# Effect of formalin preservation on the fermentation characteristics, chemical composition and protein properties of maize silage

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The effect of maturity stage, expressed in terms of moisture content (54,5  $\pm$  3,2%; 66,0  $\pm$  5,2%; and 76,3  $\pm$  1,8%), and the effect of different rates of formalin treatment (0; 2,57  $\pm$  0,64; 5,10  $\pm$  0,89; and 7,59  $\pm$  1,46 g formaldehyde/100 g crude protein) on the fermentation characteristics, in vitro dry matter digestibility, chemical composition and protein properties of maize silage were investigated. Formalin treatments were performed with and without the addition of 1 l formic acid per ton wet material ensiled. Results in general indicate that fermentation characteristics (pH, lactic acid, total volatile fatty acid, acetic acid and butyric acid content), chemical composition (crude protein, acid-detergent fibre and water soluble carbohydrates), and in vitro dry matter digestibility were improved as the moisture content at ensiling was decreased. Formalin treatment up to a rate of 5,10 ± 0,89% led to a deterioration in fermentation characteristics and chemical composition, with a concomitant decrease in in vitro dry matter digestibility. Treatment at a rate of 7,59  $\pm$ 1,46% on the other hand, improved fermentation characteristics with no deterioration in chemical composition. Formic acid exerted no additional effect on the fermentation characteristics and chemical composition of formalin-treated silages. Protein properties, viz. true protein content and in vitro ammonia nitrogen released, were favoured while percentage acid-detergent insoluble nitrogen and the ratio of lysine to total essential amino acids were deleteriously affected by increasing rates of formalin treatment. The results in this investigation suggest an increased feeding value of maize silage ensiled with formalin at a rate of 7,59 ± 1,46%. However, provision should be made for possible harmful effects of formaldehyde on protein availability, rumen micro-organisms and the ratio of lysine to total essential amino

Die invloed van stadium van inkuiling, uitgedruk in terme van die voginhoud (54,5  $\pm$  3,2%; 66,0  $\pm$  5,2%; en 76,3  $\pm$ 1,8%), en die invloed van verskillende peile van formalientoediening (0; 2,57  $\pm$  0,64; 5,10  $\pm$  0,89; en 7,59  $\pm$  1,46 g formaldehied/100 g ruproteïen) op die fermentasie-eienskappe, droëmateriaal-in vitro-verteerbaarheid, chemiese samestelling en proteïeneienskappe van mieliekuilvoer, is ondersoek. Formalienbehandelings het met en sonder die toediening van 1 l mieresuur per ton nat materiaal geskied. Die resultate in die algemeen toon dat die fermentasieeienskappe (pH, melksuur-, totale vlugtige vetsuur-, asynsuur- en bottersuurinhoud), die chemiese samestelling (ruproteïen, suuronoplosbare vesel en wateroplosbare koolhidrate) sowel as die droëmateriaal-in vitro-verteerbaarheid van die kuilvoer verbeter het namate die voginhoud by inkuiling afgeneem het. Formalienbehandeling tot en met 'n toedieningspeil van 5,10 ± 0,89% het ongunstige fermentasie-eienskappe en chemiese samestelling meegebring, gepaardgaande met 'n verlaagde droëmateriaal-in vitro-verteerbaarheid. 'n Behandelingspeil van 7,59 ± 1,46% het egter verbeterde fermentasie-eienskappe met geen agteruitgang in chemiese samestelling nie, tot gevolg gehad. Mieresuur het geen addisionele effek op die fermentasie-eienskappe en chemiese samestelling van formalien-behandelde kuilvoer gehad nie. Die proteïeneienskappe, naamlik die ware proteïeninhoud en die in vitro-vrystelling van ammoniakstikstof, is begunstig terwyl die persentasie stikstof in die suuronoplosbare veselfraksie en die verhouding van lisien tot totale essensiële aminosure ongunstig verander het met toenemende peile van formalienbehandeling. Die resultate van hierdie ondersoek dui op 'n verhoging van die voedingswaarde wanneer mielieplantmateriaal met 7,59 ± 1,46% formalien ingekuil word. Voorsorg sal egter getref moet word teen moontlik nadelige effekte van formaldehied ten opsigte van proteïenbeskikbaarheid, rumenmikro-organismes en die verhouding van lisien tot totale essensiële aminosure.

