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Laboratory silo type and inoculation effects on nutritional composition, fermentation, and bacterial and fungal communities of oat silage

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ABSTRACT

The objectives were to evaluate (1) the use of 2 types of experimental silos (S) to characterize whole-crop oat (*Avena sativa* L.) silage with or without addition of an inoculant (I), and (2) the effect of inoculation on the microbial community structure of oats ensiled using only plastic bucket silos (BKT). From each of 6 sections in a field, oats were harvested, treated (INO) or not (CON) with inoculant, packed into 19-L BKT or vacuum bags (BG), and ensiled for 217 d. The inoculant added contained *Lactobacillus buchneri* and *Pediococcus pentosaceus* (4×10^5 and 1×10^5 cfu/g of fresh oats, respectively). The experimental design was a complete randomized design replicated 6 times. Treatment design was the factorial combination of 2 S × 2 I. Some differences existed between BG versus BKT at silo opening (217 d), including a decreased CP (7.73 vs. 7.04 ± 0.247% of DM) and ethanol (1.93 vs. 1.55 ± 0.155) and increased lactic acid (4.28 vs. 3.65 ± 0.241), respectively. Also, WSC and mold counts were reduced in BG versus BKT for CON (1.78 vs. 2.70 ± 0.162% of DM and 0.8 vs. 2.82 ± 0.409 log cfu/fresh g) but not for INO (~1.53 and 1.55), respectively. Application of INO increased DM recovery (96.1 vs. 92.9 ± 0.63%), aerobic stability (565 vs. 133 ± 29.2 h), acetic acid (2.38 vs. 1.22 ± 0.116% of DM), and reduced NDF (65.0 vs. 67.0 ± 0.57), ADF (36.7 vs. 38.1 ± 0.60), ethanol (0.63 vs. 2.85 ± 0.155), and yeast counts (1.10 vs. 4.13 ± 0.484 log cfu/fresh g) in INO versus CON, respectively. At d 0, no differences were found for S and I on the nutritional composition and background microbial counts. *Leuconostocaceae* (82.9 ± 4.27%) and *Enterobacteriaceae* (15.2 ± 3.52) were the predominant bacterial families and unidentified sequences were predominant for fungi. A higher relative abundance of the *Davidiellaceae* fungal family (34.3 vs. 19.6 ± 4.47) was observed in INO versus CON. At opening (217 d), INO had a lower relative abundance of *Leuconostocaceae* (42.3 vs. 95.8 ± 4.64) and higher *Lactobacillaceae* (57.4 vs. 3.9 ± 4.65) versus CON. Despite several differences were found between BKT and BG, both techniques can be comparable for characterizing effects of INO on the most basic measures used in silage evaluation. The use of inoculant improved oat silage quality partially by a shift in the bacterial community composition during ensiling, which mainly consisted of an increased relative abundance of *Lactobacillaceae* and reduction of *Leuconostocaceae* relative to CON.

Key words: silage, inoculant, mini-silo type, 16S and ITS1 sequencing

INTRODUCTION

Silages are an important source of forage in the United States, representing ~44.2% of the total forage harvested in 2014 (USDA, 2015). Evaluation of additives effects on silage microbial composition and nutritional value using field-scale silos is challenging because of the difficulty of producing homogeneous silage for treatment evaluation (Cherney and Cherney, 2003). On the other hand, laboratory silos provide a more complete control of the ensiling conditions and have been widely used when multiple treatments are tested (Cherney and Cherney, 2003).

Vacuum bags and fixed volume vessels are common methods for screening additives and management practices in silage research (Johnson et al., 2005). Fixed volume vessels, with some limitations, can adequately represent on-farm silos (Cherney and Cherney, 2003). On the other hand, vacuum bags are reported to have a more consistent fermentation and are easier to prepare compared with fixed volume vessels (Johnson et al., 2005; Hoedtke and Zeyner, 2011). In contrast to studies with corn (*Zea mays* L.; Cherney et al., 2004), perennial ryegrass (*Lolium perenne* L.; Johnson et al., 2005; Hoedtke and Zeyner, 2011), and red clover (*Trifolium*

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pretense L.; Johnson et al., 2005), little is known about silo type effects on nutritional value, fermentation profile, and bacterial and fungal communities of small-grain cereals such as oats (*Avena sativa* L.).

Understanding the microbial ecology of silages is critical to identify novel microorganisms related to optimal silage making and to prevent the growth of pathogens that can compromise the animal food safety chain (Driehuis, 2012; Muck, 2013; Nishino, 2015). In this context, next-generation sequencing (**NGS**) provides a practical way to conduct amplicon specific [e.g., 16S rRNA for bacteria or the internal transcribed spacer (**ITS**) unit for fungi] high-throughput sequencing studies of microbial populations coming from a wide array of environments (Adams et al., 2009), including silages (Nishino, 2015). Expanding the use of NGS techniques to silage experiments also provides the opportunity to estimate the effect of environment and management practices (e.g., type of crop, addition of inoculants, DM concentration) on microbial population shifts. Currently, only a few reports are available on the use of NGS in characterizing silage ecology. Reports include description of the microbiome of alfalfa silage (*Medicago sativa* L.) and an unspecified grass silage (McGarvey et al., 2013; Eikmeyer et al., 2013).

The objectives of the present study were to (1) compare the effect of silo type (vacuum bags vs. plastic bucket) with or without a combination inoculant on the nutritional value, fermentation profile, and population of bacteria and fungi of whole-crop oats and (2) characterize via microbial community analysis the effects of a combination inoculant on whole-crop oats silage using plastic bucket silos. We hypothesized that (1) estimates of nutritional value, chemical analysis, fermentation profiles, and the population of bacteria and fungi are similar between vacuum bags and 19-L plastic buckets and (2) adding a combination silage inoculant improves nutritional value, preservation, and aerobic stability of the ensiled oats by causing a large shift in the composition and structure of the bacterial and fungal communities compared with untreated control oat silage.

MATERIALS AND METHODS

Experimental Site, Design, and Treatments

The experimental site (~3 ha) was located at the Center for Environmental Farming Systems in Goldsboro, North Carolina (35°23′ N; 78°1′ W). Oat (cultivar Brooks) was planted in clean-tilled seedbed on October 8, 2013, at a rate of 112 kg/ha. Fertilization followed the soil test and recommendation of the North Carolina Department of Agriculture and Consumer Services Soil Testing laboratory. A total of 90 kg/ha of N was split applied in halves (at planting and on March 18, 2014) using 30% liquid N solution.

Six plots were randomly located within the experimental site when oats were at 26% DM concentration and at heading stage. Oats were mowed with a New Holland 7450 disc mower/conditioner (New Holland Agriculture, Turin, Italy) to 7-cm stubble height on May 4, 2014, allowed to wilt in the field for 21 h to 45% DM concentration, and chopped to a theoretical cut length of 1.3 cm with a John Deere 3950 forage harvester (Moline, IL). Material collected from each plot (60 kg, fresh basis) was divided into 4 piles for a total of 24 piles.

Treatments were randomly assigned to one forage pile. Treatments were 2 mini-silo types (**S**) and 2 inoculations (**I**) in a 2 × 2 factorial arrangement replicated 6 times. For S, 0.3 kg (fresh basis) of chopped whole-crop oats were packed into 0.09 mm nylon-polyethylene (66 cm^3/m^2 of film per d O_2 permeability measured at 23°C and 0% relative humidity) embossed bags (15.2 × 30.5 cm, Doug Care Equipment Inc., Springville, CA), vacuumed and sealed with a Fast Vac vacuum machine (113 mmHg vacuum level, distributed by Doug Care Equipment Inc., Springville, CA; **BG**) or 8 kg (fresh basis) were packed into 19-L plastic buckets using an A-frame 11-Mg hand press and sealed with a rubber gasket lid and duct tape (186 kg of DM/m^3; **BKT**). For I, sterile double distilled water (**CON**) or inoculant (**INO**) Biotal Buchneri 500 (Lallemand Animal Nutrition, Milwaukee, WI) dissolved in the same water was applied at a rate of 1 mL/kg of fresh oats. Inoculant application followed the manufacturer's suggested dose of log 5.6 cfu/g of fresh oats for *Lactobacillus buchneri* ATCC number 40788 and log 5 cfu/g of fresh oats for *Pediococcus pentosaceus* plus fibrolytic enzymes from *Trichoderma reesei* (1,103, 3,145, and 50 mg of sugar released/min per g for β-glucanase, xylanase, and galactomannanase activities, respectively; FCC, 2015). Silos were stored at 23°C (±1°C) for 217 d, and weights were recorded individually at d 0 and 217 for determination of DM recovery following the Arriola et al. (2011) procedure.

Sampling Procedure

At d 0 and 217, samples (250 g, fresh basis) were taken from each individual replicate for the determination of nutritional composition, fermentation profile, and the bacterial and fungal population via standard plating techniques. In the case of d 0, samples were obtained immediately after treatment application. Additional sample subsets were collected only from BKT treatment to determine aerobic stability and the composition and structure of the bacterial and fungal

communities using NGS (3 kg and 100 g fresh basis, respectively).

Laboratory Analysis

Nutritional Analysis. From samples taken at d 0 and 217, subsamples were processed for determination of DM concentration by drying at 60°C until constant weight in a forced-air oven. Dried samples were ground to pass the 1-mm screen of a Wiley mill (A.H. Thomas, Philadelphia, PA). Ground samples were analyzed for concentration of DM (105°C for 16 h) and ash (600°C in a muffle furnace for 8 h). Concentration of NDF (Van Soest et al., 1991) and ADF (AOAC, 2000) was measured sequentially using an Ankom 200 Fiber Analyzer (Ankom Technologies, Macedon, NY). Heat-stable α-amylase was used in the NDF assay with no sodium sulfite and the results were expressed inclusive of residual ash. Oat N concentration was determined using the total Kjeldahl digestion procedure (McKenzie and Wallace, 1954). Digested samples were analyzed on a Seal AQ2 discrete auto analyzer (Seal Analytical Inc., Mequon, WI) using the standard USEPA Method 353.2 (USEPA, 1993). Crude protein was calculated by multiplying N concentration by 6.25.

