Preparation of fresh forages for incubation and prediction of nutritive value.

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ABSTRACT

In vitro and *in sacco* digestion offers a rapid and inexpensive method for evaluating nutritive value of ruminant feedstuffs. This study compared the conventional freeze dried and ground (FD) preparation with chopping to 6-mm lengths (C) and mincing (M) to represent chewed forage. *In sacco* and *in vitro* comparisons were made with perennial ryegrass (RG), white clover (WC) and *Lotus corniculatus* (LC). The mean proportions of large (>1.0mm) and soluble dry matter (DM) for the three forages were: M, 0.38 and 0.44; C, 0.76 and 0.21; FD, 0.35 and 0.34 respectively. Disappearance of DM and crude protein (CP) during the first 12 hours of rumen incubation was substantially lower for chopped forages. Minimal lag times before the onset of protein degradation were observed following mincing. Yields of ammonia from proteolysis in *in vitro* incubations were lowest for C and usually highest for M preparations. Preparation also affected yield but not proportions of VFA. These data show that ranking of forages for digestibility is affected by preparation and mincing appears to be a more suitable preparation for assays than chopping and freeze drying and grinding.

Keywords: forage preparation; particle size; *in sacco; in vitro.*

INTRODUCTION

Nutritive value is best determined by feeding animals and measuring performance relative to dietary intake. However, this procedure is costly, time consuming and is not appropriate for poor quality forages which may be fed in conjunction with other components (e.g., maize silage with pasture) or for dietary constituents (stem, leaf etc). Alternative procedures include *in sacco* and *in vitro* incubations, in which material is placed either in porous bags in the rumen of an animal prepared with a rumen fistula, or incubated with buffered rumen inoculum in the laboratory.

In vitro and *in sacco* procedures are well established and are usually carried out on forage prepared by freeze drying and grinding through a 1-mm screen. This may be appropriate for grains and high-dry-matter material, but is not necessarily suitable for ruminants grazing fresh pasture, as is normal in New Zealand.

Several studies have used fresh chopped plant material for incubations either *in sacco* (Van Vuuren *et al.*, 1991; Kolver *et al.*, 1998) or *in vitro* (Waghorn and Caradus 1994, Goplen *et al.*, 1992). An initial comparison between freeze dried and ground v. fresh minced *Lotus pedunculatus* (McNabb *et al.*, 1996) showed that mincing always resulted in a greater ($P<0.01$) dry matter loss than that of freeze drying and grinding, and that mincing allowed a greater loss of protein-bound amino acids from the bags than freeze drying and grinding. These differences, and the use of chopping in some research with fresh forages, suggested a need to properly evaluate contrasting methods of forage preparation prior to comparative investigations of forage digestion kinetics (Burke *et al.*, 2000). This paper reports the results of comparisons with leafy material from three contrasting forages in terms of dry matter disappearance from in sacco incubations and production of ammonia and volatile fatty acids from in vitro incubations. Data are also presented to indicate the extent of ammonia incorporation into bacteria *in vitro.*

MATERIALS AND METHODS

The research presented here describes the effects of forage preparation on digestion characteristics of contrasting fresh forages. Comparisons were made between minced (M), chopped (C) and freeze dried and ground (FD) preparations with white clover (*Trifolium repens*; WC), perennial ryegrass (*Lolium perenne*; RG) and *Lotus corniculatus* (LC). Measurements were also made of bacterial growth during incubations with the chopped preparation of the three forages. The forages were harvested in April 1999 from Grasslands Aorangi Research Station, Manawatu, New Zealand, and bulked material was stored at -16^0C for incubations. One Friesian cow, fed good quality lucerne hay and fitted with a permanent rumen fistula, was used for *in sacco* incubations and as a source of donor inoculum for *in vitro* incubations. Freeze dried and ground lucerne standards were included in both *in sacco* and *in vitro* incubations of the forages enabling correction for between-run variation.