**Keywords:** Amino acid composition, chemical composition, fermentation, formaldehyde, maize silage, moisture content, protein degradation.

Results from the literature suggest that the optimum stage for ensiling maize plants coincides with the maturity stage containing a moisture content of 60—70%. Ensiling at this stage not only yields the highest DM content (Huber, Graf & Engel, 1965; Huber, Thomas & Emery, 1968; Johnson, McClure, Johnson, Klosterman & Triplett, 1966; Weaver, Coppock, Lake & Everett, 1978), but also silage of a high quality (Buchanan–Smith, 1982; Johnson, Balwani, Johnson, McClure & Dehority, 1966; Weaver et al., 1978). Under practical farming circumstances, however, it is not always possible to ensile all material within these moisture limits. Trials with grass- and clover-dominant pastures (Barry &

Fennessy, 1972; Barry, Fennessy & Duncan, 1973; Waldo, 1975) indicate that high-quality silage may be obtained when low dry-matter material is treated with a formic acid/formalin mixture. The effect of formaldehyde on protein utilization (Siddons, Evans & Beever, 1979) makes an important contribution to this concept. Except for research being done by Kaiser, Terry & Dhanoa (1981), Kaiser, Osbourn, England & Dhanoa (1982) and Wilkinson, Huber & Henderson (1975), data on maize plant material ensiled with formalin are scarce. The purpose of this investigation, therefore, was to investigate the effect of formalin treatment,

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with and without the addition of formic acid, on the fermentation characteristics, chemical composition, *in vitro* dry matter digestibility (IVDMD) and some protein properties of maize silage.

#### **Materials and Methods**

The effects of moisture content, rate of formalin application and formic acid treatment on the ensilage of maize plants, grown on two differently situated lands, were investigated using a  $4\times3\times2$  factorial design. Plant material from each land was collected at three different stages of maturity, viz. with moisture contents of  $54.5\pm3.2\%$ ,  $66.0\pm5.2\%$ , and  $76.3\pm1.8\%$ . Plant material at each of these maturity stages was ensiled with formalin at four different rates of application, viz. 0;  $2.57\pm0.64$ ;  $5.10\pm0.89$  and  $7.59\pm1.46$  g formaldehyde/100 g crude protein (CP). Each rate of application was performed with and without the addition of 1 l formic acid per ton fresh material.

Plant material (50 kg) was chopped into 1-cm pieces with a forage harvester, followed by spraying with the specific chemical (formalin and/or formic acid), using a 1 l pressure sprayer. The chemicals were mixed with the plant material on a concrete floor by hand, using forks, and ensiled into 100 l drums. Room temperature during these procedures was ca. 30 °C. Some chemicals would have been lost under these circumstances. After being thoroughly compacted, the exposed top surface was sealed off by means of a plastic sheet covered with a 15-cm layer of sand.

After an ensiling period of one year a representative sample of approximately 1 kg was taken from each drum. This was obtained as mixed material which was bored out from the top to the bottom of the container, using a cylindrical steel tube (10 cm diameter, 15 cm long). The top 10 cm of the silage sample was removed and the rest retained for analysis. Part (500 g) of the sample was freeze-dried for later analysis while an aliquot was extracted for the determination of

fermentation characteristics. Dry-matter content of the silage was determined by drying a portion of the sample at 105°C for 48 h. A portion (80 g) of the silage was extracted with 320 ml of distilled water for 6 h, preserved by 2 ml saturated HgCl<sub>2</sub>. The following chemical analyses were performed on the silage extract which was filtered through two layers of cheesecloth and centrifuged at 3000 r.p.m.: pH, lactic acid (Hochella & Weinhouse, 1965), total volatile fatty acids (TVFA) (Fenner & Elliot, 1963) and acetic and butyric acids (Clancy, Wangsness & Baumgardt, 1977). Half of the steam distillate (125 ml) resulting from the determination of TVFA, was used for gas chromatographic determination of acetic and butyric acids. This portion of the distillate was adjusted to a pH of 10 with 1N-NaOH, evaporated to dryness on a steam bath, and stored. Immediately prior to analysis, the free fatty acids were liberated by dissolving the dried residue in 5 ml of a 5% phosphoric acid solution. Freeze-dried material was analysed for CP (Clare & Stevenson, 1964), true protein (Marais & Evenwell, 1983), acid-detergent fibre (ADF) (Van Soest, 1963), water soluble carbohydrates (WSC) (Shannon, 1972), IVDMD (Tilley & Terry, 1963, as adapted by Engels & Van der Merwe, 1967), in vitro ammonia release during incubation with rumen fluid (Broderick, 1978), acid-detergent insoluble nitrogen (ADF-N) (Goering, Gordon, Hemken, Waldo, Van Soest & Smith, 1972) and amino acids (Technicon technical publication, 1971). Free formaldehyde was determined on the silage extract (Chrastil & Wilson, 1975). The results were statistically analysed by means of a three-factor factorial analysis.