Water extracts were prepared by mixing 25 g of fresh or ensiled oats with 225 mL of 0.1% sterile peptone water in a 400C Stomacher blender for 3 min (Seward Ltd., Worthing, UK). The solution was filtered through 2 layers of sterilized cheesecloth and the pH of the fluid was measured with a calibrated SevenCompact pH meter fitted with an Inlab Expert Pro ISM pH electrode with an integrated temperature sensor (Mettler-Toledo LLC, Columbus, OH). Afterward, a portion of the extract was acidified to pH 2 with 50% H_2SO_4 and frozen (−30°C) for further analysis. Thawed samples were centrifuged at 8,000 × *g* for 20 min at 4°C and the supernatant was analyzed for lactic, acetic, butyric, and propionic acids, and 1,2-propanediol and ethanol concentrations (Siegfried et al., 1984) using a Waters High Performance Liquid Chromatograph system (Waters Co., Milford, MA) fitted with a Rezex RHM ion exchange column (Phenomenex, Torrance, CA) and a Waters 2414 refractive index detector. Ammonia-N concentration was measured using an adaptation of the Noel and Hambleton (1976) procedure that involved colorimetric N quantification with a Seal AQ2 discrete auto analyzer (Seal Analytical Inc.). Water-soluble carbohydrate (**WSC**) concentration was measured using the protocol by DuBois et al. (1956) using sucrose as the standard as described by Hall (2000).

Lactic Acid Bacteria, Yeast and Mold Populations, and Aerobic Stability. An aliquot was taken immediately after filtering with sterilized cheesecloth and used for enumeration of bacterial and fungal populations. Serial (10-fold) dilutions of the water extracts were done in 0.1% sterile peptone water and pour-plated in de Man, Rogosa, Sharpe agar (Oxoid, CM361, Oxoid Ltd., Waltham, MA) for lactic acid bacteria (**LAB**) and in Petrifilm Yeast and Mold Count Plates (3M Microbiology Products, St. Paul, MN). Plates were incubated for 48 h at 32°C for LAB and for 72 to 120 h at 25°C for yeast and molds.

Aerobic stability was measured only for BKT treatments by putting 3 kg of silage in an open plastic bucket (33 × 31 cm height and diameter, respectively) following the procedure described by Arriola et al. (2015). Temperature sensors (HOBO temperature data logger 64 k, Onset Computer Co., Bourne, MA) were placed at the center of the biomass, and data were recorded every 30 min for 29 d. Two additional sensors were placed in the temperature-controlled room (22.3 ± 0.23°C) to record ambient temperature. Silages were covered with 2 layers of sterile cheesecloth to prevent drying. Aerobic stability was expressed as the amount of time before silage and ambient temperatures differed by more than 2°C.

Microbial and DNA Extraction. Silage samples from BKT treatments (100 g, fresh basis) were weighed into 15.2 × 22.9 cm sterile 0.076-mm filter bags (FILTRA-BAG, VWR Co., Radnor, PA). Each bag received 150 mL of a previously sonicated (30 min) and sterile 10 m*M* potassium phosphate buffer solution at pH 7 containing Tween 20 at 0.05% (Gutierrez-Rodriguez et al., 2012). Bags containing samples were hand-massaged 6 times and sonicated for 30 min in an 8800 M Series Ultrasonic Cleaning Bath (Branson, Danbury, CT). After sonication, the supernatant was centrifuged at 18,500 × *g* for 10 min at 4°C until pellets were formed. The supernatant was discarded and the pellets were kept at –80°C awaiting DNA extraction. Extraction of DNA was done using the PowerLyzer-PowerSoil DNA isolation kit (MO BIO Labs Inc., Carlsbad, CA) following the manufacturer's recommended procedure. Resulting DNA was quantified using a NanoPhotometer Pearl (Denville Scientific Inc., Holliston, MA) and visualized by 2% agarose gel electrophoresis. The concentration of DNA for each PCR reaction was standardized for all samples at 5 ng/μL.

Next-Generation Sequencing. Extracted DNA from pellets was analyzed using the Illumina MiSeq platform for pair-end reads and 500 sequencing cycles. Amplification of the V4 hypervariable region of the 16S rRNA was achieved using the primer pair F515 (5′-CACGGTCGKCGGCGCCATT-3′) and R806 (5′-GGACTACHVGGGTWTCTAAT-3′) and the ITS-

1 region of fungi BITS (5′-*NNNNNNNN*CTACCTGCGGARGGATCA-3′) and B58S3 (5′-GAGATCCRTTGYTRAAAGTT-3′) as described by Caporaso et al. (2011) and by Bokulich et al. (2013). Amplification of the targeted region was achieved with the following reaction chemistry: 5 μL of Gotaq Green master mix (Promega, Madison, WI), 11.9 μL of DNase free water, 0.5 μL of $MgCl_2$ (50 m*M*), 0.5 μL of deoxynucleotide triphosphates (10 m*M*), 1 μL of DNA forward and reverse primers (10 μ*M*), and 5 μL of DNA template adjusted for all samples to an average final concentration of 1 ng/μL of reaction total volume. Reaction conditions for bacterial 16S amplification were as follows: an initial 95°C for 3 min, followed by 35 cycles of 95°C for 45 s, 50°C for 60 s, and 72°C for 90 s, and a final extension of 72°C for 10 min. Reaction conditions for fungal ITS-1 amplification were as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 30s, 55°C for 45 s, and 72°C for 60 s, and a final extension of 72°C for 10 min. Amplicons were mixed at roughly equivalent ratios based on electrophoretic band intensity and purified using GE Illustra MicroSpin S-300 HR columns (GE Healthcare Biosciences, Piscataway, NJ). Pooled samples were submitted to the University of California–Davis Genome Center for library preparation using the Kappa paired-end kit, cluster generation, and 250-bp paired-end sequencing (500 cycles) on the MiSeq platform.

Sequencing Analysis. Raw Illumina fastq files were demultiplexed, quality filtered (Q30), and analyzed using QIIME 1.9.1 and the GreenGenes 13.8, UNITE fungal ITS reference database. Bacterial 16S amplicon analysis (250 bp reads) was truncated at any site of more than 3 sequential bases receiving a quality score <1e-5, and any read containing ambiguous base calls or barcode/primer errors was discarded. QIIME was used to assign operational taxonomic units (**OTU**) using UCLUST, with a threshold of 97% pairwise identity. Weighted UniFrac analysis was used to determine β-diversity within treatments because this parameter is sensitive to factors that affect the relative abundance (**RA**) of OTU within inoculated and un-inoculated treatments. The OTU were classified taxonomically using a similar procedure described by Bokulich et al. (2012) using a 0.80 confidence threshold for taxonomic assignment. An ANOVA (2-sided Student's 2-sample *t*-test) was used to test significant differences in β diversity between all samples. Jackknifed principal coordinates were computed from these estimates to compress dimensionality into 3-dimensional principal coordinate analysis plots. Alpha diversity was also estimated from rarefied OTU tables to assess sampling depth coverage (Lozupone and Knight, 2005; Cole et al., 2007, 2009; Caporaso et al., 2011).

Statistical Analyses

Data were analyzed as a completely randomized design with a 2 (I) × 2 (S) factorial treatment arrangement and 6 replicates per treatment.

The model used to analyze the data was

$$Y_{ijk} = \mu + T_i + C_j + TC_{ij} + E_{ijk},$$

where μ = general mean, T_i = effect of I i, C_j = effect of S j, TC_{ij} = effect of the I i × S j interaction, E_{ijk} = experimental error.

Data were analyzed using the GLM procedure of SAS v.9.4 (SAS Institute Inc., 2003). Fixed effects in the model were I, S, and their interaction. In case a 2-factor interaction was present, the SLICE option was used to analyze the simple effects. Microbial data were converted to $\log_{10}$ to conduct statistical analysis and are presented on a fresh DM basis. Bacterial and fungal RA and the aerobic stability data were analyzed for BKT only and therefore no S factor was present in the model. Shapiro-Wilk test revealed data were normally distributed. Mean separation was based on the PDIFF procedure of LSMEANS. Treatments were considered different when $P \leq 0.05$. A trend was discussed when $0.05 < P \leq 0.10$.

To understand the overall relationships among the bacterial taxonomic profile and silage quality variables, a partial least-squares analysis (Breiman and Friedman, 1997) was conducted on measures taken at d 217. Independent variables were the RA (%) of bacterial families such as *Leuconostocaceae*, *Lactobacillaceae*, *Enterobacteriaceae*, *Streptococcaceae*, and *Microbacteriaceae*. Dependent variables were silage quality variables, such as pH, aerobic stability (h), WSC, acetic acid, lactic acid, 1,2-propanediol, ethanol (% of DM), and DM recovery (%). The analysis was performed using function 'pls' from the R package mixOmics (Lê Cao et al., 2009). A correlation plot was drawn by plotting loadings for the first 2 components for the silage quality variables and the most abundant bacterial families (*Leuconostocaceae* and *Lactobacillaceae* accounted for >99.7% RA).

RESULTS AND DISCUSSION

Before Ensiling (0 d)

Chemical Composition. We did not observe differences in pH, DM (%), OM, CP, NH_3-N, WSC, NDF, and ADF (% of DM) concentrations ($P > 0.15$ for all response variables; Table 1). The pH (~6.11 ± 0.073) and NH_3-N concentration (~0.048 ± 0.0021%) were within normal ranges for fresh oats (Mustafa and

Table 1. Chemical composition and microbial population of chopped whole-crop oats as a function of mini-silo type (S) and bacterial inoculation (I) at d 0[1]

	CON		INO[2]			*P*-value		
Item	BG	BKT	BG	BKT	SEM	S	I	S × I
DM, %	44.7	43.4	43.8	44.0	1.01	0.87	0.60	0.46
pH	6.10	6.04	6.13	6.16	0.073	0.30	0.79	0.49
% of DM								
OM	94.8	94.5	94.6	94.5	0.22	0.31	0.49	0.56
CP	6.82	7.03	7.07	6.84	0.203	0.94	0.87	0.26
NH_3-N	0.048	0.049	0.049	0.047	0.0021	0.67	0.92	0.41
WSC[3]	8.52	8.52	8.19	8.10	0.89	0.64	0.91	0.57
NDF	65.5	65.6	67.1	65.5	0.64	0.26	0.29	0.20
ADF	35.0	34.6	35.4	35.0	0.41	0.38	0.33	0.92
Log cfu/fresh g								
LAB[4]	9.37	9.28	9.31	9.19	0.129	0.57	0.91	0.42
Yeasts	5.00	4.94	4.94	4.78	0.097	0.56	0.11	0.95
Molds	3.93	3.89	4.07	3.97	0.075	0.15	0.32	0.71

[1]CON = control (water), INO = inoculant, BG = vacuum-sealed 15.2 × 30.5 cm nylon-polyethylene embossed bag, BKT = 19-L plastic bucket sealed with rubber gasket lid and duct tape.

