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Effects of an inoculant containing a *Lactobacillus buchneri* that produces ferulate-esterase on fermentation products, aerobic stability, and fibre digestibility of maize silage harvested at different stages of maturity

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ABSTRACT

The aim of this research was to study the effects of a commercial inoculant containing *Lactobacillus casei* and *Lactobacillus buchneri* that produces ferulate esterase enzymes on fermentation products, aerobic stability, microbial status, dry matter (DM) losses, and digestibility of neutral detergent fibre (aNDF-D) of maize silages ensiled at four stages of maturity. The kernel milk line (ML) was used to time the forage harvest, and 1/6 ML, 2/5 ML, 3/4 ML and black layer (BL) were observed, for harvest stages I, II, III and IV, respectively. Chopped whole plant maize was untreated or treated with *L. casei* LC32909 and *L. buchneri* LN40177, which were applied to achieve a final application rate of 1×10^4 cfu/g and 1.0×10^5 cfu/g of fresh forage, respectively. The maize was ensiled in laboratory silos for 260 days before opening. The DM content, starch and ether extract concentrations and mould count increased, whereas water activity, nitrate, ash, water soluble carbohydrates (WSC) and crude protein (CP) contents progressively decreased with increasing maturity at harvest. The 24-h and 48-h aNDF-D were similar for harvest stages I, II and III, whereas they were the lowest in harvest stage IV. The effect of inoculation decreased with increasing DM content at ensiling, and the inoculum was ineffective at the last stage of maturity, probably due to the high epiphytic lactic acid bacteria (LAB) count, low water activity and low sugar content that could have negatively influenced the inoculation outcome. The inoculation lowered the lactic acid, yeast and mould counts and increased acetic acid, 1,2-propanediol, pH, DM losses and aerobic stability in the first three harvest stages, whereas no differences were observed between the treated and untreated silages harvested at the last stage of maturity. Regardless of the treatment, the yeast count fell under the detection limit and

Abbreviations: ADF, acid detergent fibre; aNDF, neutral detergent fibre; aNDF-D, digestibility of neutral detergent fibre; a_w, water activity; BL, black layer; C, untreated control; cfu, colony-forming units; DM, dry matter; d*T*, difference between silage temperature and ambient temperature; FE, ferulate esterase; H, harvest stage; LAB, lactic acid bacteria; ML, milk line; T, treated with *Lactobacillus casei* LC32909 and *L. buchneri* LN40177; WSC, water soluble carbohydrates.

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the aerobic stability of the silage increased to over 200 h when the acetic acid content exceeded 25 g/kg DM. Furthermore, the DM losses were closely correlated to the acetic acid production and increased to 80 g/kg of DM in the treated silages harvested at the earliest stage of maturity. The potential milk production, estimated with MILK2006 model (Shaver et al., 2006, http://www.uwex.edu/ces/dairynutrition/spreadsheets.cfm), showed that the greater aNDF-D of the treated silage, which was observed in harvest stages I and III, did not counterbalance the higher DM losses attributable to the *L. buchneri* activity during ensiling, in terms of milk per Mg of original ensiled DM.

1. Introduction

Whole-crop maize silage is the major forage fibre source in dairy cow diets in Europe and in the United States, but its aerobic instability could decrease its nutritive value (Wilkinson and Davies, 2013). This problem could be prevented by the use of an inoculant containing *Lactobacillus buchneri*, a heterofermentative LAB, which could improve the aerobic stability of silages through the production of acetic acid from lactic acid during the anaerobic phase of silage conservation (Oude Elferink et al., 2001). The positive effect of this organism on aerobic stability, when added to maize silage, has been evaluated extensively on maize silage in laboratory experiments (Kleinschmit and Kung, 2006), in farm-scale experiments (Kristensen et al., 2010) and in farm surveys (Mari et al., 2009; Tabacco et al., 2011a). However, treating silages with inoculants containing *L. buchneri* alone has often led to an increase in DM losses and a slight increase in the final silage pH (Reich and Kung, 2010; Tabacco et al., 2009). Thus, selected homofermentative LAB have traditionally been used to rapidly produce lactic acid, lower pH and, consequently, improve the efficiency of the fermentation process and minimize DM and nutrient losses over conservation (Muck, 2004). Dual-purpose inoculants containing homofermentative and heterofermentative LAB have recently been developed to overcome the limitations of inoculants containing either type of bacteria alone. Beneficial effects of dual-purpose inoculants on the aerobic stability of maize silage have also been reported (Queiroz et al., 2012).

Recent studies have indicated that some isolates of *L. buchneri*, apart from producing acetic acid, can produce ferulate-esterase (FE) enzyme, which can hydrolyses feruloyl ester linkages between lignin and hemicellulose, and they are advocated to potentially improve fibre digestibility of forages during ensiling (Nsereko et al., 2008). Only a few studies have assessed the effects of the third-generation FE-producing inoculants containing *L. buchneri* on aerobic stability and on the improvement of fibre digestibility of maize (Nsereko et al., 2008; Kang et al., 2009), barley (Addah et al., 2012) and grass silages (De Boever et al., 2013). However, to the authors' knowledge, no research has been conducted to investigate the effects of these third-generation inoculants on the aerobic stability and fibre digestibility of maize silage harvested at different stages of maturity.