## **Results and Discussion**

Effect of maturity stage (moisture content)

The effect of maturity stage, as measured by the moisture content at ensiling, on the fermentation characteristics and chemical composition of maize silage, is given in Table 1. In general, silage ensiled at a low-moisture content (54,5  $\pm$ 

**Table 1** Effect of maturity stage (moisture content) on the fermentation characteristics, chemical composition and IVDMD of maize silage

		Moistur	Least significant difference			
Silage property		54,5 ± 3,2	66,0 ± 5,2	76,3 ± 1,8	P < 0.05	P < 0,01
Fermentation characteristic	cs:					
pН		4,53*	4,34 <sup>b</sup>	4,08°	0,08	0,11
Lactic acid	(g/100 g DM)	5,22*	6,11 <sup>a</sup>	6,42*	1,48	1,97
Total volatile fatty acids						
•	(g/100 g DM)	1,37*	2,40 <sup>b</sup>	4,77°	0,70	0,93
Acetic acid	(g/100 g DM)	0,81*	0,92*	3,58 <sup>b</sup>	1,09	1,45
Butyric acid	(g/100 g DM)	0,39*	0,57 a,b	0,92 <sup>b</sup>	0,40	0,53
Chemical composition	(g/100 g DM):					
Water soluble carbohydr	ates	14,01	13,47*	12,20°	2,00	2,67
ADF		28,22ª	29,95 <sup>b</sup>	37,75°	1,57	2,09
Crude protein		9,30*	10,73 <sup>b</sup>	9,45*	0,39	0,52
True protein		6,41	5,94 <sup>b</sup>	5,65 <sup>b</sup>	0,37	0,50
IVDMD	(g/100 g DM)	67,43ª	66,07*	64,12 <sup>b</sup>	1,63	2,17

a-c Values with common superscripts do not differ significantly from each other.

3,2%) exhibited depressed fermentation characteristics when compared to material ensiled at the recommended moisture content ( $66 \pm 5,2\%$ ).

A significant (P < 0.01) increase in the pH value accompanied by a significant (P < 0.01) decrease in TVFA were seen. This was accompanied by a tendency towards a decrease in the contents of lactic, butyric and acetic acids. Except for a small decrease in CP (P < 0.01) and ADF (P < 0.05), and a concomitant increase (P < 0.05) in the true protein content of the low-moisture silage, no other significant changes in chemical composition took place. The pattern, in general, suggests that low-moisture silage displays a more favourable chemical composition and IVDMD than silage made at the recommended stage.

When silage ensiled at a high-moisture content (76,3  $\pm$ 1,8%) was compared to silage ensiled at the recommended stage, fermentation activity increased. The pH significantly (P < 0.01) decreased to a value of 4.08 while TVFA and acetic acid contents significantly (P < 0.01) increased. The tendency towards a simultaneous increase in butyric acid was supported by the significant (P < 0.01) difference in butyric acid content between low- and high-moisture silage. Lactic acid content remained the same, indicating that the increase in overall fermentation activity was mainly due to an increase in an undesirable type of fermentation. Maize silage ensiled with a high initial moisture content also produced somewhat inferior results with respect to its chemical composition and IVDMD. The ADF content was significantly (P < 0.01) higher than that of silage ensiled at the recommended stage of maturity (37,75 vs. 29,95%), while IVDMD was significantly (P < 0.05) lower by only 1.95 percentage units, indicating a high degree of fibre digestibility at this stage of maturity. The high-moisture silage was also found to have a lower CP concentration (P < 0.01).

