level of anti-nutritional factors such as polyphenols, tannins and caffeine of coffee pulp. Combination of HCl-NaOH treatment reduced true protein (TP), while ensilage (with addition of molasses) after NaOH treatment increased TP (ROJAS et al., 2002).

The increasing of ash content was mainly from sodium residue added with NaOH. By using 5% NaOH (resulting in approximately 25 g sodium per kg straw), REXEN & KNUDSEN (1984) found 10 g of residual sodium as NaOH in the straw.

Urea treatment of feed also resulted in the change of feed quality. Treatment of straw with urea or ammonia not only increased digestibility but also nitrogen content in the treated material (HVELPLUND, 1998). NH_3-N recovery ranged from 70 – 80% which contributed to increasing CP content of treated feed (MCDONALD et al., 1991).

Alkali treatment with ammonia before ensiling reduced CO_2 released during the early stages of ensiling, DM and energy loss during ensiling by 5 – 10%. The treatment increased pH of treated maize (up to 8.9) and water soluble nitrogen (WSN) with the major increasing on alanine content. Ammonia treatment had no significant effect on cell wall digestibility (MCDONALD et al., 1991).

Different reports indicated that not all the extra nitrogen added at treatment was degraded but some of it was tightly bound to the straw such that it was not released in the rumen (HVELPLUND, 1998).

Although digestibility is greatly improved by alkali treatment, the product is still classified as a low energy feed with lower digestibility compared to good quality hay (REXEN & KNUDSEN, 1984).

3.4. Alkali treated fibrous feed in ration

Although the CP content of ammonia treated feed increase by the treatment, the efficiency of nitrogen utilisation is low as indicated by the high nitrogen content of excreted faeces. There are three possible explanations for this low efficiency. A reduction in microbial activity/ synthesis in the case of NH_3-treatment dues to an inadequate and uneven supply of NH_3-N to the microbes have been observed in several cases. Secondly there is fixation of part of the nitrogen on potentially digestible cell-walls which were not digested in the rumen because of the reduction in cellulolysis and the increased rate of passage due to the positive effect of the NH_3-treatment on intake. These cell walls could then be fermented in the hind gut where a secondary microbial synthesis would take place. Part of this microbial nitrogen, undigested, would thus increase the nitrogen content of faeces. Lastly, the fixation of nitrogen on indigestible cell-walls passes into the faeces and increases their nitrogen content (CHENOST & REINIGER 1989).

Efficiency of urea utilisation by the ruminant animal also depends on dietary protein and amino acids. In a review by VAN SOEST (1982) it was shown that as dietary nitrogen increases, the proportion of total urea that is used declines, with the balance being lost in the urine. At low dietary nitrogen intake, the recycling could be very efficient, the source of urea likely being the endogenous metabolism of tissue and absorbed amino acids. The escape of feed protein from the rumen fermentation offers the possibility of adding nitrogen to the urea pool in the rumen (REXEN & KNUDSEN, 1984).

Treatment of straw with NaOH or NH_3 increased the intake of straw, the milk yield and the fat content of the milk of dairy cows. Chemically treated straw could not replace high quality roughage without reducing milk production in early lactation, even if both the treated straw and the high quality roughage had the same level of energy and essential nutrients (ANDERSEN et al., 1989). This might have been due to the low level of acceptance of NaOH treated straw by the animals (REXEN & KNUDSEN, 1984). For better milk production, ANDERSEN et al. (1989) suggested to supplement treated straw with a feedstuff rich in easily digestible cell wall, since treated straw has relatively low rate of digestion.

CORDESSEE et al. (1989) stated that when adequately supplemented with minerals and vitamins, the ammoniated straw diets are able to meet the maintenance requirements of ewes. If the straw is supplemented with 300 g of concentrate of barley and soybean per day, the intake of the treated straw will be sufficient for the needs of pregnant ewes and ensure a mean growth rate of 190 to 280 g/d for suckling lambs.

In conclusion, the problems associated with the use of urea treated feed in a ration are palatability, toxicity, and the efficient utilisation of urea-N in the rumen. The utilisation of urea is influenced greatly by the kind and quantity of energy available in the diet. Adaptation period is also important and it is undesirable to give ruminants large quantities of urea (i.e. 3% of the ration) before they become accustomed to it (BARTLEY & DEYOE, 1981).

3.5. Method for the analysis of alkali treated feed

CHESSON & MURISON (1989) explained that routine chemical methods of feed evaluation are not generally applicable to the alkali treated straw. Although lignin and other components were solubilised by alkali to an extent which was determined by the straw during processing, acid lignin (ADL, CL) and other chemical determinations gave identical values for both treated and untreated straw. At present, no chemical methods that measure specifically the extent of cleavage of lignin-carbohydrate linkages are available. The available biological methods such as in vitro or in vivo digestion trial are equally applicable to both treated and untreated residues.

CHESSON & MURISON (1989) suggested two alternative methods for measuring soluble lignin after alkali treatment:

(1) By extracting the lignin solubilised either through washing with buffer or neutral detergent soluble (NDS) and then measuring the optical density of the extract at 280 nm. Absorbance value had been reported to be positively correlated with in vivo organic matter digestibility.

(2) Saponification value and optical density (OD) of the soluble fractions released by saponification, provide an indirect measure of lignin solubilisation.

Urea treatment of straw not only influences the digestibility, but also the protein value of the straw. The influence of treatment on the protein value can not be evaluated in a system based

on crude protein, because of the inability of this method to evaluate the contribution of the microbial protein produced in the rumen which on treated straw diet is the main contribution to the intestinal flow (HVELPLUND, 1998) and to predict animal performance (ORSKOV, 1982).

4. Nitrogen metabolism and microbial protein synthesis (MPS)

4.1. Nitrogen and amino acid supply for ruminant

Ruminants require nitrogen not only for microbial growth but also for the host animal. Information on nitrogen available to the microbial population from dietary degradable protein (referred to as rumen degradable nitrogen = RDN) as well as on the amount of protein that by-passes the rumen and which is referred to as undegradable protein (UDP) is, therefore important.

The different methods for assessing rates of protein degradation are revised recently (GIVENS et al., 2000) and include among other chemical procedures solubility test in an appropriate solvent, measurement of ammonia production when the protein source is incubated with rumen contents and measurement of the rate of disappearance of nitrogen when the protein source is placed into a porous synthetic fibre bag and suspended in the rumen (SMITH, 1989).

The degradation of protein depends on its solubility (TAMMINGA, 1982) and the proteolytic activity in rumen contents. These factors are influenced by pH, the optimum range being 6 to 7.5. There are indications that pH values ranging from 5.7 to 8.2 could also be optimal (BLACKBURN & HOBSON, 1960). This factor will also affect the amount of nitrogen retained by the animal (HUME, 1975).

Since microbial protein composition and digestibility is of fairly constant thus the variation of nitrogen retained by animal is largely dependent on the degradable feed protein fraction value (ANNISON, 1975; QUIGLEY et al., 1985). The amount and amino acid profile of bypass protein would thus represent important criteria for value of feedstuffs (MILLER, 1982).

VAN NEVEL et al.(1975), after calculating the amino acid composition, protein content and DNA content of mixed micro-organisms found that the rumen microbial dry matter contains about 11% nitrogen (84% being protein nitrogen), 46% carbon, 6% hydrogen and 31% oxygen.

CONE et al. (2004) estimated the percentage of rumen escape protein (REP) from 6 grasses and 16 grass silages from the proportion of undegraded protein after 24h (U_{24}) of in vitro

incubation with a *Streptomyces griseus* protease, and calculated REP according the function REP (%) = 3.3 (± 4.02) + 1.31 (± 0.19) * U_{24} (%); $R^2 = 0.71$.

MILLER (1982) predicted non ammonia nitrogen (NAN) reaching duodenum by the formula 0.0215 (digestible organic matter) + 0.65 (insoluble dietary N). The biological interpretation of this mathematical relationship was that microbial N requirement was 0.0215 g per g digestible organic matter and that all the soluble dietary N and 35% of insoluble dietary nitrogen used was degraded in the rumen.

Although reducing the degree of protein degradation in rumen could be fairly easy, the difficulty lies in combining protein in the rumen with undiminished digestibility of the surviving protein in the small gut (SMITH, 1989).

4.2. Nitrogen metabolism and microbial protein synthesis

Microbial metabolism in the rumen plays a crucial role in overall digestion, and the efficiency of microbial conversion would be a determining factor in the overall efficiency of feed utilisation by the ruminant animals (VAN NEVEL & DEMEYER, 1996).

There is overwhelming evidence that rumen bacteria obtain most (50 – 80%) of their amino-acid requirements by de novo synthesis from ammonia and various carbon sources produced during the fermentation of carbohydrates (NOLAN & LENG, 1983). Therefore, it is necessary to find a strategy to maximise the synthesis of microbial protein in the rumen from readily available, inexpensive sources and thereby to reduce the requirements of true protein in the diet (QUIGLEY et al., 1985).

Nitrogen metabolism and its products in rumen have been reviewed by NOLAN & LENG (1983). The flux is shown in Figure 4.1. Sources of ammonia into the pool are peptides and amino acids, miscellaneous soluble material, gaseous N_2 and ammonia derived from protozoa. The routes of ammonia loss from the pool include incorporation into microbial cell, outflow in digesta and absorption by rumen wall.

The use of the ammonia for microbial protein synthesis is successful when the rumen ammonia is on optimal level and energy is not limiting. The microbial cell yield/kg digestible organic matter should be maximum (HUME, 1975).

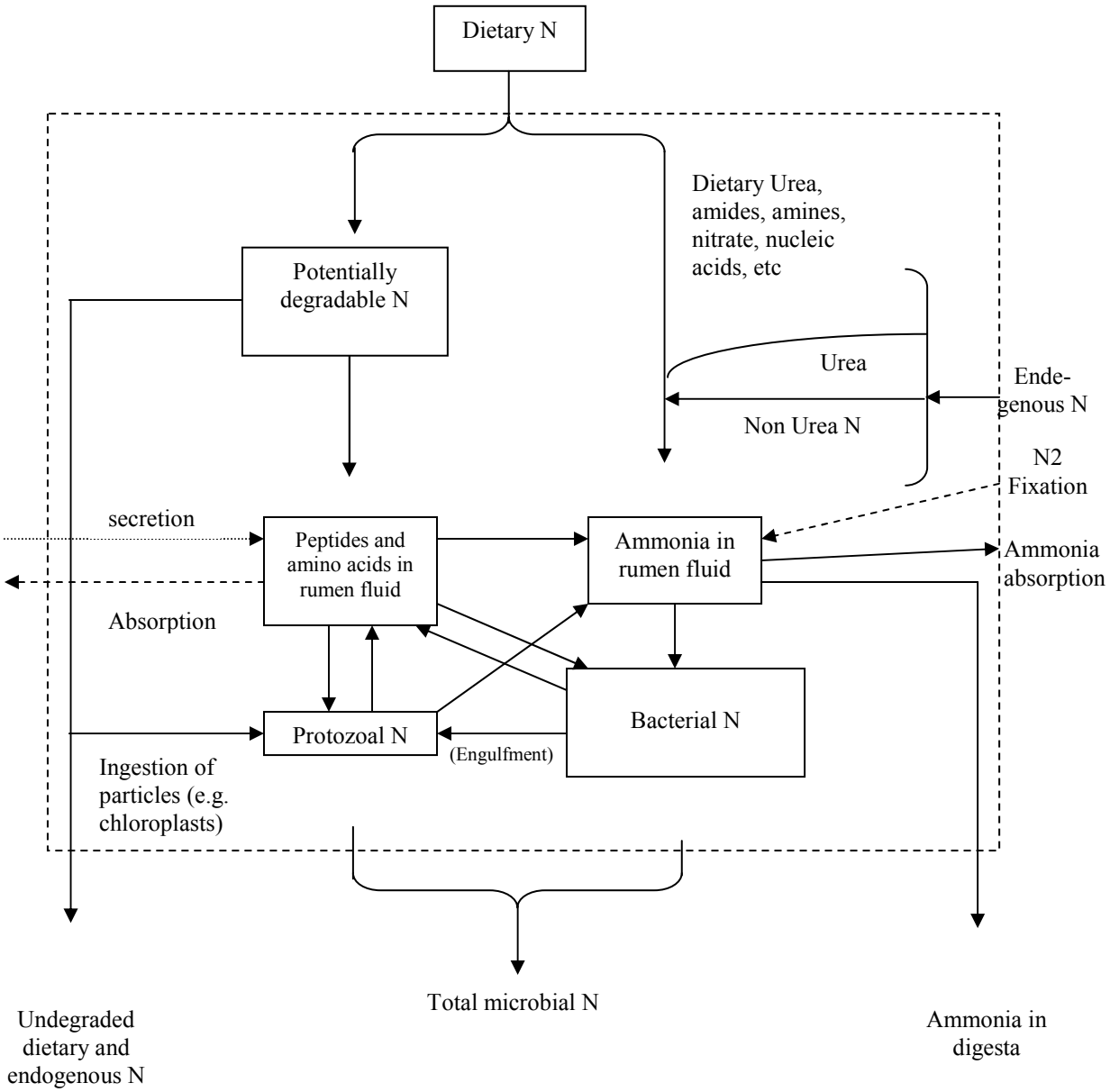


Figure 4.1: A Model of the metabolism of N in the rumen
Source: NOLAN & LENG (1983)

SATTER & SLYTER (1972) and SATTER & SLYTER (1974) found that the maximum yields of rumen microbial protein in vitro occurs at rumen ammonia concentrations of about 8 mg and 5.6 mg N/100 ml respectively. While NIKOLIC et al. (1975) found that the overall utilisation rate of ammonia-N is not significantly affected by mean concentrations of ammonia between 1.6 mg and 16.7 mg N/100 ml. The overall rate of protein synthesis in vitro also appears to be largely unaffected by the concentrations of ammonia in the incubation vessel. However, net protein synthesis as indicated by utilisation of added non-protein nitrogen and changes in ammonia concentration during incubation decrease as the supply of non-protein nitrogen was reduced. It is suggested that protein catabolism may be increased at low concentrations of

ammonia-N, and that the concentration of ammonia-N optimal for net protein production should be about 6 mg/100 ml. The wide range of ammonia requirements suggested for maximal microbial growth (0.56 mg N - 19.6 mg N/100 ml) agrees with findings by SMITH (1989).

SMITH et al. (1975) suggested that a slow release of NH_3 in the rumen would favour its efficient use as a nutrient by the rumen bacteria. Among the compounds studied was glycosyl-urea, which was shown to lead to a slower accumulation of ammonia in the rumen of the sheep than urea. These compounds are, however, destroyed progressively, with longer incubation and only about half of either the urea or glucose could be recovered after 30 h.

Besides rumen ammonia concentration, the amount of energy fermented is another important factor determining the amount of protein synthesised in the rumen. Short chain fatty acids (SCFA) are the principal products of energy fermentation in the rumen, and their rate of formation provides one of the most reliable indices of the amount of energy fermented (SMITH ET AL., 1975). NOLAN & LENG (1983) stated that the continuity in supply plus the amount of substrates available influences the efficiency of microbial protein synthesis.

Even though transamination is an effective pathway for amino-acid synthesis (ANNISON, 1975), nitrogen forms other than ammonia are required for optimal fibre digestion and microbial growth (CARRO & MILLER, 1999). OLDHAM (1981) and TAMMINGA (1982) reported that about 25 – 50% of microbial nitrogen requirements were from sources other than ammonia. These could presumably be intact amino acids or peptides which originate either from food protein, or protein recycled to the rumen (largely salivary muco-proteins) or from turnover of bacterial and protozoal protein within the rumen (OLDHAM, 1981). Therefore, high quality nutrient sources, including proper combinations of (rumen degraded protein) RDP and (rumen un-degraded protein) RUP are necessary to maximise rates and efficiency of microbial protein synthesis and body weight gain in calves (QUIGLEY et al., 1985).

Although the supply of amino acids (AA) to stimulate growth and yield of rumen bacteria are known, the precise combination of the AA has not been elucidated (ATASOGLUT et al., 2004).

The balance between nitrogen and carbon source supply is important for optimal protein use on microbial protein synthesis. BEEVER et al. (1986) and CZERKAWSKI (1986) found that ruminally degraded nitrogen to organic matter fermented (OMF) ratio approximated 25 g N/kg OM. DEWHURST et al. (2000) termed this proper combination of fermentable energy and

degradable nitrogen in rumen as 'synchronising', a process which could be altered by changing dietary ingredients, altering the relative times of feed ingredients, dosing specific forms of energy and nitrogen into the rumen or a combination of both approaches.

The degradation rates of the protein and carbohydrate fractions are pre-determined by in sacco studies (SINCLAIR et al., 1993; HENDERSON et al., 1998) in order to calculate the synchronicity index. The proportion of 25 mg N/kg fermented OM was proposed as synchronising index (SI) 1 (DAVIES et al., 1998).

In applying the principal of synchronising index, JETANA et al. (2000) studied the effect of protein and energy supplementation. They did not find any effect on the flow of nitrogenous compounds to the duodenum, i.e., total-N, non ammonia-N and rumen undegradable-N. Differences in the ratio of microbial protein to energy or total duodenal protein to energy among dietary treatments were also not significant.

4.3. Efficiency of microbial protein synthesis (EMPS)

Microbial organisms have different growth rates. The quantities of microbial protein synthesised daily in the rumen and their passage to the lower digestive tract are related to the amount of substrate available, substrate compositions and production level of the animal. In addition, turnover rates of digesta in the rumen and generation times of the micro-organisms, which varies, also play an important role in EMPS (DEWHURST et al., 2000; HARMEYER et al., 1975). The existence of protozoa, which are predators of bacteria, is another influencing factor (SMITH, 1989).

Ample data of microbial protein synthesis efficiency expressed as the relationship between organic matter fermented in the rumen and microbial cell yield have been generated since HUNGATE (1965). The available information indicates that the anaerobic nature of ruminal fermentation imposes energetic constraints on the conversion of digestible organic matter to microbial cells (HAGEMEISTER et al., 1981).

HUME (1970) in a study in which sheep were fed protein free diets found that the flow of protein to duodenum corresponded to a yield of 27 g microbial-N/kg fermented organic matter. It was also found that the cell yield is variable, and influenced by the nature of the micro-organisms and substrates, and by the dilution rate. A positive relationship between cell yield and propionate has been reported.

BEEVER et al. (1986) found more efficient synthesis of microbial cells (30 – 45 g microbial-N/kg organic matter apparently digested (OMAD) in the rumen) on high quality grazed grass ration than that found by HUME (1970). SMITH (1989), who used 262 diets in cattle or sheep showed that, on average, 32 g N/kg OMAD in the rumen (corresponding to approximately 24 g N per kg truly fermented) were incorporated into microbial N compounds, with a substantial coefficient of variation of 39 per cent. ABEL et al. (2002) found a lower microbial N/kg organic matter fermented (19 g) in Rusitec fermenter fed with silage based ration compare to others in vivo results reported above.

Because the bacteria are a part of the insoluble phase, they are included in dry matter measurements of substrate utilisation. Unless corrected, this inclusion confounds with efficiency estimates of microbial utilisation of substrate (VAN SOEST, 1982). True fermented organic matter (TFOM) corrected the microbial inclusion. It is equal to carbohydrate fermented plus cell organic matter produced during fermentation (DEMEYER et al., 1995). The TOFM term is also used instead of OMD to express EMPS. YANG et al. (2004) for example, found that microbial efficiency ranged from 18 – 20g N/kg of TFOM.

Instead of as organic matter proportion, efficiency of microbial synthesis can also express as proportion of hexose's fermented (HF). The amount of hexose theoretically fermented (HF) can be calculated from the yield of SCFA according to $C_6H_{12}O_{6\ ferm} = 0.5\ Ac_{net} + 0.5Pr_{net} + Bu_{net} + Va_{net}$. The amount of fermented organic matter (FOM) is then calculated in (g/d) as 162 x HF (DEMEYER & VAN NEVEL, 1975; DEMEYER et al., 1995). WOLIN (1960) & VAN SOEST (1994) added lactate to the formula ($C_6H_{12}O_{6\ ferm} = 0.5\ Ac_{net} + 0.5Pr_{net} + Bu_{net} + 0.5\ La_{net} + Va_{net}$). VAN NEVEL et al. (1975) reported that 60 g of cellular dry matter are formed per mol C6 fermented (or 370 g cell/kg HF), while BERGNER & HOFMANN (1996) calculated of 300 g cellular DM/kg HF.

It is also possible to express organic matter fermentation in terms of microbial adenosine triphosphate (ATP) production, which could be demonstrated in vitro (HAGEMEISTER et al., 1981). VAN SOEST (1982) suggested that the synthesis of one mol acetate would produce two ATP molecules while the synthesis of one mol of propionate via the succinate route, would produce three ATP. The acrylate route might only produce one ATP (although this is not certain). The two pathways could be equal. Butyrate formation might produce three ATP per mol, and formation of a mol of methane would produce one ATP.

VAN NEVEL et al. (1975) reported of 15 g microbial cell synthesis per mol ATP production. HESPELL & BRYANT (1979) found higher net efficiency of cell growth for mixed rumen cultures (about 19 – 20 g of cells per mol of ATP), while SMITH (1989) found 16 g microbial cells per mol of ATP.

Efficiency of microbial protein synthesis could also be assessed from metabolic hydrogen. About 90% of the metabolic hydrogen during fermentation is recovered in SCFA, CH_4, H_2 and lactic acid. 10% are not accounted for and are probably used in the synthesis of microbial dry matter. One gram of cellular dry matter requires of 0.0061 mol 2H (13 g microbial- N/mol H_2) (VAN NEVEL et al., 1975).

ALDERMAN & COTTRILL (1993) expressed EMPS based on MCP/FME, where MCP was microbial crude protein synthesis and FME fermentable metabolisable energy. It was found that MCP/FME is 9 g for maintenance of sheep and cattle, 10 g for growing sheep and cattle and 11 g for late pregnancy or lactating ewes and dairy cows. FME (MJ) is calculated as 0.65 FOM (fermentable organic matter).