In US and Europe, corn is commonly harvested for silage in horizontal silos when the milk line (ML) ranges from 3/5 to 1/4. In agricultural practice, however, forage maize is often harvested earlier or later than the optimum time for harvesting (Herrmann et al., 2005; Windle et al., 2014). More common reasons for harvest to occur outside of the recommended range of maturity are unfavourable weather conditions, inadequate capacity to harvest large amounts of forage in a short period of time, unavailability of custom harvest equipment at the optimal harvest time, errors when choosing the maturity class of hybrids, or lack of monitoring whole plant moisture or kernel milk line during the filling period (Windle et al., 2014). Harvesting too early can be unfavourable, because the energy content of maize silage is lower due to incomplete starch accumulation in the kernels (Wiersma et al., 1993). In contrast, late harvesting at the black layer (BL) stage may result in a higher proportion of starch, but in reduced fibre digestibility (Wiersma et al., 1993). The NDF degradability of whole crop maize decreases progressively from the early to late maturity stages, despite the decline in NDF content (Johnson et al., 1999; Opsi et al., 2013), and this is due to the decline in digestibility of the stover with progressive maturity, which is associated with decreasing non-structural carbohydrates and increasing fibre and lignin concentrations (Bal et al., 2000). Furthermore, as maturity advances, the ensilability characteristics become progressively worse (*i.e.* higher DM content and more difficulties in achieving high packing density, and lower fermentable sugars that make LAB activity more difficult; Buxton and O'Kiely, 2003) and maintaining aerobic stability of silage during consumption becomes more challenging (Hu et al., 2009). Since energy is the primary contribution of maize silages to dairy cattle rations, the availability of a LAB inoculum that enhances fermentation increases aerobic stability after silo opening, and increases the proportion of digestible NDF that could be beneficial to ensile maize at more advanced stages of maturity and thus maximizes starch production (Opsi et al., 2013).

Therefore, the objective of this research was to evaluate whether the use of an FE-producing silage inoculant could increase aerobic stability and fibre digestion in maize silage, especially when its harvest was delayed to more advanced stages of maturity than 1/2 kernel milk line.

2. Materials and methods

2.1. Treatments

The trial was performed at the experimental farm of the University of Turin in the western Po plain, northern Italy (44°53′N, 7°41′E, 232 m above sea level, annual mean temperature 11.7 °C, and annual average rainfall 739 mm). A maize hybrid (Eleonora, Pioneer Hi-Bred Italia Srl, Gadesco Pieve Delmona, Cremona, Italy) was grown on a 1.15 hectare field and was harvested, as chopped whole crop, at four different stages of maturity (*i.e.* harvest stages I, II, III, and IV, at different kernel milk lines). The experimental field was divided into three blocks to obtain three replications per treatment (one in each block). Each block was split into four (80 m × 12 m) plots, which were then randomly assigned to one of the four harvest stages. The kernel milk line (ML) was set to ten plants per plot, according to Afuakwa and Crookston (1984), and averaged 5/6 ML, 3/5 ML, 1/4 ML and black layer (BL), for the four harvest stages, respectively. The whole crop maize was chopped with a precision forage harvester (Claas Jaguar 950 equipped with an 8-row Orbis head, Claas, Harsewinkel, Germany) to a theoretical cut length of 10 mm. The chopped material from each plot was divided into two representative 50-kg piles. One pile was untreated (C) and the other pile treated (T) with the 11CFT inoculant (Pioneer Hi-Bred Italia Srl, Gadesco Pieve Delmona, Cremona, Italy). The inoculant was applied at the recommended rate of 1 g/Mg of fresh forage to achieve a final estimated application rate of 1×10^4 cfu/g of *L. casei* strain LC32909 and 1.0×10^5 cfu/g of *L. buchneri* strain LN40177. The microbial inoculant was diluted in deionized water and applied using a hand sprayer at a rate of 2 mL/kg of forage by spraying uniformly onto the forage that was constantly hand mixed. The fresh maize forages were sampled at each harvesting time (one sample from each of the three untreated and the three treated piles) prior to ensiling, after the inoculum was applied. The untreated and treated forages were then ensiled (about 12 kg of wet forage) in 20 L plastic silos equipped with a lid that only enabled gas release. The forages were packed by hand and final packing densities, on a wet basis, were 609 ± 33, 593 ± 18, 588 ± 31, and 474 ± 31 kg/m^3, for harvests I, II, III and IV, respectively. The silos were weighed, conserved at 20 ± 1 °C and opened after 260 d. At opening, each silo was weighed, and the contents were mixed thoroughly and sub-sampled to determine the DM content, the chemical composition, the fermentation profile, the microbiological counts and the aerobic stability.

The DM losses due to fermentation were calculated as the difference between the weight of the forage placed in each plastic silos at ensiling and the weight of the silage at the end of conservation, corrected for the DM content of the forage and its respective silage.

2.2. Chemical and microbial analyses

Each pre-ensiled sample was split into three sub-samples, whereas each silage sample was split into five sub-samples. The first sub-sample was analysed immediately for the DM content by oven drying at 80 °C for 48 h (Dulphy et al., 1999). The second sub-sample was dried for qualitative analyses in a forced-draft oven to a constant weight at 65 °C, air equilibrated, weighed and ground in a Cyclotec mill (Tecator, Herndon, VA, USA) to pass a 1 mm screen. The dried pre-ensiled and silage samples were analysed for total nitrogen (TN), according to the Dumas method (method number 992.23, AOAC, 2005), using a Nitrogen analyser Micro-N (Elementar, Hanau, Germany), for crude protein (CP) (total N × 6.25), for ash by ignition (method number 942.05, AOAC, 2005), for water soluble carbohydrates (WSC) by the phenol sulphuric acid method according to Dubois et al. (1956), and for ether extract (EE) using the Soxhlet method according to AOAC (method number 920.39; AOAC, 2005).

