The effects of microbial inoculation on short-to-long fermentation and aerobic stability of grass-legume silage ensiled in big bales

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Key words: grass-legume; microbial inoculant; silage; fermentation

Abstract
Wilted (35.9 % DM and 2.6% WSC) moderately difficult to ensile grass-legume mixture (red clover, alfalfa and timothy 50:20:30) was ensiled in cylindrical big bales (1.2 m hight and 1.2 m diameter) with a weight about 700 kg. The crop was ensiled for 8, 32 and 120 days with or without a microbial inoculation (SiloSolve® FC containing *Lactococcus lactis* DSM 11037/1k2081 and *Lactobacillus buchneri* DSM 22501/1k20738 applied at 1.5×10^5 CFU g^{-1} forage). Ten bales per treatment were prepared for each fermentation period. After each period of ensiling the big bales were opened, sampled, and tested for fermentation parameters, yeast and mould counts and aerobic stability. Weight loss during fermentation and aerobic exposure were recorded and DM losses were calculated. After 8 days of fermentation, the addition of SiloSolve® FC showed only significant positive effects on pH, acetic and butyric acid, while after 32 and 120 days of fermentation periods, significant improvements of adding SiloSolve® FC were observed across all parameters investigated. The results show that SiloSolve® FC is an effective treatment to reduce ammonia, ethanol, and butyric acid production, to control yeast and mould growth, and to improve acetic acid levels with a resulting improved aerobic stability of grass-legume mixture fermented in big bales. Total lactic acid bacteria increased significantly and an appreciable decrease number of yeasts were detected in the inoculated silage at all fermentation time points and after aerobic exposure if compared with untreated silage. Reduction in yeast and mould population during anaerobic phase of silage fermentation and during silage aerobic exposure period appears to be the main reason for the improvement aerobic stability of the inoculated silage. Improved fermentation, reduced DM loss during fermentation and during aerobic exposure periods lead to increase nutritive value of the inoculated silage.

Introduction
Grass and legumes are the mainstay of livestock and can provide high quality feed for the dairy cows and fattening cattle (Lüscher et al., 2014). Progress in improving silage quality and nutrient use efficiency, advances in plant breeding, and additives manipulating silage fermentation made silage of topmost importance feed for the ruminants. The crop at ensiling contains both aerobic and anaerobic microorganisms as well as a range of yeasts and moulds that affect silage quality. The mechanism of ensiling involves the conversion of water-soluble carbohydrates (WSC) by epiphytic and/or inoculated lactic acid bacteria (LAB) into lactic acid (LA) and other organic acids and volatile organic compounds (VOC) under anaerobic conditions (Muck, 2012). However, when the silo is opened, aerobic conditions prevail during feed-out time; the silage is subject to aerobic microbial growth and is potentially unstable. All silages exposed to air will deteriorate more or less as a result of aerobic yeast and fungi activity during feed-out (Schmidt and Kung, 2010). Aerobic deterioration usually results in DM and important nutritional components loss and negatively affects the hygienic quality of silages resulting in the accumulation of pathogenic organisms and their toxins in the silage. The accumulation of degradation products can negatively affect food quality. Microbial additives containing lactic acid bacteria (LAB) are commonly used for silage preservation to achieve a rapid pH drop through organic acid production, and some strains have demonstrated their efficacy to improve aerobic stability and increase microbial quality of silage by inhibiting spoilage moulds and yeasts (Tabacco et al. 2011). Making round-bale silage is an attractive option suitable for small amounts of forage to be ensiled, sealing silage over a very short period of time than is possible for silage stored in other silo types. Therefore, this study was aimed at evaluating the efficacy of a dual strain silage inoculant with respect to fermentation quality, dry matter loss and aerobic stability after 8, 32 and 120 days of fermentation in big bales of grass/legume mixture silage.