High levels of concentrate in dairy cattle ration decrease microbial protein production per 100 g organic matter apparently digested in the rumen. The utilisation of fermented energy available for microbial synthesis is reduced by more than 50% if diet contains more than 50% of their energy intake as sucrose. Increasing feeding frequency, however, raise microbial protein synthesis except in cases when ration contains high roughage (HAGEMEISTER et al., 1981).

High fibre ration is more efficient than concentrate for ATP production because high concentrate ration produce more lactic acid. Under physiological conditions (35 – 70% concentrate in the ration; NFE:CF ratio of 2.3 – 3.3) there would be a constant rate of microbial protein synthesis of around 22 g bacterial protein per 100 g fermentable organic matter (HAGEMEISTER et al., 1981). MILLER (1982) also found that pure roughage rations are more efficient than roughage-concentrate mixtures and dry feed is more efficient than silage feed.

TAMMINGA (1982) stresses that the production of microbial biomass in the rumen, if expressed per unit of organic matter fermented, is not constant but would among other factors vary depending on growth rate, chemical composition of the substrate, chemical composition of the produced biomass and ruminal pH.

4.4. Method of MPS measurement

A wide range of approaches have been used to identify microbial protein in rumen contents (both in vitro and in vivo) and in digesta flowing at the omasum, abomasum or duodenum, though all have limitations (DEWHURST et al., 2000).

OBISPO & DEHORITY (1999) and DEWHURST et al. (2000) classified some available methods of microbial protein assessment:

(1) Protein-free diets which measured microbial protein on protein free diet and assumed all protein flowing into duodenum was from microbes;
(2) Distinguishing feed and microbial protein on the basis of amino acid profiles;
(3) Used of endogenous or exogenous markers to label microbial protein including ^{35}S, ^{15}N, ^{3}H-Leucine, ^{32}P, diaminopimelic acid (DAPA), RNA, purine and pyrimidine bases;
(4) Near infrared reflectance spectroscopy.

DEWHURST et al. (2000) explained that each of these markers had its own problems including safety (radioisotopes), cost (^{15}N and amino acid profiles), difficulty of analysis (RNA and DNA) and presence of feeds, thereby lacking specificity to the microbial fraction (DAPA, nucleic acid and their bases). Moreover, the isotopic techniques have serious shortcomings as to convenience and adequacy (MORRIS et al., 1975).

DEHORITY (1995) suggested that an ideal microbial marker should not be present in the feed, not be absorbed, be biologically stable, have a relatively simple assay procedure, occur in a similar percentage between the various types of microbes (i.e., bacteria, protozoa, and fungi), be a constant percentage of the microbial cell in all stages of growth, and have all form flowing at a similar rate (i.e., free and bound).

The details on the principal and some results of measurement techniques of microbial protein synthesis and its outflow are given below.

1. External Markers

The ^{15}N is used such as to assess amino acid transamination by rumen bacterial or incorporation of NH_3 into microbes. ANNISON (1975) focused his attention on the formation of glutamic and aspartic acid in the rumen. By hypothesising that glutamate dehydrogenase (GLDH) is the major ammonia fixation mechanism in ruminal bacteria; the amino acid synthesis was followed using $^{15}NH_3$. Analysis of rumen contents at intervals of 1 and 3 h

indicated the occurrence of the transamination reaction. The pattern of ^{15}N labelling in the amino acids obtained from hydrolysates of whole rumen contents showed considerable variation between the two investigated sheep. In both cases, however glutamic and aspartic acids were heavily labelled.

BODA & HAVASSY (1975) studied the nitrogen pool that is used by sheep. After intravenous administration of ^{15}N-labelled urea in sheep fed a low nitrogen diet (7.6 g N/d) and a higher nitrogen diet (24 g N/d, 42% urea-N), it was found that for both diets, the largest part of unretained ^{15}N (24 – 56%) is excreted in urine within 24 hours. Small amount (1.2 – 4.2%) of ^{15}N is excreted after 48 hours. On the 3^{rd} – 9^{th} day, ^{15}N excretion decreased. ^{15}N secretion into the digestive tract within the first 24 hours was lower compared to the excreted in urine (less than 1% of the administered dose). It was suggested that the ^{15}N that is not excreted in the urine was utilised in the nitrogen pool of the sheep within the period of 9 days post urea-^{15}N infusion.

The principal of MPS measurement using ^{35}S is explained by HUME (1975). The technique involves labelling the sulphur-containing amino acids of ruminal microbial protein with ^{35}S by infusing $Na_2{}^{35}SO_4$ into the rumen. The $Na_2{}^{35}SO_4$ can be administered either as a continuous infusion or as a single dose. In both cases a total dose of approximately 240 μCi is given. The animals are fed at frequent intervals (one or two hourly) by automatic feeder to establish steady state conditions. Duodenal digesta (250 ml) is collected from a re-entrant cannula 24, 30, 38 and 54 h after the single dose or after commencement of the continuous infusion. The collected digesta is fractionated by differential centrifugation and the microbial fraction washed with physiological saline to minimise contamination by plant particles. The percentage of microbial protein (% MP) in duodenal digesta is calculated as 1/ (M/D) x 100; where M is microbial fraction and D the unfractional duodenal digesta.

The applicability of ^{35}S incorporation as a measure of microbial protein synthesis in the rumen is critically evaluated by HARMEYER et al. (1975). The technique is based on the assumptions that the S/N ration in microbial protein is constant and the ratio of ^{35}S incorporated/mg protein-N synthesised is constant. However, for results obtained on this basis to be interpreted as newly synthesised microbial protein only, it also has to be assumed that there is no ^{35}S incorporation into microbial protein already present in the rumen and the breakdown of microbial protein in the rumen it-self, e.g. by cell lysis is insignificant.

HARMEYER et al. (1975) verified the assumption above using a serial experiment. The amount of substrate contained in the incubation mixture had a significant effect on microbial growth. When 8 grams of substrate were added microbial nitrogen precipitated with tungstic acid (TCA) increased by approximately 100% during 6 h of incubation. With no substrate added microbial nitrogen originally present decreased by approximately 17%, indicating that cell lysis was predominating.

It is necessary to determine the S content of the medium as well as the incorporation of radioactivity into amino acids from both supernatant and protein precipitate if ^{35}S is to be used as a tracer (DURAN et al., 1975).

An important objection against the ^{35}S and ^{15}N methods is that they do not take into account the direct incorporation of amino acid and peptide into microbial cells. This direct incorporation of amino acid and peptide N could be 20% of total N (VAN NEVEL et al., 1975). Therefore, in both these methods the microbial synthesis could be underestimated.

VAN NEVEL et al. (1975) proposed a procedure to estimate microbial synthesis in the rumen based on the incorporation of ^{32}P. The method is based on the fact that the phosphate pool is large and the turnover low, and that the phosphorus concentration of the microbial dry matter is constant in the experimental conditions. Results obtained with this method correspond with those from other methods. The average total P content of rumen microbial dry matter as determined on centrifuged and washed rumen contents obtained after a fasting period of at least 24 h is constant 0.93 ± 0.04%. From the P content of the microbial dry matter and mg P incorporated, synthesised microbial dry matter could easily be calculated.

MILLER (1982) tried the most widely used isotopes such as ^{35}S, ^{14}C, ^{32}P and ^{15}N, but did not calculate microbial growth from rates of incorporation or loss of isotope. The microbial growth was measured from the simplest method of digesta NAN flow to the duodenum and the microbial fraction in the digesta was calculated from the ratio between isotopic concentration in the digesta and that in isolated microbial preparation.

2. Internal Marker

PUCHALA & KULASEK (1991) determined microbial protein synthesis in the rumen by quantification of nucleic acids entering the duodenum and excretion of purine derivatives in urine (allantoin, uric acid, hypoxanthine and xanthine). Their results indicate a high

correlation between allantoin excreted in the urine and the amount of microbial nucleic acids entering the duodenum (r = 0.87). A high correlation is also noted between total purine derivatives excreted in the urine and the amount of microbial nucleic acids (r = 0.78) entering duodenum. A regression equation y = 15.50 * x – 1.21 (using allantoin nitrogen (g/d) excreted in the urine) was proposed for estimating microbial nitrogen synthesis (g/d) in the rumen of sheep. It was also observed that the ratio of allantoin-N to creatinine-N in the urine spot sample is not a suitable marker for microbial protein production in the rumen.

Dependency of the purine to N ratio on the rumen outflow rates showed that this ratio could not be considered as a constant as it changes with conditions of bacterial growth. This variability represents an important limitation for those cases where direct estimation of this ratio is not carried out, such as when microbial protein synthesis is estimated from the excreted urinary purine derivatives. The rumen outflow rate is the main factor that influences the N composition of rumen bacteria. An increment of this rate is associated with an increase of the concentration of purines in bacteria and a decrease of their true protein content. These relationships partially explain the differences in chemical composition between liquid associated bacteria (SAB) and solid associated bacteria (LAB) (RODRÍGUEZ et al., 2000).

AHARONI & TAGARI (1991) collected N derived from purines in digesta for ^{15}N determination. Kinetic measurements of ^{15}N enrichment in the purine nitrogen fraction in a continuous culture in vitro together with ^{15}N enrichment measurements in other N fractions could offer a sensitive way to define differences in N metabolism characteristics between different protein sources.

SHEM et al. (1999) used allantoin as a marker and found that excretion of allantoin increases with level of dry matter intake (DMI). Microbial N supply and efficiency of microbial N supply follow the same trend (4.1 – 35.5 g N/day and 5.5 – 17.4 g N/kg digestible organic matter in the rumen) for banana leaves and 5% urea treated maize stover, respectively.

The use of RNA as an internal marker for microbial biomass in the rumen is based on the fact that RNA is a part of the protein synthesising complex in the cell. In pure cultures RNA to protein ratio of rumen bacteria increases with specific growth rate and there is no difference from 0 to 8 h after feeding in sheep (BATES et al., 1985).

Since dietary nucleic acids (RNA and DNA) are largely degraded in the rumen the components reaching the duodenum are mainly of microbial origin. RNA is the preferred

marker. It is less variable and gives a more appropriate measure for both bacteria and protozoa. However, there are a number of chemical methods for determining RNA and these do not always give similar values as indicated by the varying RNA contents of bacterial preparations from different laboratories. The RNA to N ratio of bacteria is also influenced by diet and by the time after feeding. Therefore, average RNA to N content of digesta and microbial preparation must be determined simultaneously for each animal and diet combination (MILLER, 1982).

THOMAS (1973) reviewed a number of studies in which bacterial cell yields were determined using the bacterial protein marker diaminopimelic acid (DAPA). For a range of 27 diets the average bacterial synthesis resulted in 32.1 g N/kg organic matter being fermented. Bacterial synthesis (g N/kg organic matter fermented) ranged from 15.3 to 53.1 g N/kg. These data imply that it is not possible to predict cell yields with confidence (low precisions).

MILLER (1982) reported that DAPA (∝,ε-diaminopimelic acid) is the most widely used natural marker for microbial protein. It is a component of cell wall glycopeptide of certain Gram-negative bacteria. It is not found in protozoa or the usual animal feedstuffs. The proportion of bacterial N in duodenal digesta NAN is determined from the ratio (DAPA to N in digesta)/(DAPA to N in bacteria). Since DAPA to N ratio of rumen bacteria varies with bacterial species, diet and time, and host animal it is essential that the DAPA to N ratio of bacterial fractions be determined simultaneously with that of duodenal digesta. The method does not measure protozoal N, resulting in an unknown underestimation of microbial N and consequently an overestimation of undegraded feed N. The reverse error can also be encountered.

All methods, whether using natural or isotopic markers, have to assume that the isolated microbial preparation is representative of the whole mass of microbial passing to the small intestine, and the marker remain with the microbial mass both in the rumen and during passage to the duodenum. It is difficult to prove that these assumptions are met. The common approach had been to compare the results given by independent markers (MILLER, 1982) which resulted from a wide range of experiments.

4.5. Limitation of available methods

There are several limitations and assumptions which could not be fulfilled by available microbial protein synthesis measurement methods. With all MPS measurement approaches a metabolic parameter is applied which is closely related to the synthetic processes occurring in the micro-organisms (HARMEYER et al., 1975). It needs to be stressed, however, that a constant and reproducible relation can only be expected when microbial fermentation is at its maximum. These correlations are not constant if microbial growth becomes sub-maximal due to shortage of substrate, accumulation of end products or conditions unfavourable to micro-organisms. In all other cases incorporation methods tend to overestimate the synthesis of microbial protein and its passage to the abomasums.

The bacterial population of the rumen might be thought of as occurring within at least three distinct compartments which are the rumen fluid, the food particles and the rumen wall. Most in vitro quantitative studies on efficiency of bacterial protein synthesis had been carried out using strained rumen liquor where bacteria in rumen fluid bacteria are dominant. This fact should be borne in mind as a serious limitation of the practical importance of such experiments (SMITH, 1989). A number of studies have now demonstrated large differences in the composition of rumen microbes according to whether they are isolated from rumen liquids (liquid associated bacteria, LAB) or solids (SAB). The stimulus for this work is the potential for inaccurately calculating microbial synthesis if marker to N ratio is derived from non-representative samples of rumen bacteria (DEWHURST et al., 2000).

RODRÍGUEZ et al. (2000) for example, used the purine and ^{15}N tracer method to estimate microbial protein synthesis and found that the concentration of purines and ^{15}N was lower in SAB than LAB, whereas the opposite was observed for the concentration of amino acids (mg/g DM).

In studies of microbial protein flow and the efficiency of microbial protein synthesis as well as in studies of microbial colonisation, RODRÍGUEZ et al. (2000) used LAB samples. Because LAB represents only a low proportion of the rumen bacterial population (LEGAY-CARMIER & BAUCHART, 1989), the use of ^{15}N : N or purine ratios of LAB results in underestimation of the microbial production potential of feeds.

DEWHURST et al. (2000) suggested that the term microbial which was used in MPS calculation could be misleading since estimates were generally made using marker ratios solely for bacteria that were isolated from rumen contents. Thus they were really an estimate of bacterial protein and other microbes contributing to protein flow were not included, except, to a degree, where marker might have passed via bacteria through another microbial pool. An exception was where AEP (aminoethylphosphonic acid) was used to mark the microbial fraction leaving the rumen, in combination with bacterial marker (HUTTON et al., 1971). It had been, however, argued that protozoa were largely sequestered in the rumen of animals fed high fibre diets and might not make a major direct contribution to microbial protein flow to small intestine (WELLER & PILGRIM, 1974; COLEMAN et al., 1980).

On calculating MPS through microbial outflow, DEWHURST et al. (2000) found that there are two distinct problems, namely estimating the amount of material leaving the rumen and assessing the proportion of microbial protein. The use of fistulated cows has extended these problems through low replication. TITGEMEYER (1997) in a review showed that an average of 12 animals per treatment group is required to get treatment differences of 10% in microbial efficiency.

Furthermore, DEWHURST et al. (2000) stated that getting accurate estimates of microbial synthesis is also a challenge when doing in vitro studies. In vitro systems have the advantage that representative sampling is assured and the outflow of both particulate and liquid phases could be accurately controlled (STERN et al., 1978; MERRY et al., 1987).

Estimations of post-rumen microbial supply of total AA in the new protein systems for ruminant feeding are derived from measures of the efficiency of microbial protein synthesis (expressed as g microbial N synthesised/kg organic matter fermented or digested in the rumen), assuming a constant proportion of AA in the microbial crude protein. Nevertheless, results of the present study show that the AA to N ratio decreases as the rumen outflow rate increases (RODRÍGUEZ et al., 2000).

The direct estimation of the microbial protein synthesis in the rumen requires the use of markers. The agreement between different markers and the accuracy are generally not satisfactory (DEWHURST et al., 2000). A wide range of values have been reported for the ratio of purine-N to total-N in experiments conducted both in vivo and in vitro, and most were in the range 0.08 to 0.12 (DEWHURST, 1989).

MORRIS et al. (1975) rejected the use of some isotope as markers because for example the ^{15}N technique measured only N-incorporation from NH_3 and disregards microbial protein synthesised directly from amino-N. Similarly, the ^{35}S technique is based on the premise that all the S-amino acids in rumen microorganisms are synthesised de novo. The ^{32}P incorporation technique assumes that the specific activity of the precursor pool for cell synthesis equals that of the extra cellular medium. The contribution of protozoa to total or net microbial protein synthesis is an important variable that has not been adequately considered.

VAN NEVEL et al. (1975) also expressed their objection to the ^{35}S and ^{15}N methods which do not take into account the direct incorporation of amino acid or peptide N into microbial cells. This direct incorporation of amino acid or peptide N could be 20% of the total N incorporated. Therefore, in both these methods the microbial synthesis could be underestimated.

Based on limitations of each method of MPS estimation, DEWHURST et al. (2000) suggested a molecular technique that enables a quantitative assessment of microbial biomass and, hence, rumen microbial protein outflow. However, the methods are still to be developed.

5. Methane productions and its effect on environment

5.1. Sources of methane

Based on model calculation, which are calibrated against global concentration distributions of CH_3CCl_3, total emission of methane from all sources (as an average during the 1980's) amounts to 630 ± 63 Tg/a (Tg/a = 1 terragram/annum = 10^{12} g/a = 10^6 t/a) (CRUTZEN, 1995). Over the last decade the methane content in the atmosphere had arisen by an average of approximately one percent per year (BLAKE & ROWLAND, 1986).

Contribution of agriculture and animal farming on methane emissions have been reviewed by PELCHEN & PETERS (1994). Agriculture produces 53% of total methane emissions, of which 21% is from animal production alone. Taking into consideration the methane from the greenhouse effect, agriculture has a 10% contribution to global warming, and animal farming 4%. Wet rice cultivation (32%) is the major contributor of methane emission followed by animal production (21%). The reminder is from burning biomass, swamps and anthropogenous sources (SEILER & CONRAD, 1987). The source of methane according to different authors is shown in Table 5.1.

Table 5.1: Contribution of different sources to total methane emissions

	Methane emissions							
Sources	10^6 t/a	%	10^6 t/a	%	10^6 t/a	%	10^6 t/a	%
Rice farming	120	32	280	38	30-59	14	100	19
Animal farming	80	21	100-220	22	72-99	28	105	20
Swamps	50	13	190-300	33	13-57	10	140	27
Burning biomass	60	16			53-97	24	25	5
Misc.	67	18	18-94	7	58-84	23	154	29
Total	377	100	588-594	100	226-395	100	524	100
Researchers	SEILER & CONRAD (1987)		EHHALT & SCHMIDT (1978)		SEILER (1984)		TYLER (1987)	

Source: PELCHEN & PETERS, 1994

There are variations in the estimates of the contribution of animal farming to methane emission. This is possibly due to the exclusion of between feed variations when calculating the estimates. Seasonal variations in quality and quantity of the food supply as well as species of animals also have an influence on the estimates. The ages of the animals, performance

grades of feeding intensities should be accounted for in experimental trials and not through extrapolation or prediction as done in most studies (PELCHEN & PETERS, 1994).

CRUTZEN et al. (1986) ranked methane as the most important pollutant gas from animal farming. Ninety-six percent of the methane emitted from animal farming world-wide comes from ruminants of which, 74% is from cattle, 8% from water buffalo and sheep, and the remaining from camels and goats. LENG (1992) divided the total animal methane emission to atmosphere into direct production of methane in fermentative digestion of ruminants and indirect when faecal materials decompose anaerobically.

Estimates of methane emissions from animals of developed and developing countries are shown in Table 5.2.

Table 5.2: Estimates of methane emissions from animals of developed and developing countries

Animal Type and regions	World Population (x 10^6)	CH_4 Prod (kg/hd/year)	Total CH_4 Prod (Tg)
Cattle, developed countries	573	55	31.8
Cattle, developing countries	653	35	22.8
Buffaloes	142	50	6.2
Sheep, developed countries	400	8	3.2
Sheep, developing countries and Australia	738	5	3.7
Goats	476	5	2.4
Camels	17	8	1.0
Pigs, developed countries	329	1.5	0.5
Pigs, developing countries	445	1.0	0.4
Horses	64	18	1.2
Mules, Asses	54	10	0.5
Humans	4,670	0.05	0.3
Wild ruminants and large herbivores	100-500	1-50	2-6
Total			76-80

Adapted from CRUTZEN et al. (1986)

Approximately 75% of the total methane emissions are from the Northern hemisphere. Within the northern hemisphere, much of the emissions are from areas between the 20^oN and 30^oN, while peak emissions are also observed from areas between the 40^oN and 50^oN and the 20^o and 30^oS latitudes. High levels of emission within the 40^oN and 50^oN could be explained by the large number of cattle in India and China, the water buffalo of South - East Asia, and

goats in India. Emissions in areas within the 40°N and 50°N could be from milking cows and sheep, but from the other cattle in the regions where the higher feeding level over-compensate for the relatively low numbers of animals. The vast numbers of cattle in Brazil and Argentina, and the sheep in Australia and South Africa could explain the emissions in the Southern hemisphere (PELCHEN & PETERS, 1994).

5.2. Methanogenic process in animal

Methane production in the fore-stomach of ruminants is the most important source of methane emission to the environment by living organisms. For instance an adult cow produces about 200 litres of CH_4 per day. The methane production could either be due to cleavage of acetate by acetoclastic acetogens, or by reduction of CO_2, CH_3OH or formate to CH_4 utilising H_2 (NOLLET & VERSTRAETE, 1996).

Methane represents a loss of energy that could be used for maintenance or production for the individual animal. Animal nutritionists have therefore always been interested in minimising the proportion of loss (PELCHEN & PETERS, 1994). The physiological processes of methane formation by bacteria are shown in Figure 5.1.

