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Bacterialcommunityandfermentationpatternassociatedwithensilageprocessofwilted, un-wiltedoatsilagein Afghanistan

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Abstract
Aims:*To assess the variation in bacterial communities and determine the effects of wilting, storage period and molasses inoculant on the bacterial community and fermentation pattern of oats silage. Fermentation pattern, colony counts and denaturing gradient gel electrophoresis (DGGE) profiles were determined. The lactic acid content was higher than acetic acid in all silages; however the lactic acid to acetic acid ratio decreased with storage time. Thisalteration from lactic to acetic acid was not prevented even with a combination of wilting and molasses inoculant.The DGGE analyses suggest that facultativelyheterofermentative lactic acid bacteria (Lactobacillus Lactis and Lactobacillus plantarum) were involved in the shift to acetic acid fermentation.*
Significant impacts: *The bacterial community looks stable compared to fermentation products over the course of long storage periods in oat silage. Acetic acid fermentation in oat silage can be a result of the changes in bacterial metabolism rather than community structure.*
Keywords*:silage,oat,PCR, DGGE, microbiota.*

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I. Introduction

Silage is fermented animal feed, which formed by bacterial activity and other chemical changes in green forage stored in the absence of air. Silage can be fed to cattle, sheep and other such ruminants, and fermented high-moisture stored fodder which or used as a biofuel feedstock for anaerobic digesters(Macmaster et al.1969) . It is fermented and stored in a process called ensilage, ensiling, and is usually made from grass crops, including maize, oats, sorghum or other cereals (Parvin, et al.2009). There are increasing efforts to develop appropriate ensilage procedure, because the feeding of well-preserved silage is estimated to advance the productivity of livestock in the sub tropics (Ercolini,et al. 2004). The oat (Avena sativa) is a species of cereal grain grown for its seed and ruminant nutrition (Afridi, et al. 2002). The conservation of forages as silage is asignificant source of nutrients for livestock feeding in many countries including Afghanistan. The concentration of fermentation products in silage is determined both by the initial chemical composition of the crop itself and also by the type(s) of microorganisms which advance during the storage period.

The overall objective of silage production is to preserve the inventive quality of the preserved crop as much as possible. To this end, additives have been used for several decades to direct the fermentation process towards the production of lactic acid as the main fermentation product (Rossi et al.2001). Addition of molasses will improve fermentation in Oats silage,improvement should observe for a combination of Lactic acid bacteria (LAB) and molasses. Wilting is a conventional technique to suppress the growth of undesirable bacteria in silage. Demanding wilting may result in a lowering of acidification, but proteolytic Clostridia will be more restricted than non-proteolytic LAB and other microorganisms (McDonald et al. 1991). It has been argued that wilting is essential for tropical grass ensiling, with DM content greater than 300-g/ kg shown to be crucial for assuring acceptable DM recovery after ensiling (Nussio 2005).Tjandraatmadja et al. (1990) measured LAB populations in the silages of various tropical grasses, including Avena sativa. They found that Lactobacillus plantarum was the predominant bacterium, followed by homofermentativePediococcus sp. and heterofermentative lactobacilli like L. brevis and L. fermentum. While this is similar to findings in temperate silages, most silage examined contained lactic acid, rather than acetic acid, as the major fermentation product.

Advanced molecular biological techniques have been used to understand the structure of complex microbial communities. Indeed, DNA-based profiling techniques such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism have been applied across a range of microbial habitats. Although culture-independent approaches are believed to have less bias than culture-based approaches (Ercolini 2004), only a small number of studies have employed culture-independent approaches to investigate the

ensilage process (Rossi and Dellaglio 2007; McEniry et al. 2008; Wang and Nishino 2008).

The present study aimed to assess the variation in bacterial communities and determine the effects of wilting, storage period and molasses inoculant on the bacterial community and fermentation pattern of oats silage and to maintain the quality and extend Oat silage making in Afghanistan by using culture and culture-independent methods.

