Effect of level of formic acid and urea on microbial evaluations of common reed *Phragmitis communis* silage

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Abstract

This study was conducted in a laboratory to investigate the effect of addition of different levels of formic acid and urea on microbial populations of common reed silage. Reed plants were cut into 2-3 cm, treated at ensiling with 10% of debis and 3 levels of formic acid (FM), 0.5, 1 and 1.5% and urea (U), 0, 1 and 2%, packed in double nylon bags and kept for 60 days. Results showed that increasing level of FM from 0.5 to 1 and 1.5% decreased ($P<0.01$) number of anaerobic by 0.83 and 1.94, lactic acid bacteria by 0.93 and 1.95, molds by 0.77 and 1.02 $\log^{10}$ cfu/g of wet silage respectively. Results also showed that there was a decline in numbers of yeasts ($P<0.05$) and molds ($P<0.01$) with increasing level of addition of urea from 0 to 1 and 2%, numbers were 5.28, 4.87 and 4.46 for yeast and 6.27, 4.49 and 3.25 $\log^{10}$ cfu/g of wet silage respectively. Numbers of aerobic, anaerobic and lactic acid bacteria, yeast and molds in samples of reed samples were also affected by interaction effect between level of addition of FM and urea.

Key words/ common reed, silage, formic acid, urea, microbial population.

تأثير مستويات حامض الفورميك واليوريا في التقييم الميكروبي لسايلج القصب البري *Phragmitis communis*

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الخلاصة

اجريت الدراسة في المختبر للتحري عن تأثير اضافة مستويات مختلفة من حامض الفورميك واليوريا في المجتمعات الميكروبية لسايلج القصب البري. قطعت نباتات القصب الى 2-3 سم, وعوملت عند السيلجة بالدبس (10%) و ثلاث مستويات من اليوريا 0 و 1 و 2% وحامض الفورميك 0.5 و 1 و 1.5%. عبئت النماذج في اكياس نايلون مزدوجة وحفظت لمدة 60 يوم. اظهرت النتائج ان زيادة مستوى اضافة حامض الفورميك الى نماذج القصب عند السيلجة من 0.5 الى 1 و 1.5% أدت الى حصول انخفاض ($P<0.01$) في اعداد البكتريا اللاهوائية بمقدار 0.83 و 1.94, وبكتيريا حامض اللاكتيك بمقدار 0.93 و 1.95 والخمائر بمقدار 1.57 و 1.91 والأعفان بمقدار 0.77 و 1.02 لوغاريتم10 وحدة مكونة للمستعمرات/غم سايلج رطب على التوالي. واظهرت النتائج ايضا حصول انخفاض معنوي في اعداد الخمائر والأعفان ($P<0.01$) بزيادة مستوى اضافة اليوريا من 0 الى 1 و2%, اذ بلغ اعداد الخمائر 5.28 و4.87 و4.46 واعداد الأعفان 6.27 و4.49 و3.25 لوغارتيم10 وحدة مكونة للمستعمرات/غم سايلج رطب على التوالي. كما تأثرت اعداد لبكتيريا الهوائية واللاهوائية وبكتيريا حامض اللاكتيك والخمائر والأعفان في نماذج سايلج القصب البري بالتداخل بين مستوى اضافة حامض الفورميك واليوريا.

كلمات مفتاحية: قصب, سايلج, حامض الفورميك, يوريا, عشائر ميكروبية.

Introduction

In many areas human and animal diets were highly correlated due to limited arable lands, because any increase in lands allocated for planting green roughages will lead to shrink those may allocated for planting cereal crops. Therefore, it is very important to search for alternative source of crops to feed farm animals. Common reed *Phragmitis communis* is considered the most separated plants. Al-

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Sultany (6) concluded that good quality reed silage can be made by addition of urea and molasses.

Silage was defined as any plant materials undergone anaerobic fermentation. Filya (10) reported that ensiling was affected by many factors such as epiphytic microbial populations, harvest conditions and sugar content. Adesogan, et. al., (3) indicated that the major goal of silage making is to preserve silage material with minimum nutrient loss.

El-Talty, et. al., (9) considered ensiling as a continuous competition in absence of oxygen between microorganisms on substrates found in the ensiled materials and additives. During ensiling water soluble sugars (WSC) were used by microbial societies to produce lactic acid, the main factor responsible for preservation, plant proteins were extensively degraded to amino acids and ammonia (17). In addition of lactic acid bacteria (LAB) other microorganisms compete on substrates. Then inhibition undesirable microbes may account for a goal of producing good quality silages.

Many chemical and biological additives were developed to improve silage quality. Nagel and Broderick (18) demonstrated that formic acid is known as an effective additive. Addition of formic acid to silage material has been reported to have positive effects on fermentation (25). However, formic acid as a fermentation inhibitor can restrict the fermentation by its ability to decrease the pH during ensiling (14).