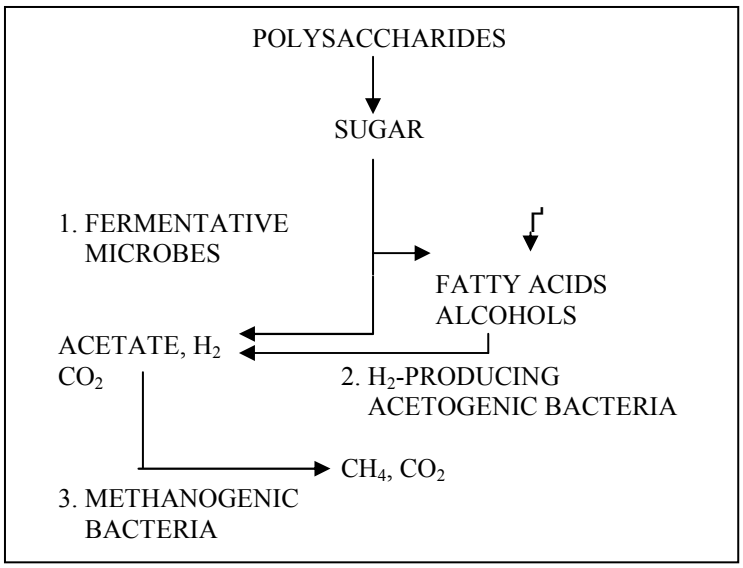


Figure 5.1.: The methanogenic process
Adapted from BRYANT (1979)

According to RATH & GÄDEKEN (1991) the following groups of bacteria take part in methane formation: (1) bacteria that fermented polysaccharides, (2) bacteria that get their energy from alcohol and organic acids, forming acetate and H_2, and (3) the actual methanogens, which produce methane from CO_2 and H_2. While NOLLET & VERSTRAETE (1996) classified the H_2 consuming bacteria as (1) sulphate-reducing bacteria (SRB) use H_2 to reduce SO_4^{2-} to H_2S, (2) hydrogenotropic methane-producing bacteria (MPB) using H_2 to reduce CO_2 to CH_4 and (3) reductive acetogens (RAC) use H_2 to reduce CO_2 to CH_3COOH. A competition between these three bacterial groups exists for the common H_2 substrate. This results generally in the dominance of one group above the other two.

In the rumen 5 – 15% of the gross energy in feed is believed to be lost by hydrogenotrophic methanogenesis (CZERKAWSKI, 1969). Inducing Hydrogen utilising reductive acetogenesis in the rumen could therefore result in better utilisation of the carbon sources fed to the ruminants. This would lead to an increased efficiency in feed utilisation resulting in reduced animal feed requirement.

BALDWIN (1965) and DEMEYER & HENDERICKX (1967) suggested that the primary precursors of methane in the rumen appear to be formate (via CO_2 and H_2), CO_2 and H_2. CAROLL & HUNGATE (1955) proposed formic acid as one of the most important hydrogen donors. However, comparing theoretical production rates of methane and formic acid in the rumen, they concluded that other substrate could also be involved.

Although, WOLIN et al. (1963) had succeeded in demonstrating the synthesis of methane in cell free extracts of *M. omelianskii* , the pathway of methane synthesis at that time was still unknown. Ferredoxin was believed to be the stimulator in the system. The terminal reaction in methanogenesis appeared to involve the reductive cleavage of methylcobalamin to form methane and cobalamin.

The molar proportion of methane formation from hexose was explained by WOLIN (1960) as

$$57.5\ (C_6H_{12}O_6) \rightarrow 65\ Ac + 20\ Pr + 15\ Bu + 60\ CO_2 + 35\ CH_4 + 25\ H_2O$$

Where Ac was acetate, Pr was propionate and Bu was butyrate.

The production of acetate and butyrate from glucose results in the production of 2 and 1 mol of H_2/mol of acid, respectively. When propionic acid is the end-product, 1 mol of H_2 is taken up. Thus, there is an excess of hydrogen which is normally used for reduction of CO_2 to CH_4. Although methane production is the reaction that provides energy for growth to the methane

bacteria it nevertheless constitutes a major loss to the host animal If the methane production could be inhibited, then the food energy could be used for increased production. The manipulation of rumen fermentation is an example of the inhibition of methane production for economic reasons (CZERKAWSKI, 1973).

Factors influencing of the ruminant methane production are rumen SCFA proportion, presence of ciliate population (WHITELAW et al., 1984), composition of ration, digestibility, feed intake and energy supply (PELCHEN & PETERS, 1994). According to LENG (1992) the efficiency of fermentative digestion in the rumen and the efficiency of conversion of feed to product (e.g. milk, beef, draught power) influence the level of methane output relative to product output of ruminants.

Furthermore, WHITELAW et al. (1984) observed that rumen SCFA proportion changes from a high-propionate pattern under ciliate-free conditions to a low-propionate, high-butyrate pattern in the presence of ciliates. CH_4 production is higher in the presence of rumen ciliates. The increased loss of energy as CH_4 in the faunated animals amounted to 4.8 MJ/100 MJ of energy intake. Stoichiometric estimates of CH_4 production derived from observing SCFA proportions agrees with those measured in respiration chambers. On average, the stoichiometric CH_4 values overestimated CH_4 production by a factor of 1.08. The proportion of propionic acid is inversely related to CH_4. Rumen ciliates, by modifying the rumen VFA proportions results in increased CH_4 production in faunated animals.

Furthermore LENG (1982) clarified that feeds that allow a high efficiency of microbial protein cell synthesis produce low amounts of methane per unit of feed digested. In cattle fed based on poor quality forages, a number of essential microbial nutrients might be deficient and microbial growth efficiency in the rumen is low. Under these conditions CH_4 production may represent 15 – 18% of digestible energy. Correction for the deficiencies may reduce this to as low as 7%.

Methane emission rates tend to follow a behavioural pattern with peak emission rates corresponding to peaks in eating activity. Emission rates decrease when sheep are ruminating. Methane emission averages 20.3 l/d/animal (LOCKYER & CHAMPION, 2001).

5.3. Effect of methane to the environment

The greenhouse effect, or increase in world temperature is due to accumulation of gases – the so called "greenhouse gases" – in the atmosphere. This is clearly ascribed to the major industrial countries as some 50% of the increased retention of energy by the atmosphere is a result of the accumulation of carbon dioxide from combustion of fossil fuel. Industrialised countries use 70% of the world's oil production and it had been much higher in the past (LENG, 1992).

Animal farming contributes to air pollution and the greenhouse effect around the world. In relation to air pollution agriculture is also responsible for approximately three quarters of the global emissions of ammonia (NH_3). It also adds insignificant shares of carbon dioxide (CO_2) and around one-fifth of the methane (CH_4) emissions to the global budget of greenhouse gases (SEILER & CONRAD, 1987).

Methane plays an important role in the earth's radiation budget, and has a 19 percent share in the greenhouse effect. It is thus in second place after carbon dioxide (SEILER & CONRAD, 1987). Although methane is one of the trace gases and insignificant in quantity, compared with carbon dioxide it makes 25 times more substantial contribution to the greenhouse effect because of its greater effect per molecule (DANTUS, 1998).

The rates of accumulation of methane and carbon dioxide in the world's atmosphere have changed dramatically in the last 10 years. Prior to this, the rise in world temperatures and composition of the atmosphere changed only by a small percent, but there now seems to be an increasing rate of gas accumulation. The contamination of the atmosphere with carbon dioxide, methane and other gases must be reduced or the future of the earth is threatened (LENG, 1992).

5.4. Strategy to reduce methane production from animal agriculture

For the ruminant, methane production means an important energy loss (8 – 10% of the gross energy ingested with food). There is possibility of lowering methane production through addition of substrates that are able to compete for H_2 with CO_2 (MCDONALD et al., 1995)

Conversion of the CO_2-CH_4 fermentating ruminants to a CO_2-acetate fermentation would simultaneously benefit agricultural production and significantly diminish the contribution of ruminants to global CH_4. Conversion of CO_2 to acetate instead of CH_4 would increase the microbial production of a rumen fermentation product that is absorbed into the blood and used as a major source of carbon and energy by cattle and sheep. Biotechnology could be applied to replace methanogenic microbial community of the rumen with a community that produces the CO_2-acetate fermentation (MILLER, 1991).

PELCHEN & PETERS (1994) suggested some strategies to reduce methane emissions by reducing the number of animals, intensifying the feeding, use of high-performance animals, prevention of methano-genesis, and use of bovine recombinant somatropin (BST). Improving feed efficiency and utilisation by increasing degradability and supplementation of low quality roughage which contributes to 75% of world rumen methane production would not only increase product output but also reduce methane production per unit of consumed feed (LENG, 1992).

LENG (1992) suggested that developing mechanisms that increase the efficiency of animal production at high and economic stocking rate and decreasing methane production per unit of product output by 50% will be a potential approach for scientists in countries with high quality pastures. High production rates per animal are often incompatible with the optimisation of production of animal products per hectare. High stocking rates reduces production rate per animal but increase production per hectare. This is often the most profitable approach but is in most cases environmentally harmful as it increases methane emissions per unit output of animal products.

DEMEYER & HENDERICKX (1967) observed that C_{18} unsaturated fatty acids inhibits methane production from various substrates by mixed rumen bacteria in vitro. This inhibition is not due to competition for available hydrogen, but to a toxic effect towards methanogenic bacteria. *Cis*-unsaturated fatty acids are much more active than *trans* isomers or saturated fatty acids. With cis isomers, toxicity increases with the number of double bonds. Various esters are inactive, indicating the importance of the free carboxyl group. This finding could be explained by assuming a physicochemical mechanism of inhibition. With pyruvate as substrate, inhibition of methane production was accompanied by stimulated propionic acid production.

As methane and propionic acid are produced by different bacteria, inhibition of methane production could be the indirect result of a stimulation of propionic acid-producing bacteria in the washed cell suspension symbiosis (DEMEYER & HENDERICKX, 1967).

Methane inhibitors both in nature or synthetic compounds of feed have been studied extensively. Supplementation of coconut oil at proportions of 3.5 and 7% DM sheep rations suppressed in vivo methane production by 28 and 73%, respectively, as related to the non supplemented diet (MACHMÜLLER & KREUZER, 1999). However, the supply of these lipids to ruminants is quite restricted due to their adverse side-effects, particularly on fibre digestion (MACHMÜLLER et al., 2000).

MACHMÜLLER et al. (2000) tried a relatively low dietary lipid proportion and found that coconut oil and sunflower seeds decreased energy loss via methane by up to 25%. These compounds had a potential as natural methane-suppressing feedstuffs in ruminant fattening diets, particularly in comparison with free polyunsaturated plant oils or synthetic methane inhibitors. Concerning the feeding value for fattening ruminants, crystalline fat was slightly superior to other supplements. When regarding the effect of methane release and energy balance together, coconut oil appeared to be the most beneficial choice.

Alkali treatment of cereal straw increased the volume of methane produced per animal per day, but decreased the methane by up to about 21 l/kg digested feed OM. The intensity of inhibition is dependent on the straw type and the alkali used. Maximum inhibition was obtained with wheat straw treated with ammonia which reduced methane in the control period from 58 l/kg OMAD to 37 l/kg OMAD after treatment of the straw. It was also proven that a methane inhibitor did not work for gram-negative bacteria because of the cell wall protection by outer membrane. Therefore were propionate was produced. Supplementation of long chain fatty acid (LCFA) reduced methane by decreasing OMD. Several factors such as the lower rumen digestibility of the crude fibre fraction, the loss of methanogenes attached to ciliates and the loss of protozoa as producers of hydrogen and formate, the precursors of methane, could be responsible for the methane reducing effect of defaunation (MOSS et al., 1994)

Sodium fumarate might also be a useful dietary additive for ruminants, because it diverts some H_2 from CH_4 production and is able to stimulate proliferation of cellulolytic bacteria and digestion of fibre (LOPEZ et al., 1999).

LESLIE & O'HARA (2003) applied a methane inhibitor vaccine. The methane vaccine discourages 'methanogenic archae' ancient living organisms which inhabite the animal's rumen and produce methane by breaking down feed. The commercial vaccine could reduce methane emission by about 20% or total saving equivalent to that which would result from a reduction of 300,000 ton in carbon dioxide in Australia.

SAR et al. (2004) used an appropriate combination of nitrate with β1-4 galacto-oligosaccharides or nisin to abate rumen methanogenesis. Addition of β1,4-galacto-oligosacharides or nisin to nitrate abates rumen methanogenesis with a decrease in nitrate/ nitrite toxicity and tends to improve some rumen fermentation parameters. However, dosage of nitrate (1.3 g $NaNO_3$/kg 0.75 of BW) chosen in this study was high enough to induce blood methemoglobinemia and sub-clinical toxicity (TAKASHI & YOUNG, 1991).

6. Experiment 1. Nutritional properties of fresh, ensiled and urea-treated cocoa pods (*Theobroma cocoa,* LINN)

6.1. Introduction

Cocoa pod is a by-product of cocoa plantation. Up to 2 kg air-dry (87%DM) cocoa pod meal can be yielded by producing a kilogram of dry cocoa beans (DUKE, 1983). The world production of cocoa beans is estimated to be about 3 million tons per year (ICCO, 2003), implying a potential yield of 6 million tons of cocoa pods per annum.

On a dry matter basis, cocoa pods contain 9% crude ash, 10% crude protein, 2 – 3 % crude lipids and 35% crude fibre, leaving a residual fraction of approximately 43% N-free extractants (BARNES & AMEGA, 1984). The fibre fraction of cocoa pods contains 35% cellulose, 11% hemicelluloses, 6% pectin and 15% lignin (SOBAMIWA & LONGE, 1994). The nutrient composition of cocoa pods resembles that of king grass (*Pennisetum purpureum*, cultivar king grass; SUTARDI, 1988) and may be superior to palm press fibre and rice straw as a source of fibre in dairy cattle's ration (TOHARMAT et al., 1997). A 100 kg of cocoa pod meal have been observed to have the same feeding value as 96 – 97 kg chopped green corn (DUKE, 1983). The high content of lignocellulosic material in cocoa pods, however, results in low digestibility (∅RSKOV, 1982). Theobromine, a toxic alkaloid present in cocoa plants, may also limit its use in ruminant nutrition (ICCO, 2000). However, biological, physical and chemical treatments may be used to improve the feeding value of severely lignified agriculture by-products (SUNDST∅L & OWEN, 1984).

Strong alkali such as NaOH and strong acid such as H_2SO_4 have been applied successfully in the treatment of fibrous by-products (KLOPFENSTEIN, 1978; KLOPFENSTEIN, 1994; OWEN et al, 1984). Ammonia treatment has been shown to be as effective as NaOH in cleaving alkali-labile lignocarbohydrate linkages and may additionally supply nitrogen for the rumen microbes (SCHIERE & IBRAHIM, 1989; SUNDST∅L et al, 1993; CHENOST & KAYOULI, 1997). Likewise, urea can be applied as a source of ammonia (CHENOST, 2001).

Urea yields ammonia after its enzymatic ureolytic cleavage. Most of the abundantly occurring enzyme urease is of microbial origin and its activity is strongly sensitive to pH, reaching maximal activity at a pH of about 7, whereas at lower or higher pH urea will be split to lesser extents (WBC, 2003). During the ensilage process the pH of the silage is reduced by the production of weak organic acids, which may increase the nutrient availability of fibrous

material but at the same time will reduce urease activity, thus inhibiting the effect of ammonia treatment. On the other hand, high urea doses may create a strong alkalic pH-milieu which would inhibit any fermentative acid production (WILKINSON, 1984).

The effect of ensiling and of urea treatment on nutritional properties of cocoa pods particularly focussing on nutrient contents and in vitro gas production using rumen inocula has not yet been investigated. This experiment is directed to study nutritive changes in urea treated compared to untreated cocoa pod. The in vitro gas test aimed at studying the effect of urea on the activity of rumen microbes, at finding the optimum level to achieve maximum organic matter digestibility and metabolisable energy contents as well as to find out its toxic level.

6.2. Material and methods

6.2.1. Urea treatments

The cocoa pods (CPs) of AFR (Forastero) cultivar were collected after harvesting the beans. The cocoa trees at the Cikasungka Cocoa Plantation in Bogor-Indonesia were about 20 years old and ranged from 1.5 to 3 m height at the time of the harvest. The pods were collected from cocoa plants on February15th, 2001. The cocoa pods were randomly subdivided into five treatments, each of three replications which were fresh cocoa pods (C) and four levels of urea applications: 0 (U0), 10 (U1), 20 (U2) and 30 (U3) g/kg fresh materials respectively (w/w).

All treatments were processed manually. Firstly, cocoa pods were sliced into about 2 mm thickness and samples of 2 kg each were weighed. The pods in the control treatment (C) were sun dried immediately, while the other samples were put into 40 x 50 cm and 0.12 cm thin polybags. Urea was added layer by layer in order to achieve homogeneous mixtures. The bags were then pressed to reduce the air contents and sealed with nylon tape. Finally, the bags were stored at room temperature. After 14 days the bags were opened and the contents sun dried (about 18 hours light intensity). The dried material was then ground with a laboratory bur mill to pass a 0.5 mm sieve for further analyses.

6.2.2. Chemical analysis

The pH and DM were determined directly in fresh cocoa pods and in the ensiled, while all other parameters were measured in sun-dried samples. The pH was measured according to NAUMANN & BASSLER (1997). Ten grams fresh samples were mixed with 100 ml distilled water and stirred with a laboratory blender for 3 min. The pH was measured in the filtrate with an electrode (Mettler Toledo In lab 417).

Contents of dry matter (DM), crude ash (ASH), crude protein (CP), crude lipid (XL), and crude fibre (CF) were analysed according to the conventional Weende procedure (NAUMANN & BASSLER, 1997).

Non protein nitrogen (NPN) was determined by precipitation of true protein (TP) with tungstic acid, filtration and determination of the insoluble nitrogen in the residue. NPN was calculated as difference between total crude protein nitrogen and true protein nitrogen (LICITRA et al., 1996).

Residual urea (UR) was measured according to NAUMANN & BASSLER (1997). The sample was cleared with Carrez-solution I and II and agitated using an automatic shaker (Co Köttermann type 5627) for 30 minutes after the addition of water. The suspension was then filtrated. After the addition of 4-Dimethylamino-benzaldehyde, the absorbance in the filtrate was measured at 436 nm wavelength (Co. Eppendorf 1101 M) and the amount (concentration) calculated by plotting the absorbance value in a urea standard curve.

Non ammonia nitrogen (NAN) was determined using the same method as used by CARRO & MILLER (1999). The sample was wetted with distilled water, adjusted with 1 M NaOH to pH above 10, and dried at 90^{o}C for 16 h to remove NH_3-N. The N-content of the dried residues accounting for NAN content was determined by micro Kjeldhal (NAUMANN & BASSLER, 1997).

The amino acid (AA) composition was measured first by ion exchange chromatograph. The cocoa pod samples (250 mg) were hydrolysed with 50 ml phenolic hydrochloric acid at 110^{o}C for 24 hours. The hydrolysed sample was transferred into a volumetric flask and adjusted to pH 2.2 with 7.5 M NaOH under cooling temperature at which condition all amino acids existed as cations. The volume was filled up to 250 ml after adding an internal standard (norleucine). The sample was centrifuged for 10 min at 15 000 g. The supernatant was

analysed using an amino acid analyser LC 3000 (Co Eppendorf Biotroniks). The sulphur containing amino acids (methionine and cysteine) must be oxidised before hydrolysis with 5 ml performic acid (0.5 ml H_2O_2) and 4.5 ml 88% phenolic formic acid.

The determination of neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were carried out according to VAN SOEST et al. (1991). The contents of hemicellulose, cellulose, and crude lignin were calculated by difference. Hemicellulose were calculated as the difference between NDF and ADF, cellulose as the difference between ADF and ADL and crude lignin was calculated by subtracting ash residue (muffle-oven at 550^{o}C) from ADL.

Theobromine content was analysed according to NAUMANN & BASSLER (1997). Theobromine was extracted with chloroform and the extract was dried and then resolved in water and treated with silver-nitrate solution. The free saltpetre acid was titrated with sodium hydroxide. Theobromine content was calculated according to the formula:

Theobromine (% DM) = (ml NaOH x 18)/(mg sample weight x %DM)

Where 18 is the coefficient of conversion for each ml 0.1 N NaOH used in titration.

6.2.3. In vitro gas test

In vitro gas production was measured applying the Hohenheim Gas Test (MENKE et al., 1979). Approximately 200 mg sample (of about 90% DM) was weighed and incubated with 30 ml rumen fluid-buffer solution (1:2) in a 100 ml glass syringe. The syringes were put in a double wheel plat and rotated using a rotor in a 39^{o}C water bath. The fermentation process was stopped after 24 hours of incubation. The amounts of gas produced (Gb) in the syringes were read. The gas production was calculated for 200 mg DM.

6.2.4. Statistical analysis

The experiment was completely random designed with fresh cocoa pods as control and 4 levels of urea applications. Each treatment was repeated 3 times. The linear model of complete random design is

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$$

Where Y_{ij} is an observation value at treatment i and replication j, μ is the overall mean of observations, τ_i is additive effect of urea treatment i, and ε_{ij} is the residual error of observation treatment i and replication j. Significant differences between treatments were analysed using analysis of variance (ANOVA) and continued with Tukey's test. Analyses were done using SPSS statistical software version 10.0.

6.3. Results

6.3.1. Chemical compositions

The pH and the results of proximate analyses of cocoa pods (CPs) are shown in Table 6.1. Ensilage (U0) reduced pH of CPs from 5.35 in the control (C) to 4.72. Addition of urea significantly increased pH (up to 8.38). However, there was no significant difference between U2 and U3. Dry matter (DM) contents tended to be reduced by ensiling with or without urea.

Table 6.1: PH and crude nutrient contents of cocoa pods

Parameter	Treatment				
	C	U0	U1	U2	U3
PH	5.35^{b}	4.72^{a}	7.93^{c}	8.20^{d}	8.38^{d}
DM (%)	18.98	18.06	18.06	18.42	18.24
ASH (% DM)	6.55^{a}	8.43^{b}	6.32^{a}	5.81^{a}	5.49^{a}
XL (%DM)	0.50	0.55	0.40	0.40	0.46
CF (%DM)	52.30^{b}	42.09^{a}	47.18^{ab}	49.98^{b}	46.83^{ab}

Different superscripts in the same line indicate significant differences ($p< 0.05$)

Crude ash content of cocoa pods increased by ensilage compared to the control. However, addition of urea (U1 to U3) resulted in the same crude ash value as in the control. No significant difference was found in crude lipid content (XL). Inversely to crude ash, crude fibre content (CF) was decreased by ensilage. Urea addition tended to increase CF but still to a lower level than the control.