Neutral detergent fibre (aNDF) was analysed using a Raw Fiber Extractor (FIWE, VELP Scientifica, Usmate Velate, Italy) with the addition of heat-stable amylase (A3306, Sigma Chemical Co., St. Louis, MO) and expressed on a DM basis including residual ash, as described by Van Soest et al. (1991). Acid detergent fibre (ADF) and acid detergent lignin were analysed (Robertson and Van Soest, 1981) and expressed on a DM basis including residual ash. Acid detergent lignin was determined after a 4 h sample immersion in sulphuric acid (720 g/kg) following the ADF procedure.

The digestibility of aNDF (aNDF-D) was measured using the in vitro procedure described by Goering and Van Soest (1970), with some modifications, which included the addition of heat-stable amylase. Forage subsamples of 0.5 g of dried pre-ensiled material and silages were weighed in 100-mL flasks (Schott Duran, Wertheim/Main, Germany). A medium, composed of a buffer, a macromineral solution, a micromineral solution, and resazurin as the redox state indicator of the system were introduced into the flasks together with a reducing solution to create an anaerobic environment. The flasks were then introduced into a water bath and purged with CO_2 to obtain complete anaerobiosis. Rumen fluid was collected from a dry cow fed 2 kg of concentrate per day and given *ad libitum* access to grass/alfalfa mixed hay (550 g/kg aNDF; 140 g/kg CP), stirred, filtered through four layers of cheesecloth and inoculated in each flask to start sample fermentation. Sub-samples were incubated for 24 or 48 h at 39.5–40 °C. Four replicates were incubated per treatment for each incubation period. After incubation, the content of each flask was introduced into raw fibre extractor tubes (FIWE, VELP Scientifica, Usmate Velate, Italy), boiled in a neutral detergent solution that included heat-stable amylase (Number A3306; Sigma Chemical Co.) and filtered through crucibles (Robu Glass Filter–ROBU H3, Borosilicate 3.3, 30 mL–Por. 2, Hattert, Germany) to determine aNDF. The residuals were then rinsed 3 times with boiling water, dried overnight at 103 °C and weighed to calculate aNDF-D at 24 and 48 h. The results were expressed as the average of the four replications at each interval.

The starch concentration was determined according to the AOAC methods (method number 996.11; AOAC, 2005), using a K-TSTA assay kit (Megazyme International, Bray, Ireland). Nutrient analysis and aNDF-D data of the fresh and ensiled maize were used as inputs for Milk2006 (Shaver et al., 2006) to estimate the net energy for lactation at 3× maintenance (NEL−3×) and the milk production per Mg of DM (kg/Mg). In vitro aNDF-D at 48 h of incubation was used as the cell wall digestibility value required by the model for the calculations. The potential milk yield of the silages was estimated taking into account the DM losses that occurred during conservation.

The third sub-sample was extracted, as a wet sample, using a Stomacher blender (Seward Ltd, Worthing, UK) for 4 min in distilled water at a water-to-sample material (fresh weight) ratio of 9:1, and filtered through four layers of cheesecloth. The filtrate was immediately analysed for the nitrate (NO_3) content, through semi-quantitative analysis, using Merckoquant test strips (detection limit 100 mg NO_3/kg of fresh matter, Borreani and Tabacco, 2008), pH, and ammonia content using a specific electrode (DX217, Mettler Toledo, Novate Milanese, Italy). In order to conduct the microbial counts, 30 g of wet sample were transferred into sterile homogenization bags, suspended 1:10 (w/v) in a peptone physiological salt solution (PPS: 1 g of neutralized bacteriological peptone and 9 g of sodium chloride per litre) and homogenized for 4 min in a laboratory Stomacher blender. Serial dilutions were prepared and the mould and yeast numbers were determined using the pour plate technique with 40.0 g/L of Yeast Extract Glucose Chloramphenicol Agar (YGC agar, DIFCO, West Molesey, Surrey, UK) after incubation at 25 °C for 3 and 5 d for yeast and mould, respectively. The mould and yeast colony forming unit (cfu) were enumerated separately, according to their macromorphological features, on plates that yielded 1–100 cfu. The lactic acid bacteria were determined on MRS agar (Merck, Whitehouse Station, NY) with added natamycin (0.25 g/L), by incubating Petri plates at 30 °C for 3 d under anaerobic conditions, according to Spoelstra et al. (1988).

In order to determine the fermentation profile of the silages, a fourth sub-sample was extracted using a Stomacher blender for 4 min in H_2SO_4 0.05 M at an acid-to-sample material (fresh weight) ratio of 5:1. The lactic and monocarboxylic acids (acetic, propionic, and butyric), ethanol and 1,2-propanediol were determined by high performance liquid chromatography (Borreani and Tabacco, 2008). The water activity (a_w) was measured at 25 °C on a fresh sample using an AquaLab Series 3TE (Decagon Devices Inc., Pullman, WA), which adopted the chilled-mirror dew point technique.

Finally, a fifth sub-sample of silage (about 4 kg) was subjected to an aerobic stability test. The silages were allowed to aerobically deteriorate at room temperature (24.9 ± 1.1 °C) in 10 L polystyrene boxes that were protected from dust contamination with a single layer of aluminium cooking foil. The room temperature and the temperature of each silage were measured each hour by a mini temperature logger (Escort Intelligent Mini, Escort Data Logging Systems Limited, Auckland, NZ). Aerobic stability was defined as the number of hours the silage remained stable before rising more than 2 °C above room temperature. Other aerobic stability indices were expressed as dT, which is the difference between silage temperature and ambient temperature, and the hourly accumulated dT (°C) after 168 h of aerobic exposure. After 168 h of aerobic exposure, the silages were sampled to quantify the fermentative and microbiological (yeast and mould count) changes that had taken place during exposure to air.