The overall data in Table 1 indicate that the effect of maturity stage on chemical composition and IVDMD was relatively small and did not appear to influence feeding value to any great extent. Its effect on fermentation characteristics, however, does suggest some effect on feeding value. It is known that an increase in fermentation activity as well as a shift to undesirable fermentation will suppress DM intake (Wilkinson, Wilson & Barry, 1976). This may have serious consequences on the feeding value of maize ensiled at a high-moisture maturity stage. The intake of maize silage ensiled at a low-moisture content, on the other hand, will be favoured by the lower degree of fermentation. Ensiling at this stage, however, is normally avoided due to higher losses in the field during harvesting (Weaver et al., 1978; Van der Merwe, 1981), as well as improper compaction. This results in increased aeration which leads to a decline in feeding value (Huber et al., 1968; Weaver et al., 1978). With bunkers currently replacing traditional tower silos, effective compaction can be accomplished, thereby leading to the ensilage of maize plants with a lower moisture content. The predicted higher feeding value and lower handling costs may compensate for higher field losses.

## Effect of formalin treatment

The effect of increasing the rate of formalin treatment on the fermentation characteristics, chemical composition, IVDMD

and protein properties of maize silage, is given in Table 2. The data indicate that increasing rates of application, up to a value of  $5,10 \pm 0,89\%$ , resulted in a decrease in total fermentation accompanied by a shift towards undesirable fermentation. The pH increased significantly (P < 0.01) to a value of 4,35. This took place concomitantly with a significant (P < 0.01) decrease in the lactic acid content. At the same time, TVFA content increased significantly (P < 0.05)from 3,27 to 4,20%, including a non-significant increase in the contents of acetic and butyric acids. Except for a small, but significant (P < 0.05) increase in CP, chemical composition reflected the undesirable change in fermentation pattern. Formalin (5,10  $\pm$  0,89%) caused a negative change in chemical composition and IVDMD. The WSC content decreased significantly (P < 0.05), while the ADF content increased significantly (P < 0.01) from 31.8 to 36.7%. IVDMD decreased (P < 0.01) by 2,7 percentage units.

Except for a significant (P < 0.01) increase in pH and a significant (P < 0.01) decrease in lactic acid content, an application rate of 2.57  $\pm$  0.64% formalin exerted no other significant changes on fermentation characteristics or chemical composition. The overall trend for individual parameters, however, coincided with that of the 5.10  $\pm$  0.89% treatment. Formalin application at a rate of 7.59  $\pm$  1.46%, however, depressed total fermentation without inducing undesirable fermentation. The pH increased (P < 0.01) to a value of 4.84 while the contents of lactic acid (P < 0.01), TVFA (P < 0.01), and acetic acid (P < 0.05) were significantly reduced. This resulted in silage with no significant change in chemical composition or IVDMD, except for a small decrease (P < 0.05) in CP content.

The data in Table 2 furthermore indicate that increasing rates of formalin treatment improved some protein properties. The true-protein content was significantly (P < 0.01)increased from 4,67 to 7,01%; a fraction representing 78,6% of the CP content. This was accompanied by a significant decrease in the amount of ammonia released during in vitro incubation, which suggests an increased stability against ruminal degradation (Broderick, 1978). Formalin treatment, on the other hand, also had a detrimental effect on some protein properties. Increasing rates of formalin application resulted in increased ADF-N, which suggests increased unavailability of bypass-protein (Goering et al., 1972). In Table 3, the composition of essential amino acids in maize silage is compared to that of fishmeal, as presented by Feedstuffs Ingredient Analysis Table (Allen, 1986). Increasing rates of formalin application significantly decreased the ratio of lysine to total essential amino acids. The ratios of arginine, threonine, isoleucine, leucine, and phenylalanine were significantly increased. In addition to these effects on protein properties, the data in Table 2 show that increasing rates of formalin application led to increasing levels of free formaldehyde. The increase became significant at the  $7,59\pm1,46\%$ rate of application. High levels of free formaldehyde may inactivate rumen micro-organisms, depressing the DM intake (Wilkinson et al., 1976).