II. Materials & Methods

2.1 Preparation of silage

First growth of Oats (Avena sativa) was manually harvested at the heading stage in April 2016 and wilted for 5 days with occasional turning, For laboratory-scale preparations, the wilted and un wilted oats were chopped by a forage cutter at a theoretical length of 13 mm. Samples weighing 300 g, than 600 ml molasses juice was added to selected oat silage andwere packed in plastic bags, and the air was removed using a vacuum sealer. The size, thickness and oxygen permeability of the pouch were 270 x 400 mm, 0.075 mm and 44 ml m-2 atm-1 per day, respectively. Silos were made in triplicate and stored at ambient temperature. Storage was terminated after 15, 30, 90 and 180 days (Nishino et al, 2012).Ambient temperatures were about 25–30 0C and 15–25 0C in the first and second 30 days of ensiling, respectively. The temperatures declined to about 5–15 0C in the last 90 days of the whole 180-day period (Nishino et al, 2012). Chemical composition of fresh and wilted Oats was determined as in table1.

Table1. Chemical composition of direct cut and wilted Oats used in experiments

Items	Direct cut	Wilted
Dry matter (g /1kg)	187	476
NDF (g kg)1 DM	619	648
ADF (g kg)1 DM	373	395
WSC (g kg)1 DM	26.5	43.7

NDF; neutral detergent fiber, ADF; acid detergent fiber, WSC; water-soluble carbohydrates

2.2 Chemical analyses and account of viable microorganisms

The DM content of oat and silages was determined by oven drying at 60oC for 48 h. Dried samples were ground to pass through a 1-mm screen using a sample crusher and milled samples were used to determine water soluble carbohydrate (WSC), neutral detergent fiber and acid detergent fiber contents (Nishino et al. 2004). The pH value, lactic acid, volatile fatty acids and ethanol were determined in cold water extracts (Nishino et al. 2007). Bacteria count of LAB was performed by a pour plate technique by using MRS agar. Cycloheximide was added to MRS agar at 50 mg l-1. Yeasts and molds were counted on spread plates of potato dextrose agar added with chloramphenicol at 50 mg l-1. The pH of potato dextrose agar was adjusted to 3.5 by adding sterilized lactic acid solution after autoclaving. All plates were incubated for 3 days at 30oC.

2.3 Denaturing gradient gel electrophoresis

Frozen samples were melted and mixed with 10 times the volume of decontaminated, physiologically buffered saline. Extracts were prepared by vigorous shaking for 10 min at room temperature and centrifuged at 8000 g for 15 min to obtain microbial pellets. A commercial kit (DNeasy tissue kit; Qiagen, Germantown, MD, USA) was used to purify the bacterial DNA. Polymerase chain reaction (PCR) was carried out to amplify the V3 region of the bacterial 16S rRNA gene with the GC-clamp forward primer GC357f (5/-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3/) and the reverse primer 517r (5/-ATTACCGCGGCTGCTGG-3/). The PCR mixture contained 25 mmol l-1 Tris (hydroxymethyl) methyl aminopropanesulphonic acid (pH 9.3), 50 mmol l-1 KCl, 2.0 mmol l-1 MgCl2,0 dNTP, 2.5U Taq polymerase, and 1.0 lmol-1 each of primer and DNA template. The PCR programme comprised an initial denaturation at 95oC for 10 min and 30 cycles of denaturation at 93oC for 30 s; annealing at 65oC (first 10 cycles), 60oC (second 10 cycles), and 55oC (last 10 cycles) for 30 s; and extension at 72oC for 1 min; and a final extension at 72oC for 5 min (Pedro et al. 2001). The reaction was conducted in a PCR thermal cycler (TP-600; Takara Bio Inc., Shiga, Japan). The GC-clamp PCR products were separated according to their sequences with a D Code Universal Mutation System (Bio-Rad Ltd, Tokyo, Japan). Samples were applied into 100 g l)1 (w/v) polyacrylamide gradient gels in a buffer containing 20 mmol l-1 Tris–acetate and 0.5 mmol l-1 EDTA–2Na (pH 8.5). Denaturing gradient gels were prepared with 25–50% urea and form amide (7 mol l-1 urea and 400 ml -1 form amide as 100% denaturants). Electrophoresis was conducted with a constant voltage of 150 V for 12 h at 60oC. After electrophoresis, the gels were stained with distilled water containing SYBR Green I (Cambrex Bio Science Inc., Rockland, ME, USA) and photographed under UV illumination.