Methods and Study Site
A homogenous plot of a grass/legume crop (red clover, alfalfa and timothy 50:20:30) was divided into two blocks and was mown with a disk mower-conditioner harvester and set to place windrows. Wilted up to 35.9 % DM, the grass/legume crop was picked up with a round baler with the stationary cutting blades engaged

and baled into a 1.2 m hight and 1.2 m diameter cylindrical bales with a weight of about 700 kg. Immediately after baling, the bales were carted from the field to the storage area individually wrapped and then labelled. Six layers of plastic film were used for wrapping the big bales to ensure anaerobic storage until opening. Thirty untreated (CTR) bales and 30 SiloSolve® FC (FC) treated bales were prepared for each fermentation period (8, 32 and 120 days). SiloSolve® FC containing *Lactococcus lactis* DSM 11037/1k2081 and *Lactobacillus buchneri* DSM 22501/1k20738 applied at 1.5×10^5 CFU g^{-1} forage. The inoculant was dissolved in tap water and applied as 2 g inoculant/tonne of fresh forage. The blend of the bacterial strains was applied during the harvesting process and using a commercial pump. All 60 big bales were individually weighed after wrapping and again after 8, 32 and 120 days of storage for measuring fresh weight losses for calculating DM loss. Five big bales from each treatment and each fermentation period chosen at random were sampled and tested for nutritional and fermentation parameters, and yeasts and mould count. Aerobic stability was measured using temperature sensors inserted into the centre of other five replicate, uncovered big bale silages, and defined as the time difference for the bale temperature to exceed a threshold of 3 °C above ambient. At the end of the aerobic stability test, big bales were individually weighed for determining fresh weight loss and silages were sampled and tested for DM content, pH, yeast and mould count. Data were statistically analysed as a randomized complete block by using the GLM procedure of SAS.

Results

Mean DM and WSC content of wilted grass/legume reached the targeted value of 35.9% and 2.65 %, respectively. The number of epiphytic LAB was 4.48 $\log_{10}$ cfu/g of pre-ensiled fresh grass/legume. Molds and yeasts were detected at 5.00 $\log_{10}$ cfu/g and 5.70 $\log_{10}$ cfu/g, respectively. Chemical composition, fermentation products and microbial analyses data show that there were clear differences between the inoculant treated and control silages (Table 1). DM content, DM loss and fermentation products data (lactic acid, alcohols and proportion of ammonia-N) during the 8 days ensiling period showed no significant differences between the treatments in the rate and type of fermentation, despite a significantly lower pH of the treated silage. Appreciable decreased number of yeast and mould ($P < 0.01$) were detected in the inoculated silage when compared with untreated silage. After 32 and 120 days of fermentation, DM content, lactic and acetic acid concentrations were higher ($P < 0.01$) in inoculant treated silage compared to control silage. Inoculant treatment during 32 days fermentation reduced DM loss, pH value, ammonia, alcohols and butyric acid concentration compared to untreated silages. At day 8, 32 and 120 of fermentation LAB number in the inoculated silages was higher ($P < 0.01$) and number of yeast and mould lower ($P < 0.01$) when compared with untreated silage.

Table1. Fermentation characteristics and microbiological parameters of grass/legume mixture ensiled in big bales with or without inoculantion with SiloSolve® FC after different periods of fermentation

Days of fermentation	**8**			**32**			**120**		
Treatment	CTR	FC	P	CTR	FC	P	CTR	FC	P
DM, g/kg	340.9	342.8	ns	327.7	334.7	**	317.6	328.7	**
DM loss, g/kg	56.3	48.4	ns	94.8	58.6	**	120.9	70.1	**
Crude protein, g/kg DM	180.6	184.7	ns	162.4	175.8	*	139.4	168.2	**
pH	5.28	5.00	**	4.67	4.50	**	4.37	4.21	**
N-NH_3 fraction, g/kg total N	30.82	30.58	ns	43.6	33.7	**	60.08	39.31	**
Alcohols, g/kg DM	6.05	5.26	ns	9.03	6.83	**	10.27	7.03	**
Lactic acid, g/kg DM	19.92	26.62	ns	33.45	57.18	**	51.63	70.22	**
Acetic acid, g/kg DM	10.95	13.46	*	14.89	23.35	**	21.13	32.91	**
Butyric acid, g/kg DM	0.72	0.16	**	1.37	0.28	**	1.09	0.26	**
Propionic acid, g/kg DM	0.20	0.23	ns	0.25	0.39	*	0.20	0.59	**
LAB, log CFU/g	7.06	8.90	**	8.17	9.88	**	8.02	9.41	**
Yeast, log CFU/g	3.54	2.35	**	2.94	1.06	**	2.33	1.00	**
Mould, log CFU/g	3.17	1.92	**	2.64	1.59	**	2.44	1.22	**