The overall data in Tables 2 and 3 suggest that a  $7.59\pm1,46\%$  formalin treatment may benefit the utilization of maize silage. Although the chemical composition and

**Table 2** The effect of formalin treatment during ensiling on the fermentation characteristics, chemical composition, IVDMD and protein properties of maize silage

		Rate	of application (g	formaldehyde/10	00 g CP)	Least sig differ	
Silage property		0 ± 0	$2,57 \pm 0,64$	5,10 ± 0,89	7,59 ± 1,46	P < 0,05	P < 0,01
Fermentation characteristic	cs:						
pН		3,88ª	4,12 <sup>b</sup>	4,35°	4,84 <sup>d</sup>	0,10	0,13
Lactic acid	(g/100  g DM)	8,90ª	6,32 <sup>b</sup>	4,74 <sup>b,c</sup>	3,43°	1,77	2,39
Total volatile fatty acids							
	(g/100 g DM)	3,27*	3,64 a,b	4,20 <sup>b</sup>	1,50°	0,83	1,13
Acetic acid	(g/100 g DM)	2,26ª	2,48*	3,26*	0,53 <sup>b</sup>	1,30	1,76
Butyric acid	(g/100 g DM)	0,64	0,63*	0,934	0,53*	0,47	0,64
Chemical composition	(g/100 g DM):						
Water soluble carbohydra	ates	13,98**	12,16 <sup>a,b</sup>	11,33 <sup>b</sup>	15,07°	2,39	3,24
ADF		31,77**	33,32*	36,72 <sup>b</sup>	30,89°	1,88	2,54
Crude protein		9,49*	9,84 a,b	10,04 <sup>6</sup>	8,92°	0,46	0,63
IVDMD	(g/100 g DM)	66,74*	65,69ª,b	64,04 <sup>b</sup>	65,73 <sup>a,b</sup>	1,80	2,45
Protein properties:							
True protein	(g/100  g DM)	4,67*	5,64 <sup>b</sup>	6,70°	7,01°	0,43	0,56
In vitro release of ammor	nia						
nitrogen (g/100	g true protein/24 h)	2,834	1,91 <sup>b</sup>	1,31 b,c	0,98°	0,82	1,12
ADF-N	$(g/100 g N)^{1}$	8,2	12,8	17,3	21,9		
Free formaldehyde	(mg/kg)	26,50°	45,14*	114,29*	408,2 <sup>b</sup>	126	171

a-d Values with common superscripts do not differ significantly from each other.

**Table 3** Ratios of individual essential amino acids to total essential amino acids <sup>1</sup> of formalin-treated maize silage and of fishmeal

	(i	Rate of formaldehyde	Fishmeal	Least significant difference			
Amino acid	0	$2,47 \pm 0,56$	4,95 ± 1,13	7,43 ± 1,69	(white)	P < 0.05	P < 0,01
Lysine	0,093*	0,072 <sup>b</sup>	0,063 <sup>b</sup>	0,056 <sup>b</sup>	0,15	0,020	0,027
Histidine	0,197*	0,181 a.b	0,172 <sup>b,c</sup>	0,156°	0,07	0,020	0,027
Arginine	0,063*	0,068 a,b	0,077 <sup>b,c</sup>	0,081°	0,15	0,010	0,013
Threonine	0,078*	0,083 a,b	0,087 <sup>b</sup>	0,090 <sup>b</sup>	0,09	0,008	0,011
Valine .	0,131*	0,123*	0,134*	0,135*	0,11	0,014	0,019
Methionine	0,075*	0,079*	0,070*	0,074*	0,06	0,011	0,015
Isoleucine	0,098*	0,101 a,b	0,103 <sup>a,b</sup>	0,107 <sup>b</sup>	0,11	0,006	0,008
Leucine	0,173*	0,190 <sup>a,b</sup>	0,191 <sup>b</sup>	0,195 <sup>b</sup>	0,16	0,017	0,023
Phenylalanine	0,091	0,100 <sup>b</sup>	0,103 <sup>b</sup>	0,108 <sup>b</sup>	0,10	0,008	0,011

<sup>\*-</sup>c Values with common superscripts do not differ significantly from each other.

IVDMD were not improved, fermentation activity was strongly inhibited without inducing undesirable fermentation. This may stimulate dry-matter intake which seems to be one of the great obstacles in the utilization of high-moisture silage (Wilkinson et al., 1976). The data also suggest that formalin treatment at this rate may have the additional advantage of inhibiting protein breakdown during ensiling as

well as degradation of true-protein in the rumen. These effects of formalin may increase duodenal supply of amino acids, thereby increasing animal performance. High ratios of ADF-N measured at this rate  $(7,59 \pm 1,46\%)$  of treatment, however, suggested that protein availability was decreased, due to irreversible bonding with formaldehyde (Siddons et al., 1979). Furthermore, the already low ratio of lysine to

<sup>&</sup>lt;sup>1</sup> Values were calculated by means of the following linear regression, obtained from the data of this experiment: y = 0.082 + 0.019x (r = 0.89; P < 0.01).