[2]Biotal Buchneri 500 (Lallemand Animal Nutrition, Milwaukee, WI) delivering *Lactobacillus buchneri* 40788, and *Pediococcus pentosaceus* at 400,000 and 100,000 cfu/g of fresh oats, respectively.

[3]WSC = water-soluble carbohydrates.

[4]LAB = lactic acid bacteria.

Seguin, 2003) or corn (Schmidt and Kung, 2010). The WSC (~8.33 ± 0.89%) concentration was sufficient for an adequate fermentation process during ensiling (Liebensperger and Pitt, 1988) and within the expected range for small-grain cereals at heading stage (6.2–11%, Kennelly and Weinberg, 2003). We found a low CP concentration (~6.94 ± 0.203%), considering the fertilization management in this study and also compared with previously reported values for oats at the heading stage (13.2 and 15.5%; Helsel and Thomas, 1987; Kilcher and Troelsen, 1973, respectively); nevertheless, values as low as 4.0 have been reported at the same physiological stage (Haile, 1984). The reason for the low CP values is not completely clear. Concentrations of NDF and ADF were ~65.9 (±0.64%) and ~35.0 (±0.41%), respectively.

Background Microbial Population. We did not find differences ($P > 0.11$) on the initial population of LAB, yeasts, and molds for S and I ($P > 0.11$; Table 1). The background LAB population was high enough (~9.29 ± 0.129 log cfu/g of fresh oats) to provide an adequate LAB concentration for spontaneous fermentation during ensiling (Pahlow et al., 2003). Epiphytic populations of yeasts and molds were within the range of values typically reported before ensiling (~4.9 ± 0.097 and 3.47 ± 0.075 log cfu/g of fresh oats, respectively; Pahlow et al., 2003). This coincides with previous reports of increased yeast counts during the wilting of forages due to soil contact and favorable conditions for nonfermentative yeast species (McDonald et al., 1991).

Rarefaction analysis indicated that coverage of bacterial (Appendix Figure A1) and fungal (Appendix Figure A2) diversity was sufficient to evaluate the bacterial and fungal community composition of fresh and ensiled whole-crop oats from BKT treatments. Sampling saturation of OTU was met for CON and INO in both bacterial and fungal analysis. The majority of 16S sequences belonged to phyla *Firmicutes* or *Proteobacteria*, each one representing 83.6 and 16.2% of total sequences on average, respectively. Using NGS, McGarvey et al. (2013) reported that *Proteobacteria* and *Firmicutes* were the most abundant phyla, accounting for 89.6 and 8.1% of total sequences, respectively, in 10% bloom alfalfa wilted for 5.5 h to a final DM of 35%.

We did not observe INO effects ($P > 0.30$) on the 68 bacterial families detected at d 0, with *Leuconostocaceae* being the most abundant at 83.0%, followed by *Enterobacteriaceae* at 15.2%, and lower than 0.6% for *Streptococcaceae*, *Aeromonadaceae*, and *Pseudomonadaceae* (Figure 1). Relative abundances of bacterial genera identified in our study were 4.95% of genus *Weissella* for the *Leuconostocaceae* family, 1.64% of genus *Erwinia*, and 0.84% of genus *Cronobacter* for the *Enterobacteriaceae* family, and 0.60% of genus *Pseudomonas* for the *Pseudomonadaceae* family. *Weissella paramesenteroides* has been previously reported as part of the epiphytic population of wilted grass (Tohno et al., 2012), *Erwinia herbicola* in fresh Italian ryegrass (*Lolium multiflorum*, Heron et al., 1993), and *Pseudomonas syringae* in wilted Italian ryegrass (Li and Nishino, 2011). Brusetti et al. (2006) reported the relative dominance of *Weissella confusa* at d 0 in whole corn using length heterogeneity PCR. However, McGarvey et al. (2013) reported a minor

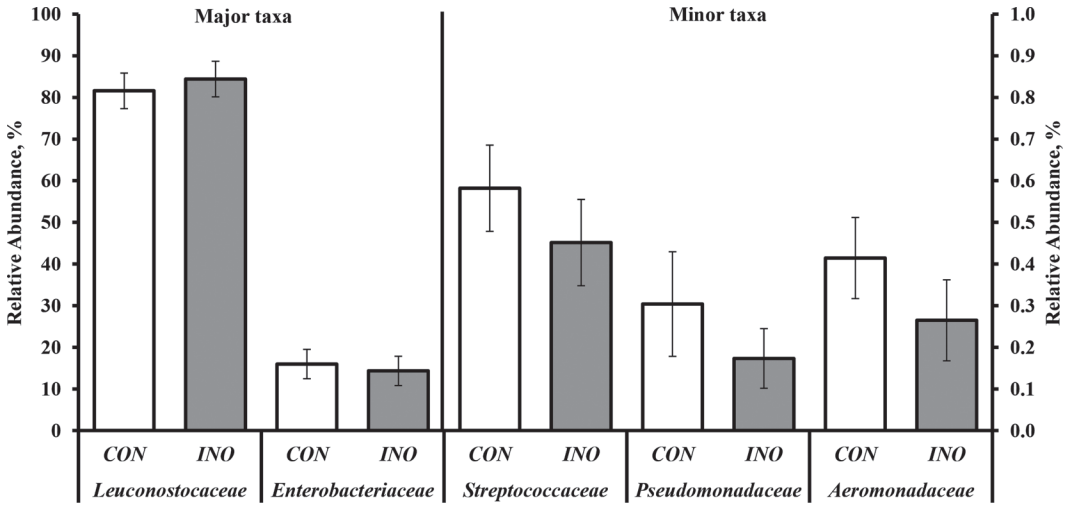

Figure 1. Effect of bacterial inoculation on bacterial families identified from 16S rDNA sequences extracted from chopped whole-crop oats at d 0. Major taxa consist of families with mean relative abundances of >2.5% and minor taxa consist of the top 5 families with mean relative abundances of >0.2 and <2.5%. CON = control (water), INO = inoculant, BKT = 19-L plastic bucket sealed with rubber gasket lid and duct tape. Bars represent means ± SEM. Inoculant used was Biotal Buchneri 500 (Lallemand Animal Nutrition, Milwaukee, WI) delivering *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* at 400,000 and 100,000 cfu/g of fresh oats, respectively.

presence of *Leuconostocaceae* in wilted alfalfa (<0.5%). Interestingly, using NGS, Williams et al. (2013) showed that the most prevalent bacterial families in a romaine lettuce study were also *Enterobacteriaceae* and *Leuconostocaceae*, with the former dominating in June and the latter in August and October, which suggests that seasonality may be a factor to consider when comparing studies assessing the microbial diversity of forages.

The predominant ITS detected sequence belonged to the *Ascomycota* phyla at 41.4% and was followed by lower *Basidiomycota* (1.4%) and *Zygomycota* (0.2%) phyla. Nevertheless, the majority of remaining fungal sequences at 56.9% RA were unidentified. We found an INO effect ($P < 0.05$) on the RA of the *Davidiellaceae* family (34.3 vs. 19.6 ± 4.47%) and unidentified *Pleosporales* (3.16 vs. 1.47 ± 0.40%) when compared with CON (Figure 2). Nevertheless, it is not clear unclear how INO could have caused these differences at d 0. No INO effect ($P > 0.30$) in any of the other 56 fungal families was detected. The family with the highest RA was *Davidiellaceae* (26.9 ± 4.47%), and it was followed by *Pleosporaceae* (5.09 ± 2.083%) and unidentified *Pleosporales* (2.32 ± 0.395%). Minor taxa (i.e., RA <2.5%) detected were *Nectriaceae*, incertae sedis *Pleosporales*, and *Mycosphaerellaceae*, unidentified *Ascomycota*, and *Debaryomycetaceae*. Few studies have reported the fungal diversity of standing forage crops and none quantified the relative differences. Breton and Zwaenepoel (1991) observed the presence of the genera *Alternaria*, *Cladosporium*, *Fusarium*, *Penicillium*, *Ascochyta*, *Phoma*, and *Phaeoseptoria* on fresh unspecified fescue grass, with an uncertain dominance of *Cladosporium* spp., which is a member of the *Davidiellaceae* family and an ubiquitous mold (Scudamore and Livesey, 1998). *Alternaria* spp. and *Fusarium* spp. are molds part of the *Pleosporaceae* and *Nectriaceae* families, respectively (Gräfenhan et al., 2011; Ariyawansa et al., 2015). Using denaturing gradient gel electrophoresis, Li and Nishino (2011) reported the presence of *Davidiella tassiana* (*Davidiellaceae*) in wilted Italian ryegrass. More information is needed to identify and quantify patterns in the fungal communities inhabiting forages, especially when mycotoxin-producing fungi are present.