* $P < 0.05$; ** $P < 0.01$; ns: non-significant

SiloSolve® FC reduced the development of yeast and mould inside silage and limited weight loss during the aerobic exposure for the all fermentation periods (Table 2). Reduction in yeast and mould population during anaerobic phase of silage conservation and during aerobic exposure appears to be the main reason for the improved aerobic stability of the inoculated big bale silage (Table 2).

Table 2. Aerobic exposure results of grass/legume mixture ensiled in big bales with or without inoculantion with SiloSolve® FC after different days of storage.

Days of fermentation	**8**			**32**			**120**		
Treatment	CTR	FC	P	CTR	FC	P	CTR	FC	P
Fresh weight loss, %	28	20	*	4.64	2.63	*	3.66	1.64	**
DM, g/kg	318.6	327.2	*	309.3	321.7	**	302.9	317.6	**
pH	7.16	5.95	**	9.15	6.04	**	5.00	4.31	**
Yeasts, Log_{10} CFU/g	7.75	3.21	**	6.60	1.91	**	5.60	1.12	**
Moulds, Log_{10} CFU/g	7.02	3.62	**	6.02	3.71	**	6.87	4.13	*
Aerobic stability, h	0	0	ns	57	198	**	346	720	**

* $P < 0.05$; ** $P < 0.01$; ns: non-significant

Results of temperature changes inside the big bales during exposure to air over time are shown in Figure1, and Figure 2. Big bales, fermented for 8 days, did not differ in temperature between treatments, as the temperature of both treatments was above the threshold from the beginning of the aerobic exposure. Big bales, fermented for 32 days, showed a faster increase in temperature of the CTR bales than of the FC bales from day 5 up to day 18 of exposure to air. Inside big bale silage fermented for 120 days, CTR bales had a significantly higher temperature than of the FC bales from day 10 up to the end of aerobic stability test. Moreover, the temperature decreased continuingly inside FC bales during the aerobic exposure period.

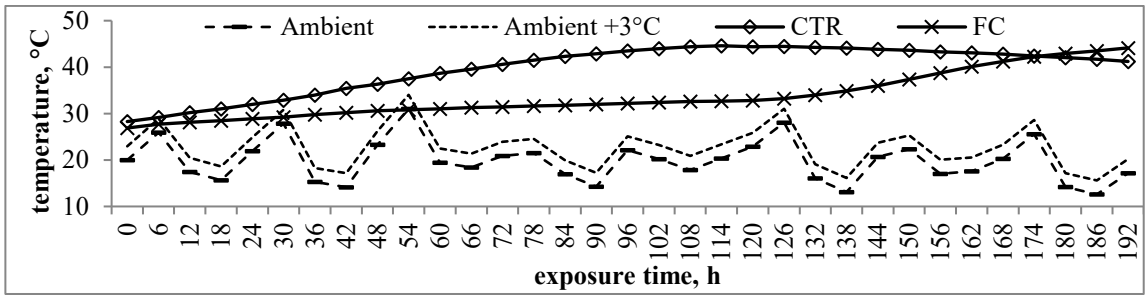

Figure 1. Temperature changes inside grass/legume big bales (8 days of fermentation) during 8 d exposure to air period