2.3. Statistical analysis

All the microbial counts were $\log_{10}$ transformed to obtain log-normal distributed data. In order to calculate the averages, the values below the detection level (detection levels: 10 yeast/g and 10 mould/g of silage) were assigned a value corresponding to half of the detection level (*i.e.*, 5 yeast/g and 5 mould/g of silage).

The chemical compositional data of fresh forages and fermentation characteristics, chemical characteristics and aerobic stability indices of silages were analysed with the GLM procedure according to the model for a 4 × 2 factorial treatment design:

$$Y_{ij} = \mu + H_i + I_j + (H \times I)_{ij} + e_{ij}$$

where μ = overall mean; H_j = effect of harvest stage; I_i = effect of inoculation; $(H \times I)_{ij}$ = effect of interaction between the harvest stage and inoculation; and e_{ij} = error term. Significance was defined with a 0.05 probability level.

The aerobic stability data, yeast count, and DM losses were pooled together across trials and were regressed on the acetic acid concentration as the independent variable. A regression analysis was performed to select the best regression model at $P<0.05$. All the above statistical analyses were performed using the version 17 of SPSS for Windows (SPSS Inc., Chicago, USA).

3. Results

3.1. Chemical characteristics of pre-ensiled maize forages

The chemical composition of the freshly treated and untreated chopped maize forages prior to ensiling, is shown in Table 1. Harvest stage affected almost all the analysed parameters, except for the fibre components (hemicellulose, aNDF, ADF and Lignin). The DM content, starch and ether extract concentrations and mould count increased, whereas water activity, nitrate, ash, WSC and CP contents progressively decreased with increasing maturity at harvest. The aNDF-D 24-h and 48-h were similar for harvest stages I, and II, whereas they were the lowest in the IV harvest stage.

Table 1
The chemical composition and microbial populations of maize forages at ensiling.

Harvest stage	I	II	III	IV	SEM	P value
DM, g/kg	276[d]	325[c]	385[b]	439[a]	12.9	<0.001
pH	5.66[ab]	5.47[b]	5.68[ab]	6.28[a]	0.066	<0.001
a_w	0.991[a]	0.991[a]	0.982[b]	0.973[c]	0.002	<0.001
Nitrate, mg/kg DM	1798[a]	1489[b]	1206[c]	517[d]	103	<0.001
NH_3–N, g/kg DM	0.09[a]	0.08[a]	0.05[b]	0.06[b]	0.001	0.027
LAB, log cfu/g of fresh forage	6.44[c]	6.57[c]	7.76[a]	7.29[b]	0.147	<0.001
Yeasts, log cfu/g of fresh forage	6.33[c]	6.39[c]	7.04[a]	6.80[b]	0.081	0.003
Moulds, log cfu/g of fresh forage	6.01[c]	6.09[c]	6.42[b]	7.03[a]	0.103	<0.001
Ash, g/kg DM	42[a]	41[a]	35[b]	36[b]	0.733	<0.001
WSC, g/kg DM	155[a]	131[b]	75[c]	35[d]	10.3	<0.001
Starch, g/kg DM	286[c]	304[c]	362[b]	391[a]	9.61	<0.001
Ether extract, g/kg DM	29[b]	30[ab]	31[ab]	33[a]	0.610	0.033
CP, g/kg DM	82[a]	81[a]	77[b]	77[b]	0.561	0.002
Hemicelluloses, g/kg DM	189	197	188	192	2.80	0.644
ADF, g/kg DM	217	199	198	207	3.01	0.073
Lignin, g/kg DM	35[a]	26[b]	28[b]	35[a]	1.15	0.001
aNDF, g/kg DM	406	397	385	399	3.96	0.308
aNDF-D 24-h, g/kg aNDF	495[ab]	520[a]	487[b]	408[c]	9.99	<0.001
aNDF-D 48-h, g/kg aNDF	600[a]	603[a]	568[b]	534[c]	7.68	0.001

C = untreated silage; T = silage treated with *L. casei* strain LC32909 (1×10^4 cfu/g of fresh forage) and with *L. buchneri* strain LN40177 (1.0×10^5 cfu/g of fresh forage); a_w = water activity; DM, dry matter; LAB, lactic acid bacteria; NH_3–N, ammonia nitrogen; CP, crude protein; WSC, water soluble carbohydrates. Means with different letters (a–d) in a row differ significantly ($P < 0.05$).

3.2. Fermentation characteristics of treated and untreated silages

The fermentation end products, pH, yeast and mould counts, DM losses and aerobic stability of the treated and untreated maize silage samples taken after 260 days of ensiling, are presented in Table 2.

The DM content of the silages was similar to the values observed at ensiling, and the inoculum had no effect. The water activity, nitrate content, and ammonia were unaffected by the inoculum and the values of the first two ones decreased with increasing stage of maturity at harvest. Lactic acid was lower in the treated silages than in the untreated ones, whereas acetic acid, 1,2-propanediol, and pH were higher, in the first three harvest stages. No differences were observed between the treated and untreated silages harvested at the BL stage of maturity (harvest stage IV) for these fermentation products and pH. The propionic acid content was higher in the treated silages of harvest stages I and II, whereas the values were similar between the treated and untreated silages for harvest stages III and IV. The ethanol content decreased with increasing harvest maturity, with no differences between the treated and untreated silages. The lactic to acetic acid ratio was lower than 2.0 in the treated silages of harvest stages I, II, and III, whereas this ratio was higher than 4.5 in all other silages.

The number of yeasts was markedly lower and aerobic stability was markedly higher in the inoculated silages compared to the untreated silages in harvest stages I, II, and III, whereas no differences were observed in the last harvest stage. Mould count was unaffected by either the harvest stage or LAB inoculation.