Each feed was prepared by three methods: chopping with a knife to 6-mm lengths (C), minced to a particle size resembling forage chewed by sheep using a Kenwood chef household electric mincer fitted with a screen plate with 12-mm holes (M), and by freeze drying and grinding through a 1-mm sieve (FD). The nine sample preparations were used for both *in vitro* and *in sacco* incubations as follows:

In vitro incubations

In vitro incubations with chopped and minced preparations required about 2.5g/bottle fresh (wet) weight and 0.5 g of freeze dried and ground material to be weighed into 50ml vented sealed bottles. Each preparation was prepared with four replicates at each of the 4 incubation times (0, 2, 8 and 24 hours) so that each feed comprised 16 bottles of each of M, C and FD preparations. Incubations were undertaken on three days over a six-day period. On the day of incubation, the bottles containing forage substrate were warmed to 39^0C in an incubator, 12ml of McDougalls

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buffer (saturated with carbon dioxide) was added with 0.5ml cysteine sulphide reducing agent and 3ml rumen liquor (strained through cheesecloth). Incubations were maintained under anaerobic conditions and mixed continuously (90 oscillations/minute).

Four bottles of each forage preparation were removed from the incubator at 0, 2, 8 and 24 hours. The pH was measured and aliquots of supernatant were collected for determination of ammonia and VFA.

In sacco incubations

In sacco incubations required about 30g fresh (wet) M and C material, and 5.5g FD material to be weighed into dacron bags (10 x 10cm) with 35mm pore size. These were sealed and duplicate samples of each of the nine forage preparations were prepared for incubation for 0, 2, 4, 8, 12 and 24-hour intervals. After removal from the rumen, bags were washed under cold water until no further colour appeared and dried at 60^0C for 24 hours. Dried bags were weighed and ground to a fine powder for analysis by near infrared reflectance spectroscopy (NIRS).

A separate series of incubations were undertaken with WC, RG and LC chopped to 6-mm lengths to determine bacterial growth and N content. These data were used to define the gross release of plant N in vitro as that entering microbial biomass relative to N accumulation as dissolved ammonia. Ammonia and VFA analyses have been described by Burke *et al.* (2000).

Dry matter and protein disappearance from *in sacco* incubations were plotted and digestion characteristics defined by fitting non-linear models using the Marquardt procedure (Burke *et al.*, 2000). Particle size distribution of forages was determined by wet sieving (Waghorn 1986).

The significance of differences among treatment means was assessed by analysis of variance using GLM (general linear models) procedures (SAS, version 6).

RESULTS

Forage composition (Table 1) shows the forages had high concentrations of crude protein, and although ryegrass had a substantial neutral detergent fibre (NDF) concentration in the DM, all forages were green, leafy and lush when harvested. As expected, preparation had a major impact on the particle size distribution of the forages (Table 2). Mincing resulted in the largest amount of soluble DM released, while the particles were reasonably evenly distributed over the five different sieves. In contrast, chopping yielded 10-27% soluble DM, with about 50% of particle DM being larger than 4mm in length. Freeze drying and grinding the forages resulted in very few particles being greater than 4mm and 28-39% of particles were larger than 1mm. RG had less soluble DM than WC or LC for all preparations.

The loss of DM from *in sacco* bags is illustrated in Figure 1 and Table 3 and crude protein (CP) losses in Table 3. Preparation method had major effects on DM loss, with chopped material having a smaller ($P<0.05$) initial loss of soluble DM and CP ('A' values) but a greater ($P<0.05$) amount of insoluble degradable DM and CP ('B' values). This resulted in distinctly separate disappearance curves, particularly for RG, with M and FD showing a greater disappearance of DM and CP during the first 12 hours. Differences in fractional disappearance rates (k) between preparation methods were not significantly different, but CP disappearance rate was greater ($P<0.05$) for WC than RG or LC. Substantial lag times (2.5-3.5 hours) for the disappearance of DM and CP were observed with freeze dried and ground RG and LC, and for CP with chopped RG (Table 3). No lag times occurred with WC.