Jaakkola et al., (13) found out that direct acidification with organic acid, such as formic acid, restricts fermentation, increases residual WSC and decreases production of volatile fatty acids (VFA) and protein degradation.

Formic acid is widely used as an additive due to low production costs, high acidification capacity and a broad spectrum activity against bacterial pathogens and selected fungal pathogens (17, 20). Accordingly, the current study aimed to evaluate the effect of addition of different levels of formic acid and urea on fermentation and nutritive quality of reed silage via changes in microbial populations.

Materials and methods

Making reed silage:

This study was carried out in nutrition laboratory based on preparing 400-500 g samples of common reed silages with 4 replicates. Reed plants (40.84% dry matter, 15.22% ash, 5.71% crude protein, 3.74% ether extract, 44.95% crude fiber and 24.67% nitrogen free extract) were obtained from the area nearby Animal Production Department-Alqasim Green University. Plants were chopped into 2-3 cm of length. Treatment solutions were prepared by addition of debis (date honey) as a source of WSC at level of 10%, and three levels of both formic acid, 0.5, 1 or 1.5% and urea as a source of nitrogen, 0. 1 or 2%. Table 1 shows the percentages of components of silage samples.

Table 1- Components of reed silage (%).

Level of formic acid (%)	0.5			1			1.5		
Level of urea (%)	0	1	2	0	1	2	0	1	2
Chopped reed plant	90	89	88	90	89	88	90	89	88
Debis (date's honey, %)	10	10	10	10	10	10	10	10	10

Quantities of additives were estimated on dry matter basis of reed plants and tap water was added to ensure dry matter content of about 30% in treated materials. Samples were tightly packed in double nylon sacs, compacted by hand to exclude the air. Samples were then moved to pit silos, well covered, filled up with soil and stored for 60 days. When this period was passed sacs were moved again to laboratory to perform microbial analysis.

Microbial analysis:

Microbial analysis of silage samples included total count of aerobic, anaerobic and lactic acid bacteria, yeasts and molds. Microbial analysis of silages were performed in duplicate shortly after each bags were opened. Each of silage samples (1 g) were blended with 9 ml of sterilized peptone water (29). The suspensions were serially diluted (10^{-1} to 10^{-10}) in peptone water as described by Harrigan and McCane (1976). 100-μL aliquots of each dilution were spread onto selective medias as follows:

Aerobic bacteria were counted on nutrient agar (28 g dissolved into 1L of distilled water, Nissui-seiyaku, Tokyo, Japan) and autoclaved at 37ºC for 24 hours (26). Plates were incubated in an incubator at 37ºC for 24–48 hours. The same steps were performed to count anaerobic bacteria but plates were incubated anaerobically at 37ºC for 24–48 hours (4).

Lactic acid bacteria (LAB) were counted on deMan, Rogosa, and Sharpe agar (28 g of media dissolved into 1L of distilled water, Difco Laboratories, Detroit, MI, USA) after incubation at 37°C for 48 h under anaerobic conditions (2). Yeasts were counted on malt extract agar (28 g dissolved into 1L of distilled water, Nissui-seiyaku, Tokyo, Japan). Plates were incubated aerobically at 25°C for 72 h (5). Molds were counted on Potato dextrose (19.5 g dissolved into 1L of distilled water, Nissui-seiyaku, Tokyo, Japan). Plates were incubated aerobically at 25°C for 72 h (4).

Colonies were counted as viable numbers of microorganisms from plates containing a minimum of 30 and a maximum of 300 colonies. All analysis was carried out using the aseptic technique by using sterilized equipment and solutions to prevent contamination. Numbers of microorganisms are expressed as colony-forming units per g of wet silages and were log transformed.

Statistical analysis:

Data obtained were analyzed as a factorial experiment in completely randomized design by analysis of variance using statistical analysis system, SAS (23).

Results and discussion

Table 2 shows microbial populations of reed silages as affected by addition of different levels of FM and urea. With exception of aerobic bacteria, results revealed that numbers of other microorganisms were affected (P<0.01) by addition of acid. A decline (P<0.01) in numbers of anaerobic bacteria by 0.83 $\log^{10}$ cfu/g fresh silage was observed due to increasing level of addition of acid from 0.5 to 1%. With increasing acid level to 1.5%, more decline in numbers of these bacteria was recorded and reached to 1.94 $\log^{10}$ cfu/g fresh silage.

Table 2– Effect of addition of different levels of formic acid and urea on microbial populations of common reed silage ($\log^{10}$ cfu/g fresh silage ± SE).