2.4 Sequence Analyses OfDNA Bands

Selected bands were excised from the DGGE gels and soaked in 10 ml of sterilized distilled water to dissolve DNA. Eluted DNA was re amplified by PCR using 357f (without GC-clamp) and 517r primers, and the PCR products were then purified with a commercial clean-up kit (Geneclean kit; Qbiogene, Carlsbad, CA, USA). Purified DNA was subjected to a sequence reaction using the Big Dye Terminator ver. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), followed by automated base sequencing with the ABI PRISM 310A (Applied Biosystems). To determine the closest relatives of the partial 16S rDNA sequences, a search of the GenBank DNA database was conducted using the Blast(http://www.ncbi.nlm.nih.gov/BLAST). Percentage of identities ranged from 87 to 100%.

Statistical analysis:Two-way analysis of variance was used to determine the effects of wilting level, molasses storage period and of LAB. Tukey's multiple comparison method was used to detect differences between means. All statistical analyses were performed with jmp software (ver. 7, SAS Institute, Tokyo, Japan).

III. Results& Discussion

The direct cut and molasses inoculated oat silage attained a pH of 3.9 and 4.1 after 15 and 30 days of fermentation respectively (Table 2). Further pH reduction with prolonged ensiling was not found. However, the levels of lactic acid, acetic acid and ethanol continued to increase over 180 days of storage. The production was more with lactic acid than acetic acid in all oat silage; however the lactic acid to acetic acid ratio decreased with direct cut and molasses at storage time. This alteration from lactic to acetic acid was not prevented even with a combination of wilting and molasses inoculant. The lactic acid and ethanol contents of wilted oat silage also increased with storage time. However, there was little change in acetic acid content, and the L/A ratio was unaffected by the continuation of ensiling. Values for the L/A ratio were higher on day 30 and lower on day 180 in molasses inoculant and direct cut oat than that in wilted oat silage. Although the L/A ratio varied with the storage period, lactic acid accounted for more than 50% of the fermentation acid content of all silages. The WSC content decreased with storage time. This reduction was particularly intensive during the first 15 days of storage in direct cut and molasses inoculant oat silage respectively. Nevertheless, the consumption of WSC across the entire ensiling period was similar in the direct cut, molasses inoculant oat and wilted silages.

Table 2. Effect of wilting, storage period and molasses addition on fermentation product and microbial counts of oat silage

	DM	WSC	pH	LA	AA	ET	L/A	LAB	YT
Direct cut silage									
Day 15	290	5.15	4.1	11.4	4.8	0.91	2.38	7.94	<2.00
30	312	3.96	4.1	12.4	5.29	1.14	2.78	7.94	<2.00
90	297	4.13	4.14	12.3	6.19	2.38	1.99	7.8	<2.00
180	269	3.1	4.2	13.1	10.6	2.46	1.24	7.22	<2.00
Wilted Silage									
Day 15	433	10.5	5.31	7.82	5.1	0.9	1.53	7.74	<2.00
30	449	5.56	5.22	8.03	4.81	1.19	1.67	7.98	<2.00
90	444	4.07	4.63	11.8	5.74	2.63	2.06	7.5	2.51
180	405	3.61	4.55	13.3	7.36	2.21	1.81	6.91	<2.00
Molasses added direct cut silage									
Day 15	298	6.83	3.9	13.8	5.3	0.98	2.26	8.24	<2.00
30	323	5.55	3.9	13.2	6.1	1.16	2.62	8.22	<2.00
90	312	4.81	4.64	12.5	7.3	2.44	1.88	8.11	<2.00
180	290	3.6	4.5	12.9	10.14	2.65	1.22	7.8	<2.00
SE	5.6	4.43	0.09	1.02	2.46	1.68	0.16	0.12	
Levels of Significance									
Wilting (W)	**	NS	*	*	NS	NS	**	NS	_
Molasses (M)	NS	*	**	**	**	NS	NS	**	_
Storage Period (S)	NS	**	NS	**	**	**	**	NS	_
W x M	**	NS	**	**	NS	NS	NS	*	_
W x S	NS	**	*	*	**	*	**	NS	_
M x S	NS	**	*	*	**	**	*	NS	_
W x M x S	NS	**	*	*	NS	**	NS	NS	_