<sup>&</sup>lt;sup>1</sup> Tryptophan is not included.

Table 4	Two-factor	interactions	with r	espect	to diffe	erent	treatments	on	the
fermentati	on characte	ristics and c	hemica	l compo	osition	of ma	aize silage		

	Silage treatment interactions						
	Formalin	Formic acid	Formic acid				
	vs	vs	vs				
Silage property	moisture content	formalin	moisture content				
Fermentation characteristics:							
pН	***	NS <sup>b</sup>	NS				
Lactic acid	NS	NS	NS				
Total volatile fatty acids	NS	NS	NS				
Acetic acid	NS	NS	NS				
Butyric acid	NS	NS	NS				
Chemical composition:							
Water soluble carbohydrates	NS	NS	NS				
Acid-detergent fibre	NS	NS	NS				
Crude protein	NS	NS	NS				
IVDMD	NS	NS	NS				
Free formaldehyde	NS	NS	NS				

 $<sup>^{\</sup>bullet} P < 0.01.$ 

total essential amino acids in maize silage was further reduced at this rate of formalin application. If this reflected a similar change in the amino acid composition of the digestible bypass-protein, the composition of essential amino acids would be deleteriously affected. In addition, the possibility of a toxic free-formaldehyde level at this rate of application must be taken into account. Wilkinson (1976) proposed an application rate of 3—5 g formaldehyde/100 g CP as a safe and effective working range for grasses while Tayler & Wilkins (1976) found a reduction in intake, but no change in live mass-gain when ryegrass silage, treated with 11 g formaldehyde/100 g CP was fed to calves. More *in vivo* research is needed to quantify the contribution of these effects.

Interactions between formalin treatment, formic acid treatment and moisture content

Two-factor interactions between formalin treatment, formic acid treatment and moisture content at the stage of ensiling are shown in Table 4. The data show a significant interaction (P < 0.01) between formalin treatment and moisture content with respect to pH, but not to any of the other parameters. Furthermore, no interactions were found between formic acid and formalin and formic acid and moisture content with respect to any of the parameters, which shows that addition of 1 l formic acid per ton wet material could not prevent the detrimental effect of high-moisture contents or low rates of formalin application. This seems to be in contrast to the findings of Kaiser et al. (1981), who found no clostridial activity in maize silage treated with relatively low levels of formalin (<60 g formaldehyde/kg CP) together with 2 l formic acid per ton fresh material. This, however, may suggest that formic acid should be added at levels higher

than 1 l per ton wet material to be effective against undesirable fermentation.

#### Conclusions

Results from this investigation suggest that the stage of maturity, as reflected by a moisture content of between  $54.5 \pm 3.2\%$  and  $76.3 \pm 1.8\%$ , influenced chemical composition and IVDMD of maize silage only to a small extent. Fermentation characteristics, however, were clearly favoured in the drier material, but deleteriously affected in material with an increasing moisture content. As a result, a higher DM intake and feeding value may be predicted for silage with a higher DM content. Ensiling with low and intermediate application rates of formalin (2,57  $\pm$  0,64% and 5,10  $\pm$ 0,89%) stimulated an undesirable type of fermentation with detrimental effects on chemical composition, and the addition of 1 l formic acid per ton wet material did not prevent this. Treatment with a relatively high rate of formalin (7.59 ± 1,46%), however, not only prevented this detrimental effect, but also inhibited extensive fermentation. This may lead to a higher intake and a higher feeding value of silage. Formalin treatment at a rate of 7,59 ± 1,46% increased protein quality by diminishing protein breakdown during ensiling and diminishing protein degradation by rumen organisms. Indications of a simultaneous decrease in protein availability and in the lysine:total essential amino acids ratio, and an increased level of free formaldehyde, however, might have off-set the beneficial effects of formalin treatment at this rate to some extent.

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<sup>&</sup>lt;sup>b</sup> Not significant.

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