Silage microbes	Level of formic acid (%)			Level of urea (%)			P	
	0.5	1	1.5	0	1	2	FM	U
Aerobic bacteria	6.02 ± 0.18	5.84 0.25 ±	6.28 0.21 ±	6.14 0.18 ±	5.62 0.26 ±	6.38 0.16 ±	NS	NS
Anaerobic bacteria	9.76[a] 0.04 ±	8.93[b] ± 0.02	7.82[c] 0.05 ±	8.86 0.23 ±	8.75 0.24±	8.91 0.25±	**	NS
LAB	9.57[a] ± 0.02	8.64[b] 0.03 ±	7.62[c] 0.02 ±	8.65 0.24 ±	8.59 0.24 ±	8.59 0.22 ±	**	NS
Yeasts	6.03[a] 0.37 ±	4.46[b] 0.14 ±	4.12[b] 0.14 ±	5.28[a] 0.45 ±	4.87[a] 0.33 ±	4.46[b] 0.15 ±	**	*
Molds	5.26[a] 0.53 ±	4.49[b] 0.50 ±	4.24[b] 0.29 ±	6.27[a] 0.37 ±	4.49[b] 0.31 ±	3.25[c] 0.17 ±	**	**

Means having different letters at the same row are significantly different at * (P<0.05) ** (P<0.01)

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That decline in numbers of anaerobic bacteria may be a result of increasing acidification by formic acid, the pKa of formic acid is 3.75, and that of lactic acid 3.85. With the pKa defined as the negative common logarithm of the acid dissociation constant, these values show that formic acid is twice stronger than lactic acid, the stronger acids produced during silage fermentation (27). Similar result was obtained by Akintokun, et. al., (5), where, numbers of bacteria were decreased due to acidity produced by fermentation of WSC. Cao, et. al., (8) reported that organic acids produced during ensiling process reduced pH, low pH inhibited the growth of some microorganisms such as aerobic bacteria, yeasts and molds.

Decline in numbers of anaerobic bacteria can be explained by a decrease in pH. Yang, et. al., (30) showed that numbers of bacteria including LAB were reduced with a decrease in pH caused by high lactic acid production. Moreover, formic acid lowered the pH immediately after its addition and worked by reducing the activity of saccharolytic enterobacteri and clostridia bacteria (16).

Results also showed that there was a gradual significant ($P<0.01$) decline in numbers of LAB with addition of FA at level of 0.5, 1 and 1.5%, mean numbers were, 9.57, 8.64 and 7.62 log^{10} cfu/g fresh silage respectively. This result is consistent with that obtained by Rowghani and Zamiri (21), in which FA had an antibacterial effect on some bacterial species, included the LAB.

Decline in numbers of LAB may attributed to the limiting effect of FA on silage fermentation (7, 19). However, Hapsari, et. al., (11) showed that addition of formic acid did not affect the population of LAB in Napier grass silage. The difference in level of addition of FA, 0.15 vs. 0.5-1.5% in the later and current studies may explain the inconsistency of the effect of addition of FA on numbers of LAB.

Results revealed also that increasing level of FA gradually decreased ($P<0.01$) numbers of yeasts and molds. Number of yeasts decreased from 6.03 in samples of reed silage prepared with addition of FA at level of 0.5% to 4.46 and 4.12 log^{10} cfu/g fresh silage in samples prepared with addition of acid at level of 1 and 1.5% respectively. Reduction of molds numbers from 5.26 to 4.49 and 4.24 log^{10} cfu/g fresh silage was observed in samples of silage prepared with addition of acid at level of 0.5, 1 and 1.5% respectively.

Significant decline in numbers of yeast and molds in a current study may due to presence of higher acidic medium in the silage, hence growth of aerobic microbes was impeded. McDonald, et. al., (17) considered low pH in silage a probable reason for a decline in numbers of yeasts. Weinberg (28) attributed that decline of yeasts and molds numbers to the effect of volatile fatty acids which directly and effectively affect acidic medium of the silage.

Regarding effect of level of addition of urea on microbial populations of common reed silage, results of the current study showed that this effect was limited to numbers of yeasts and molds. Numbers of yeasts ($P<0.05$) and molds ($P<0.01$) were decreased with increasing level of addition of urea from 0, 1 and 2%, numbers were 5.28, 4.87 and 4.46 for yeasts, 6.27, 4.49 and 3.25 log^{10} cfu/g fresh silage respectively. Similar results were obtained by Abid (1) in rice straw silage. Decline in numbers of yeasts and molds was attributed to the antifungal effect of urea (15)

Table 3 shows the effect of interaction between level of formic acid and urea on microbial populations of common silage. As shown numbers of aerobic, anaerobic bacteria and LAB ($P<0.05$) and yeasts and molds ($P<0.05$) were affected by that interaction. Lower numbers of aerobic bacteria (4.77 log^{10} cfu/g fresh silage) were observed in samples of silage treated with both acid and urea at level of 1%, whereas, numbers of that bacteria tended to decrease with increasing level of FA from 0.5 to 1 and 1.5% without addition of urea (0%), numbers were 6.53, 6.19 and 5.71 log^{10} cfu/g fresh silage respectively. This result is in line with