3.3. Chemical characteristics of treated and untreated silages

Table 3 shows the effects of harvest stage and inoculation on the chemical characteristics and aNDF-D of the treated and untreated maize silages. The harvest stage affected all the analysed parameters, except for aNDF-D 24-h, and the trends were similar to those described for fresh chopped forages. Furthermore, the differences between harvest stages were also observed for aNDF, ADF and Lignin. Inoculation only affected the ash and starch contents: the ash content was higher, whereas the starch content was lower in the treated than in the untreated silages in all harvest stages.

3.4. Microbial and fermentation quality after air exposure

The fermentation products, yeast and mould counts and the differences between the silage temperature and ambient temperature (d*T*), after 168 h of aerobic exposure, are reported in Table 4. The treated silages of harvest stages I, II, and III were aerobically stable for more than 168 h, and they had pH, mould count, lactic and acetic acid contents that could be considered almost similar to values observed at silage opening. Differences were observed in yeast count, which increased to values greater than 2 log cfu/g of silage. Aerobic deterioration had already taken place within 168 h of air exposure in the treated silage of harvest stage IV and in all the untreated silages and, as a consequence, an increase in the yeast and mould counts, silage temperature and pH, and a decrease in the content of fermentation products was observed compared to the treated silage.

Table 2
The pH, fermentation end products, DM losses, yeast and mould counts and aerobic stability of treated (T) and untreated (C) maize silages after 260 days of conservation.

Harvest stage	I		II		III		IV		SEM	*H* effect	*I* effect	$H \times I$
Treatment	C	T	C	T	C	T	C	T				
DM, g/kg	265	258	310	312	378	374	430	426	13.3	<0.001	0.875	0.713
pH	3.56	3.86	3.57	3.77	3.79	3.88	3.78	3.81	0.024	<0.001	<0.001	<0.001
a_w	0.985	0.986	0.987	0.986	0.981	0.979	0.971	0.972	0.001	<0.001	0.988	0.811
Nitrate, mg/kg DM	913	957	470	471	296	259	0	0	72.3	<0.001	0.950	0.842
NH_3–N, g/kg DM	1.5	1.5	1.4	1.6	1.4	1.6	2.2	2.1	0.091	0.046	0.712	0.798
Lactic acid, g/kg DM	104	54	86	59	54	44	66	64	3.86	<0.001	<0.001	<0.001
Acetic acid, g/kg DM	21	46	18	30	12	29	12	13	2.33	<0.001	<0.001	<0.001
Propionic acid, g/kg DM	3.6	8.8	0.0	4.9	2.3	2.0	2.4	2.3	0.578	0.001	0.002	0.007
Butyric acid, g/kg DM	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.012	0.418	0.332	0.418
Ethanol, g/kg DM	19	22	15	15	13	11	11	11	0.862	<0.001	0.908	0.157
1,2-Propanediol, g/kg DM	0.5	0.8	1.0	7.5	0.0	10.9	0.1	0.1	0.882	<0.001	<0.001	<0.001
Lactic to acetic acid ratio	4.9	1.2	4.9	1.9	4.5	1.5	5.5	5.0	0.353	<0.001	<0.001	<0.001
DM losses, g/kg DM	54	83	51	66	28	51	31	39	3.82	<0.001	<0.001	0.106
Yeast, log cfu/g of fresh forage	0.96	0.69	1.40	0.69	4.06	0.69	2.55	2.41	0.242	<0.001	<0.001	<0.001
Mould, log cfu/g of fresh forage	1.41	1.91	1.13	1.51	1.74	1.71	2.34	1.55	0.085	0.132	0.912	0.151
Aerobic stability (h)	153	246	94	226	39	242	95	81	16.5	<0.001	<0.001	<0.001

H = harvest stage effect; *I* = inoculum effect; $H \times I$ = interaction; C = untreated silage; T = silage treated with *L. casei* strain LC32909 (1×10^4 cfu/g of fresh forage) and with *L. buchneri* strain LN40177 (1.0×10^5 cfu/g of fresh forage); a_w = water activity; DM, dry matter; NH_3-N, ammonia nitrogen.

Table 3
Nutritional characteristics of treated and untreated maize silages after 260 days of conservation.

Harvest stage	I		II		III		IV		SEM	*H* effect	*I* effect	*H* × *I*
Treatment	C	T	C	T	C	T	C	T				
Ash, g/kg DM	44	47	42	43	35	36	34	36	1.01	<0.001	0.002	0.193
WSC, g/kg DM	8	6	9	12	12	12	9	9	0.651	0.026	0.747	0.611
Starch, g/kg DM	286	261	339	338	392	363	406	369	10.2	<0.001	0.003	0.308
Ether extract, g/kg DM	29	29	29	32	31	31	33	32	0.386	<0.001	0.296	0.074
CP, g/kg DM	82	84	79	80	78	78	76	76	0.569	<0.001	0.125	0.524
Hemicelluloses, g/kg DM	162	168	150	147	144	150	133	141	2.49	<0.001	0.171	0.533
ADF, g/kg DM	234	250	215	219	205	202	186	190	4.59	<0.001	0.239	0.470
Lignin, g/kg DM	46	50	31	30	30	31	32	33	1.84	<0.001	0.655	0.944
aNDF, g/kg DM	396	409	365	365	349	352	319	338	6.46	<0.001	0.243	0.791
aNDF-D 24-h, g/kg aNDF	421	434	453	444	431	411	422	418	4.22	0.062	0.518	0.536
aNDF-D 48-h, g/kg aNDF	501	544	540	539	503	552	496	480	7.07	0.029	0.112	0.147

H = harvest stage effect; *I* = inoculum effect; *H* × *I* = interaction; C = untreated silage; T = silage treated with *L. casei* strain LC32909 (1×10^4 cfu/g of fresh forage) and with *L. buchneri* strain LN40177 (1.0×10^5 cfu/g of fresh forage); CP, crude protein; WSC, water soluble carbohydrates.