Silo Opening (217 d)

Chemical Composition. We did not find differences in DM (~42.95 ± 0.93%), OM (~94.2 ± 0.22% of DM), and NH_3-N concentrations (~0.131 ± 0.0039% of DM; $P > 0.25$ for all response variables; Table 2). We found a greater CP concentration in BG versus BKT (7.73 vs. 7.04 ± 0.247% of DM, respectively; $P = 0.01$) in accord with lower NH_3-N (as % of total N) for BG versus BKT (10.7 vs. 11.7 ± 0.45, respectively; $P = 0.01$). We hypothesize that differences in CP are the result of greater temperature in BKT versus BG that increased proteolysis during the aerobic phase of ensiling (McDonald et al., 1991; Rooke and Hatfield, 2003), due to biomass differences (8.0 vs. 0.3 kg, fresh basis, respectively; McDonald et al., 1966). Also, slightly increased respiration losses from BG versus BKT could have occurred, increasing CP and tending to increase

NDF (65.5 vs. 66.5 ± 0.57% of DM, respectively; $P = 0.08$). Silo pH at opening was not different in BG versus BKT (4.34 vs. 4.32 ± 0.026, respectively; $P = 0.68$, Table 3). A similar rate of pH decline has been reported in the literature for vacuum bags (Johnson et al., 2005; Hoedtke and Zeyner, 2011).

We found lower NDF (65.0 vs. 67.0 ± 0.57% of DM, respectively; $P = 0.002$) and lower ADF (36.7 vs. 38.1, ± 0.60% of DM, respectively; $P < 0.03$; Table 2) in INO versus CON. We hypothesize that the lower fiber concentrations in INO are a consequence of the presence of the exogenous fibrolytic enzymes from *T. reesei* found in the inoculant product, which has the potential to solubilize fiber, releasing mainly WSC available for fermentation during ensiling (Kung et al., 2003). Our results agree with the results of Arriola et al. (2011) who used the same inoculant product and reported a lower NDF concentration in corn silage when the inoculated silage was compared with noninoculated silage (42 vs. 44, respectively; % of DM). However, other reports from experiments using the same inoculant indicate no effect on NDF concentration of corn (Reich and Kung, 2010; Queiroz et al., 2013) or bermudagrass silage (Adesogan et al., 2004; Arriola et al., 2015). Reports are limited on small-grain cereal silage trials using the inoculant product used in this study or with the same combination of bacterial species. Zahiroddini et al. (2006) used a comparable inoculant product containing *L. buchneri* and *P. pentosaceus* (Biotal Inc., Eden Prairie, MN) applied at a lower total LAB dose (1.19×10^5 cfu/g fresh forage) to barley (*Ordeum vulgare* L.) at the soft dough stage. The authors reported no effect of inoculant addition on NDF concentration. The fungal source of the enzymes contained in the inoculant was not disclosed (Zahiroddini et al., 2006). We hypothesize that relative immaturity of the oats used in this study (heading stage, before seed starch deposition) resulted in a NDF more susceptible to enzymatic hydrolysis, as less mature forage is more amenable to fibrolytic enzyme action (Romero et al., 2013).

We found an interaction effect of S × I on WSC concentration ($P = 0.021$; Table 2). In INO, WSC concentration was not different between BG and BKT (~1.53 ± 0.162% of DM); however, in CON we found greater

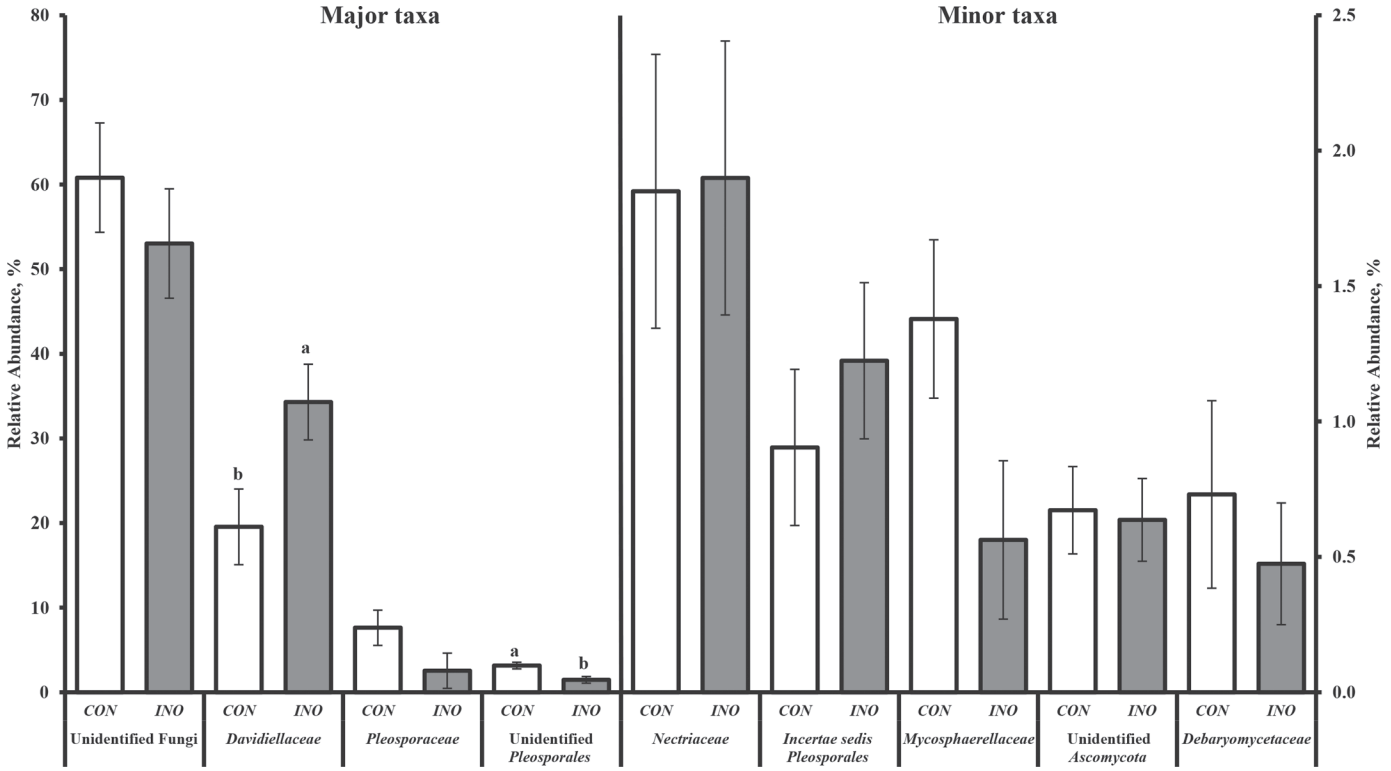

Figure 2. Effect of bacterial inoculation on fungal families identified from internal transcribed spacer (ITS) region sequences extracted from chopped whole-crop oats at d 0. Major taxa consist of families with mean relative abundances of >2.5% and minor taxa consist of the top 5 families with mean relative abundances of >0.2 and <2.5%. CON = control (water), INO = inoculant, BKT = 19-L plastic bucket sealed with rubber gasket lid and duct tape. Bars represent means ± SEM. Inoculant used was Biotal Buchneri 500 (Lallemand Animal Nutrition, Milwaukee, WI) delivering *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* at 400,000 and 100,000 cfu/g of fresh oats, respectively. Within each taxon, bars with different letters differ ($P < 0.05$).

Table 2. Nutritional composition of chopped whole-crop oats as a function of mini-silo type (S) and bacterial inoculation (I) at d 217[1]

	Treatment				*P*-value		
Item	CON	INO[2]	Mean	SEM	S	I	S × I
DM, %				0.93	0.77	0.90	0.72
BG	43.2	43.0	43.1				
BKT	42.6	43.0	42.8				
Mean	42.9	43.0					
OM, % of DM				0.22	0.25	0.32	0.52
BG	94.1	94.1	94.1				
BKT	94.2	94.5	94.4				
Mean	94.1	94.3					
CP, % of DM				0.247	0.01	0.94	0.45
BG	7.81	7.64	7.73^{A}				
BKT	6.93	7.14	7.04^{B}				
Mean	7.37	7.39					
NH_3-N, % of DM				0.0039	0.83	0.68	0.63
BG	0.128	0.131	0.130				
BKT	0.131	0.131	0.131				
Mean	0.130	0.131					
NH_3-N, % of total N				0.45	0.04	0.62	0.17
BG	10.3	11.1	10.7^{B}				
BKT	11.8	11.5	11.7^{A}				
Mean	11.1	11.3					
NDF, % of DM				0.57	0.08	0.002	0.42
BG	67.7	65.3	66.5				
BKT	66.2	64.7	65.5				
Mean	67.0^{a}	65.0^{b}					
ADF, % of DM				0.60	0.71	0.03	0.62
BG	38.0	36.9	37.5				
BKT	38.1	36.4	37.3				
Mean	38.1^{a}	36.7^{b}					
WSC,[3] % of DM				0.162	0.002	<0.001	0.021
BG	1.78^{B}	1.45	1.61				
BKT	2.70^{A,a}	1.60^{b}	2.15				
Mean	2.24	1.53					

$^{A,B;a,b}$Means with different uppercase letters within a column and lowercase letters within a row are different, $P \leq 0.05$.

[1]CON = control (water), INO = inoculant, BG = vacuum-sealed 15.2 × 30.5 cm nylon-polyethylene embossed bag, BKT = 19-L plastic bucket sealed with rubber gasket lid and duct tape.

[2]Biotal Buchneri 500 (Lallemand Animal Nutrition, Milwaukee, WI) delivering *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* at 400,000 and 100,000 cfu/g of fresh oats, respectively.

[3]Water-soluble carbohydrates (WSC).

WSC in BKT versus BG (2.70 vs. 1.78 ± 0.162% of DM, respectively). Accordingly, lower WSC was observed in INO versus CON within BKT (1.60 vs. 2.70 ± 0.162% of DM, respectively), but in BG, WSC was not different for INO and CON (~1.61 ± 0.162% of DM). Addition of inoculant resulted in a more consistent residual WSC value, which is the net balance between WSC supplied (including enzyme released) and used for fermentation by microbes. The reasons behind this response remain unclear, but increased *L. buchneri* count application has been related to decreased WSC concentrations in a meta-analysis of small-grain and grass silage experiments (Kleinschmit and Kung, 2006a). Higher residual WSC is nutritionally desirable as it is rapidly digestible in the rumen (Van Amburgh et al., 2015) but also carries a higher risk of yeast spoilage during silo opening if not enough acetate or propionate is present (McDonald et al., 1991).