The results for N-fractions are shown in Table 6.2. Total N and N-fractions were not significantly influenced by the ensilage process, whereas urea treatment increased these parameters in a dose dependent manner. However, NAN and UR were not significantly increased from U1 to U2, whereas further addition of urea to U3 level increased NAN and UR

contents almost two and threefold respectively. NAN minus UR (NAN – UR) tended to decrease from U0 to U3 in contrast to TP values which increased with the addition of urea.

Table 6.2: N-Fractions of cocoa pods (%N in DM)

Fraction	Treatments				
	C	U0	U1	U2	U3
Total N	1.35[a]	1.69[a]	2.90[b]	3.98[c]	6.77[d]
NAN	1.22[a]	1.57[a]	2.16[b]	2.73[b]	5.21[c]
NH_3	0.13[a]	0.12[a]	0.74[b]	1.25[c]	1.56[d]
UR	0.07[a]	0.03[a]	0.74[ab]	1.35[b]	3.86[c]
NAN - UR	1.15	1.54	1.42	1.38	1.36
TP	1.28[a]	1.21[a]	1.73[b]	1.93[c]	2.04[d]
AA	0.78	1.11	nd	0.83	nd

NPN = non protein nitrogen; TP = true protein; UR = residual urea; NAN = non ammonia nitrogen; AA = amino acids; nd = not determined. Different superscripts in the same line indicate significant differences ($p < 0.05$).

AA (calculation based on N-contents of measured amino acid) found in U0 was higher than in the control and in U2. No analysis has been made for U1 and U3. The amino acid composition changed as a result of ensilage (Table 6.3). This effect was more pronounced for proline, aspartic acid, and glutamine. Amino acids of U2 treated CPs was similar or slightly higher than for the control except for tyrosine, phenylalanine and lysine which were reduced.

Table 6.3: Amino acid contents of cocoa pods (mg/g DM)

Amino Acid	Treatment		
	F	U0	U2
Cysteine	1.10	1.65	1.26
Aspartic acid	5.64	8.47	6.01
Methionine	1.24	1.74	1.21
Threonine	2.90	4.23	3.23
Serine	3.33	4.81	3.39
Glutamine	7.11	10.89	7.76
Proline	4.18	9.37	4.88
Glycine	2.90	4.76	3.54
Alanine	3.80	5.46	4.36
Valine	3.65	5.49	4.22
Isoleucine	2.84	3.81	2.59
Leucine	4.64	6.38	4.49
Tyrosine	3.54	4.11	2.82
Phenylalanine	4.62	5.12	3.44
Histidine	1.89	2.48	2.17
Lysine	3.89	3.88	3.26
Arginine	2.71	3.89	3.42
Total amino acids	60.00	86.56	62.02

The analysed cell wall contents are shown in Table 6.4. There was no significant effect of ensilage and urea on NDF. ADF was decreased by urea treatment whereas ADL was increased by ensilage.

Table 6.4: Cell wall constituents of treated CPs (% DM)

Parameter	Treatment				
	C	U0	U1	U2	U3
NDF	79.48	82.04	82.59	81.92	80.07
ADF	63.62^{bc}	65.57^{c}	60.51^{ab}	60.66^{ab}	57.35^{a}
ADL	29.08^{a}	34.82^{b}	29.33^{a}	29.87^{a}	27.73^{a}
Hemicellulose	15.85	16.47	22.08	21.26	22.72
Cellulose	34.54^{b}	30.76^{a}	31.18^{ab}	30.79^{a}	29.62^{a}
Crude lignin	28.64	33.13	29.17	29.66	27.40

Different superscripts in the same line indicate significant differences ($p < 0.05$).

Hemicelluloses tended to increase by urea additions while cellulose was reduced in all urea treatments (U0 – U3). There were no significant effects of the treatment on crude lignin content.

The contents of theobromine are shown in Figure 6.1. There was no significant effect of the treatments on theobromine content of cocoa pods. Treatment U1 resulted in the lowest and the control in the highest theobromine contents (0.32 vs. 0.37 g/kg).

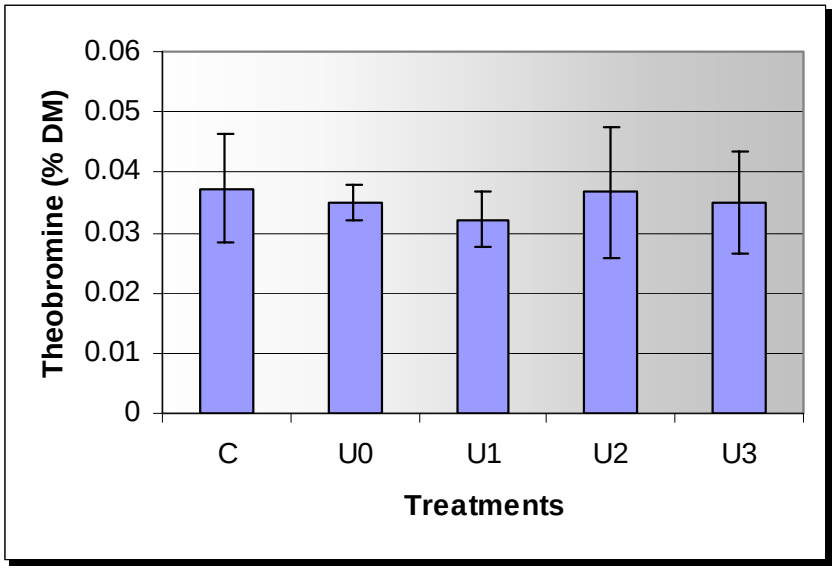


Figure 6.1: Contents of theobromine in cocoa pods
(n = 15; average ± standard deviation)

6.3.2. In vitro digestibility

In vitro gas production (Gb) of CPs is shown in Figure 6.2. Ensilage with or without urea treatment increased Gb significantly, treatment U3 excepted which decreased Gb sharply.

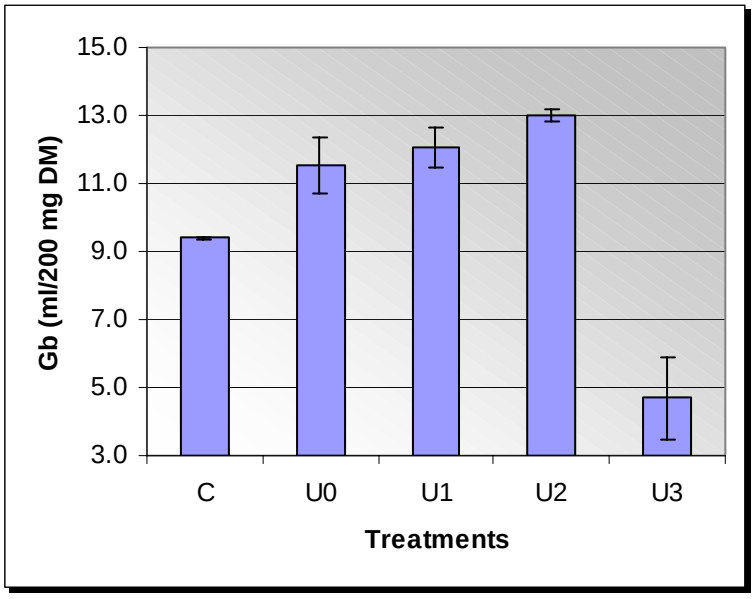


Figure 6.2: Gas productions of cocoa pods
(n = 15; average ± standard deviation)

6.4. Discussion

The proximate analyses of the cocoa pods indicated lower contents of CP (Total-N x 6.25), ash and crude lipid but higher CF than those observed by BARNES & AMEGA (1984). Theobromine content was also lower (0.032 vs. 0.32%) than reported by BARNES & AMEGA (1984). This may have been caused by the inclusion of pod husks in the samples. Although there was no effect of treatment, theobromine content of samples used in this study was very low and could be considered as being unaffective in ruminant nutrition. At present, there are no reports on theobromine toxicity in animals due to cocoa pods (ICCO, 2000).

The amount of N being lost from the time of addition to that of analysis increased with increasing levels of urea. While 10 g, 20 g and 30 g of urea had been added per kg fresh cocoa pods corresponding to 3.2 g, 6.4 g and 9.7 g urea-N per kg DM, only 1.7 g, 2.8 g and 5.6 g total-N (feed plus urea) were found in U1, U2 and U3 treatments respectively. Percentages of

N losses as the ratio of urea-N added were 47%, 56% and 42% for U1, U2 and U3 respectively. It indicates an increased ureolytic activity (assuming N-losses as NH_3). This is confirmed by the similar trend in ammonia values. According to CHENOST (2001), two-third of ammonia released from ureolysis is volatile and lost, and only one third of the ammonia binds on the forage cell wall.

The amount of N lost is also related to pH. Increasing urea level from U2 to U3 did not increase pH significantly. Possibly the time of incubation (14 days) was too short. Ureolytic activities are maximal at pH 7.0. The higher the pH the higher the ammonia-N lost. At an extremely high temperature of 90°C and pH > 10, all ammonia is lost. According to this feature ammonia is used to determine NAN (CARRO & MILLER, 1999). According to REXEN & KNUDSEN (1984) the alkali process on feed depends on temperature, pressure, alkali concentration and reaction time.

The evaluation of TP as tungstic acid precipitable protein seems not to be valid for urea treated fibrous feed. TP may be overestimated and NPN underestimated since not all of the NPN might be separated from TP (LICITRA et al., 1996).

Total N in U0 was slightly higher than in the control. It may partly be the result of indirect enrichment of N due to organic matter degradation during ensilage. Both, the decreased pH and the low ammonia concentration are indicators for an acid fermentation of ensiled cocoa pods (U0). Possibly the substrate for fermentation was too low for a stronger acid production which would have led to an even lower pH.

The decrease in ADF for treatment U1 – U3 in comparison to control is associated with lower cellulose and partially compensatory higher hemicellulose contents. The quality of the fibre fraction of urea treated cocoa pods was therefore modified and should be higher and/or faster degradable in the microbial rumen environment.

This is confirmed by in vitro gas production which was increased from treatments U0 to U2. The sharp decrease of Gb with U3 must be explained with a too high and therefore toxic ammonia effect on the microbes in the in vitro test. According to SMITH (1989), at pH above 7, urea splitting in the rumen results predominantly in NH_3 which is toxic to the rumen if present in large amounts. However, at pH below 7 a high concentration of non toxic NH_4^+ predominates.

For calculation of OMD and ME from in vitro gas production, cocoa pods are grouped to dry fodder or hay and the following formulas are applied:

$$OMD\ (\%) = 16.49 + 0.9042\ Gb + 0.0492\ (CP \text{ or } TP \text{ or } (NAN - UR) \times 6.25) + 0.0387\ XA$$

$$ME\ (MJ/kg\ DM) = 2.43 + 0.1206\ Gb\ (ml) + 0.0069\ (CP \text{ or } TP \text{ or } (NAN - UR) \times 6.25) + 0.0187\ XL$$

Where Gb is in ml, while TP, ASH and XL are in g/kg DM (MENKE & STEINGASS (1987). The results of these calculations are shown in Table 6.5.

Table 6.5: OMD (%) and ME (MJ/kg DM) of cocoa pods

Parameter	Treatment				
	C	U0	U1	U2	U3
OMD					
CP (TN x 6.25)	31.79[a]	35.35[b]			
TP (TP-N x 6.25)	31.48[b]	33.90[c]	35.20[cd]	36.48[d]	29.10[a]
NAN – UR (NAN – UR x 6.25)	31.06[ab]	34.90[b]	34.00[b]	34.72[b]	27.02[a]
ME					
CP (TN x 6.25)	4.25[a]	4.65[b]			
TP (TP-N x 6.25)	4.21[b]	4.44[b]	4.71[c]	4.91[c]	3.96[a]
NAN – UR (NAN – UR x 6.25)	4.15[a]	4.58[b]	4.54[b]	4.66[b]	3.67[a]

Different superscripts in the same line indicate significant differences ($p < 0.05$)

For C, the formula renders almost the same OMD- and ME-values with either CP or TP as dependent variables whereas (NAN-UR) delivers somewhat lower values. The lower values calculated for U0 when TP is used instead of CP can be explained with the relatively big difference between CP and TP.

CP cannot be used in the formula for the urea treated samples. TP and (NAN-UR) give the same trend as the gas production values, and can therefore be used to express the protein value of urea treated cocoa pods. Except U0, applying TP in the formulas leads to higher OMD and ME contents than (NAN–UR). Presumably some NPN is precipitated with tungstic acid resulting in higher TP than (NAN-UR) values. The values calculated by applying (NAN-UR) are therefore suggested to be the nearest evaluations of the real feeding values of the samples. However, the OMD and ME values may be underestimated due to considering TP instead of CP.

The response of Gb, OMD and ME on urea applications followed the equations shown in Table 6.6. The maximum Gb was reached at approximately 67 g urea per kg DM cocoa pods. About the same urea level was estimated for OMD and ME maximum with (NAN–UR) as dependent variable.

Table 6.6: Response of OMD and ME on urea applications

No	Formula	Peak	n	r^2	F
	Based on Gb	U (g/kg DM)			
1	$Gb = 11.0306 + 0.067U - 0.0005U^2$	67	12	0.86	0.000
	Based on %TP (TP-N x 6.25)				
2	$OMD = 33.4659 + 0.0743\ U - 0.0004\ U^2$	93	12	0.84	0.000
3	$ME = 4.3889 + 0.0113\ U - 0.00006\ U^2$	94	12	0.84	0.000
	Based on (NAN – UR-N) x 6.25				
4	$OMD = 34.3873 + 0.0401\ U - 0.0003\ U^2$	67	12	0.75	0.000
5	$ME = 4.5191 + 0.0065\ U - 0.00005\ U^2$	65	12	0.72	0.000

The use of TP as dependent variable resulted in maximal OMD and ME at approximately 93 g urea per kg DM cocoa pods (Figure 6.3). The optimal level of urea found was higher than that for treated straw reported by WILLIAMS et al. (1984) and CHENOST (2001) (40 and 53 g/kg DM respectively). It may have been caused by the higher lignocellulosic material in cocoa pods compared to rice straw.

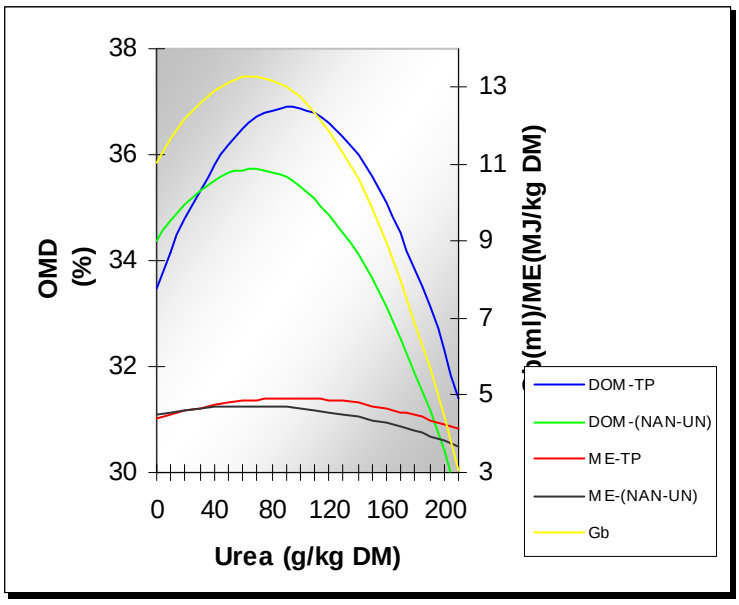


Figure 6.3: OMD and ME of CPs as response to urea treatment

6.5. Conclusions

From this experiment, it can be concluded that:

1. The treatments (ensilage with or without urea) improve the nutritive value of cocoa pods by weakening the ligno-cellulose or –hemicellulose bonds which then can easier be penetrated by rumen microbes, resulting in higher in vitro gas production, as well as higher calculated OMD and ME values compared to the control. The protein value of cocoa pods, expressed as TP or (NAN-UR) was also increased.
2. The (NAN-UR) value is suggested to be the nearest evaluation of the real protein value of urea treated cocoa pods.
3. The optimum level of urea to reach Gb maximum was 67 g/kg DM cocoa pods. The optimum level to reach maximal OMD (%) and ME (MJ/kg DM) based on TP as protein value was 93 – 94 g urea per kg DM, while using (NAN-UR) was reached at an urea level of 66 g/kg DM cocoa pods.

7. Experiment 2. Effect of substituting barley grain by urea treated cocoa pod on microbial metabolism and feed degradation in rumen simulation technique (Rusitec)

7.1. Introduction

According to previous studies, urea treatment of cocoa pod (14 kg per 100 kg DM) gave the best result compared to other treatments as measured through gas production. Relative to untreated cocoa pod, the urea treatment increased gas production (Gb) by 38%.

A too high consumption of dietary urea would however be toxic for the animal (HELMER & BARTLEY, 1971; BARTLEY & DEYOE, 1981; VAN SOEST, 1982). Since the amount of urea used in our previous study to treat the cocoa pods were higher than that applied by CHENOST (2001) for rice straw equivalent to 5.3 kg urea per 100 kg DM or WILLIAMS et al. (1984) who applied 4 kg urea 100 kg barley straw, it seems necessary to test the possible toxicity of urea treated cocoa pod in a more physiological rumen model such as Rusitec.

According to SMITH (1974) urea toxicity can be minimised by ensuring an adequate matched supply of available energy instead of replacing urea with more expensive NPN sources. In this study, the urea treated cocoa pods were mixed with different amounts of barley as an energy source and constant amounts of these mixtures were combined with constant amounts of hay in Rusitec.

7.2. Objectives

The study aimed at investigating the effects of replacing barley/soybean meal-mixture by urea treated cocoa pod in ruminant feed rations on methane release, SCFA production and N-metabolism as well as the efficiency of protein synthesis of rumen microbes. The specific objective was:

1. To study the effect of barley/soybean meal-mixture substitution by urea treated cocoa pod on fermentation characteristics particularly pH, methane production, ammonia concentration, SCFA production, and microbial growth in Rusitec.
2. To control the possible toxicity of urea treated cocoa pod on rumen microbes.
3. To study the effect of the substitution level on rate of disappearance of DM and nutrients from the feed used as substrate in Rusitec.

7.3. Material and methods

7.3.1. Urea treated cocoa pod

The cocoa pods (CPs) of AFR (Forastero) cultivar were collected after harvesting the beans. The cocoa trees at the Cikasungka Cocoa Plantation in Bogor-Indonesia were about 20 years old and ranged from 1.5 to 3 m height at the time of the harvest. The pods were collected from cocoa plants on February15th, 2001. The cocoa pods were treated with urea (20 g/kg fresh materials or equal to 140 g/kg DM (w/w)).

The treatment was processed manually. Firstly, cocoa pods were sliced into about 2 mm thickness and samples of 2 kg each were weighed. The pods were put into 40 x 50 cm and 0.12 cm thin polybags. Urea was added layer by layer in order to achieve homogeneous mixtures. The bag was then pressed to reduce the air contents and sealed with nylon tape. Finally, the bag was stored at room temperature. After 14 days the bag was opened and the contents sun dried (about 18 hours light intensity). The dried material was then ground with a laboratory bur mill to pass a 0.5 mm sieve for further analyses.

7.3.2. Experimental ration.

The experiment was conducted in rumen simulation technique fermenters. The fermenters were supplied with 10 g of hay without or with 4 g of concentrate daily. Barley grain enriched with extracted soybean meal (to achieve the same crude protein (CP) content as urea treated cocoa pod) was used as standard concentrate. The standard concentrate was gradually replaced with increasing levels of treated cocoa pod. In the following the concentrate is termed with the proportion (%) that is replaced by treated cocoa pod.

The resultant six treatments were (T1) hay, (T2) hay + concentrate 0, (T3) hay + concentrate 25, (T4) hay + concentrate 50, (T5) hay + concentrate 75, and (T6) hay + concentrate 100. The nutritional composition of the feed components is shown in Table 7.1.

Table 7.1: Nutritional composition of the feed components

Component	DM	ASH	CP	XL	CF	NDF	ADF
	%	% DM					
Hay	93.4	7.92	15.8	1.32	30.2	57.3	33.9
Barley	87.6	2.48	12.7	2.48	7.26	30.8	8.46
Soy bean meal extr.	89.2	7.12	52.2	1.82	4.66	10.6	5.86
Treated cocoa pod	90.5	8.58	15.9	0.74	51.9	76.9	59.4

The daily amounts of the different feed components supplied to six fermenters in Rusitec and their chemical composition are shown in Table 7.2.

Table 7.2: Composition of the feed rations

Ingredients	T1	T2	T3	T4	T5	T6
Hay (g)	10	10	10	10	10	10
Barley (g)	0	2.4	1.8	1.2	0.6	0
Soy bean meal extr.(g)	0	1.6	1.2	0.8	0.4	0
Treated cocoa pod (g)	0	0	1.0	2.0	3.0	4.0
Mineral mix (g)	0.15	0.15	0.15	0.15	0.15	0.15
Nutrient contents						
DM (%)	93.5	91.9	92.1	92.3	92.5	92.7
ASH (%DM)	9.73	7.64	8.02	8.40	8.78	9.15
CP (% DM)	15.6	15.9	15.8	15.8	15.7	15.7
XL (% DM)	1.30	1.60	1.49	1.37	1.26	1.14
CF (% DM)	29.7	23.6	26.7	29.8	32.8	35.9
NDF (% DM)	56.4	48.9	52.2	55.5	58.8	62.1
ADF (%DM)	33.3	26.5	30.0	33.6	37.0	40.5

7.3.3. Rusitec procedure

The study was conducted in Rusitec fermenters following the procedure which has been developed by CZERKAWSKI & BRECKENRIDGE (1977). The trial was repeated three times (3 replications) with 6 fermenters for each run. Each run lasted 14 days including day 0 to day 9 as preliminary phase and day 10 to 14 as the collecting phase. The Rusitec procedure is explained below:

Water bath and Fermenter

Water bath was set at 39^{o}C using a thermostat heater. Water circulated through a circulating pump. The water bath contained 6 fermenters. Each fermenter had a volume of 1060 ml and was filled with 690 ml rumen fluid which was collected from two rumen fistulated steers. A nylon bag containing 80 g of rumen solid fraction was introduced into a covered bottle at

day 0 together with two bags containing hay and concentrate respectively. The cover and the bottom of the bottle had several holes to allow free fluid movement when introduced into the cylindric fermenter. The bottle cover was connected to a wheel allowing an automatic stroke frequency of the bottle of about 400 times per hour. The fermenter was filled with buffer and closed. Nitrogen gas was blown for about 2 minutes to pull oxygen out and to maintain anaerobic conditions. The overflow of the fermenter was connected to a 1000 ml Erlenmeyer containing 1 ml $HgCl_2$. The gas produced was collected in a gas bag.