In vitro incubations enabled ammonia production to be determined (Table 4). WC exhibited a linear yield of ammonia over 24 hours, with minced material yielding the highest rate of production. Mincing also resulted in the most rapid ammonia yield with LC, but for RG, FD exhibited the most rapid production. At 2 hours, there were significant differences ($P<0.05$) between all preparation methods for RG, and between M and C for WC and LC. At 8 hours, most preparations affected ($P<0.05$) the yield of ammonia within forages and these differences remained at 24 hours, except for LC, where C and FD yielded similar quantities of ammonia (Table 4).

Over 24-hours of incubation, up to 49% of plant N (minced WC) was released as ammonia, but only 19-28% of N from the tannin-containing LC was degraded to ammonia (Table 4).

The microbial growth during *in vitro* incubations of chopped forages resulted in an accumulation of 6.2-10.7% plant N into microbial N after 24 hours (Table 6). Initial incorporation of N (indicating microbial growth) was similar for all forages, but after 24 hours, N incorporation was greater with WC than RG or LC. By 24 hours, the proportion of ammonia released from plant N incorporated into bacteria was 22% for WC, 18% for RG and 29% for LC.

Mean VFA yields from *in vitro* incubations (mM/g plant DM) after 24h average 4.3 (C), 5.6 (M) and 5.4 (FD) and 5.5, 5.3 and 4.5 for WC, RG and LC respectively. Although C resulted in a lower yield than either M or FD for each forage, the yield of VFA from M v. FD varied for the three forages. Differences in rates of VFA production were evident by 8 hours of incubation, after which the rates were similar for all preparations except for slower production from freeze dried and ground LC (Figure 4). The ratio of acetate:propionate indicates the glycogenic potential of the VFA mixture and this ratio was not affected by the type of preparation.

TABLE 1: Composition (% DM) of fresh white clover (WC), ryegrass (RG) and *Lotus corniculatus* (LC) determined by NIRS analysis.

	DM	CP	Lipid	Ash	ADF	NDF	CHO	Tannin	OMD	ME
WC	17.1	29.8	4.5	12.4	20.1	26.6	10.2	0.0	82.1	12.2
RG	17.9	22.5	4.6	11.1	22.9	48.3	8.1	0.0	79.1	11.8
LC	18.1	20.9	4.2	7.6	20.7	27.6	12.3	2.3	73.0	10.9

CP: Crude protein; ADF: Acid Detergent Fibre; NDF Neutral Detergent Fibre; CHO: Carbohydrate; OMD: Organic Matter Digestibility; ME: Metabolisable Energy (MJ/ kg DM)

TABLE 2: Particle size distribution (% distribution in dry matter) of white clover (WC), ryegrass (RG) and *Lotus corniculatus* (LC) prepared by mincing (M), chopping (C) and freeze drying and grinding (FD).

Sieve size	>4mm	1-4mm	<1mm	Solubles
M				
WC	13.7	19.8	14.2	52.3
RG	15.6	31.1	26.2	36.1
LC	14.8	20.5	22.8	42.0
C				
WC	52.7	17.9	4.1	25.4
RG	49.1	38.7	2.2	10.1
LC	48.2	21.0	3.6	27.2
FD				
WC	0.2	34.4	28.7	36.6
RG	4.2	34.7	31.6	29.6
LC	0.3	27.3	35.9	36.4

Solubles: Fraction of plant DM that was not accounted for on sieves or in residues

TABLE 3: Forage dry matter (DM) and crude protein (CP) degradation characteristics derived from *in sacco* incubations as defined by soluble (A) and degradable insoluble (B) pools, fractional disappearance rate (k: h^{-1}), and lag time (h) for white clover (WC), ryegrass (RG) and *Lotus corniculatus* (LC) prepared by mincing (M), chopping (C) and freeze drying and grinding (FD).