Fermentation Parameters and Aerobic Stability. We found greater DM recovery in INO versus CON (96.1 vs. 92.9 ± 0.63%; $P < 0.001$, respectively; Table 3). The DM recovery was not different for BKT and BG (~94.5 ± 0.63%; $P = 0.18$). Similar DM recovery values have been reported for treated (with the same inoculant) and nontreated corn (Schmidt and Kung, 2010; Reich and Kung, 2010; Queiroz et al., 2013) and bermudagrass silages (Adesogan et al., 2004; Arriola et al., 2015). Contrary to our findings, Zahiroddini et al. (2006) reported lower DM recovery for barley silage when a similar inoculant was added compared with noninoculated (88.6 vs. 92.5%) with also a greater final pH (3.92 vs 3.60). The DM in Zahiroddini et al. (2006) was 35.6 compared with 43% in the present experiment, which could explain different responses in DM recovery due to the depressed fermentation typical of low-moisture silages (McDonald et al., 1991) and reflected

in the final pH values for each study (~3.8 vs. 4.3, respectively). Greater DM recovery in INO compared with CON could be partially explained by a lower ethanol production (0.63 vs. 2.85 ± 0.155% of DM, respectively; $P < 0.001$) indicating less inefficient secondary fermentation by yeasts and heterofermentative bacteria (Pahlow et al., 2003) and a similar production of lactic acid that ensured rapid acidification (3.89 vs. 4.03 ± 0.241% of DM; $P = 0.56$, respectively). The final pH was lower in INO versus CON (4.25 vs. 4.41 ± 0.026; $P < 0.001$, respectively).

The slight extra acidity of INO came from an increased acetic acid production typical of *L. buchneri* fermentations, compared with CON (2.38 vs. 1.22 ± 0.116% of DM, respectively; $P < 0.001$; Muck and Kung, 2007). Zahiroddini et al. (2006) also reported greater acetic acid concentration when a similar inoculant was applied in barley silage compared with untreated (2.57 vs. 1.95% of DM) and similar lactic acid concentrations. The production of 1,2-propanediol in INO versus CON (0.692 vs. 0.013 ± 0.053% of DM, respectively; $P < 0.001$) confirms successful growth of *L. buchneri* as 1,2-propanediol is produced along with acetic acid and traces amount of ethanol from lactic acid degradation when silage pH reaches a value lower than 5.8 during the later stages of fermentation (>56 d of ensiling; Oude Elferink et al., 2001; Kleinschmit and Kung, 2006b).

Differences were observed between BKT and BG for lactic acid (4.28 vs. 3.65 ± 0.241% of DM; $P = 0.02$, respectively) and ethanol concentrations (1.55 vs. 1.93 ± 0.155% of DM; $P = 0.02$, respectively; Table 3). No differences between BKT and BG were found for pH, acetic acid, and 1,2-propanediol (~4.33 ± 0.026, 1.80

Table 3. Fermentation parameters of chopped whole-crop oats as a function of mini-silo type (S) and bacterial inoculation (I) at d 217[1]

	Treatment				*P*-value		
Item	CON	INO[2]	Mean	SEM	S	I	S × I
DM recovery, %				0.63	0.18	<0.001	0.50
BG	92.3	95.9	94.1				
BKT	93.6	96.3	94.9				
Mean	92.9[b]	96.1[a]					
pH				0.026	0.68	<0.001	0.30
BG	4.40	4.27	4.34				
BKT	4.42	4.23	4.32				
Mean	4.41[a]	4.25[b]					
Lactic acid (L), % of DM				0.241	0.02	0.56	0.15
BG	3.90	3.40	3.65[B]				
BKT	4.17	4.39	4.28[A]				
Mean	4.03	3.89					
Acetic acid (A), % of DM				0.116	0.08	<0.001	0.08
BG	1.22	2.16	1.69				
BKT	1.21	2.59	1.90				
Mean	1.22[b]	2.38[a]					
1,2-Propanediol, % of DM				0.053	0.41	<0.001	0.21
BG	0.025	0.634	0.330				
BKT	0.000	0.749	0.375				
Mean	0.013[a]	0.692[b]					
Ethanol, % of DM				0.155	0.02	<0.001	0.84
BG	3.06	0.80	1.93[A]				
BKT	2.65	0.45	1.55[B]				
Mean	2.85[a]	0.63[b]					
L:A ratio				0.124	0.20	<0.001	0.69
BG	3.24	1.58	2.41				
BKT	3.46	1.70	2.58				
Mean	3.35[a]	1.64[b]					
L:(A+OH) ratio[3]				0.075	0.03	0.51	0.82
BG	0.93	0.96	0.95[B]				
BKT	1.09	1.15	1.12[A]				
Mean	1.01	1.06					

[A,B;a,b]Means with different uppercase letters within a column and lowercase letters within a row are different, $P \leq 0.05$.

[1]CON = control (water), INO = inoculant, BG = vacuum-sealed 15.2 × 30.5 cm nylon-polyethylene embossed bag, BKT = 19-L plastic bucket sealed with rubber gasket lid and duct tape.

[2]Biotal Buchneri 500 (Lallemand Animal Nutrition, Milwaukee, WI) delivering *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* at 400,000 and 100,000 cfu/g of fresh oats, respectively.

[3]OH = ethanol plus 1,2-propanediol.

± 0.116, 0.353 ± 0.053, respectively; $P > 0.08$). In contrast, Hoedtke and Zeyner (2011) reported a higher lactic acid concentration in vacuum bags compared with fixed volume vessels for wilted perennial ryegrass (6.5 vs. 2.0% of DM) and similar concentrations of acetic acid and ethanol. Differences between our study and Hoedtke and Zeyner (2011) may be explained by the type of epiphytic microbial communities originally present in the forage sources used. Interestingly, very high butyric acid (6.6% of DM) and propionic acid concentrations (4.3% of DM) were reported in Hoedtke and Zeyner (2011), suggesting the presence of *Clostridia* and propionic acid bacteria, respectively. Propionic and butyric acids were not detectable in this study for any treatment (<0.014% of DM). The lactic to acetic acid ratio indicates a more homolactic fermentation in CON compared with INO (3.35 vs. 1.64 ± 0.124; $P < 0.001$; Table 3), in its traditional interpretation. However, when using a ratio that also includes the alcohol produced during fermentation (lactic acid to acetic acid plus 1,2-propanediol plus ethanol ratio; L:(A+OH) ratio), no difference was observed between CON and INO (~1.04 ± 0.075; $P = 0.51$). A difference was observed for the L:(A+OH) ratio between BKT and BG (1.12 vs. 0.95 ± 0.075; $P = 0.03$).

Aerobic stability was only measured for BKT because the amount of forage in vacuum bags was lower than needed (>2 kg fresh) for conducting an aerobic stability study. Nevertheless, we envision using vacuum bags as a first screening tool for large number of treatments before moving to intensive analysis such as aerobic stability or metagenomics. The INO increased aerobic stability compared with CON (Figure 3; 565 vs. 133 ± 29.2 h, respectively; $P < 0.001$). Greater aerobic stability in INO was most likely the result of increased acetic acid production by *L. buchneri* found in the inoculant product, which has the potential to reduce spoilage after silo opening by inhibiting undesirable fungi (Kleinschmit and Kung, 2006a). Similar improvements of aerobic stability have been reported for corn (Schmidt and Kung, 2010; Reich and Kung, 2010; Queiroz et al., 2013) and bermudagrass silages (Arriola et al., 2015). Nevertheless, Zahiroddini et al. (2006) reported no differences in aerobic stability by applying a similar inoculant to barley silage ensiled for 61 d compared with untreated.

Silage Microbial Population. We observed a higher population of LAB (Table 4) in INO versus CON (6.39 vs. 5.65 ± 0.255 log cfu/fresh g; $P = 0.006$), suggesting the successful establishment of *L. buchneri* as seen by an increased acetic acid production while maintaining a similar lactic acid concentration compared with CON. Previous studies have reported also increases in LAB counts in corn silage when the same inoculant was used (Reich and Kung, 2010; Schmidt and Kung, 2010). Zahiroddini et al. (2006) reported an increase in LAB counts when a similar inoculant was applied to barley silage (7.96 vs. 8.95 log cfu/ fresh g). Similar counts of LAB were observed between BKT and BG (~6.02 ± 0.255 log cfu/fresh g; $P = 0.26$). No LAB counts were reported by Johnson et al. (2005) or Hoedtke and Zeyner (2011).

At d 217, a significant shift occurred compared with d 0 with almost all 16S sequences being part of the *Firmicutes* phylum (99.8% of total sequences) for both CON and INO treatments. The *Proteobacteria* and *Actinobacteri*a phyla represented only 0.07 and 0.03% of total sequences, respectively. Using NGS, McGarvey et al. (2013) reported also a similar population shift, with *Firmicutes* and *Proteobacteria* being the most abundant phyla and accounting for 70.6 and 26.9% of total sequences, respectively, in alfalfa ensiled for 40 d. Furthermore, Eikmeyer et al. (2013) also described the relative dominance of the *Firmicutes* phylum in an unspecified grass ensiled after 14 and 58 d (86 and 87%, respectively) using NGS. We found that INO addition largely increased the RA of the *Lactobacillaceae* family when compared with CON in the ensiled material (57.4 vs. 3.9 ± 4.65%, respectively) and reduced the *Leuconostocaceae* family (42.3 vs. 95.8 ± 4.64%, respectively; $P < 0.001$; Figure 4). Eikmeyer et al. (2013) observed an increase in the RA of the *Lactobacillus* genus (*Lactobacillaceae* family) in grass treated with

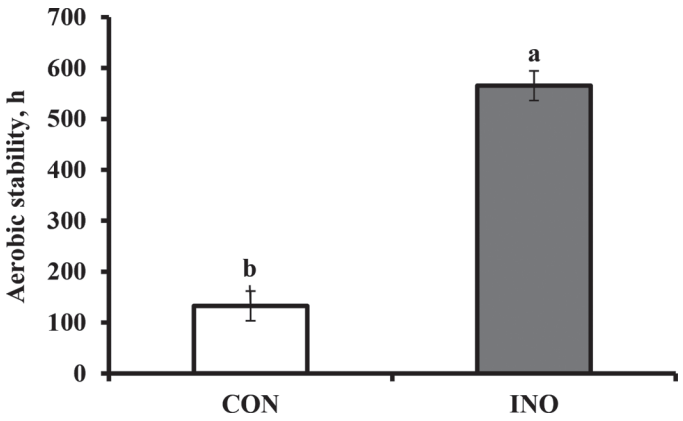

Figure 3. Effect of bacterial inoculation on aerobic stability (elapsed hours before silage and ambient temperatures, 22.3°C, differed by >2°C) of chopped whole-crop oats ensiled in a BKT and opened after 217 d of fermentation. CON = control (water), INO = inoculant, BKT = 19-L plastic bucket sealed with rubber gasket lid and duct tape. Bars represent means ± SEM. Inoculant used was Biotal Buchneri 500 (Lallemand Animal Nutrition, Milwaukee, WI) delivering *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* at 400,000 and 100,000 cfu/g of fresh oats, respectively. Bars with different letters differ ($P < 0.001$; SEM = 29.2).