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observations of Saarisalo, et. al., (22) in which authors referred to a decrease in numbers of 5.9 log^{10} cfu/g fresh silage due to addition of FA at level of 4L/t in 1st and 2nd experiments respectively. Numbers of anaerobic bacteria showed a gradual significant ($P<0.05$) decrease with increasing level of FA regardless to level of urea, numbers were 7.89, 7.71 and 7.86 log^{10} cfu/g fresh silage when acid was added at high level (1.5%) in comparison with 8.95, 8.86 and 8.99 log^{10} cfu/g fresh silage at medium level of acid (1%) and 9.73, 9.66 and 9.89 log^{10} cfu/g fresh silage at lower level of acid (0.5%), for 0, 1 and 2% levels of urea respectively.

Numbers of LAB followed similar trend as in anaerobic bacteria of gradual significant ($P<0.05$) decrease with increasing level of FA regardless to level of urea as previously shown in table 2, a decline in numbers of LAB was 1.95 log^{10} cfu/g fresh silage due to increasing level of addition of FA from 0.5 to 1.5%. In another study, there were a decline in numbers of LAB of 2.1-2.2 log^{10} cfu/g fresh silage of timothy-meadow fescue herbage (22).

Numbers of yeasts and molds were also affected ($P<0.05$) by the effect of interaction between level of formic acid and urea added to reed plant at ensiling. From one side, numbers of yeasts were decreased with addition of high level of urea, 4.52, 4.56 and 4.30 log^{10} cfu/g fresh silage together with the 0.5, 1 and 1.5% levels of FA respectively. Abid (1) observed similar trend of decline in numbers of yeasts and molds in rice straw silage due to increasing level of urea as affected by its antifungal effect (15). From the other side, numbers of yeasts were decreased ($P<0.05$) with increasing level of addition of acid, 7.27, 4.76 and 3.81 log^{10} cfu/g fresh silage in samples of reed silage prepared with addition of 0.5, 1 and 1.5% levels of formic acid without addition of urea respectively.

This trend can be explained on basis of correlation between increasing level of pH and pH of silage (24). Low pH in turn inhibits growth of yeasts and molds (17). McDonald, et. al., (17) and Randby (20) referred to the ability of FA to acidify silage medium and its antifungal activity against wide spectrum of bacteria and fungi. Numbers of molds followed similar trend of changes, where, lower numbers were counted in samples of reed silage prepared by addition of high level of urea (2%) with 0.5, 1 and 1.5% levels of FA, 3.18, 2.78 and 3.79 log^{10} cfu/g fresh silage. Whereas, samples prepared with these levels of acid without addition of urea recorded higher numbers of molds.

Table 3- Effect of interaction between level of addition of formic acid and urea on microbial composition of common reed silage (log^{10} cfu/g fresh silage ± SEM).

Level of formic acid (%)	0.5			1			1.5			P
Level of urea (%)	0	1	2	0	1	2	0	1	2	
Aerobic bacteria	6.53^{a} 0.41±	5.53^{bc} 0.02±	6.01^{ab} 0.21±	6.19^{ab} 0.27±	4.77^{c} 0.24±	6.57^{a} 0.02±	5.71^{ab} 0.11±	6.57^{a} 0.40±	6.56^{a} 0.41±	*
Anaerobic bacteria	9.73^{a} 0.09±	9.66^{a} 0.09±	9.89^{a} 0.01±	8.95^{b} 0.01±	8.86^{b} 0.07±	8.99^{b} 0.02±	7.89^{c} 0.08±	7.71^{c} 0.08±	7.86^{c} 0.12±	*
Lactic acid bacteria / LAB	9.60^{a} 0.04±	9.59^{a} 0.04±	9.51^{a} 0.00±	8.73^{b} 0.06±	8.61^{b} 0.05±	8.59^{b} 0.05±	7.61^{c} 0.04±	7.58^{c} 0.04±	7.66^{c} 0.06±	*
Yeasts	7.27^{a} 0.22±	6.3^{a} 0.23±	4.52^{cd} 0.40±	4.76^{c} 0.26±	4.05^{cd} 0.29±	4.56^{cd} 0.00±	3.81^{d} 0.22±	4.25^{cd} 0.22±	4.30^{cd} 0.27±	**
Molds	7.31^{a} 0.21±	5.29^{bc} 0.48±	3.18^{e} 0.17±	6.38^{ab} 0.43±	3.57^{de} 0.40±	2.78^{e} 0.25±	5.06^{c} 0.64±	4.62^{cd} 0.43±	3.79^{de} 0.23±	**

Means having different letters at the same row are significantly different at * ($P<0.05$) ** ($P<0.01$)

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