Buffer (McDOUGAL, 1948) was pumped continuously using an automatic pump at a flow rate of 620 ml/d. Buffer composition is detailed in appendix 2.2. The fermenter was maintained under anaerobic conditions by infusing N_2 gas each time it was opened. The arrangement of fermenters, pump and thermostat heater in the Rusitec system is shown in Figure 7.1.

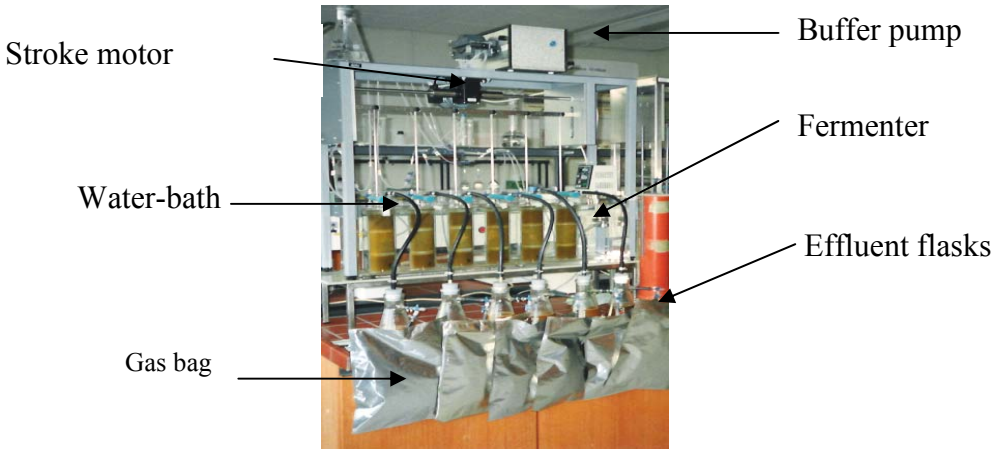


Figure 7.1: Rumen simulation technique instruments

Ration

The daily ration was put into two nylon bags. A 60 µm pore size bag was used for concentrate and a 100 µm pore size bag for hay. The bag containing rumen solid fraction was removed after 24h, while the feed ration was incubated for 48 hours. The residual ration in the nylon bag served to calculate the rate of disappearance and was therefore washed with 60 ml buffer which was returned to the fermenter.

^{15}N infusion

^{15}N-Urea with 95% enrichment was used as a tracer for the determination of microbial growth. The amount of 0.2259 g $(^{15}NH_2)_2CO$ was weighed and solved in 100 ml distilled

water. Five ml of the solution was added to 5 l of fresh buffer. The amount of ^{15}N injected daily was calculated as

$$^{15}N \text{ (mg/d)} = (0.2259 \text{ g/100 ml})(1000 \text{ mg/1g})(5 \text{ ml/5000 ml})(28/60)(95/100)(\text{ml overflow/d})$$

$$= 0.00100149 \text{ mg/ml (ml overflow/d)}.$$

The quasi-steady state condition in the fermenters with a nearly constant ^{15}N-concentration of the ammonia pool was achieved after 5 days.

Parameters

Overflow, gas production, pH, redox potential and NH_3 concentration were measured daily. Protozoal counts and gas composition were analysed every two days during the preliminary phase and daily during the collection phase. The SCFA and ^{15}N-NH_3 were analysed in the overflow samples of the collection phase, while the disappearance rate of the feed ration was measured during the collection phase by analysing the residual contained in the nylon bag after fermentation.

7.3.4. Rate of disappearance

The DM-disappearance rate of the feed ration was determined by subtracting the residual ration in nylon bags after 48 h of incubation from the amount of ration introduced into the fermenter. The disappearance of OM, CP, CF, NDF and ADF was determined by analysing the residual solids for crude ash (XA), crude protein (CP) and crude fibre (CF) according to NAUMANN & BASSLER (1997) and for NDF and ADF according to VAN SOEST et al. (1991).

7.3.5. Amount and composition of fermentation gas

The composition of produced fermentation gas was analysed by gas chromatography (SimadzuGC-8A) in collected gas samples on a packed silica gel column, at 80°C injection port and detector temperature and 40°C column temperature (isothermal), TC-detector (Simadzu C-R1B) and argon as carrier gas (DA COSTA GOMEZ, 1999). Gas samples were taken from the gas bag using a 1 ml syringe.

A standard gas consisting of CO_2, N_2, CH_4 and O_2 in the proportions of 65, 30, 4 and 0.9% respectively was used. The peak of graph was achieved at the 2nd minute for H_2, after 2.85 minutes for N_2, 3.89 minutes for CH_4 and 20.95 minutes for CO_2.

7.3.6. Short chain fatty acid (SCFA)

A 10 ml sample of the effluent was centrifuged in a Martin Christ centrifuge (Type UJ 1S) for 10 minutes at 3900 ***g***. The supernatant was separated from the sediment. An internal standard solution consisting of 4% 2-methyl valeric acid in formic acid (w/w) was prepared. 250 µl of the internal standard solution was mixed with 5 ml supernatant using a vortex. About 1.5 ml of the mixture was transferred to a covered centrifuge cup and centrifuged at 15000 ***g*** (Biofuge A, Heraues Sepatech) for 10 minutes. 1.4 µl of the centrifuged sample was injected to GC using a 10 µl syringe.

SCFA was detected using a gas chromatograph (GC-14B, Simadzu) equipped with a packed column (10% Carbowax 20 MTPA SP 1000 with 1% H_3PO_4 on Cromosorb WAW 80/100) and flame ionisation detector connected to a chromato-integrator (D-2000 Merck-Hitachi). H_2 at 120 kPa pressure served as carrier gas. The injection oven temperature was set at 170^oC while the column and detector temperatures were set at 120^oC and 220^oC respectively.

The peak curve was measured after a retention time (min) of approximately 3.33 for C2 (acetate), 4.90 for C3 (propionate), 5.66 for iC4 (iso-butyrate), 7.51 for nC4 (butyrate), 9.23 for iC5 (iso-valerate), 12.73 for nC5 (valerate) and 14.64 for the internal standard.

Additionally to the internal standard, an external standard was also used which consisted of (µmol/ml) 30 acetic acid, 20 propionic acid, 1 iso-butyric acid, 10 butyric acid, 2 iso-valeric acid and 3 valeric acid. To determine the concentration of SCFA, a combined internal/external standard procedure was applied.

7.3.7. Microbial protein synthesis (MPS)

Microbial protein synthesis (MPS) was determined by the continuous infusion technique applying ^{15}N-urea as a tracer (ABEL et al., 1990). Three drops of silicon oil (anti foam), 8 drops of Thymolphthalein (2% in ethanol solution) or methyl red, 10 ml boric acid buffer (in 1 N KCl solution adjusted with 1 N KOH to pH 9.5) and 4.5 ml 1N KOH were added to

100 ml effluent (pooled 10% collection phase overflow samples). The solution was distilled and the distillate collected in a beaker glass containing 0.1 N H_2SO_4. Basically 0.1 N H_2SO_4 would be enough to bind the NH_3 released. The amount of 0.1 N H_2SO_4 needed to bind NH_3 was calculated as the concentration of NH_4-N/100 ml sample divided by 1.401 plus 1 ml. The beaker volume was filled to 40 ml with distilled water. The distillation process was terminated when 200 – 250 ml of distillate were collected which occurred in approximately 12 minutes.

Part of the distillate volume (equal to approximately 0.5 mg nitrogen) was evaporated. The amount of distilled volume to be evaporated was calculated as the volume of distillate in the beaker divided by NH_4-N/100 ml rumen fluid and divided by 2. This amount was transferred into a 50 ml beaker glass and evaporated at a temperature of 60^{o}C to give a volume of 3 – 4 ml. The temperature was then increased to 100^{o}C to further reduce the volume to750 µl. The remaining solution was pipetted into a tin cup and further dried at 105^{o}C. The cup was then formed to a small pellet and placed into a pallet. The pellets were analysed for ^{15}N enrichment by means of a mass spectrometer (Finnigan MAT Delta C, Bremen, connected to an elemental analyser, Fisons 1108 Rodano, Milano).

Assuming steady state conditions, microbial nitrogen was derived according to the following equation:

$$\text{Microbial N (mg/d)} = r_i \times ((s_i/s_p) - 1)$$

Where r = infusion rate (mg/d), s_i = ^{15}N-excess in infusion, s_p = ^{15}N-excess in NH_4-N pool. Microbial protein synthesis (MPS) in mg/d can be calculated as microbial-N divided by (8/100), assuming 8% N in microbial cells (CZERKAWSKI, 1986). The efficiency of microbial protein synthesis (EMPS) is calculated according to

$$\text{EMPS (mg/g OMAD)} = \text{MPS/OMAD}$$

Where OMAD is organic matter apparently degraded.

7.3.8. Experimental design and statistical analysis

The trial had a randomised block design with 6 rations in 6 fermenters as treatments and 3 runs as block. The linear model used for the analysis was:

$$Y_{ij} = \mu + \tau_i + \beta_j + \varepsilon_{ij}$$

Where, Y_{ij} is the observation value of ration i and block j, μ is the overall mean of observations, τ_i is the additive effect of ration i, β_j is the effect of block j and ε_{ij} is experimental error of ration i and block j.

7.4. Results

7.4.1. Fermentation characteristics

The effect of replacing the barley/soybean meal-mixture with urea treated cocoa pod on fermentation in Rusitec is shown in Table 7.3.

Table 7.3: Effect of replacing barley/soybean meal-mixture by urea treated cocoa pod on fermentation characteristics in Rusitec

Parameter	Treatment					
	T1	T2	T3	T4	T5	T6
PH	6.63^{d}	6.51^{a}	6.52^{ab}	6.54^{b}	6.59^{c}	6.63^{d}
Protozoa (cts/ml)	17475	17868	16310	14267	13672	12436
CH_4 (mmol/d)	5.45^{a}	7.51^{de}	7.71^{e}	6.95^{cd}	6.38^{bc}	6.02^{ab}
NH_4^+ (mmol/l)	5.64^{a}	8.31^{d}	7.42^{c}	7.28^{c}	6.68^{b}	6.48^{b}
SCFA (mmol/d)	24.88^{a}	35.61^{b}	33.59^{b}	31.94^{ab}	30.94^{ab}	28.78^{ab}
acetate	14.46^{a}	18.84^{b}	18.47^{b}	17.85^{ab}	17.80^{ab}	17.26^{ab}
propionate	6.07^{a}	9.20^{b}	8.57^{b}	7.76^{ab}	7.52^{ab}	6.59^{a}
iso-butyrate	0.17	0.25	0.18	0.16	0.15	0.20
n-butyrate	3.02	5.14	4.58	4.45	3.94	3.41
iso-valerate	0.60^{a}	1.12^{d}	0.98^{cd}	0.88^{bc}	0.79^{abc}	0.70^{ab}
n-valerate	0.56	1.06	0.81	0.84	0.75	0.63
Microbial cells (mgDM/d)	700^{a}	859^{c}	821^{b}	782^{b}	793^{b}	776^{b}

There was a significant decrease in pH due to the addition of concentrate (T2 – T5). However, the substitution of barley/soybean meal-mixture by treated cocoa pod resulted in a gradual increase in pH at a rate that was nearly proportional to the increased cocoa pod proportion in the ration (T3 – T6). A 100% replacement of barley/soybean meal-mixture by urea treated cocoa pod resulted in a pH similar to that in T1 with hay as the sole substrate.

Addition of concentrate (barley/soybean meal-mixture) to hay based ration (T2) tended to increase protozoal counts. Replacing the concentrate with urea treated cocoa pods, however, reduced the protozoal counts inversely to the cocoa pod level below the value of T1.

Methane production was significantly increased by the addition of concentrate (T2 – T5). The increment could be reduced to the T1 level by replacing 100% concentrate with urea treated cocoa pods (T6).

Although ammonium production was decreased by the replacement of barley/soybean meal-mixture with urea treated cocoa pods (T3 – T6), the level was still higher than T1.

Total and partial SCFA production showed the same trend (significantly or just by tendency). Addition of concentrate (T2 – T6) increased the values compared to the pure hay ration T1. Replacement of barley/soybean meal-mixture with urea treated cocoa pod (T3 – T6) decreased the production inversely to the cocoa pod level and approached the T1 level at the 100% substitution (T6).

Microbial cell production was also enhanced by the addition of concentrate (T2 – T6). However, urea treated cocoa pod (T3 – T6), resulted in lower increment compared to the barley/soybean meal-mixture, T2.

7.4.2. Development during Rusitec

The average pH, concentration of NH_4^+ and counts of protozoa during Rusitec are illustrated in Figure 7.2. Daily pH ranged from 6.4 to 6.8. The pH tended to fluctuate during the preliminary phase (day 0 – 9) and then stabilised during the collecting phase (day 10 – 14). Ammonium production also showed the same trend and ranged from 4.8 – 9.0 mmol/l. The number of protozoa decreased with increasing time. The average count of protozoa at day 0 was about 50000/ml and was reduced to a tenth in the collection phase.

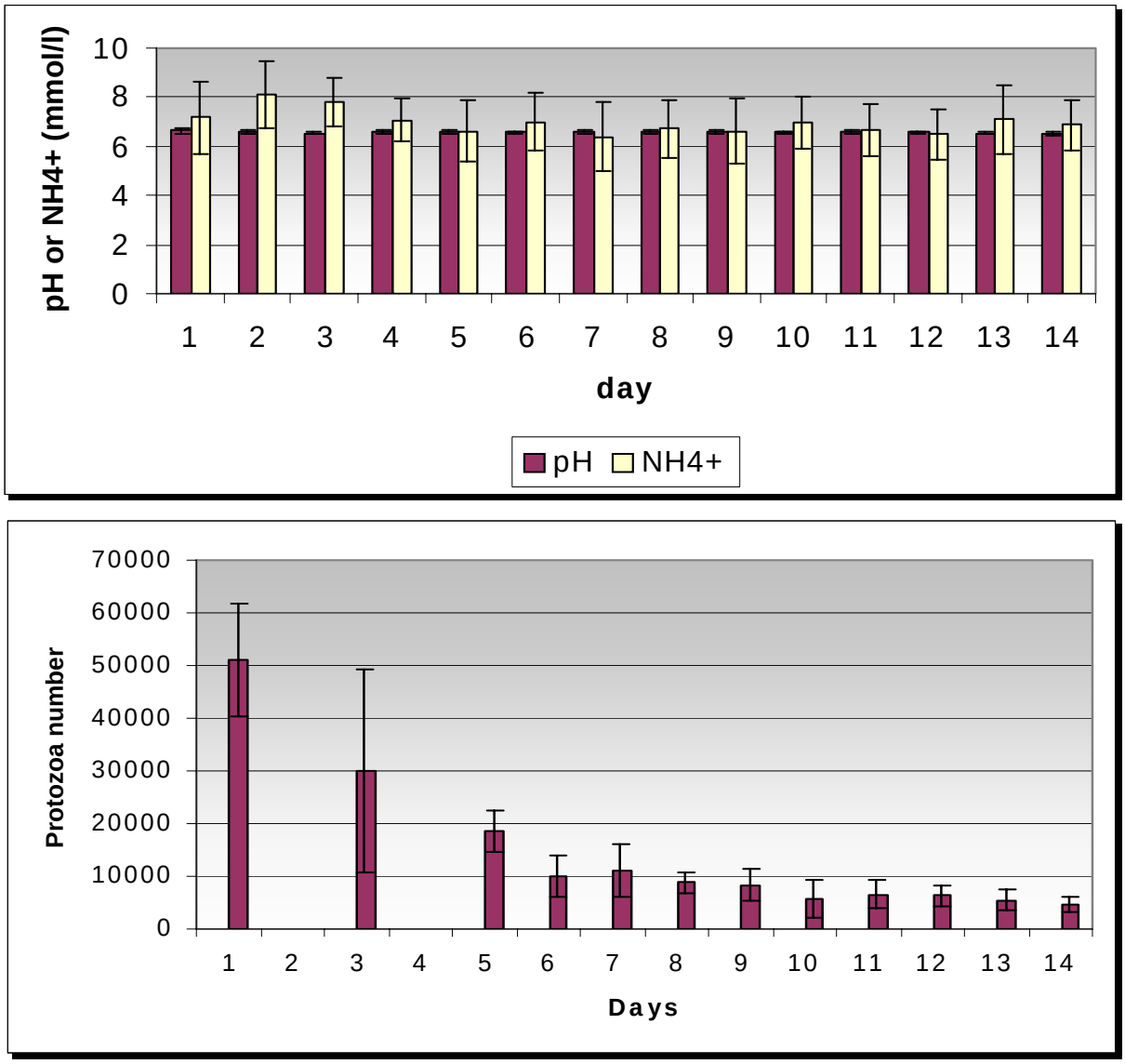


Figure 7.2.: The mean pH, NH_4^+ concentration and protozoa counts during Rusitec

7.4.3. Rate of disappearance

The rate of disappearance of DM and nutrients is shown in Table 7.4. There were no significant differences in hay disappearance between the treatments. The disappearance of DM of concentrate T3 (substitution of 25% barley/soybean meal-mixture by urea treated cocoa pod) was not significantly different from that in T2, however, increasing the proportion of urea treated cocoa pod (T4 – T6) resulted in a gradual decrease in DM disappearance. The OM disappearance of concentrate also tended to decrease with increasing amounts of cocoa pod, while the disappearance of CP decreased significantly when barley/soybean meal-

mixture was partially or completely replaced by cocoa pod. The fibre fractions of concentrate disappeared in most cases in higher amounts with the cocoa pod containing rations.

The disappearance of DM and CP of the total feed ration increased compared to the pure hay ration (T1) when concentrate regardless of substitution level was added. There was no clear effect of the substitution level on fibre disappearance. The hay/concentrate 0-ration (T2) showed significantly lower crude fibre and ADF disappearance than the hay/concentrate 100-ration (T6), whereas, the lowest NDF disappearance was observed for the pure hay ration T1.

Table 7.4: Disappearance of dry matter and nutrients of the feed ration in Rusitec

Parameter	Treatment					
	T1	T2	T3	T4	T5	T6
Disapp. hay	g/d					
DM	4.741	4.344	4.622	4.675	4.395	4.577
OM	4.216	3.818	4.057	4.129	3.879	4.001
CP	0.773	0.716	0.724	0.734	0.698	0.730
CF	1.168	1.008	1.131	1.074	1.002	1.066
ADF	1.202	1.029	1.174	1.153	1.053	1.108
NDF	1.908	1.699	1.961	1.958	1.755	1.868
Disapp. concentrate	g/d					
DM		1.779^{c}	1.566^{bc}	1.393^{ab}	1.270^{a}	1.169^{a}
OM		1.721	1.483	1.266	1.098	0.960
CP		0.272^{b}	0.215^{a}	0.183^{a}	0.212^{a}	0.223^{a}
CF		0.016^{a}	0.083^{a}	0.146^{ab}	0.266^{b}	0.438^{c}
ADF		0.002^{a}	0.019^{a}	0.042^{a}	0.130^{a}	0.306^{b}
NDF		0.436^{a}	0.400^{a}	0.396^{a}	0.466^{a}	0.651^{b}
Disapp. ration	g/d					
DM	4.741^{a}	6.123^{b}	6.188^{b}	6.068^{b}	5.665^{b}	5.747^{b}
OM	4.216	5.539	5.540	5.395	4.978	4.962
CP	0.773^{a}	0.988^{b}	0.939^{b}	0.917^{b}	0.910^{b}	0.953^{b}
CF	1.168^{ab}	1.024^{a}	1.214^{ab}	1.219^{ab}	1.268^{ab}	1.504^{b}
ADF	1.202^{ab}	1.031^{a}	1.193^{ab}	1.196^{ab}	1.183^{ab}	1.413^{b}
NDF	1.908^{a}	2.135^{ab}	2.362^{ab}	2.354^{ab}	2.220^{ab}	2.519^{b}

The proportions of the disappeared DM and nutrients (apparent degradability) are shown in Table 7.5. There were no significant differences in hay degradability between treatments. The inclusion of urea treated cocoa pod decreased the degradability of DM, OM, CP and NDF of the concentrate whereas it was increased for CF and ADF.

The substitution of up to 75% barley/soybean meal-mixture by cocoa pod (T5) did not significantly decrease the degradability of DM and OM of the ration. The substitution of 100% barley/soybean meal-mixture did not affect the degradability for CP, CF, ADF and

NDF of the feed ration. Compared to pure hay (T1), the addition of concentrate decreased the degradability of the total rations.