Table 4
Yeast and mould counts, aerobic stability indices and fermentative end products of treated (T) and untreated (C) maize silages after 168 h of aerobic exposure.

Harvest stage	I		II		III		IV		SEM	*H* effect	*I* effect	*H* × *I*
Treatment	C	T	C	T	C	T	C	T				
DM, g/kg	276	255	314	312	368	367	434	435	13.2	<0.001	0.034	0.036
Yeast, log cfu/g of fresh forage	7.84	2.54	7.88	2.17	8.48	3.16	8.13	8.03	0.559	<0.001	<0.001	<0.001
Mould, log cfu/g of fresh forage	2.37	1.33	4.18	1.71	4.12	1.18	3.29	2.29	0.307	0.412	0.001	0.393
d*T* (°C)	6	0	13	0	12	0	12	10	1.22	0.008	<0.001	0.037
Accumulated d*T* (°C)	198	0	771	0	1050	0	698	734	91.7	0.002	<0.001	0.003
pH	3.83	3.80	4.19	3.74	4.74	3.86	4.39	4.14	0.084	0.032	0.004	0.116
Nitrate, mg/kg DM	880	891	124	206	174	263	0	0	71.3	<0.001	0.030	0.344
NH_3–N, g/kg DM	0.9	1.4	0.4	1.5	0.8	1.1	1.2	1.2	0.088	0.455	0.004	0.120
Lactic acid, g/kg DM	72	69	25	71	20	49	19	37	5.47	0.005	0.008	0.169
Acetic acid, g/kg DM	9	47	2	32	2	34	1	2	3.64	<0.001	<0.001	<0.001
Propionic acid, g/kg DM	0.0	4.3	0.0	4.7	0.0	1.0	0.0	1.8	0.519	0.343	0.003	0.346
Butyric acid, g/kg DM	0	0	0	0	0	0	0	0	–	–	–	–
Ethanol, g/kg DM	0	21	0	17	0	3	0	0	1.73	<0.001	<0.001	<0.001
1,2-Propanediol, g/kg DM	0.2	0.3	0.1	8.7	0.0	1.8	0.0	0.0	0.618	<0.001	<0.001	<0.001

H = harvest stage effect; *I* = inoculum effect; *H* × *I* = interaction; C = untreated silage; T = silage treated with *L. casei* strain LC32909 (1×10^4 cfu/g of fresh forage) and with *L. buchneri* strain LN40177 (1.0×10^5 cfu/g of fresh forage); Accumulated d*T*, the hourly accumulated d*T* after 168 h of aerobic exposure; DM, dry matter; d*T* = difference between silage and ambient temperature; NH_3–N, ammonia nitrogen.

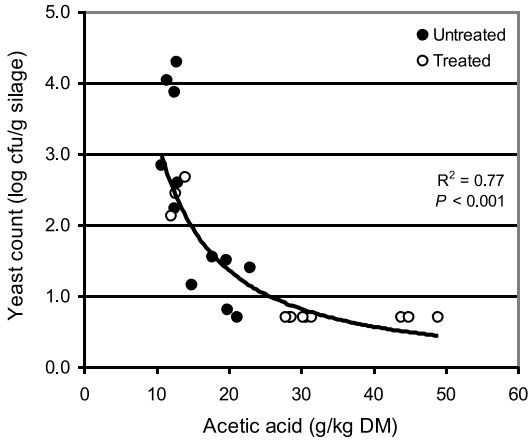

Fig. 1. Relationship between acetic acid content and yeast count of silages conserved for 260 days. Untreated, control silage; treated, silage treated with *L. casei* strain LC32909 (1×10^4 cfu/g of fresh forage) and with *L. buchneri* strain LN40177 (1.0×10^5 cfu/g of fresh forage). The values of yeast below the detection level (10 yeast/g) were assigned a value corresponding to half of the detection level to calculate the average.

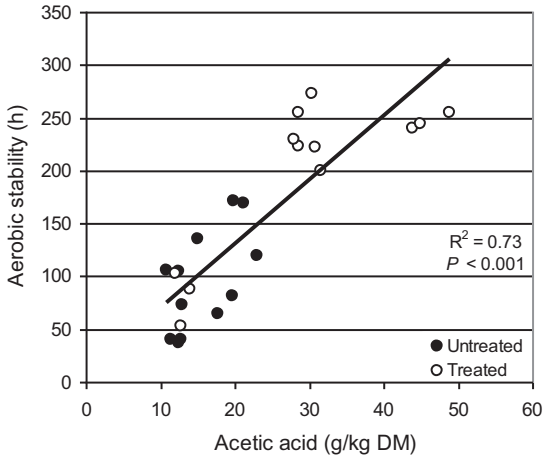

Fig. 2. Relationship between acetic acid content and aerobic stability of silages conserved for 260 days. Untreated, control silage; treated, silage treated with *L. casei* strain LC32909 (1×10^4 cfu/g of fresh forage) and with *L. buchneri* strain LN40177 (1.0×10^5 cfu/g of fresh forage).