Table 4. Microbial counts of chopped whole-crop oats as a function of mini-silo type (S) and bacterial inoculation (I) at d 217[1]

	Treatment				*P*-value		
Item	CON	INO[2]	Mean	SEM	S	I	S × I
Lactic acid bacteria, log cfu/fresh g				0.255	0.26	0.006	0.29
BG	5.66	6.67	6.16				
BKT	5.64	6.12	5.88				
Mean	5.65[b]	6.39[a]					
Yeast, log cfu/fresh g				0.484	0.09	<0.001	0.43
BG	3.89	0.47	2.18				
BKT	4.36	1.72	3.04				
Mean	4.13[a]	1.10[b]					
Molds, log cfu/fresh g				0.409	0.01	0.53	0.04
BG	0.80[B]	1.42	1.11				
BKT	2.82[A]	1.68	2.25				
Mean	1.81	1.55					

[A,B;a,b]Means with different uppercase letters within a column and lowercase letters within a row are different, $P \leq 0.05$.

[1]CON = control (water), INO = inoculant, BG = vacuum-sealed 15.2 × 30.5 cm nylon-polyethylene embossed bag, BKT = 19-L plastic bucket sealed with rubber gasket lid and duct tape.

[2]Biotal Buchneri 500 (Lallemand Animal Nutrition, Milwaukee, WI) delivering *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* at 400,000 and 100,000 cfu/g of fresh oats, respectively.

L. buchneri (6 log cells/g of fresh grass) after 14 d (34 vs. 31%) and more after 58 d (67 vs. 35%) of ensiling when compared with untreated silage, respectively. As a result, the *Lactococcus* (*Streptococcaceae* family), and to a lesser degree, the *Leuconostoc* and *Weisella* genera (*Leuconostocaceae* family) were more abundant in the untreated silages (Eikmeyer et al., 2013). *Lactobacillaceae* also had the highest RA (41.2%) in the alfalfa silage study of McGarvey et al. (2013). In CON, *Weissella* and *Leuconostoc* were the prevailing identifiable genera for *Leuconostocaceae* (6.83 and 4.54%, respectively) and for *Lactobacillaceae* was *Pediococcus* (2.43%). In the case of INO, the predominant genus for *Lactobacillaceae* was *Lactobacillus* (56.9%) and *Weissella* and *Leuconostoc* were the prevailing identifiable genera for *Leuconostocaceae* (4.11 and 2.82%).

Principal coordinate analysis plots indicated a clear separation and difference in the distribution and structure of the bacterial community at d 0 and 217 and within d 217, between the CON and INO (Figure 5, panel A). This was further confirmed by the *t*-test statistical analysis performed for the weighted β-diversity analysis, confirming the turnover of species within treatments and time points (data not shown). Although some bacterial taxa were shared between time points and treatments, the overall community structure was changed for the whole-crop oats silage treated with INO.

A trend was observed for greater yeast populations in BKT versus BG (3.04 vs. 2.18 ± 0.484 log cfu/g of fresh oats; $P = 0.09$), although the biological meaningfulness in a silage context can be debatable. We found an interaction effect of S × I on mold counts ($P = 0.04$; Table 4). Inoculant application resulted in similar populations of molds for BKT versus BG (~1.55 ± 0.409 log cfu/g of fresh oats); however, in the absence of inoculant the population of these microorganisms was 100-fold higher

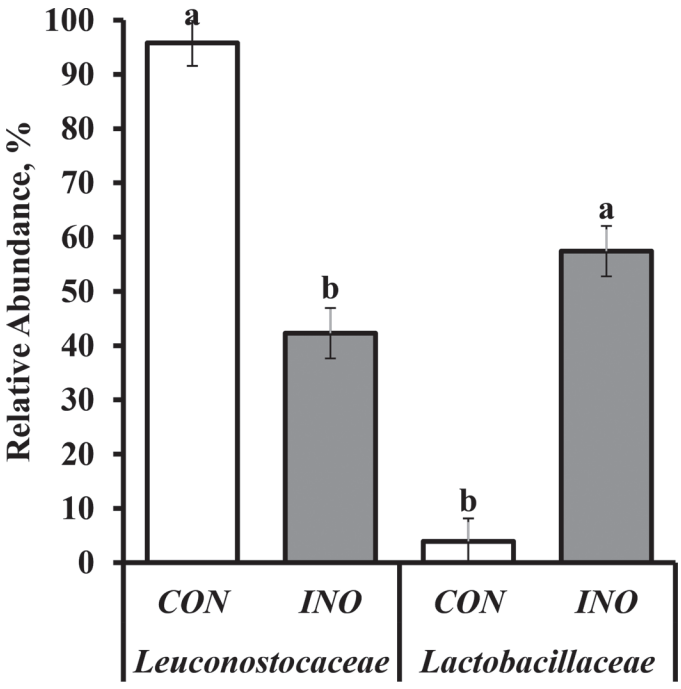

Figure 4. Effect of bacterial inoculation on bacterial families identified from 16S rDNA sequences extracted from chopped whole-crop oats ensiled for 217 d. CON = control (water), INO = inoculant, BKT = 19-L plastic bucket sealed with rubber gasket lid and duct tape. Bars represent means ± SEM. Inoculant used was Biotal Buchneri 500 (Lallemand Animal Nutrition, Milwaukee, WI) delivering *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* at 400,000 and 100,000 cfu/g of fresh oats, respectively. Within each taxon, bars with different letters differ ($P < 0.05$).

in BKT versus BG (2.82 vs. 0.8 ± 0.409 log cfu/fresh g, respectively). These differences could be explained due to lower availability of oxygen during the fermentation process in BG compared with BKT (Johnson et al., 2005). No yeast and mold counts were reported by Johnson et al. (2005) or Hoedtke and Zeyner (2011).

We found lower yeast populations in INO versus CON samples (1.10 vs. 4.13 ± 0.484 log cfu/g of fresh oats; $P < 0.001$, respectively); nevertheless, mold populations were not different for INO and CON (~1.68 ± 0.409 log cfu/g of fresh oats; $P = 0.53$). The same inoculant used in this study has been reported to reduce yeast populations when compared with noninoculated treatments in corn silage (Kleinschmit and Kung, 2006b; Schmidt and Kung, 2010; Reich and Kung, 2010) but not in bermudagrass silage (Adesogan et al., 2004; Arriola et al., 2015). Mold populations have also been reported to decrease by the same inoculant when used to ensile corn (Schmidt and Kung, 2010) but not in other reports (Reich and Kung, 2010; Arriola et al., 2011). For bermudagrass, Adesogan et al. (2004) and Arriola et al. (2015) reported no differences in mold counts compared with noninoculated silage. For barley silage, Zahiroddini et al. (2006) reported no differences in yeast counts (<2.00 log cfu/fresh g) and mold counts (undetectable) with a similar inoculant. Lower populations of yeast and molds in INO samples are a consequence of higher acetic acid concentrations as described in Table 3, and leading to higher aerobic stability when compared with CON samples (Figure 3). Acetic acid exerts antimicrobial action against yeasts and molds by penetrating their membrane in its undissociated form at silage pH, releasing H^+ in the cytosol, which causes expenditure of ATP by yeast to maintain homeostasis (Walker, 1998; Danner et al., 2003).

In this study at d 217 for BKT, the predominant fungal phylum was *Ascomycota* (97.4%) and it was followed by lower than 1% for both *Basidiomycota* and *Zygomycota*. The remaining 1.48% were unidentified fungal sequences. Most of the fungi reported by May et al. (2001) in corn silage using denaturing gradient gel electrophoresis also belonged to the *Ascomycota*, followed by *Basidiomycota* and to a lesser degree to *Zygomycota*. A tendency was observed for INO to increase the RA of an unidentified sequence of the *Ascomycota* phyla when compared with CON (11.3 vs. 0.5 ± 4.00%, respectively; Figure 6). No effect of INO ($P > 0.21$) was observed for *Pichiaceae* (~61.9 ± 20.68%), *Trichocomaceae* (~7.9 ± 6.71%), an unidentified sequence from the *Saccharomycetales* order (~13.9