Table 7.5: Apparent degradability of the feed ration in Rusitec (%)

Parameter	Treatment					
	T1	T2	T3	T4	T5	T6
Degradability Hay						
DM	49.91	45.72	48.65	49.20	50.12	48.18
OM	48.99	44.36	47.14	47.97	49.39	46.48
CP	49.33	50.64	49.63	48.93	48.40	52.26
CF	41.38	35.69	40.04	38.02	38.64	37.74
ADF	37.99	32.52	37.10	36.44	36.99	35.01
NDF	35.61	31.71	36.61	36.54	36.37	34.86
Degradability Conc.						
DM		50.59^{d}	44.12^{c}	38.99^{bc}	35.65^{ab}	32.28^{a}
OM		50.43^{d}	43.71^{c}	37.60^{b}	33.22^{ab}	29.00^{a}
CP		46.30^{c}	36.66^{ab}	31.29^{a}	37.26^{ab}	38.67^{b}
CF		6.57^{a}	12.72^{ab}	13.70^{ab}	19.68^{ab}	23.31^{b}
ADF		0.59	2.55	3.48	10.00	14.20
NDF		43.18^{b}	27.48^{a}	20.84^{a}	21.73^{a}	23.39^{a}
Degradability Ration						
DM	49.91^{b}	47.03^{ab}	47.42^{ab}	46.41^{ab}	46.15^{ab}	43.79^{a}
OM	48.99^{c}	46.08^{bc}	46.17^{bc}	45.06^{abc}	44.87^{ab}	41.63^{a}
CP	52.26^{b}	47.81^{a}	45.45^{a}	44.45^{a}	46.87^{a}	46.34^{a}
CF	41.38	33.36	34.89	31.37	32.14	31.97
ADF	37.99^{b}	29.85^{a}	30.44^{ab}	27.26^{a}	27.60^{a}	26.58^{a}
NDF	35.61	33.53	34.66	32.43	31.92	30.94

7.5. Discussion

The ammonium concentration in the liquid phase (4.8 to 9.0 mmol/l) and the pH (6.4 to 6.8) during the experiments show that the conditions in the fermenters were within the physiological range for rumen microbes (SATTER & SLYTER, 1974; MCDONALD et al., 1995). However, the counts of protozoa in the rumen fluid (4×10^4 to 6×10^4 per ml) were less than is usual for the normal rumen physiology where up to 10 million protozoa per ml may be expected (MCDONALD, et al., 1995). The protozoa were even decreased in Rusitec to about 5000 counts/ml during the collection phase. Apart from methodological reasons associated with the discrepancy between protozoal generation interval and substrate exchange, the survival of only one tenth of the initial introduced protozoa during the collection phase might also have been caused by the restricted substrate available for protozoa when high fibre diets are used (WELLER & PILGRIM, 1974; COLEMAN et al., 1980).

The amounts of microbial cell synthesis measured in Rusitec are comparable to theoretical expectation (Table 7.6). The amount of synthesised microbial cells can be calculated using the basic stochiometry of SCFA production. According to DEMEYER &VAN NEVEL (1975) and DEMEYER et al. (1995) the amount of hexose fermented (HF) can be calculated:

$$HF = \text{hexose fermented (mol/d)} = (A + P)/2 + B + V$$

Where A is acetate, P is propionate, B is butyrate and V is valerate expressed in mol/d repectively. The amount of hexose fermented (g/d) is calculated as 162 x HF. The fermentation of 100 g carbohydrate (hexose) is assumed to result in the synthesis of 30 g microbial cells (MC; BERGNER & HOFMANN, 1996).

Table 7.6: Stoichiometry of fermentation in Rusitec

Parameter	Treatment					
	T1	T2	T3	T4	T5	T6
Microbial cell (MC) measured in Rusitec (mg/d)	**700^{a}**	**859^{c}**	**822^{bc}**	**783^{b}**	**793^{b}**	**776^{b}**
Hexose Fermented (HF)						
HF (mmol/d)	13.9^{a}	20.3^{e}	19.1^{de}	18.1^{cd}	17.4^{bc}	16.0^{b}
HF (g/d)	2.25^{a}	3.28^{e}	3.09^{de}	2.93^{cd}	2.82^{bc}	2.60^{b}
MC calculated from HF(mg/d)	**675^{a}**	**985^{e}**	**928de**	**881^{cd}**	**845^{bc}**	**779^{b}**

MC synthesis measured for the different treatments was similar to those theoretically calculated on the basis of HF.

The efficiency of microbial metabolism depends on the amount of microbial cells as well as the amounts of methane per unit of fermented substrate. It can be expressed in different terms. Calculated values of microbial N in relation to organic matter apparently degraded (OMAD), to truly digested organic matter (TOMD = truly degraded organic matter), to hexose fermented, to SCFA and to ATP are shown in Table 7.7. The highest efficiencies for microbial growth can be stated for the pure hay ration (T1). In most cases, the addition of concentrate 0 led to a significant reduction in EMPS, whereas the replacement of increasing barley/soybean meal-mixture by urea treated cocoa pod caused a slight recovery of EMPS reaching almost similar values with concentrate 100 (T6) and hay (T1).

Table 7.7: Efficiency of microbial protein synthesis expressed in different terms

Parameters	Treatments					
	T1	T2	T3	T4	T5	T6
TOMD (HF + MC) g/d	2.95^{a}	4.14^{e}	3.91^{de}	3.72^{cd}	3.61^{bc}	3.37^{b}
Microbial-N/OMAD (mg/g)	13.3^{b}	12.4^{ab}	11.9^{ab}	11.6^{a}	12.9^{ab}	12.5^{ab}
Microbial-N/TOMD (mg/g)	19.1^{c}	16.6^{a}	16.8^{a}	16.9^{a}	17.7^{ab}	18.5^{bc}
Microbial-N/HF (mg/g)	25.1^{c}	21.0^{a}	21.3^{a}	21.5^{a}	22.7^{ab}	24.0^{bc}
Microbial-N/SCFA (mg/mmol)	2.27^{c}	1.93^{a}	1.96^{ab}	1.97^{ab}	2.07^{abc}	2.16^{bc}
Microbial-N/ATP* (mg/mmol)	1.27^{b}	1.09^{a}	1.10^{a}	1.10^{a}	1.15^{a}	1.20^{ab}
CH_4/SCFA (mmol/mmol)	0.22	0.21	0.23	0.22	0.21	0.21
CH_4/OMAD (mmol/g)	1.29	1.37	1.40	1.29	1.28	1.23
CH_4/TOMD (mmol/g)	1.84	1.83	1.99	1.88	1.78	1.82
CH_4/NDF disappearance (mmol/g)	2.86^{ab}	3.55^{c}	3.29^{bc}	2.97^{abc}	2.89^{ab}	2.42^{a}

*) ATP = 2 Acetate + Propionate + 3 Butyrate (mmol/day)

With the exception of methane per unit NDF disappearance the methane release remained unaffected by the different treatments. However, less methane was produced per unit NDF disappearance when concentrate 100 (T6) was supplied to the fermenters instead of concentrates 0 (T2), 25 (T3) and 50 (T4).

It may also be noted that the lower amounts of produced microbial protein observed when cocoa pod containing concentrates served as the substrate, were associated with a slightly but not significantly increase in EMPS.

7.6. Conclusions

It was found that the substitution of barley/soybean meal-mixture by urea treated cocoa pod up to 100% in hay based rations did not decrease the rate of ration DM disappearance, and fibre degradation even tended to be increased.

Eventhough the microbial N-fixation decreased with the inclusion of cocoa pod in the rations, the efficiency of microbial N-fixation was increased in line with the cocoa pod level. Methane release per unit NDF disappearance decreased inversely to the cocoa pod level.

Although urea treated cocoa pod cannot replace barley/soybean meal-mixture equivalently in feed rations for ruminants, in fact, the inclusion of urea treated cocoa pod at the expense of higher fermentable feed components will reduce the fermentation intensity thereby yielding lower amounts of SCFA and microbial protein for the host animal. Nevertheless, the urea

treated cocoa pod can be used as a feedstuff for low performance ruminants as a substitute for barley/grain meal-mixture or as a supplement to hay based rations without reducing the efficiency of microbial synthesis.

No toxicity effects were observed with the inclusion of urea treated cocoa pod in the ration as to a dramatically disturbed fermentation or a too high concentration of ammonia.

8. Experiment 3. Comparison of two different methods for the determination of microbial growth in the rumen simulation technique (Rusitec)

8.1. Introduction

Microbial protein (MP) is the major crude protein fraction leaving the rumen when high NPN diets are fed to ruminants. It is therefore important to quantify its synthesis. Several available methods have been used to assess microbial protein synthesis. OBISPO & DEHORITY (1999) and DEWHURST et al. (2000) classified these methods into: (1) Measurement of microbial protein on protein free diets and assuming that all protein flowing to the duodenum is of microbial origin (2) distinguishing feed and microbial protein on the basis of amino acid profiles; (3) use of endogenous or exogenous markers such as ^{35}S, ^{15}N, ^{3}H-Leucine, ^{32}P, diaminopimelic acid (DAPA), RNA and purine and pyrimidine bases to label microbial protein material and (4) near infrared reflectance spectroscopy.

These wide ranges of approaches for microbial protein identification have limitations and have been reviewed by DEWHURST et al. (2000). It is discussed that each of the methods has its own problems including safety (radioisotopes), cost (^{15}N and amino acid profiles), difficulties of analysis (RNA and DNA) and contamination of feeds, thereby lacking specificity to the microbial fraction (DAPA, nucleic acid and their bases).

ABEL et al. (1990) measured microbial protein synthesis in Rusitec by using small quantities of ^{15}N and applying the continuous tracer infusion technique. The method is based on the rate of incorporation of nitrogen into microbes through the ammonia pools. However, VAN NEVEL et al. (1975) had expressed their objection to the ^{15}N based methods which do not take into consideration the direct incorporation of amino acid or peptide nitrogen into microbial cells. The direct incorporation of amino acid or peptide nitrogen could amount to 20% of the total nitrogen incorporated. With the ^{15}N-based methods the microbial synthesis could therefore be under-estimated.

8.2. Objective

The objective of this investigation was to compare the indirect continuous tracer infusion technique and the direct measurement of isolated microbes in Rusitec. With both methods stable isotope nitrogen (^{15}N) served as a tracer.

8.3. Material and methods

Three series of Rusitec were run. The Rusitec system consisted of 6 simultaneously operating 1060 ml fermenters. At the beginning of each run, each fermenter was filled with 690 ml rumen fluid and 80 g of solid fraction of rumen content taken from rumen fistulated Jersey steers. The solid fraction was contained in a nylon bag. Simultaneously, two other nylon bags one of 100 μm pore size containing 10 g hay and another of 60 μm pore size containing 4 g concentrate (barley, extracted soybean meal, urea treated cocoa pods and mineral mixtures in different proportions as shown in table 8.2) were introduced. Urea was used to treat cocoa pod with a level of 140 g/kg DM. The fermenters were then filled to volume with pre-warmed incubation buffer and operated during the following days according to the established procedure of Rusitec (CZERKAWSKI & BRECKENRIDGE, 1977). The bag containing the solid ruminal fraction was removed after 24h and replaced by bags containing the feed ration. The ration containing bags were removed after 48h of incubation.

Six different rations containing (% DM) CP 16, NFE 41 to 52, CF 24 to 36, NDF 49 to 62 and ADF 27 to 41 were used in this experiment. The nutritional composition of the feed rations is shown in table 8.1.

Table 8.1: Nutritional composition of the feed components

Ingredient (g)	DM	ASH	CP	XL	CF	NDF	ADF
	%	% DM					
Hay	93.4	7.92	15.8	1.32	30.2	57.3	33.9
Barley	87.6	2.48	12.7	2.48	7.26	30.8	8.46
Soy bean meal	89.2	7.12	52.2	1.82	4.66	10.6	5.86
Cocoa pod	90.5	8.58	15.9	0.74	51.9	76.9	59.4

The daily amounts of the different feed components supplied to six fermenters in Rusitec and their chemical composition are shown in Table 8.2.

The experiment was repeated 3 times. Each run consisted of 9 days of preliminary phase and 5 days of collection phase. Each fermenter was infused with about 620 ml buffer (MCDOUGAL, 1948) daily using an automatic pump. The overflows were collected in 1000 ml Erlenmeyer flasks connected to each fermenter, while the gas was collected in gas bags.

Table 8.2: Composition of the feed rations

	Ration					
	R1	R2	R3	R4	R5	R6
Ingredients						
Hay (g)	10	10	10	10	10	10
Barley (g)	0	2.4	1.8	1.2	0.6	0
Soy bean meal extr. (g)	0	1.6	1.2	0.8	0.4	0
Cocoa pod (g)	0	0	1.0	2.0	3.0	4.0
Mineral mix (g)	0.15	0.15	0.15	0.15	0.15	0.15
Nutrient contents						
DM (%)	93.5	91.9	92.1	92.3	92.5	92.7
ASH (%DM)	9.73	7.64	8.02	8.40	8.78	9.15
CP (% DM)	15.6	15.9	15.8	15.8	15.7	15.7
XL (% DM)	1.30	1.60	1.49	1.37	1.26	1.14
CF (% DM)	29.7	23.6	26.7	29.8	32.8	35.9
NDF (% DM)	56.4	48.9	52.2	55.5	58.8	62.1
ADF (%DM)	33.3	26.5	30.0	33.6	37.0	40.5

^{15}N-urea with 95% enrichment was used as a tracer for microbial growth measurements. The amount of 0.2259 g $(^{15}NH_2)_2CO$ was weighed and solved in 100 ml distilled water. Five millilitres of the solution was added to 5 l of fresh buffer. The amount of ^{15}N injected daily was calculated as

$$^{15}\text{N (mg/d)} = (0.2259 \text{ g}/100 \text{ ml})(1000 \text{ mg}/1\text{g})(5 \text{ ml}/5000 \text{ ml})(28/60)(95/100)(\text{ml overflow/d})$$

$$= 0.00100149 \text{ mg/ml (ml overflow/d)}.$$

The quasi-steady state condition in the fermenters with a nearly constant ^{15}N-concentration of the ammonia pool was achieved after 5 days.

The proximate nutritional compositions of the feed rations and of the solid residues after fermentation in Rusitec were analysed according to the conventional Weende method (NAUMANN & BASSLER, 1997). A differential fibre analysis according to VAN SOEST et al., (1991) was also performed. SCFA were detected using a gas chromatograph (GC-14B, Simadzu) equipped with a packed column (10% Carbowax 20 MTPA SP 1000 with 1% H_3PO_4 on Cromosorb WAW 80/100) and flame ionisation detector connected to a chromato-integrator (D-2000 Merck-Hitachi). Hydrogen at 120 kPa pressure served as carrier gas. The injection oven temperature was set at 170^{o}C while the column and detector temperatures were set at 120^{o}C and 220^{o}C respectively. NH_3-N was measured by means of an electrode.

After continuous infusion of ^{15}N-urea, the microbial synthesis was measured either indirectly by measuring the ^{15}N-enrichment of the ammonia pool or directly by analysing the ^{15}N-enrichment in microbial isolate. Details of the two methods are described below.

Methane production was measured by gas chromatography (SimadzuGC-8A) in collected gas samples on a packed silica gel column, at 80^{o}C injection port and detector temperature and 40^{o}C column temperature (isothermal), TC-detector (Simadzu C-R1B) and argon as carrier gas (DA COSTA GOMEZ, 1999).

Indirect method

The estimation of MPS from N-turnover of the ammonia pool was done using the same method described by ABEL et al. (1990). Three drops of silicon oil (anti foam), 8 drops of Thymolphthalein (2% in ethanol solution) or methyl red, 10 ml boric acid buffer (in 1 N KCl solution adjusted with 1 N KOH to pH 9.5) and 4.5 ml 1N KOH were added to100ml effluent. The solution was distilled and the distillate collected in a beaker glass containing 0.1 N H_2SO_4. Basically 0.1 N H_2SO_4 would be enough to bind the NH_3 released. The beaker volume was filled to 40 ml with distilled water. The distillation process was terminated when 200 – 250 ml of distillate was collected which occurred in approximately 12 minutes.

Part of the distillate volume containing approximately 0.5 mg nitrogen was evaporated. The amount of distilled volume to be evaporated was calculated as the volume of distillate in the beaker divided by NH_4-N/100 ml rumen fluid and divided by 2. This amount was transferred into a 50 ml beaker glass and evaporated at a temperature of 60^{o}C to give a volume of 3 – 4 ml. The temperature was then increased to 100^{o}C to further reduce the volume to750 µl. The remaining solution was pipetted into a tin cup and further dried at 105^{o}C. The cup was then formed to a small pellet and placed into a pallet. The pellets were analysed for ^{15}N enrichment by means of a mass spectrometer (Finnigan MAT Delta C, Bremen, connected to an elemental analyser, Fisons 1108 Rodano, Milano).

Assuming steady state conditions, microbial nitrogen was derived according to the following equation:

$$\text{Microbial N (mg/d)} = r_i \times ((s_i/s_p) - 1)$$

Where r = infusion rate (mg/d), s_i = ^{15}N-excess in infusion, s_p = ^{15}N-excess in NH_4-N pool. Microbial protein synthesis (MPS) in mg/d can be calculated as microbial-N divided by (8/100), assuming 8% N in microbial cells (CZERKAWSKI, 1986). The efficiency of microbial protein synthesis (EMPS) is calculated according to

$$\text{EMPS (mg/g OMAD)} = \text{MPS/OMAD}$$

Where OMAD is organic matter apparently degraded.

Direct isolation method

Sample reconstitute: The isolation method to estimate microbial protein synthesis in this study used the same principle as described by CARRO & MILLER (1999). The samples taken from the overflow and the solid residuals contained in the nylon bag on day 10 and 12 of Rusitec were well mixed using a low speed blender to reconstitute the total digesta. The sample was used to isolate bacterial pellets (TB). The sample was kept in an ice bath during the preparation to prevent microbial activity.

Isolation process: Samples of reconstituted digesta were strained using two layers of cheese cloth. The strained fluid was centrifuged for 10 min at 500 ***g*** and 4^{o}C. The supernatant was centrifuged again for 25 minutes at 18 000 ***g***, 4^{o}C to retain the bacterial pellet. The sediment was washed by re-suspension in NaCl solution (9 g/l) followed by centrifugation for 25 minutes at 18 000 ***g***, 4^{o}C. This sediment was washed again by re-suspension in distilled water and then again centrifuged for 25 minutes at 18 000 ***g***, 4^{o}C. The resultant pellet was freeze dried.

The freeze dried isolate was put into a tin cup and the cups were formed to pellets. The enrichment of ^{15}N in the isolate was detected using a mass spectrometer (Finnigan MAT Delta C, Bremen, connected to an elemental analyser, Fisons 1108 Rodano, Milano).

Assuming steady state conditions, microbial protein synthesis (MPS) and efficiency of microbial protein synthesis (EMPS) were derived as described above, however, inserting ^{15}N-excess of the microbial isolate instead of the ^{15}N-excess of the ammonia pool into the formula.

8.4. Results

The results of Rusitec are shown in Table 8.3. The values represent means of pooled samples per treatment and collection phase (n = 18). Organic matter apparently degraded (OMAD) and fermentation characteristics in the fermenters are shown in Table 8.3. The amount of organic matter apparently degraded varied around 5.1 g with a maximum 5.8 g and a minimum around 4.1.

There were no significant differences in pH among the rations used. The big difference between maximum and minimum values was caused by the effects of the different rations.

The average daily methane production ranged from 5.21 to 8.47 mmol with an average of 6.7 mmol. Ammonium concentrations varied from 5.11 to 8.63 mmol/l with an average of about 6.8 mmol/l. SCFA production ranged from 22.5 to 37.6 mmol/d.

Table 8.3: OMAD and fermentation characteristics in Rusitec (n = 18)

Parameters	dimension	average	maximum	minimum	STD
OMAD	g/d	5.10	5.79	4.13	0.56
PH		6.56	6.65	6.49	0.06
Protozoa	cts/ml	6240	9691	3465	1795
CH_4	mmol/d	6.70	8.47	5.21	0.96
NH_4^+	mmol/l	6.80	8.63	5.11	0.97
SCFA	mmol/d	31.0	37.6	22.5	4.16
Acetate	mmol/d	17.4	20.0	13.5	1.87
Propionate	mmol/d	7.62	9.75	5.50	1.24
i-Butyrate	mmol/d	0.18	0.30	0.08	0.06
n-Butyrate	mmol/d	4.09	5.98	2.44	0.99
i-Valerate	mmol/d	0.84	1.16	0.52	0.19
n-Valerate	mmol/d	0.78	1.49	0.42	0.28

Incorporation rate of ^{15}N into microbes (% atom excess) which was measured directly in microbial isolates and indirectly through the ammonia pool is given in Table 8.4.

Table 8.4: Average values of ^{15}N enrichment (% atom excess), microbial protein synthesis and their efficiency using the direct and indirect method

Parameter	Direct			Indirect		
	average	maximum	minimum	average	maximum	minimum
^{15}N enrichment (%)	0.61	0.68	0.56	0.88	1.00	0.81
Microbial-N (mg/d)	90.3	100.1	80.2	63.1	70.2	54.3
Microbial-N/OMAD (mg/g)	17.8	21.8	15.5	12.4	14.9	10.9

The ^{15}N enrichment in bacteria isolate (direct) was lower than in the ammonia pool (indirect). This resulted in higher bacterial-N fixation calculated for the direct microbial isolate method compared to the indirect approach. Correspondingly, microbial-N per gram of organic matter apparently degraded was higher when ^{15}N-enrichment was taken from the bacteria isolate instead of taking it from the ammonia pool.

8.5. Discussion

Microbial-N measured by means of ^{15}N-enrichment in the microbial isolate was higher than that determined from the ammonia pool. It may support the argument of VAN NEVEL et al. (1975) that the method based on the rate of incorporation of nitrogen through the ammonia pool (indirect) does not take into consideration the direct incorporation of amino acid or peptide nitrogen into microbial cells. The direct incorporation of amino acids or peptide nitrogen may amount to 20% of the total nitrogen incorporated. In this experiment, however, unaccounted nitrogen incorporation as proportion of total nitrogen incorporated reached 33% (different value between direct and indirect method). Feed particle contamination in bacterial isolates may have contributed to the lower ^{15}N-enrichment thus leading to higher calculated microbial N-incorporation with the direct method.

A comparison of the measured MPS values with the microbial protein synthesis to be expected theoretically from the stoichiometry of rumen fermentation may be helpful. The amount of synthesised microbial cells can be calculated from SCFA production. According to DEMEYER et al. (1995) the amount of hexose fermented (HF) can be calculated:

$$HF = \text{hexose fermented (mol/d)} = (A + P)/2 + B + V$$

Where A is acetate, P is propionate, B is butyrate and V is valerate expressed in mol/d respectively. The amount of hexose fermented (HF; g/d) is calculated as 162 x HF (mol/d).

The fermentation of 100 g hexose is assumed to result in the synthesis of 30 g microbial cells (BERGNER & HOFMANN, 1996).

If the average SCFA values from table 8.3 are taken for the formula above and assuming 8% nitrogen in microbial cells (Czerkawski, 1986), 68 mg microbial-N are theoretically expected per day. This is lower than the average microbial-N measured with the direct method but higher than that calculated with the indirect method.