3.5. Relationship between acetic acid content and yeast count, silage aerobic stability and DM losses

Overall, the analysis of the data showed that the production of acetic acid decreased with increasing DM content of the crop at ensiling, and the inoculation with *L. buchneri* almost doubled the amount of acetic acid produced compared to the relative untreated silages of harvest stages I, II, and III, whereas there was no difference in acetic acid content between the treated and untreated silages harvested at the highest DM content of 440 g/kg (harvest stage IV). Regardless of the treatment, when the acetic acid concentrations were related to the yeast counts at silo opening, it was noted that the yeast count dropped under the detection limit when the acetic acid content exceeded 25 g/kg DM (Fig. 1) and, as a consequence, the aerobic stability of the silage increased to over 200 h (Fig. 2). Furthermore, shifting fermentation to a more heterolactic pathway increased DM losses to 80 g/kg, and a close relationship between acetic acid content and silage DM losses was observed (Fig. 3).

3.6. Estimated energy value and milk production

The potential milk yield of the pre-ensiled and post-treated and untreated ensiled maize, which were estimated through the MILK2006 model, are reported in Fig. 4. The potential milk production (kg/Mg DM) of fresh forages was similar for the first three harvest stages while slightly lower values were observed for the last harvest stage. Because of the DM losses, ensiling reduced the potential milk production of the original harvested DM, and this reduction was more evident for the treated silages of maturity stages I, II, and III, whereas no differences were observed between the treated and untreated silages for the last stage of maturity.

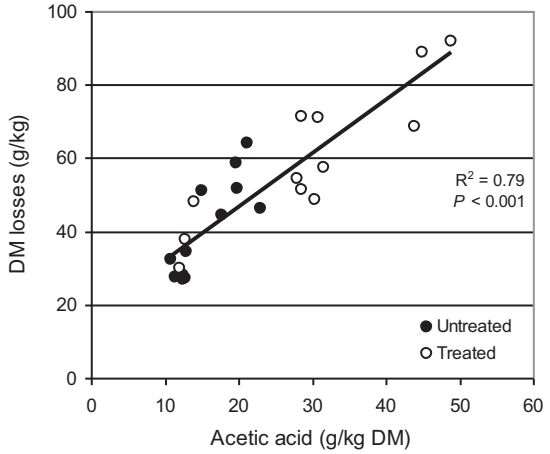

Fig. 3. Relationship between acetic acid content and DM losses of silages conserved for 260 days. Untreated, control silage; treated, silage treated with *L. casei* strain LC32909 (1×10^4 cfu/g of fresh forage) and with *L. buchneri* strain LN40177 (1.0×10^5 cfu/g of fresh forage).

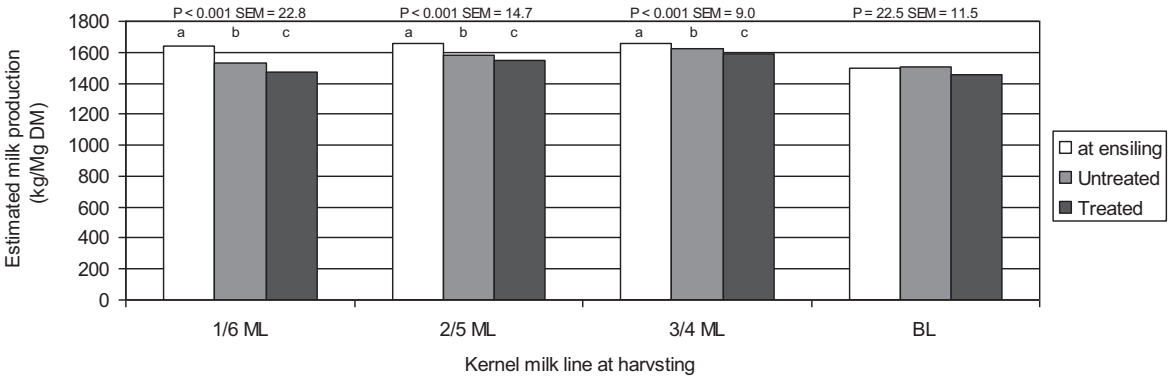

Fig. 4. The estimated milk yield per Mg of pre-ensiled harvested DM and of untreated and treated whole plant maize silages. The DM losses due to fermentation during conservation were taken into account when the estimations were made with the MILK2006 model. Untreated, control silage; treated, silage treated with *L. casei* strain LC32909 (1×10^4 cfu/g of fresh forage) and with *L. buchneri* strain LN40177 (1.0×10^5 cfu/g of fresh forage).

4. Discussion

It has been investigated in this paper whether the use at ensiling of a third generation of silage inoculants that contain a mixture of facultative heterofermentative (*L. brevis*) and obligate heterofermentative FE producing (*L. buchneri*) LAB strains could enhance silage fermentation, aerobic stability and fibre degradation of whole plant maize, harvested at successive stages of maturity, from the early kernel milk line to the black layer. As also observed by other authors (Johnson et al., 2002; Hu et al., 2009; Opsi et al., 2013), the stage of maturity had a great influence on the chemical, nutritional and microbiological quality of whole-crop maize at harvesting. In the present study, the DM content and starch concentrations increased, whereas a_w, WSC content, CP content, and aNDF-D 24-h and 48-h progressively decreased with increasing maturity at harvest. The results are consistent with data from Bal et al. (2000) and Johnson et al. (2002), who reported that the main reason for these changes in whole-crop maize, with advancing harvest stage of maturity, is the increase in the starch concentration of the cob during grain filling. The lack of an effect of stage of maturity on the whole-crop aNDF and ADF concentrations observed in the present experiment, was probably due to the increase in grain proportion being counter-balanced by an increase in the stover NDF and ADF contents, as previously reported by other authors (Johnson et al., 1999). Although the aNDF and ADF contents remained static with advancing maturity, the aNDF-D 24-h and 48-h were lower at the most advanced stage of maturity (kernel black layer, harvest stage IV), thus confirming data previously reported by other authors (Johnson et al., 2002; Lewis et al., 2004), who found that NDF digestibility was lower at later maturity stages, mainly due to the declined digestibility and the increased lignin concentration of the stover portion of maize silage, and the formation of ferulate molecules that cross-link arabinoxylans and lignin via both ester and ether linkages, as reported by Jung and Allen (1995).