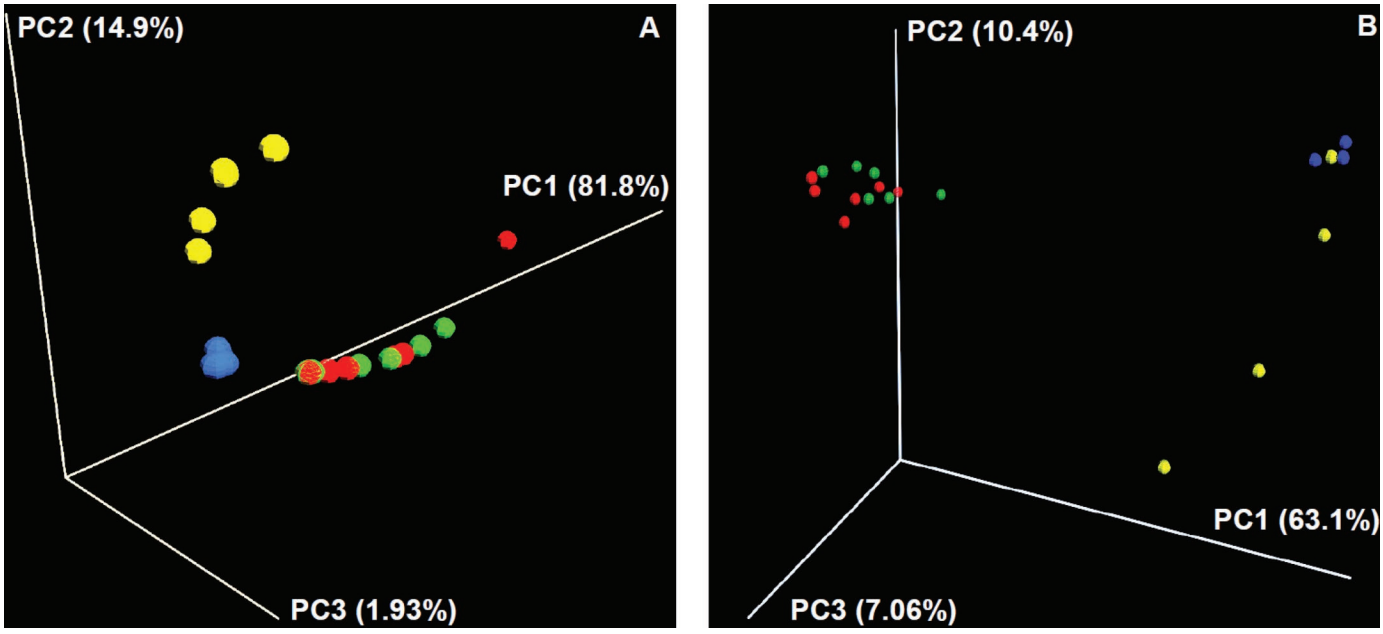

Figure 5. Principal coordinate (PC) analysis plots for bacterial and fungal families coming from whole-crop oats ensiled in a BKT with (INO) or without (CON) an inoculant and opened after 217 d of fermentation. CON = control (water), INO = inoculant. BKT = 19-L plastic bucket sealed with rubber gasket lid and duct tape. Inoculant used was Biotal Buchneri 500 (Lallemand Animal Nutrition, Milwaukee, WI) delivering *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* at 400,000 and 100,000 cfu/g of fresh oats, respectively. Panel A represent bacterial β diversity (weighted-UniFrac). Green dots indicate INO replicates at 0 d, whereas the red circles indicate the CON replicates at 0 d. Yellow circles indicate INO replicates at 217 d, whereas blue circles indicate the CON replicates at 217 d. Panel B represent fungal β diversity (Bray-Curtis). Green dots indicate INO replicates at 0 d, whereas the red circles indicate the CON replicates at 0 d. Yellow circles indicate INO replicates at 217 d, whereas blue circles indicate the CON replicates at 217 d. Color version available online.

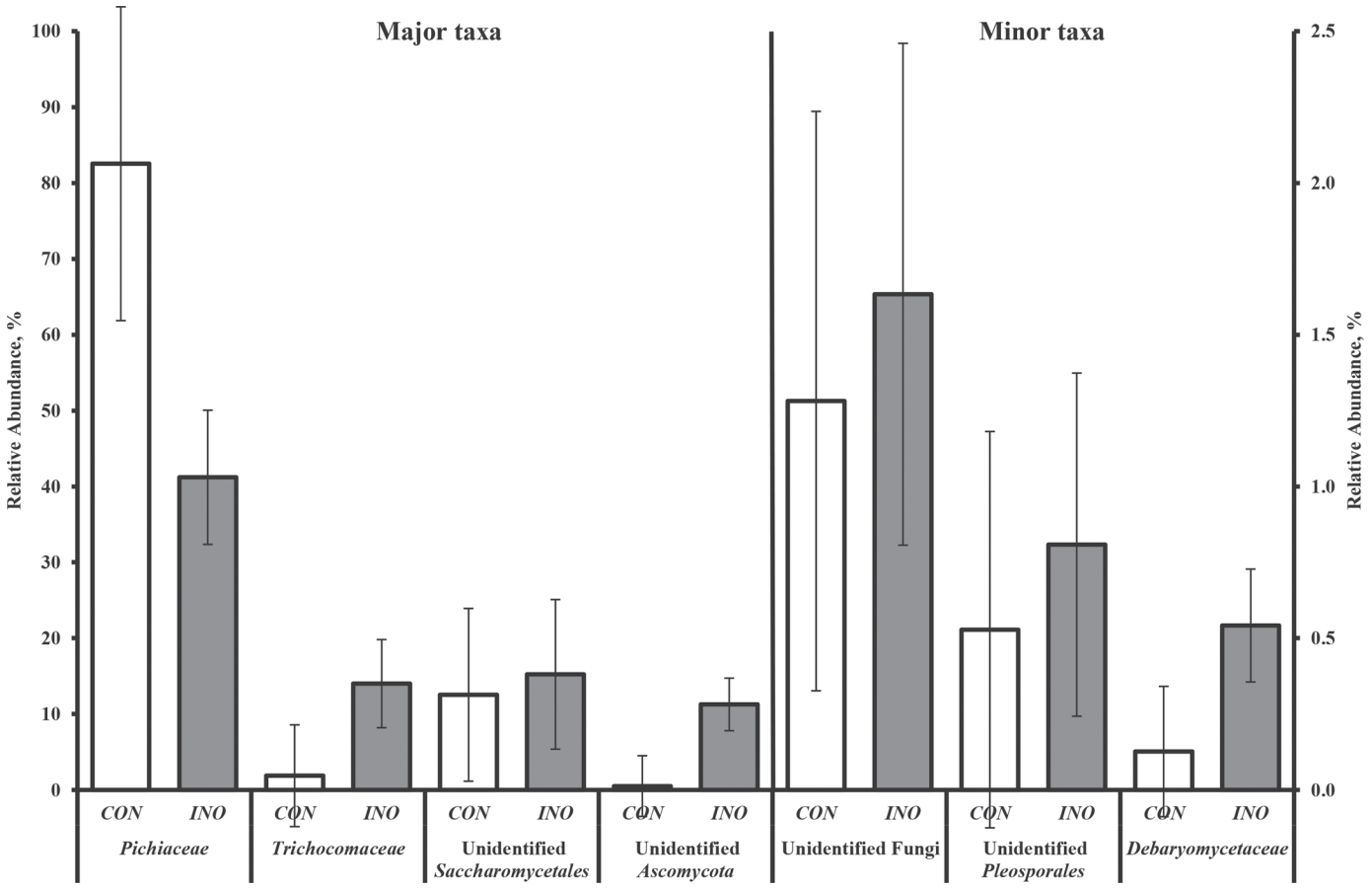

Figure 6. Effect of bacterial inoculation on fungal families identified from internal transcribed spacer region sequences extracted from chopped whole-crop oats ensiled for 217 d. Major taxa consist of families with mean relative abundances of >2.5% and minor taxa consist of the top 5 families with mean relative abundances of >0.2 and <2.5%. CON = control (water), INO = inoculant, BKT = 19-L plastic bucket sealed with rubber gasket lid and duct tape. Bars represent means ± SEM. Inoculant used was Biotal Buchneri 500 (Lallemand Animal Nutrition, Milwaukee, WI) delivering *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* at 400,000 and 100,000 cfu/g of fresh oats, respectively.

± 11.39%, respectively), and unidentified fungi (~1.5 ± 0.96%), when compared with CON. Few studies have assessed the fungal diversity in ensiled forages, and none quantified the relative differences. May et al. (2001) reported the presence of *Aspergillus clavatus* (*Trichocomaceae*), *Candida* spp. (*Debaryomycetaceae*), and *Pichia anomola* (*Pichiaceae*) in corn silage treated or not with an inoculant containing *Lactobacillus plantarum*, *Enterococcus faecium*, and *L. buchneri* applied at a rate of 5.7 log cfu/g and a second undescribed inoculant. In a corn silage ensiled for 3 mo, May et al. (2001) was only able to observe a fainter band for all the other fungi except for a band that may be *Gomphus floccosus* (*Gomphaceae*) for the combination inoculant treatment compared with untreated corn silage. In that study, the undescribed inoculant denaturing gradient gel electrophoresis band pattern (i.e., fungal diversity) was undistinguishable from the untreated silage. Li and Nishino (2011) reported only the presence of *Saccharomyces cerevisiae* in wilted Italian ryegrass ensiled for 120 d with the disappearance of the *D. tassiana* band observed in the pre-ensiled crop. Similarly, in this study, *Davidiellaceae* was only found at d 0 but disappeared at d 217. Using principal coordinate analysis plots we observed a clear separation and difference in the distribution and structure of the fungal community at d 0 and 217 but no clear separation within both sampling dates between CON and INO (Figure 5, panel B). This was further confirmed by having similar β-diversity analysis between the INO and CON samples (data not shown). More research needs to be conducted to improve our knowledge of fungal communities in silages.

Relationship Between Silage Bacterial Taxonomic Profile and Quality Variables. The correlation plot is reported in Figure 7. The position over the 2 dimensions on the graph indicates how variables clustered. The angle included between the arrows pointing at 2 variables determines the correlation between

the variables: (very) sharp angles define (strong) and positive correlations, squared angles define a null correlation, (very) obtuse angles define (strong) negative correlations. *Lactobacillaceae* was positively correlated with acetic acid, aerobic stability, and DM recovery. *Lactobacillaceae* was the most abundant bacterial family in INO, which includes *L. buchneri,* and *Lactobacillus* was the most predominant genus for that family in this study. The positive correlation of DM recovery and *Lactobacillaceae*, may be explained by the negative correlation between *Lactobacillaceae* and ethanol and pH, reflected in the lower ethanol production and pH in INO versus CON. *Leuconostocaceae* were positively correlated with pH and ethanol. *Leuconostocaceae* are heterofermentative LAB, usually producing lactic acid, CO_2, and ethanol during glucose metabolism by the pentose phosphate pathway (Endo et al., 2014). We found weak correlations between lactic acid, 1,2-propanediol, and WSC with bacterial RA.

