Analysis of the effects of early or late air stress during storage and inoculation on the microbial community composition of corn silage by next-generation sequencing

L. Kung Jr.[1], E.B. da Silva[2,*], R.M. Savage.[1], S.A. Polukis.[1], M.L. Smith[1], A.C.B. Johnson[1], M.A. Miller[3].

*[1]Department of Animal and Food Sciences University of Delaware, Newark, DE, 19716, United States, [2]University of São Paulo, Piracicaba, SP, 13418-900, Brazil, *Coordination for the Improvement of Higher Education Personnel (CAPES), Brasília, Brazil., [3]Phaseolus Consulting, Wyndmoor, PA, 19038, United States. Email: ericabs@usp.br*

Keywords: corn silage, inoculant, next-generation sequencing.

Introduction There is a lack of knowledge on the impacts of air stress on silage when it occurs early during the ensiling process versus during the late stages of ensiling. *Lactobacillus buchneri* produces antifungal compounds that can inhibit microorganisms that cause spoilage and, therefore, can possibly control the detrimental effects of air infiltration during storage and feedout. We aimed to analyze the effects of air stress during the early *vs.* late stages of ensiling on the microbial communities of corn silage by next-generation sequencing (NGS), and if an inoculant (*L. buchneri* 40788 + *Pediococcus pentosaceus* 12455) could inhibit microorganisms stimulated by air stress.

Materials and Methods Whole corn plant was harvested at 34% DM and untreated (C) or treated with Biotal Buchneri 500 (INO; application rate of *Lactobacillus buchneri* 40788, 400,000 cfu/g fresh forage and *Pediococcus pentosaceus* 12455, 100,000 cfu/g; Lallemand Animal Nutrition, Milwaukee, WI, USA). Four replicated silos (7.5 L, 224 kg of DM/m^3) for each treatment were not air stressed (NO), air stressed early (EARLY, 3 h/wk for wk 1-9), or air stressed late (LATE, 3 h/wk for wk 10-19). Bucket silos used for air stress challenge had three holes of 1.60 cm diameter. Silages were analyzed after 126 d of ensiling for the composition of bacterial and fungal communities by the sequencing of the V4-V5 region of the 16S rRNA and ITS1 region, respectively, using the Illumina MiSeq platform. Quality filtering, primer trimming, operational taxonomic units picking at 97% similarity (Greengenes for bacteria and UNITE for fungi) was done on QIIME. Distance matrices were calculated to build PCoA plots using the Phyloseq package in R. Statistical analysis of the matrices was done by permutational multivariate analysis of variance method using the Vegan package. Relative abundance (RA) data were analyzed as a completely randomized design with a 2 × 3 factorial arrangement of treatments with the fixed effects of additive, air stress, and their interaction using JMP.

Results and Discussion The distance matrices, built for both bacterial and fungal communities, showed similar statistical results. The treatments did not have the same centroid ($P < 0.01$) and differences in dispersion were caused by the treatments ($P < 0.02$). The ensiling process modified both the bacterial and fungal communities of the forage, as fresh forage samples clustered separated from silages (Figure 1). The bacterial community of C-NO and C-LATE were similar, and more different from C-EARLY (Figure 1a). Silages C-NO, INO-NO, INO-EARLY, and INO-LATE clustered together, whereas C-LATE and C-EARLY had a fungal community more distinct from the other treatments (Figure 1b). The inoculant did not affect the overall bacterial community composition of silages at genus level but there was an effect of air stress (Figure 2). Silages air stressed later during ensiling had the highest (97.6%), EARLY stressed the lowest (83.37%), and NO intermediate (96.7%) RA of *Lactobacillus* ($P < 0.05$). There was a tendency ($P = 0.10$) for an

air stress effect on the RA of *Acetobacter*. Silages NO and LATE had numerically lower RA of *Acetobacter* than EARLY (average of 0.2% *vs.* 10.2%). Bacteria from the genus *Acetobacter* can assimilate acetic and lactic acids and can initiate the aerobic spoilage of corn silage. Both air stress and inoculation modified the fungal communities of silages. Silages that were air stressed EARLY or LATE during storage had lower ($P < 0.05$) *Candida* RA than NO (average of 30.7 *vs.* 63.3%). Silages INO had higher ($P < 0.05$) RA of *Candida* than C (54.6 *vs.* 28.6%). Silages INO, independent of air stress challenge, had a RA of *Monascus* close to zero. On C silages, the fungi *Monascus* was not observed on C-NO silages and had low RA on C-EARLY (11.4%), but it had a high RA on C-LATE (79.6%) ($P < 0.05$).