DM, dry matter (g kg $^{-1}$); LA, lactic acid (g kg $^{-1}$ DM); AA, acetic acid (g kg $^{-1}$ DM); ET, ethanol (g kg $^{-1}$ DM); L / A, lactic to acetic acid ratio; LAB, lactic acid bacteria (log CFU g^{-1}); YT, yeasts (log CFU g^{-1})

Values are means of triplicate silages. Factorial analysis was performed to determine the effects of wilting, molasses addition, storage period and their interactions. NS; P> 0.05, *; P <0.05, **; P <0.01. Molasses was added at 10 g kg g^{-1}, which supplied 4.21 g sugars kg (glucose equivalent) at ensiling

A level of LAB more than 109 CFU g-1 and 108 CFU g-1was found in silages with molasses

inoculatedand fresh silages up to day 90 respectively, beyond which the numbers declined to around 107 CFU g-1 (Table 3). No yeasts and molds were found in all the types of silages storage period, except that wilted silage had yeasts (102 CFU g-1) on days 15 and 90, respectively.

Although bands for various enterobacterial species, such as Pantoeaagglomerans (bands 1 and 4), Acinetobacter sp. (band 2) and Pantoeaananatis (bands 3 and 5), were observed on the DGGE gel in the case of direct-cut and wilted materials, bands for these species were not detectable after ensiling (Fig. 1). Distinct bands indicative of Morganellamorganii (band 6),Lact.plantarum (band 7), Enterococcus sp. (band 8), Pantoea sp. (band 9), L. lactis (band 10) and Enterococcus faecium (band 13) were observed in the case of direct-cut silage. On days 30 and 90, prominent bands for M. morganii (band 12) and Clostridium botulinum (band 11) were also observed. The DGGE patterns for direct-cut silage to which were similar to wilted silage; however, bands indicative of M. morganii (band 6), Enterococcus sp (band 8),Pantoea sp. (band 9) and Enterococcus faecium (band 13) were faint in the case of wilted silage. A distinct band was observed for Lactococcusgarvieae (band 17) in the case which molasses was added

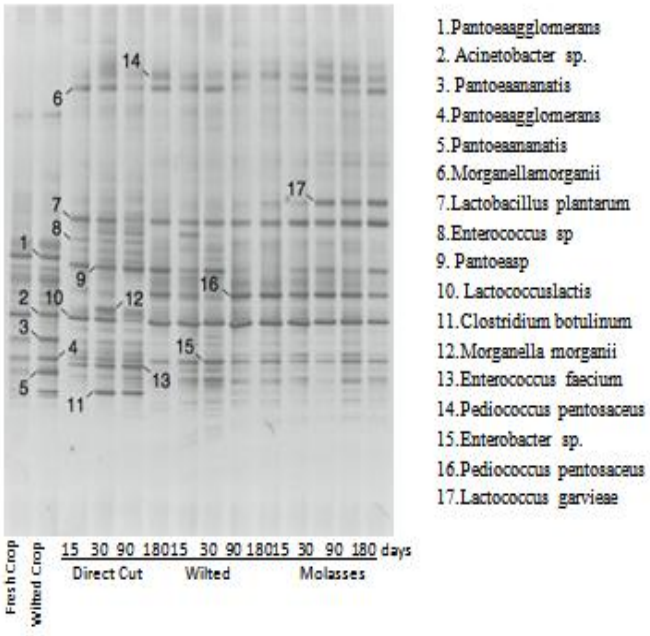

Figure1. Bacterial community associated with direct cut, wilted and Molasses inoculant Oat silage. Silos were opened at 15, 30, 90 and 180 days after packaging. Denaturing gradient gel electrophoresis was carried out at a constant voltage of 150 V for 12 h at 60C, and a number of DNA bands were excised and sequenced.