Forage	Preparation	A	B	k	Lag
Dry Matter					
WC	M	42	54	0.13	0.0
	C	20	76	0.12	0.0
	FD	36	55	0.18	0.0
RG	M	37	49	0.11	0.0
	C	9	81	0.07	0.0
	FD	37	53	0.11	3.4
LC	M	49	47	0.07	0.2
	C	14	69	0.11	0.0
	FD	38	49	0.19	3.5
Crude Protein					
WC	M	39	59	0.15	0.0
	C	9	90	0.12	0.0
	FD	35	63	0.15	0.0
RG	M	31	67	0.11	0.0
	C	1	97	0.11	2.5
	FD	39	59	0.11	2.5
LC	M	49	47	0.07	0.0
	C	8	90	0.08	0.0
	FD	37	61	0.10	3.6

TABLE 4: Net ammonia production (Mean±SEM) from in vitro incubation of white clover (WC), ryegrass (RG) and *Lotus corniculatus* (LC) (mM NH_3/ mM plant N) prepared by mincing (M), chopping (C) and freeze drying and grinding (FD).

		Incubation time (hours)		
Forage	Preparation	2	8	24
WC	M	37±1.6[a]	166±5.2[a]	494±13.4[a]
	C	27±1.6[b]	132±6.0[b]	375±15.5[b]
	FD	32±1.9[ab]	142±5.2[b]	427±13.4[c]
RG	M	63±1.6[a]	165±5.2[a]	365±13.4[a]
	C	52±1.6[b]	153±5.2[a]	290±13.4[b]
	FD	41±1.9[c]	220±5.2[b]	470±13.4[c]
LC	M	42±1.6[a]	153±5.2[a]	281±13.4[a]
	C	37±1.6[b]	51±6.0[b]	212±13.4[b]
	FD	42±1.6[ab]	103±5.2[c]	183±13.4[b]
		Main Effects		
WC		32±1.0[a]	147±3.2[a]	432±8.2[a]
RG		52±1.0[b]	179±3.0[b]	375±7.7[b]
LC		40±1.0[c]	102±3.2[c]	225±7.7[c]
	M	48±1.0[a]	161±3.0[a]	380±7.7[a]
	C	39±1.0[b]	112±3.3[b]	292±8.2[b]
	FD	38±1.1[b]	155±3.0[a]	360±7.7[a]

[abc] For each parameter within main effects, means within columns having superscript letters in common are not significantly different (P > 0.05).

FIGURE 1: Dry matter (DM) loss during *in sacco* incubations of white clover, Ryegrass. and *Lotus corniculatus* prepared by mincing (M), chopping (C), and freeze drying and grinding (FD).