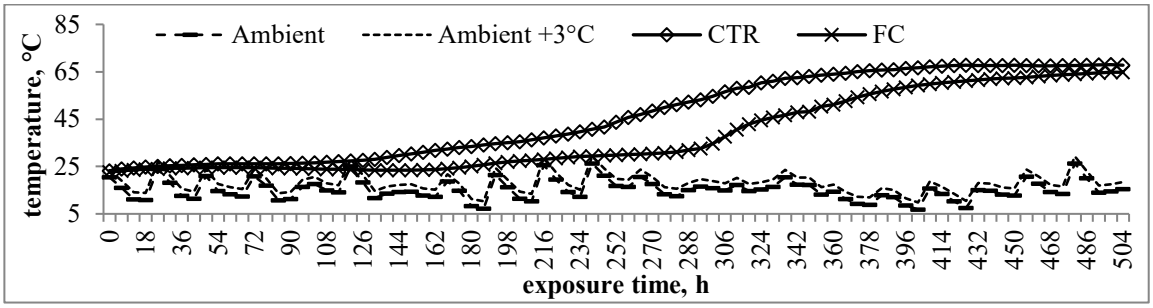

Figure 2. Temperature changes inside grass/legume big bales (32 d of fermentation) during 21 d exposure to air period

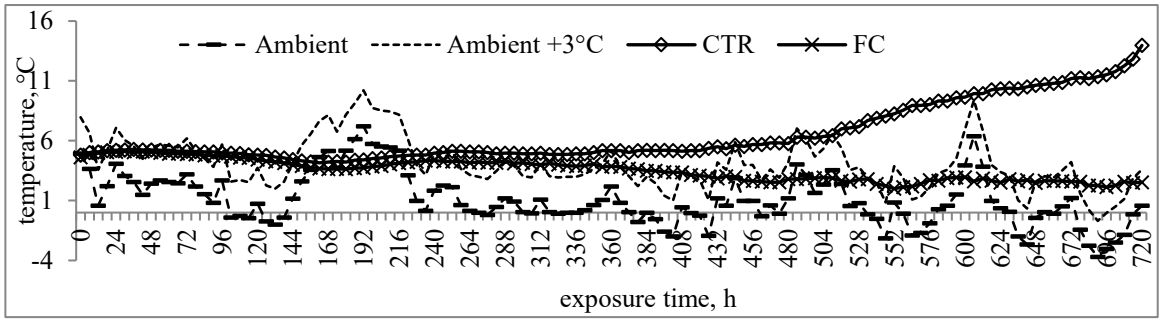

Figure 3. Temperature changes inside grass/legume big bales (120 d of fermentation) during 30 d exposure to air period

Discussion and Conclusions

Recently, dual-purpose inoculants containing homo-fermentative and hetero-fermentative bacteria have been developed to improve the speed of fermentation and the aerobic stability. In our study, inoculated silage had significantly lower pH value and higher levels of lactic acid and acetic acid. Acetic acid has antifungal properties and can inhibit the growth of yeasts and moulds under aerobic conditions (Đorđević et al., 2017). In our experiment, concentration of ethanol was lower in the inoculted silage. High levels of ethanol are often associated with high levels of yeast. Lactic acid, acetic acid, and water-soluble carbohydrates are the main sources of energy for the microorganisms involved in the first phase of silage deterioration during aerobic exposure. The oxidation of these nutrients results in the production of carbon dioxide and water, with the evolution of heat and an increase in silage pH (Kung et al., 2018). Borreani and Tabacco (2010) reported that increases in temperature at the silo face are clearly linked to aerobic activity of mould growth. Previous studies have reported that combining specific strains of *L. buchneri* and *L. lactis* as a silage inoculant can efficientely control yeast and mould growth in grass and maize silages (Copani et al., 2018a,b). The present study indicated that inoculated silages had significantly better aerobic stability by delaying aerobic deterioration time, compared with the control silage. These results are directly related to the inhibition of growth of yeasts and moulds. The present results are in agreement with several previous studies, where inoculation with *L. buchneri* and *L. lactis* improved aerobic stability in various crops (Galo et al., 2018). The results confirm that applying bacterial inoculant containing *L.buchneri* and *L. lactis* can effeciently control yeast and mould growth and improve aerobic stability of grass/legume ensiled in big bales. The evaluation of the extension of the visible mouldy areas combined with temperature measurements could be used to obtain a good indication of the health status of the silage during feed out. Reduction in yeast and mould population during anaerobic phase of silage fermentation and during silage aerobic exposure period appears to be the main reason for the improvement aerobic stability of the inoculated silage. Improved fermentation, reduced DM loss during fermentation and during aerobic exposure periods lead to increase nutritive value of the inoculated silage.

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