8.6. Conclusions

For the range of rations used, it can be concluded that microbial-N synthesis in Rusitec measured by the direct method (based on ^{15}N-incorporation in microbial isolates) is higher than that determined with the indirect method (based on the rate of incorporation of nitrogen into microbes through the ammonia pool). The direct method also results in higher microbial-N synthesis compared to the theoretically expected microbial-N synthesis.

9. Summary

Three series of experiments have been conducted in order to evaluate the potential of cocoa pods as a feedstuff for ruminants.

The first study comprised five treatments (C = cocoa pod fresh; U0 = ensiled without urea; U1, U2 and U3 = addition of urea 10, 20 and 30 g/kg fresh cocoa pods respectively and then ensiled) and three replications in a complete random design. It was found that ensilage improves the feed value of cocoa pod by weakening the ligno-cellulose or –hemicellulose bonds, resulting in higher in vitro gas production, as well as higher calculated organic matter digestibility (OMD) and metabolisable energy (ME) contents compared to the fresh cocoa pods. The protein value of cocoa pods, expressed as true protein (TP) or non ammonia nitrogen minus urea-N (NAN-UR) was also improved. Additions of urea affected the composition of the fibre fraction as to the proportion of NDF, ADF and ADL and the content of TP was slightly increased. However, (NAN–UR) was lower than in the non urea treated ensiled samples. Urea treatment increased Gb and calculated OMD and ME compared to ensiled or fresh cocoa pod.

The (NAN-UR) value is suggested to be the nearest evaluation of the real protein value of urea treated cocoa pods.

The optimum level of urea for maximal Gb was 67 g/kg DM of cocoa pod. The optimum level to reach maximal OMD (%) and ME (MJ/kg DM) based on TP as protein value was 93 – 94 g urea per kg DM, while using NAN-UR as protein value, the OMD- and ME-maxima were reached at an urea level of 65 - 67 g/kg DM cocoa pod.

The second experiment aimed at investigating the effects of replacing a barley/soybean meal-mixture by urea treated cocoa pod in feed rations on methane release, SCFA production as well as the amount and efficiency of microbial-N fixation in Rusitec. The experiment included six different rations (T1 = 10 g/d hay; T2 = T1 + 4 g/d barley-soybean mixture (barley); T3 = T1 + 3 g barley + 1 g cocoa pod); T4 = T1 + 2 g barley + 2 g cocoa pod; T5 = T1 + 1 g barley + 3 g cocoa pod; T6 = T1 + 4 g cocoa pod) and three runs of Rusitec in a block random design. Cocoa pod was treated with 20 g urea per kg fresh material.

It was found that the substitution of a barley/soybean meal-mixture by urea treated cocoa pod up to 100% in a hay based ration did not decrease the rate of ration DM disappearance, and fibre degradation even tended to be increased.

Eventhough the microbial N-fixation decreased with the inclusion of cocoa pod in the rations, the efficiency of microbial N-fixation was increased in line with the cocoa pod level.

In conclusion, urea treated cocoa pod cannot replace an iso-nitrogenous barley/soybean meal-mixture equivalently in feed rations for ruminants. The inclusion of urea treated cocoa pod at the expense of higher fermentable feed components will reduce the fermentation intensity thereby yielding lower amounts of SCFA and microbial protein for the host animal.

In the third experiment, two methods for the determination of microbial protein synthesis (MPS) in Rusitec were applied and compared. With both methods stable isotope nitrogen (^{15}N) served as a tracer. The direct measurement of microbial-N in bacteria isolates resulted in higher MPS compared to the indirect method based on N-turnover in the ammonia pool. The results obtained are compared and discussed in relation to theoretical stoichiometric data of rumen fermentation.

10. References

ABEL, HJ.; COENEN, G.; IMMIG, I., 1990: Untersuchungen zum Einfluß von Fett- und Stärkezulagen auf den mikrobiellen Stoffwechsel im Pansensimulationssystem RUSITEC. J. Anim. Physiol. Anim. Nutr. 64, 62 – 73.

ABEL, HJ.; IMMIG, I.; HARMAN, E., 2002. Effect of adding caprylic and capric acid to grass on fermentation characteristics during ensiling and in the artificial rumen system RUSITEC. Anim. Feed Sci. Technol. 99, 65 – 72.

ADAMAKO, D.,1995: Non-traditional uses of cocoa in Ghana. Eighth Meeting of Advisory Group on the World Cocoa Economy. 26 - 30 June 1995. Yaounde, Cameroon. pp 79 – 85.

AHARONI, Y.; TAGARI, H., 1990: Use of nitrogen-15 determinations of purine nitrogen fraction of digesta to define nitrogen metabolism traits in the rumen. J. Dairy Sci. 74, 2540 – 2547.

ALDERMAN, G.; COTTRILL, B.R., 1993: Energy and Protein Requirements of Ruminants. CAB International. Wallingford, UK.

ANDERSEN, P.E.; KRISTENSEN, V. F.; HERMANSEN, J., 1989: The use of treated straw for feeding dairy cattle in Scandinavia. In: CHENOST, M.; REINIGER, P., (Eds.): Evaluation of Straws in Ruminant Feeding. Elsevier Applied Science, London and New York, pp. 86 – 97.

ANNISON, E.F., 1975: Microbial protein synthesis in relation to amino-acid requirements. In: Studies on non-protein nitrogen for ruminants II. Proceedings of a Research Co-ordination Meeting and Panel. Vienna, 18 – 22 February 1974, 141 – 173.

ARAMBEL, M.J.; BARTLEY, E.E.; DENNIS, S.M.; DUFVA, G.S.; NAGARAJA, T.B.; NUZBACK, D.E.; RIDDEL, D.O., DAYTON, A.D.; GALITZER, S.J., 1987: Evaluation of several methods for estimating microbial nitrogen concentration in the rumen. Nutr. Reports. Int. 35, 25 – 38.

ATASOGLU, C.; GULIYE, A.Y.; WALLACE, R.J., 2004: Use of stable isotopes to measure de novo synthesis and turnover of amino acid-C and –N in mixed micro-organisms from the sheep rumen in vitro. British J. Nutr. 91, 253 – 261.

BALDWIN, R.L., 1965: Pathway of carbohydrate metabolism in the rumen. Proceeding of the 2nd International Symposium on the Physiology of Digestion in the Ruminant. Ames, Iowa. Agustus, 1964. pp. 379 – 389.

BARNES, A.R.; AMEGA, W.K., 1984: Utilization of cocoa husk meal by growing-finishing pigs. 9th International Cocoa Research Conference, Lomé, Togo, 12-18 February 1984. pp. 449 – 454.

BARTLEY, E.E.; DEYOE, C.W., 1981: Reducing the rate of ammonia release by the use of alternative non-protein nitrogen sources. In: HARESIGN, W.; COLE, D.J.A., (Eds.): Recent Developments in Ruminant Nutrition. Butterworths, London. pp. 99 – 114.

BATES, D.B.; GILLET, J.A.; BARAO, S.A.; BERGEN, W.G., 1985: The effect of specific growth rate and stage of growth on nucIeic acid-protein values of pure cultures and mixed ruminal bacteria. J. Anim. Sci. 61, 713 - 724.

BEEVER, D.E.; LOSADA, H.R.; CAMMEL, S.B.; EVANS, R.T.; HAINES, M.J., 1986: Effect of forege species and season on nutrient digestion and supply in grazing cattle. British J. Nutr. 56, 209 – 226.

BERGNER, H.; HOFMANN, L., 1996: Bionergetik und Stoffproduktion Landwirtschaftlicher Nutztiere. Harwood Academic Publishers. Singapore.

BESLE, J.M.; CHENOST, M.; TISSERAND, J.L.; LEMOINE, J.P.; FAUTI, F.; SALEH, H.; GRENET, N., 1990: Ammoniation of straw by urea:extent of ureolysis and improvement of nutritive value with moderate water addition. Reprod. Nutr. Dev., suppl.2, 174.

BLACKBURN, T.H.; HOBSON, P.N., 1960: The degradation of protein in the rumen of the sheep and redistribution of the protein nitrogen after feeding. British J. Nutr. 14, 445.

BLAKE, D.R.; ROWLAND, F.S., 1986: World-wide Increase in Tropospheric Methane 1978-1983. Journal of Atmospheric Chemistry. 4, 43 - 62.

BODA, K., 1990: Non-conventional foodstuffs in the nutrition of farm-animals. Elsevier, Amsterdam.

BODA, K.; HAVASSY, I., 1975: Urea-^{15}N conversion after intravenous administration in sheep. In: Studies on non-protein nitrogen for ruminants II. Proceedings of a Research Co-ordination Meeting and Panel. Vienna, Germany. 18 – 22 February 1974. pp. 99 – 109.

BRANDT, M., 1979: Versuche zur Quantifizierung der mikrobiellen Proteinsynthese im Pansen (mit Hilfe von 15N) bei Verwendung harnstoffhaltiger Rationen. Kiel : VDI-Gesellschaft Agrartechnik, 115 p [Dissertation]

BRYANT, M.P., 1979: Microbial methane production – Theoritical aspects. J. Anim. Sci. 48, 193 – 201.

CAROLL, E.J.; HUNGATE, R.E., 1955: Formate dissimilation and methane production in bovine rumen contents. Arch. Biochem.Biophys. 56, 525 – 536.

CARRO, M.D.; MILLER, E.L., 1999: Effect of supplementing a fibre basal diet with different nitrogen forms on ruminal fermentation and microbial growth in an in vitro semi-continuous culture system (RUSITEC). British J. Nutr. 82, 149 – 157.

CHENOST, M., 2001: Optimizing the use of poor quality roughages through treatments and supplementation in warm climate countries with particular emphasis on urea treatment. http://www.fao.org/ag/AGA/AGAP/FRG/ECONF95/HTML/CHENOST.HTM.

CHENOST, M.; KAYOULI, C., 1997: Roughage utilization in warm climates. Animal Production and Health Paper 135. FAO, Rome.

CHENOST, M.; REINIGER, P., 1989: Evaluation of straws in ruminant feeding. Proceeding of a Workshop: “Use of lignocellulose containing by-products and other plant residues for animal feeding”. Commission of the European Communities. Elsevier, London.

CHESSON, A.; MURISON, S., 1989: Biochemical evaluation of straw as a feedstuff for ruminants. In: CHENOST, M.; REINIGER, P., (Eds.): Evaluation of Straws in Ruminant Feeding. Elsevier Applied Science, London and New York, pp. 124 - 133.

COLEMAN, G.S.; DAWSON, R.M.C.; GRIME, D.W., 1980: The rate of passage of ciliate protozoa from the ovine rumen. Proc. Nutr. Soc. 39, 6.

COLUMBIA ENCYCLOPEDIA. 2003. Cocoa. http://yahooligans.yahoo.com/reference/encyclopedia/entry?id=7837

CONE, J.W.; VAN GELDER, A.H.; KAMMAN, A.A.M.; HINDLE, V.A., 2004: Rumen escape protein in grass and grass silage determined with a nylon bag and an enzymatic technique. Anim. Feed Sci. Technol. 111, 1 – 9.

CORDESSEE, R.; FACI, R.; MUNOZ, F.; ALIBES, X.; GUESSOUS, F., 1989: Long-term utilization of ammonia-treated straws for ewes in mediterranean countries. In: CHENOST, M.; REINIGER, P., (Eds.): Evaluation of Straws in Ruminant Feeding. Elsevier Applied Science, London and New York, pp. 80 – 85.

CRUTZEN, P.J., 1995: The role of methane in atmospheric chemistry and climate. Proceedings of the 8th International Symposium on Ruminant Physiology: Digestion, Metabolism, Growth and Reproduction. Ferdinand Enke Verlag, Stuttgart. pp. 291 – 315.

CRUTZEN, P.J.; ASELMAN, I.; SEILER, W., 1986: Methane production by Domestic Animals, Wild Ruminants, other Herbivorous Fauna, and Humans. Tellus 38B, 271 – 284.

CZERKAWSKI, J.W., 1969: Methane production in ruminant and its significance. World Rev. Nutr. Diet. 11, 240 – 282.

CZERKAWSKI, J.W., 1973: Manipulation of rumen fermentaion. Process Biochemistry, October 1973. pp. 25 – 30.

CZERKAWSKI, J.W., 1986: An Introduction to Rumen Studies. Pergamon Press, Oxford.

CZERKAWSKI, J.W.; BRECKENRIDGE, G. 1977. Design and development of a long-term rumen simulation technique (rusitec). British J. Nutr. 38, 371 – 374.

DA COSTA GOMEZ, C., 1999: In-vitro-Unterschungen zur reduktiven Acetogenese im Pansen. Diss.agr.Göttingen, pp. 18 - 25.

DANTUS, M., 1998: An Introduction to Global Warming. Lecture Note. http://www.cem.msu.edu/~cem181h/projects/98/global/gwintro.html

DAVIES, Z.D.; BROOKS, A.E., GRIFFITHS, G.W., THEODOROU, M.K., 1998: Assessment of fermentation characteristics of ruminant foods using an automated pressure evaluation system. In: Deauville, E.R.; Owen, E.; Adesogan, A.T.; Rymer, C.; Huntingdon, J.; Lawrence, T.L.J. (Eds.): In vitro techniques for measuring nutrient supply to ruminants. British Society of Animal Science, Occasional Publication No. 22, 190 – 192.

DEGER, H.M., 1987: Cellulose degradation: concentrated acid hydrolysis processes; application of the final products. In: Van der Meer, J.M.; Rijkens, B.A.; Ferranti, M.P.,

(Eds.): Degradation of Lignocellulosics in Ruminants and in Industrial Processes. Pp. 85 – 88.

DEHORITY, B.A., 1995: Methodology for measuring microbial growth in the rumen. In: Proc. Int. Symp. on the Nutrition Requirements of Ruminants, Universidad Federal de Vicosa, Vicosa-MG-Brazil. Pp. 121 – 137.

DEMEYER, D.I.; HENDERICKX, H.K., 1967: The effect of C_{18} unsaturated fatty acids on methane production in vitro by mixed rumen bacteria. Biochem. Biophys. Acta 137, 484 – 497.

DEMEYER, D.I.; VAN NEVEL, C.J., 1975: Methanogenesis, an integrated part of carbohydrate fermentation and ist control. In: McDonald, I.W.; Warner, A.C.I. (Eds.), Digestion and Metabolism in the Ruminant. University of New England Publishing Unit. Armidale. pp. 366 – 382.

DEMEYER, D.I.; WOESTYNE, M.V.; PRINS, R., 1995: Mikrobiologie der Verdauung. In: ABEL, HJ.; MOLNAL, S.; FLACHOWSKY, G.; JEROCH, H., (Eds.): Nutztierernährung: Potentiale – Verantwortung – Perspektiven. Gustav Fischer Verlag. Jena, 18 – 206.

DEPARTMENT OF AGRICULTURE MALAYSIA, 2001: Industrial Crop Technologies. http://agrolink.moa.my/doa/english/croptech/cocotech.html

DEWHURST, R.J., 1989: Studies on energy and nitrogen metabolism in the rumen – Investigation of less invasive techniques for these studies. Ph.D. thesis, University of Bristol.

DEWHURST, R.J.; DAVIES, D.R.; MERRY, R.J., 2000: Microbial protein supply from the rumen. Anim. Feed Sci. Technol. 85, 1 – 21.

DUKE, A.D., 1978: The quest for tolerant germplasm. In: ASA special symposium 32 "Crop tolerance to suboptimal land conditions". Am. Soc. Agron. Madison, W.I.. pp.1 – 61.

DUKE, A.D., 1983: Handbook of Energy Crops. Unpublished. http://www.hort.purdue.edu/newcrop/duke_energy/Theobroma_cacao.html

DURAN, M.; GUEGUEN, L.; PRIETO, F., 1975: Effect of source of energy and minerals on microbial protein synthesis in the rumen using labelled sulphur as indicator. In: Studies on non-protein nitrogen for ruminants II. Proceedings of a Research Co-ordination Meeting and Panel. Vienna, 18 – 22 February 1974, 29 – 42.

EHHALT, D.H.; SCHMIDT, U., 1978: Sources and sinks of atmospheric methane. Pure Applied Geophysics. 116, 452.

ENCARTA, 1996: Encarta Multimedia Encyclopedia. http://sres.anu.edu.au/associated/fpt/nwfp/chocolatetree/chocolatetree.html

ERIKSSON, O.; GORING, D.A.I.;LINDGER, B.O., 1980: Structural studies on the chemical bonds between lignin and carbohydrates in spruce wood. Wood Sci. Technol. 14, 267 – 279.

FIGUEIRA, A.; JANICK, J.; MILLER, J.N., 1993: New products from *Theobroma cocoa*: Seed pulp and pod gum. Pp. 475 – 478. In: JANICK, J.; SIMON, J.E., (Eds.), New crops. Wiley, New York. http://www.hort.purdue.edu/newcrop/proceedings1993/v2-475. html

FLACHOWSKY, G.; KAMRA, D.N; ZADRAZIL, F., 1999: Cereal straws as animal feed – possibilities and limitation. J. Appl. Anim. Res. 16: 105 – 118.

HAGEMEISTER, H.; LÜPPING, W.; KAUFMANN, W., 1981, Microbial protein synthesis and digestion in the high-yielding dairy cow. In: HARESIGN, W.; COLE, D.J.A., (Eds.): Recent Developments in Ruminant Nutrition. Butterworths. London, 31 – 48.

HARMEYER, J.; MARTENS, H.; HÖLLER, H.,1975: Incorporation of ^{35}S by rumen micro-organisms in vitro at various microbial growth rates. In: Studies on non-protein nitrogen for ruminants II. Proceedings of a Research Co-ordination Meeting and Panel. Vienna, 18 – 22 February 1974, 7 – 14.

HELMER, L.G.; BARTLEY, E.E., 1971: Progress in the utilization of urea as a protein replacer for ruminants. J. Dairy Sci. 54, 25 – 51.

HENDERSON, A.R.; GARNWORTHY, P.C.; NEWBOLD, J.R.; BUTTERY, P.J., 1998: The effect of asynchronous diets on the function of the rumen in the lactating dairy cow. Proceedings of the BSAS Annual Meeting. pp19.

HESPELL, R.B.; BRYANT, M.P., 1979: Efficiency of rumen microbial growth: Influence of some experimental factors on Y_{ATP}. J. Anim. Sci. 49, 1640 – 1648.

HOMB, T.; SUNDSTØL, F.; ARNASON, J., 1977: Chemical treatment of straw at commercial and farm level. FAO Anim. Prod. & Health Paper. 4, 25 – 37.

HRISTOV, A.N., 2002: Fractination of ammonia nitrogen isotopes by ruminal bacteria in vitro. Anim. Feed Sci. Technol. 100, 71 – 77.

HUME, I.D., 1970: Synthesis of microbial protein in the rumen 3: The effect of dietary protein. Aust. J. Agric. Res. 21, 305 – 314.

HUME, I.D., 1975: Use of ^{35}S to estimate the proportion of dietary protein degraded in the rumen. In: Studies on non-protein nitrogen for ruminants II. Proceedings of a Research Co-ordination Meeting and Panel. Vienna, 18 – 22 February 1974, 1 – 6.

HUNGATE, R.E., 1965: Quantitative aspects of rumen fermentation. In: DOUGHERTY, R.W., (Eds.): Physiology of Digestion in the Ruminant, Butterworths, London.

HUTTON, K.; BAILEY, F.J.; ANNISON, E.F., 1971: Measurement of the bacterial nitrogen entering the duodenum of the ruminant using diaminopimelic acid as a marker. British J. Nutr. 25, 165 – 173.

HVELPLUND, T., 1998: Research activities on ruminant feed evaluation in Denmark In: Kusina, N.J., (eds.): Integrated livestock/crop production systems in the smallholder Farming system in Zimbabwe. Proceedings of a workshop at the University of Zimbabwe. Harare-Zimbabwe, 13-16 January. pp. 60-72.

IBRAHIM, M.N.M.; SCHIERE, J.B., 1986: Rice straw and related feeds in ruminant rations. Proc. of an international Workshop held in Kandy, Sri Lanka, 24–28 March, 1986. Straw Utilization Project, Pub. No. 2, Sri Lanka-Netherlands Livestock Development Programme, pp 408.

ICCO 1998[b]: Is it true that 1 ha land with 900 cocoa trees could produce 1000 kg of beans every month? http://www. icco.org/questions.

ICCO, 1998: How exactly is cocoa harvested? http://www.icco.org/questions/harvest.htm.

ICCO, 1998[a]: What time of year is cocoa harvested? http://www.icco.org/questions/harvest1.htm.

ICCO, 2000: Animal feed from cocoa. http://www.icco.org/questions/animalfeed.htm.

ICCO, 2003: World cocoa bean production from 1990/1991 to 2002/2003. http://www.icco.org/questions/worldprod.htm.

ICCO, 2003[a]: Main cocoa producing countries and their cocoa bean production. http://www.icco.org/questions/production.htm.

ICCO, 2003[b]: Products that can be made from cocoa. http://www.icco.org/questions/ production2.htm.

ICCO, 2003[C]: Cocoa in Indonesia. http://www. icco.org/questions/indonesia.htm.

JETANA, T.; ABDULLAH, N.; HALIM, R.A.; JALALUDIN, S.; HO, Y.W., 2000: Effects of energy and protein supplementation on microbial-N synthesis and allantoin excretion in sheep fed guinea grass. Anim. Feed Sci. Technol. 84, 167 – 181.

KLOPFENSTEIN, T., 1978: Chemical treatment of crop residues. J. Anim. Sci. 46, 841 – 848.

KLOPFENSTEIN, T., 1994: Crop residue use in animal feed. In: Unger, P. (Ed.) Managing Agricultural Residues. Vol.1. Lewis, London. pp. 315 – 342.

KRUG, C.A.; QUARTEY-PAPAFIO, E., 1964: World Cocoa Survey. Food and Agriculture organization of the United Nations, Rome

LEBZIEN, P.; PAUL, C., 1997: Use of the near-infrared reflectance spectroscopy for the estimation of the microbial portion of non-ammonia nitrogen in the duodenum of dairy cows. Anim. Feed Sci. Technol. 68, 225 – 233.

LEGAY-CARMIER, F.; BAUCHART, D., 1989: Distribution of bacteria in the rumen contents of dairy cows given a diet supplemented with soya-bean oil. British. J. Nutr. 61, 725 – 740.