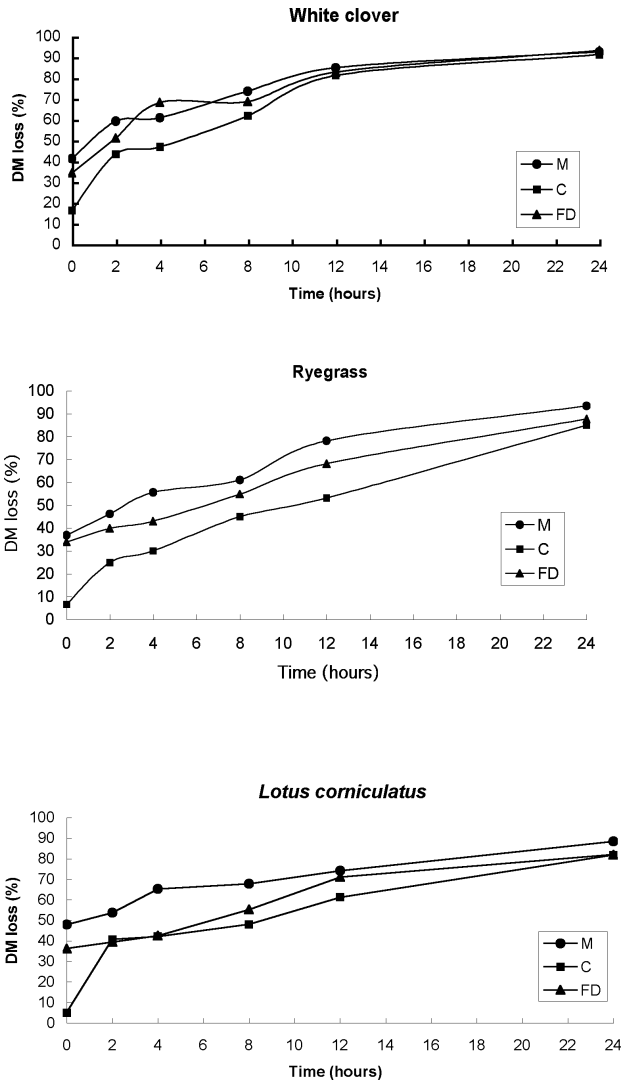

TABLE 5: Net accumulation of nitrogen in microbial biomass (mM N/ mM plant N) during incubation of chopped white clover (WC), ryegrass (RG) and *Lotus corniculatus* (LC), over time.

	Incubation time (hours)		
Forage	2	8	24
WC	27.7	69.3	107.1
RG	28.2	61.1	62.6
LC	28.6	69.2	88.3

DISCUSSION

The most important finding from this study was that the ranking of the three forages differed with each preparation (in terms of digestion losses or ammonia and VFA production). This means that a comparison between forages in both digestibility and nutritive value for ruminants will be affected by the way the forage is prepared for *in sacco* and *in vitro* digestion. It is, therefore, crucial that an appropriate preparation be conducted prior to attempts at either ranking or predicting the nutritive value of forages.

The most common procedure for sample preparation, freeze drying and grinding, does not replicate chewing of fresh or ensiled forage. The extent of particle size reduction is dependent upon forage type and the amount of chewing during eating and rumination (Ulyatt *et al*., 1986). About 60% of cell contents are released during eating (Waghorn *et al*., 1989) but particulate material in the rumen (replicated by *in vitro* and *in sacco* incubations) may have a smaller size than that in boli swallowed during eating because of continued breakdown though rumination. Typical particle size distribution of DM in boli swallowed by sheep eating fresh forages comprise 49-51% larger than 1mm and 32-38% soluble (Ulyatt *et al*., 1986), whilst rumen contents of cows fed forages comprise 42-47% over 1mm and 17-22% soluble DM (Waghorn *et al.*, 1989). The particle size distribution achieved by M (Table 2) conformed to these size ranges more closely than C or FD material. We believe that the minced preparation is more appropriate than either freeze dried and ground or chopped methods for *in vitro* and *in sacco* incubations because it mimics more closely the events occurring in the rumen.

The chopped preparation was evaluated because it had been used previously ((Van Vuuren *et al*., 1991; Kolver *et al*., 1998; Goplen *et al*., 1992). However, differences between forages appeared to be exaggerated when C preparations were used relative to M and FD. This is particularly evident with the fractional rate of DM and CP disappearance for RG and the net ammonia yield with LC. Forages with tough or waxy cuticles will have slower microbial degradation with C than with either M or FD because microbes will access the plant substrate mainly via the chopped surfaces (Wilson, 1991).

The *in sacco* incubation provided a clear contrast between rates of DM and CP loss for each feed preparation and indicates the significance of the lag occurring with some RG and LC preparations. When curves were fitted to the data, there was minimal lag response with M, and none with WC. We are not discounting the possibility of a lag period with *in vivo* digestion, but periods of 2-3 hours during which time microbial degradation is insignificant, or unlikely. The absence of a lag with minced preparations supports our preference for mincing.

Data from *in vitro* incubations complement that from *in sacco* incubations, and the effect of condensed tannins in LC became apparent by reducing the yield of ammonia, although they did not appear to inhibit bacterial growth. C also appeared to reduce proteolysis and VFA production relative to M and FD.

In summary, freeze drying and grinding or chopping into short lengths for *in sacco* and *in vitro* incubations are not appropriate preparations for fresh forages. Mincing provides a particle size distribution similar to that of chewed forage and avoided lag periods, which were associated with other preparations of the same forages. The ranking of forges in terms of both *in sacco* and *in vitro* characteristics was altered by the form of preparation and we believe that mincing provides the most appropriate preparation for screening.

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