CONCLUSIONS

At silo opening, BKT had lower CP and ethanol, and higher lactic acid, NH_3-N (N basis) concentrations, and L:(A+OH) ratio compared with BG. In addition, WSC and mold counts were reduced in BG compared with BKT for CON, but no difference was observed for INO. Because differences between BKT and BG were relatively small, although statistically significant, we conclude that both techniques of silo type are comparable for characterizing effects of INO on the most basic measures of silage quality research used in small-grain silage evaluation. Application of INO increased DM recovery, aerobic stability, acetic acid, 1,2-propanediol, and reduced NDF, ADF, ethanol, yeasts, WSC in BKT but not in BG, compared with CON. When evaluated only in BKT at d 217, INO favored the dominance of *Lactobacillaceae* compared with *Leuconostocaceae* bacterial family dominance in CON. No effects of INO

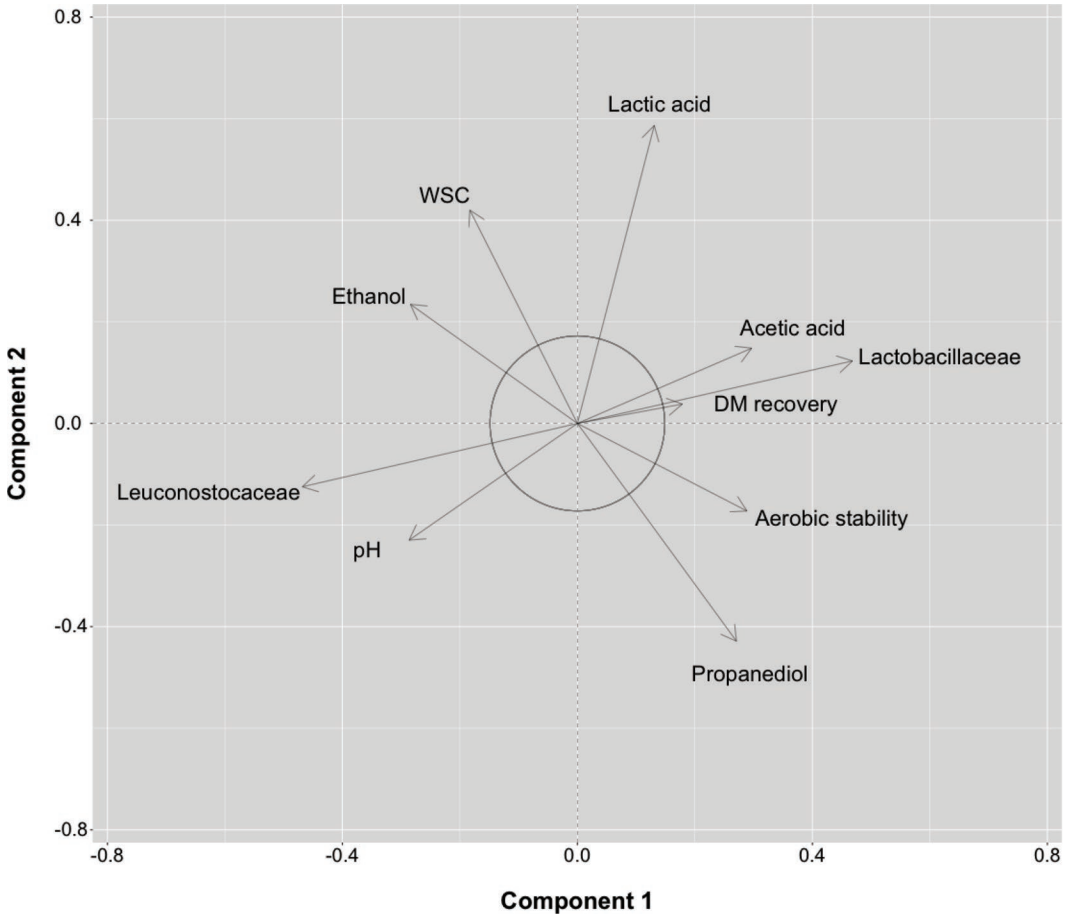

Figure 7. Correlation plot among taxonomic profile and silage quality variables. The variable loadings for the 2 components from the partial least squares analysis are reported on the axis. Position over the 2 dimensions on the graph indicates how variables clustered. The angle included between the arrows pointing at 2 variables determines the correlation between the variables: (very) sharp angles define (strong) positive correlations, squared angles define a null correlation, (very) obtuse angles define (strong) negative correlations. WSC = water-soluble carbohydrates.

were observed on the fungal community RA. The INO used in this study improved small-grain silage quality partially by a shift in the bacterial community composition during ensiling.

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APPENDIX

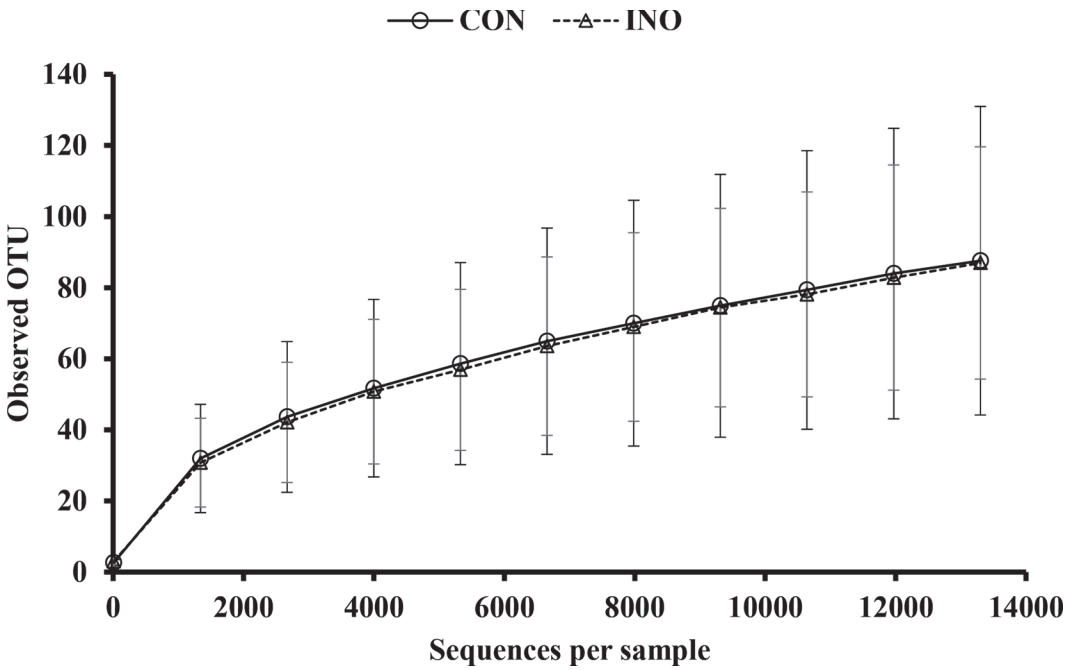

Figure A1. Rarefaction curves for bacterial operational taxonomic units (OTU) in fresh and ensiled whole-crop oats treated with an inoculant in BKT. CON = control (water), INO = inoculant, BKT = 19-L plastic bucket sealed with rubber gasket lid and duct tape. Error bars represent SEM values. Inoculant used was Biotal Buchneri 500 (Lallemand Animal Nutrition, Milwaukee, WI) delivering *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* at 400,000 and 100,000 cfu/g of fresh oats, respectively.

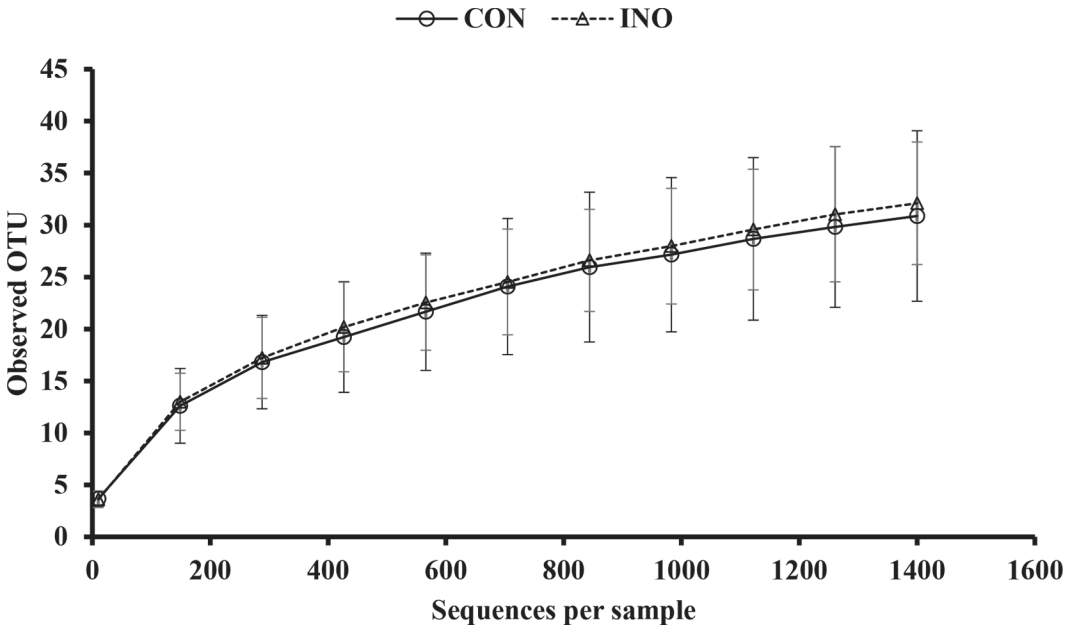

Figure A2. Rarefaction curves for fungal operational taxonomic units (OTU) in fresh and ensiled whole-crop oats treated with an inoculant in BKT. CON = control (water), INO = inoculant, BKT = 19-L plastic bucket sealed with rubber gasket lid and duct tape. Error bars represent SEM values. Inoculant used was Biotal Buchneri 500 (Lallemand Animal Nutrition, Milwaukee, WI) delivering *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* at 400,000 and 100,000 cfu/g of fresh oats, respectively.