IV. Discussion

All silages of the present study contained lactic acid as the major preservative. Although wilting to approximately 476 g DM kg-1 was previously shown to enhance lactic acid production in oat silages, acetic acid fermentation could be retained when the content was yet as low as 300 g kg-1 (Rodriguez et al. 1989; Umana et al. 1991). In the present study, we found that the L/A ratio decreased when ensiling was prolonged in direct cut and molasses inoculated oat silage. This effect was ascribed to a large increase in acetic acid rather than changes in lactic acid levels, as was seen in the results of Umana et al. (1991). However, a decrease in the L/A ratio has also been shown to be the combined result of an increase in acetic acid and a decrease in lactic acid (Rodriguez et al. 1989).

In this study, changes in the bacterial community during ensiling were detected using DGGE analysis; these changes could account for the increase in butyric acid content in direct-cut silage and for the increase in lactic acid content because of wilting and molasses addition. The appearance of Cl. botulinum was in accordance with the time from which the butyric acid content increased, and the appearance of Ped. pentosaceus and L. garvieae was in accordance with the enhanced lactic acid production because of wilting and molasses addition. However, the bacteria associated with acetic acid fermentation could not yet be clearly identified. No distinct differences were observed between the acetate-type and lactate-type silage. The most noticeable, but not straightforward, changes in the bacterial community were found in the case of M. morganii (band 6) and Pantoea sp. (band 9); their bands were faint in the case of wilted silage in the early stages of fermentation (lactate-type silage). Although DGGE analysis is not quantitative and cannot be used to determine how bacteria are metabolically active, small populations may be difficult to detect. Therefore, the changes in the DGGE patterns in this study suggested an association between

enterobacteria and acetic acid production in oat crop ensiling. Although Morganella sp., Pantoea sp., and Acinetobacter sp. have not yet been isolated from crop silage in any other studies, the DGGE analysis in our previous study showed the presence of Pantoea sp. in Italian ryegrass silage (Li and Nishino 2011a) and that of Acinetobacter sp. in whole crop maize silage (Li and Nishino 2011b). The results of studies suggested that Lact. plantarum was involved in the increase in acetic acid content because of prolonged ensiling (Parvin and Nishino 2009) because Lact. plantarum can metabolize lactic acid to acetic acid under sugar-deficient conditions (Lindgren et al. 1990).

The same metabolic process could have occurred in the case of untreated silage and wilted silage to which molasses was added because the amount of fermentation products on day 14 exceeded the WSC contents of the pre-ensiled materials. Although Lactobacillus buchneri is known to be involved in anaerobic lactic acid degradation to acetic acid (Oude Elferink et al. 2001), this LAB species was not detected during DGGE analysis, and 1,2-propanediol, a metabolite of lactic acid degradation, was not detected in this study. In untreated direct-cut silage, the ethanol content markedly increased during prolonged ensiling. The fermentation product levels had already exceeded the initial WSC content on day 15, and therefore, none of the usual sugar substrates should have remained in the silage to support a further increase in the fermentation product levels. Moreover, the bacteria responsible for the increased ethanol level were difficult to identify – except for M. morganii (band 12), which appeared in the late stages of fermentation. In this study, the DM content of the pre-ensiled crop was lower than 250 g kg)1 even after wilting; hence, few improvements were expected in the composition of fermentation products. However, this light wilting substantially suppressed acetic acid lactic acid production.

The effects of molasses were greater than those of wilting, as evidenced by the DGGE analysis, where bands indicative of M. morganii and Pantoea sp. became faint in wilted silage but not in direct-cut silage after the addition of molasses. These results indicate that, although a shift from lactic acid to acetic acid fermentation because of prolonged ensiling is unavoidable, molasses addition can be regarded as more efficient and economically feasible than wilting to promote desirable fermentation. Because the increase in acetic acid content because of prolonged ensiling was unavoidable even after using a combination of wilting and molasses addition, sugar deficiency in the pre-ensiled crop may not be the critical factor for acetic acid fermentation in tropical grass ensiling. Although this study revealed how lactic acid fermentation was enhanced by molasses addition, further studies are required to elucidate the bacteria associated with the enhancement of acetic acid fermentation.

V. Conclusions

Lactic acid can dominate the fermentation in oat silage with sufficient wilting and molasses inoculant prior to ensiling. Storage continuation may lead to high levels of acetic acid without exclusive changes in the bacterial community.

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