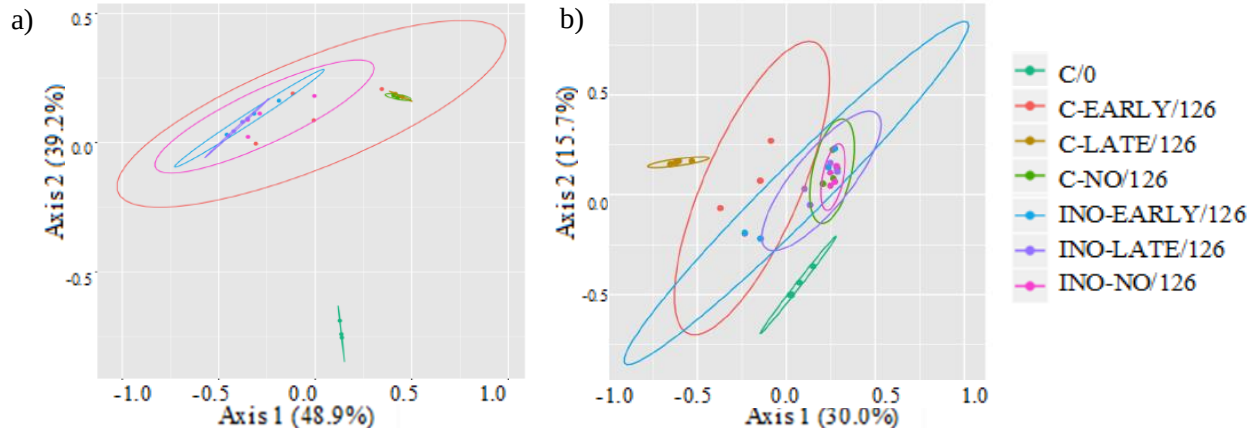

Figure 1. Principal coordinate analysis (PCoA) plots with a) weighted unifrac dissimilarity of the bacterial community and with b) Bray-Curtis dissimilarity of the fungal community. Treatments consisted of fresh forage (0 d) and corn silage (126 d), untreated (C) or treated with an inoculant (INO, *Lactobacillus buchneri* 40788, 400,000 cfu/g fresh forage and *Pediococcus pentosaceus* 12455, 100,000 cfu/g), not subjected to air stress (NO), air stressed early (EARLY, 3 h/wk for wk 1-9), or air stressed late (LATE, 3 h/wk for wk 10-19) during ensiling.

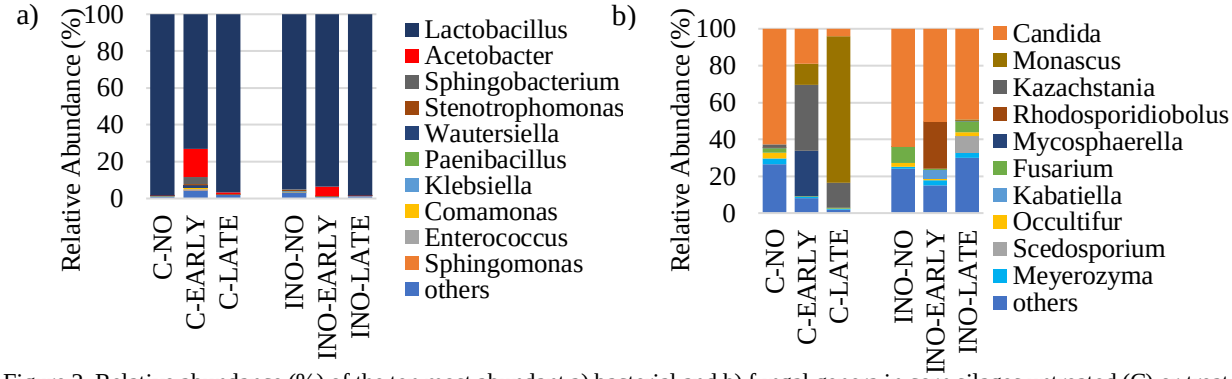

Figure 2. Relative abundance (%) of the ten most abundant a) bacterial and b) fungal genera in corn silages untreated (C) or treated with an inoculant (INO, *Lactobacillus buchneri* 40788, 400,000 cfu/g fresh forage and *Pediococcus pentosaceus* 12455, 100,000 cfu/g), not air stressed (NO), or air stressed early (EARLY, 3 h/wk for wk 1-9) or late (LATE, 3 h/wk for wk 10-19) during ensiling, as analyzed by the sequencing of the V4-V5 region of the 16S rRNA, for bacteria, and ITS1, for fungi, using the Illumina MiSeq platform.

Conclusions Air stress led to the development of undesirable microorganisms such as *Acetobacter* and molds. Silages that were air stressed during the late stages of ensiling had a fungal population characteristic of the more advanced stages of spoilage, showing that when the air stress occurs right before the silo opening it can be more prejudicial to the silage quality than when it occurs during the early ensiling stages. The additive prevented the development of undesirable microorganisms stimulated by oxygen as it maintained the fungal community of silages challenged with air stress (both early or late), similar to the fungal communities of silages that were not air stressed.