LENG, R. A., 1982: Modification of rumen fermentation. In: HACKER, J.B., (Eds.): Nutritional Limits to Animal Production from Pastures. Commonwealth Agricultural Bureaux, Farnham Royal, UK. pp.427-453.

LENG, R.A., 1992: Ruminant production and greenhouse gas emissions. Proceedings of the New Zealand Society of Animal Production, 52 (suppl.), 15-23.

LESLIE, M.; O'HARA, P., 2003: A Pastoral Greenhouse Gas Research Strategy. http://www.maf.govt.nz/mafnet/rural-nz/sustainable-resource-use/climate/pastoral-greenhouse-gas-strategy/#_Toc57524211

LEUNG, A.Y., 1980: Encyclopedia of common natural ingredients used in food, drugs, and cosmetics. John Wiley & Sons. New York.

LICITRA, G.; Hernandez, T. M.; Van Soest, P. J. , 1996: Standardization of procedures for nitrogen fractionation of ruminant feeds Anim. Feed Sci. Technol. 57, 347 – 358.

LLAMOSAS, C.A.; PEREIRA, J.M.; SOARES, M.S., 1983: Fresh cocoa husks as a substitute for elephant grass for finishing confined steers. Revista Theobroma. 13 (2), 57 – 62.

LOCKYER, D.R.; CHAMPION, R.A., 2001: Methane production by sheep in relation to temporal changes in grazing behaviour. Agriculture, Ecosystems & Environment. 86, 237 – 246.

LOPEZ, S.; VALDES, C.; NEWBOLD, C.J.; WALLACE, R.J., 1999. Influence of sodium fumarate addition on rumen fermentation in vitro. British J. Nutr. 81, 59 – 64.

MACHMÜLLER, A.; KREUZER, M., 1999: Methane suppression by coconut oil and associated effects on nutrient and energy balance in sheep. Can. J. Anim. Sci. 79, 65 – 72.

MACHMÜLLER, A.; OSSOWSKI, D.A.; KREUZER, M., 2000: Comparative evaluation of the effects of coconut oil, oilseeds and crystalline fat on methane release, digestion and energy balance in lambs. Anim. Feed Sci. Technol. 85, 41 – 60.

MCDONALD, P.; EDWARDS, R.A.; GREENHALGH, J.F.D.; MORGAN, C.A., 1995: Animal Nutrition 5th ed. Pearson Education Limited, Edinburgh Gate, Harlow.

MCDONALD, P.; HENDERSON, N.; HERON, S., 1991: The Biochemistry of Silage, 2nd ed. Chalcombe Publications, Marlow.

MCDOUGAL, E.I. 1948: Studies on ruminant saliva I. The composition and output of sheep's saliva. Biochem. J. 43, 99 – 109.

MENKE, K.H..; STEINGASS, H., 1987: Schätzung des Energetischen futterwerts aus der In vitro mit Pansensaft bestimmen gasbidung und der Chemischen analyse. II. Regressionsgleichungen. Übers. Tierernähr. 15, 59 – 94.

MENKE, K.H.; RAAB, L.; SALEWSKI, A.; STEINGASS, H.; FRITZ, D.; SCHNEIDER, W., 1979: The estimation of the digestibility and metabolizable energy content of ruminant feedstuffs from the gas production when they are incubated with rumen liquor. J. Agric. Sci. 93, 217 – 222.

MERRY, R.J.; SMITH, R.H.; MCALLAN, A.B., 1987: Studies on rumen function in an in vitro continuous culture system. Arch. Anim. Nutr. 37, 475 – 488.

MILLER, E.L., 1982: Methods of assessing proteins for ruminants, including laboratory methods. In: MILLER, E.L.; PIKE, I.H.; VAN ES, A.J.H., (Eds.), Protein contribution of feedstuffs for ruminants: application to feed formulation. Studies in the Agricultural and Food Sciences. Butterworth Scientific, London. pp. 18 – 35.

MILLER, T.L., 1991: Biogenic sources of methane. In: ROGERS, J.E.; WHITMAN, W.B. (Eds.): Microbial Production and Consumption of Greenhouse Gases: Methane, Nitrogen Oxide and Halomethanes. American Society for Microbiology, Washington D.C. pp. 175 – 188.

MORRIS, J.G.; BALDWIN, R.L.; MAENG, W.J.; MAEDA, B.T., 1975: Basic characteristics of a computer-simulated model of nitrogen utilization in the grazing ruminant. In: Studies on non-protein nitrogen for ruminants II. Proceedings of a Research Co-ordination Meeting and Panel. Vienna, 18 – 22 February 1974, 65 – 79.

MOSS, A.R.; GIVENS, D.I.; GARNSWORTHY, P.C., 1994: The effect of alkali treatment of cereal straws on digestibility and methane production by sheep. Anim. Feed Sci. Technol. 49, 245 – 259.

NAUMANN, C.; BASSLER, R., 1997: VDLUFA-Methodenbuch Band III, Die chemische Untersuchung von Futtermitteln. 3rd ed. VDLUFA-Verlag, Darmstadt, Germany.

NIKOLIC, J.A.; JOVANOVIC, M.; FILIPOVIC, R., 1975: Microbial protein synthesis by bovine rumen content in relation to ammonia concentration. In: Studies on non-protein nitrogen for ruminants II. Proceedings of a Research Co-ordination Meeting and Panel. Vienna, 18 – 22 February 1974, 43 – 54.

NOLAN, J.V.; LENG, R.A., 1983: Nitrogen metabolism in the rumen and its measurement. Nuclear Techniques for Assessing and Improving Ruminant Feeds. IAEA, Vienna.

NOLLET, L.; VERSTRAETE, W., 1996: Gastro-Enteric methane versus sulphate and volatile fatty acid production. Environmental Monitoring and Assessment. 42, 113 – 131.

OBISPO, N.E.; DEHORITY, B.A., 1999: Feasibility of using total purines as a marker for ruminal bacteria. J. Anim. Sci. 77, 3084 – 3095.

OFFER, N.W.; AXFORD, R.F.E.; EVANS, R.A., 1978: The effect of dietary energy sourcece on nitrogen metabolism in the rumen of sheep. British J. Nutr. 40, 35 – 44.

OLDHAM, J.D., 1981: Amino acid requirements for lactation in High-yielding dairy cows. In: HARESIGN, W.; COLE, D.J.A., (Eds.): Recent Developments in Ruminant Nutrition. Butterworths. London, 49 – 81.

ØRSKOV, E.R., 1982: Recent advances in knowledge of protein evaluation for ruminants. In: MILLER, E.L.; PIKE, I.H.; VAN ES, A.J.H., (Eds.), Protein contribution of feedstuffs for ruminants: application to feed formulation. Studies in the Agricultural and Food Sciences. Butterworth Scientific. London, 1 – 3.

OWEN, E.; KLOPFENSTEIN, T.; URIO, N., 1984: Treatment with other chemicals. In: SUNDSTØL, F.; OWEN, E. (Eds.): Straw and other fibrous by-products as fed. Elsevier, Amsterdam. pp. 248 – 275.

PELCHEN, A.; PETERS, K.J., 1994: Pollutant gases from animal farming - Influences on the greenhouse effect. Natural Resources and Development. Institute for Scientific Co-operation, Tübingen.40, 56-68.

PRESTON, T.R., 1995: Tropical Animal Feeding - A Manual for Research Workers. Animal Production and Health Paper. 126. FAO. Rome.

PUCHALA, R.; KULASEK, G.W., 1991: Evaluation of allantoin as a marker for microbial protein synthesis in the rumen of sheep. Proceedings of the 6th International Symposium on Protein Metabolism and Nutrition. Herning, Denmark. 9 – 14 June. Pp. 89 – 91.

PURSEGLOVE, J.W., 1968: Tropical Crops: Dicotyledons 2. Longman, London.

QUIGLEY, J.D.; SCHWAB, C.G.; HYLTON, W.E., 1985: Development of rumen function in calves: nature of protein reaching the abomasum. J. Dairy Sci. 68, 694 – 702.

RANFFT, K., 1973: Gaschromatographiche Bestimmung kurzkettiger flüghtiger Fettsäuren im Pansensaft. Arch. Tierernährung. 23 (5), 343 – 352.

RATH, D.; GÄDEKEN, D., 1991: Milchproduktion beeinflußt das Weltklima. Der Tierzüchter. 43, pp. 103.

REHM, S.; ESPIG, G., 1991: The Cultivation Plants of the Tropical and Substropics. Verlag Josef Margraf. Weikersheim, Germany.

REXEN, F.P.; KNUDSEN, K.E.B., 1984: Industrial-scale dry treatment with sodium hydroxide. In: SUNDSTØL, F.; OWEN, E., 1984: Straw and other fibrous by-products as feed. Dev. Anim. Vet. Sci. 14, 127 – 159.

REYNOLDS, S.G., 1995: Pasture-Cattle-Coconut Systems. http://www.fao.org/DOCREP/005/AC489E/AC489E00.htm#ch6.6.3.

RODRÍGUEZ, C.A.; GONZALES, J.; ALVIR, M.R.; REPETTO, J.L.; CENTENO, C.; LAMRANI, F., 2000: Composition of bacteria harvested from the liquid and solid fractions of the rumen of sheep as influenced by feed intake. British J. Nutr. 84, 369 – 376.

ROJAS, J.B.U.; VERRETH, J.A.J.; VAN WEERD, J.H.; HUISMAN, E.A., 2002: Effect of different chemical treatments on nutritional and antinutritional properties of coffee pulp. Anim. Feed Sci. Technol. 99, 195 – 204.

SAR, C.; SANTOSO, B.; MWENYA, B.; GAMO, Y.; KOBAYASHI, T.; MORIKAWA, R.; KIMURA, K.; MIZUKOSHI, H.; TAKAHASHI, J., 2004: Manipulation of rumen methanogenesis by the combination of nitrate with β1-4 galacto-oligosaccharides or nisin in sheep. Anim. Feed Sci. Technol. 115, 129 – 142.

SATTER, L.D.; SLYTER, L.L., 1974: Effect of ammonia concentration on rumen microbial protein production in vitro. British J. Nutr. 32, 199 – 208.

SATTER, L.D.; SLYTER, L.L., 1972: Effect of ammonia concentration on ruminal microbes in vitro. J. Anim. Sci. 35, pp. 273.

SCHIERE, J.B.; IBRAHIM, M.N.M., 1989: (Eds.) Feeding of urea ammonia treated rice straw. A compilation of miscellaneous reports produced by the Straw. Utilization Project (Sri-Lanka). Pudoc, Wageningen, pp. 125.

SEILER, W., 1984: Contribution of biological processes to the global budget of CH4 in the atmosphere. In: KLUG, M.; REDDY, C., (Eds.): Current Perspectives in Microbial Biology, pp. 468.

SEILER, W.; CONRAD, R., 1987: Contribution of tropical ecosystems to the global budgets of tracer gases, especially CH4, H2, CO2 and N2O. In: Dickinson, R.E. (Eds.): The Geophysiology of Amazonia. John Wiley, New York. Ch. 9, 133 – 162.

SHEM, M.N.; HOVELL, F.D.D.; KIMAMBO, A.E., 1999: Estimation of net ruminal protein synthesis from urinary allantoin excretion by bulls given tropical feeds. Anim. Feed Sci. Technol. 81, 279 – 289.

SINCLAIR, L.A.; GARNSWORTHY, P.C.; NEWBOLD, J.R.; BUTTERY, P.J., 1993: Effect of synchronizing the rate of dietary energy and nitrogen release on rumen fermentation and microbial protein synthesis in sheep. J. Agric. Sci. 120, 251 – 263.

SMITH, R.H., 1989: Nitrogen metabolism in the ruminant stomach. In: Bock, H.D.; Eggum, B.O.; Low, A.G.; Simon, O.; Zebrowska, T.; Low, A.G., (Eds.): Protein Metabolism in Farm Animals: Evaluation, Digestion, Absorption and Metabolism. VEB Deutscher Landwirtschaftsverlag, Berlin. pp. 165 – 203.

SMITH, R.H.; SALTER, D.N.; SUTTON, J.D.; MCALLAN, A.B., 1975: Synthesis and digestion of microbial nitrogen compounds and VFA production by the bovine. In: Studies on non-protein nitrogen for ruminants II. Proceedings of a Research Co-ordination Meeting and Panel. Vienna, 18 – 22 February 1974, 81 – 93.

SOBAMIWA, O.; LONGE, O.G., 1994. Utilisation of cocoa-pod pericarp fractions in broiler chick diets. Anim. Feed Sci. Technol. 47, 237 – 244.

STERN, M.D.; HOOVER, W.H.; SNIFFEN, C.J.; CROOKER, B.A.; KNOWLTON, P.H., 1978: Effects of constructural carbohydrate, urea and soluble protein levels on microbial protein synthesis in continuous culture of rumen contents. J. Anim. Sci. 47, 944 – 956.

SUNDSTØL, F.; MGHENI, D.M.; PEDERSON, I., 1993: Recent finding on upgrading of the feeding value of straw by chemical and biological methods. Proceedings of the International Conference on Increasing Livestock Production through Utilisation of Local Resources. 8 – 12 October 1993. Beijing – China.

SUNDSTØL, F.; OWEN, E., 1984: Straw and other fibrous by-products as feed. Dev. Anim. Vet. Sci. 14, 1 – 3.

SUTARDI, T., 1988: Rumus Penduga TDN. Faculty of Animal Science. Bogor Agricultural University. Unpublished.

TAKASHI, J.; YOUNG, B.A., 1991: Prophylactic effect of L-cysteine on nitrate-induce alteration in respiratory exchange and metabolic rate in sheep. Anim. Feed Sci. Technol. 35, 105 – 113.

TAMMINGA, S., 1982: Energy-protein relationships in ruminant feeding: Similarities and differences between rumen fermentation and postruminal utilization. In: MILLER, E.L.; PIKE, I.H.; VAN ES, A.J.H., (Eds.), Protein contribution of feedstuffs for ruminants:

application to feed formulation. Studies in the Agricultural and Food Sciences. Butterworth Scientific. London, 4 – 17.

TARLA, 1996 : Glorious Chocolate. http://wwwiz.com/issue05/wizd04.html.

THEANDER, O.; AMAN, P., 1984: Anatomical and chemical characteristics. In: SUNDSTØL, F.; OWEN, E., (Eds.): Straw and other fibrous by-products as feed. Dev. Anim. Vet. Sci. 14, 45 – 78.

THOMAS, P.C., 1973: Microbial protein synthesis, Proc Nutr. Soc. 32, 85 – 91.

TITGEMEYER, E.C., 1997: Design and interpretation of nutrient digestion studies. J. Anim. Sci. 75, 2235 – 2247.

TOHARMAT, T. ; TJAKRADIDJAJA, A.S. ; SURYAHADI ; SIGIT, N.A. ; PERMANA, I.G., 1997: Nutritive value of palm and cocoa plantation by-products in dairy cattle ration. Proceeding National Seminar II Nutrition and Feed Sciences, 15 – 16 July 1997. Bogor – Indonesia. pp. 57 – 58.

TRACH, N.X.; DAN, C.X.; LY, L.V.; SUNDSTØL, F., 1998: Effects of urea concentration, moisture content, and duration of treatment on chemical composition of alkali treated rice straw. Livestock Research for Rural Development. Vol. 10 (1).

TYLER, S.C., 1987: $^{13}C/^{12}C$ ratios in atmospheric methane and some of its sources. Ecological Studies. 68, 395.

VAN NEVEL, C.J.; DEMEYER, D.I., 1996: Control of rumen methanogenesis. Environmental Monitoring and Assessment. 42, 73 – 79.

VAN NEVEL, C.J.; DEMEYER, D.I.; HENDERICKX, H.K., 1975. Use of ^{32}P to estimate microbial synthesis in the rumen. In: Studies on non-protein nitrogen for ruminants II. Proceedings of a Research Co-ordination Meeting and Panel. Vienna, 18 – 22 February 1974, 15 – 20.

VAN SOEST, P. J.; ROBERTSON, J.B.; LEWIS, B.A., 1991: Methods for dietary fiber, neutral detergent fibre and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583 – 3597.

VAN SOEST, P.J., 1982: Nutritional Ecology of the Ruminant: Ruminant metabolism, nutritional strategies, the cellulolytic fermentation and the chemistry of forages and plant fibers. Durham and Downey, Inc. Portland.

VAN SOEST, P.J., 1994: Nutritional Ecology of the Ruminant (2nd Ed.). Comstock Publishing Association. Ithaca.

VAN SOEST, P.J.; ROBERTSON, J.B., 1979: Systems of analysis for evaluating fibrous feeds. In: Standardization of analytical methodology for feeds. Proceedings of a workshop held in Ottawa, Canada, 12-14 March, 1979.

VAN SOEST, P.J.; JONES, L.P.H., 1968: Effect of silica in forages upon digestibility. J. Dairy Sci. 51, 1644 – 1648.

WBC, 2003: Introduction Biochemistry: Effects of pH. http://www.worthington-biochem.com /introBiochem/effectspH.html.

WELLER, R.A.; PILGRIM, A.F., 1974: Passage of protozoa and volatile fatty acids from the rumen of the sheep and from a continuous in vitro fermentation system. British J. Nutr. 32, 341 – 351.

WHITELAW, F.G.; EADIE, J.M.; BRUCE, L.E.; SHAND, W.J., 1984: Methane formation in faunated and ciliate-free cattle and its relationship with rumen volatile fatty acid proportions. British J. Nutr. 52, 261 - 275.

WILKINSON, J.M., 1984: Farm-scale dry treatment with sodium hydroxide. In: SUNDSTØL, F.; OWEN, E., (Eds.): Straw and other fibrous by-products as feed. Dev. Anim. Vet. Sci. 14, 162 – 195.

WILLIAMS, P.E.V.; INNES, G.M.; BREVER, A., 1984: Ammonia treatment of straw via the hydrolysis of urea. Additions of soy bean (urease), sodium hydroxide and molasses. Effects on the digestibility of urea-treated straw. Anim. Feed Sci. Technol. 11, 115 – 124.

WOLIN, E.A.; WOLIN, M.J.; WOLFE, R.S., 1963: Formation of Methane by Bacterial Extracts J. Biol. Chem. 238, 2882 – 2886.

WOLIN, M.J., 1960: A theoretical rumen fermentation balance. J. Dairy Sci. 43, 1452 – 1459.

WOOD, G.A.R.; LASS, R.A., 1985: Cocoa. 4th ed. Longman. New York

YANG, W.Z.; BEAUCHEMIN, K.A.; VEDRES, D.D.; GHORBANI, G.R.; COLOMBATTO, D.; MORGAVI, D.P., 2004: Effects of direct-fed microbial supplementation on ruminal acidosis, digestibility, and bacterial protein synthesis in continuous culture. Anim. Feed Sci. Technol. 144, 179 – 193.

YOUNG, A.M., 1994: The Chocolate Tree. Smithsonian Institution Press, Washington and London .

11. Appendices

Appendix 1.1.: Contents of theobromine and gas production in cocoa pods (Experiment 1)

Treatments	Theobromine			Gas		
	n	Average (%)	Standard deviation (%)	n	Average (ml/200mg DM)	Standard deviation (ml/200 mg DM)
F	3	0.0373	0.0090	3	9.41^{b}	0.03
U0	3	0.0349	0.0030	3	11.51^{c}	0.83
U1	3	0.0321	0.0047	3	12.07^{c}	0.61
U2	3	0.0366	0.0110	3	12.99^{c}	0.19
U3	3	0.0348	0.0085	3	4.68^{a}	1.21

Appendix 2.1.: The mean pH, NH_4^+ concentration and protozoa counts during Rusitec (Experiment 2)

Day	pH		NH_4		Protozoal counts	
	Average	STD	Average	STD	Average	STD
1	6.64	0.10	7.16	1.45	50902	10717
2	6.59	0.07	8.12	1.38		
3	6.55	0.06	7.79	0.98	29898	19265
4	6.57	0.08	7.08	0.89		
5	6.57	0.06	6.63	1.25	18583	4003
6	6.56	0.06	6.98	1.18	9956	3874
7	6.57	0.06	6.39	1.40	11054	4855
8	6.58	0.06	6.71	1.16	8893	1957
9	6.58	0.06	6.61	1.32	8370	3119
10	6.56	0.06	6.99	1.07	5656	3538
11	6.57	0.07	6.69	1.06	6602	2536
12	6.56	0.07	6.50	1.02	6271	2091
13	6.55	0.07	7.11	1.41	5533	2061
14	6.53	0.07	6.86	1.01	4677	1569

Appendix 2.2. Chemical composition of buffer (McDougall, 1948)

37.2	g	$NaHCO_3$
31.0	g	$Na_2HPO_4.12H_2O$
33	ml	Solution II
1	ml	Cobalt acetate

---------- filled up to 5000 ml with aqua bidest.

Solution II

47.0	g	NaCl
57.0	g	KCl
5.3	g	$CaCl_2.2H_2O$
12.8	g	$MgCl.6H_2O$

---------- filled up to 1000 with aqua bidest.

Curriculum Vitae

Name	: Despal
Address	: Bukit Cimanggu Vila Blok W5 No. 19. Jl. Raya Baru, Bogor – Indonesia. Email: despal@hotmail.com
	: Jurusan Ilmu Nutrisi dan Makanan Ternak Fakultas Peternakan – IPB Jl. Rasamala, Dramaga – Bogor – Indonesia
Date of Birth	: 17 December 1970
Place of Birth	: Pariaman
Nationality	: Indonesian
Marital Status	: Married with 2 children

Education

1983	: Elementary School, Sibolga, North Sumatera
1986	: Junior High School, Sibolga, North Sumatera
1989	: Senior High School, Sibolga, North Sumatera
1993	: Sarjana 1 in Animal Production Science, Bogor Agricultural University, Indonesia
1999	: M.Sc. Agr. in Animal Nutrition Science, Georg-August University, Göttingen, Germany
2001 – up to present	: Registered as Ph.D. student at Institute of Animal Physiology and Animal Nutrition, Georg-August University, Göttingen, Germany
Present Occupation	: Lecturer for Dept. Animal Nutrition, Bogor Agricultural University (IPB), Indonesia