The increase in DM content and the decrease in water activity (a_w) and WSC content at ensiling with increasing maturity make whole crop maize progressively more difficult to ensile, as pointed out by Buxton and O'Kiely (2003) for many different crops. In the present experiment, other than these parameters, harvest stage also affected the microbial counts with numbers

of yeast, mould and epiphytic LAB increasing with increasing maturity at harvest. Similar results were found for primary growths of two grass swards cut at three different harvest dates by Muller (2009), who reported that later harvest stages resulted in increased yeast, mould, enterobacteria and LAB counts in the pre-conserved herbage. An increased number of epiphytic microrganism on the herbage and a reduced a_w and WSC content at ensiling could decrease the possibility of a LAB inoculant being effective (Kung, 2009). In the present experiment in which adverse conditions were present, inoculation affected the fermentation quality of maize harvested at stages of maturity I, II, and III, but no effect was observed for inoculated silages harvested at the IV stage of maturity, compared to the control silages. The metabolic activity of *L. buchneri* was evident for the first three harvest stages of maturity, with the inoculated silages having higher pH, acetic and propionic acid content s, 1,2-propanediol content, DM losses and aerobic stability, and a lower lactic acid content and yeast count. The activity of *L. buchneri*, as indicated by the changes in pH, the concentrations of fermentation end-products and DM losses during conservation, was substantially greater in the inoculated silages with a lower DM content (226 and 325 g/kg, corresponding to harvest stages I and II, respectively) compared to the control silages. The effect was reduced in the inoculated silage with a relatively high DM content (385 g/kg, stage III), and it was inconsistent in the inoculated silage with the highest DM content (440 g/kg, stage IV). These observations suggest that the competitiveness of the *L. buchneri* strain used in this research decreases with increasing DM content, compared to both the epiphytic LAB and the co-inoculated facultative heterofermentative LAB, as previously reported for grass silages by Driehuis et al. (2001). What was observed in silages harvested at the IV stage of maturity, can be explained by what Kung (2009) pointed out that an increased microbial number at ensiling, especially epiphytic LAB, could adversely affect the effectiveness of silage additives, and that a consistent increase in the number of inoculated lactic acid bacteria would be required to obtain a positive response to inoculation.

In contrast to the results of the present research, Hu et al. (2009) reported that the magnitude of the effects of *L. buchneri* inoculation for acetic acid and aerobic stability was greater in moderately high DM silage (410 g/kg) than in normal DM silage (330 g/kg). These contrasting results could be due to the different *L. buchneri* strain used in our experiment from that used in the experiment of Hu et al. (2009), which could probably be more tolerant to high DM conditions. Furthermore, Kang et al. (2009) and Schmidt and Kung (2010) suggested that other unknown reasons, such as differences in the epiphytic bacterial population, water activity, WSC concentration, cell-wall component concentrations, or different locations, hybrid selection, or stage of maturity at harvest might influence the outcome of silage inoculation.

In the present study, the treated silages had a higher aerobic stability than the corresponding untreated silages at harvest stages I, II and III, whereas no differences were observed in the silages at harvest stage IV. Furthermore, the number of hours the silages remained stable after air exposure decreased with increasing stage of maturity. This higher aerobic stability delayed the onset of aerobic deterioration in the treated silages of harvest stages I, II, and III, and in the untreated silage of harvest stage I. The explanation for the aerobic stability enhancing effect of *L. buchneri* is that this bacterium could impair the activity of yeast in relation to its ability to ferment lactic acid to acetic acid, 1,2-propanediol and ethanol (Oude Elferink et al., 2001). A higher acetic acid content contributed to reducing yeast survival during the anaerobic ensilage phase and subsequently to inhibiting the growth of yeasts during exposure of silage to air (Driehuis et al., 2001). All the inoculated silages of stages I, II, and III had a higher acetic acid content than 30 g/kg of DM, with values decreasing with increasing DM content at ensiling. The concentrations of acetic acid in the control silage of harvest stage I at the end of ensiling was higher than 20 g/kg of DM and likely contributed to the lower yeast activity and higher aerobic stability of this untreated silage after exposition to air. These results are in agreement with the findings of Kleinschmit and Kung (2006) and Der Bedrosian et al. (2012), who reported that the increase in the concentration of acetic acid was more evident in silages treated with *L. buchneri* and in untreated maize silages with a low DM content. As reported in Figs. 1 and 2, regardless of the inoculation and stage of maturity at harvest, the higher the acetic acid concentration in the silage, the lower the yeast count at silo opening and the higher the aerobic stability after exposure of silage to air. The relationship between the acetic acid content and aerobic stability found in this experiment is in agreement with data from the research by Danner et al. (2003) and Schmidt and Kung (2010), who reported high correlation values of 0.95 and 1.00, respectively.

As a consequence of the anoxic degradation of lactic acid to acetic acid, 1,2-propanediol, CO_2 and ethanol by *L. buchneri*, an extra DM loss occurred during the storage phase and they are linearly correlated to the amount of acetic acid produced in the silage (*cf.* Fig. 3). Similar results have already been reported by Borreani et al. (2014), who hypothesized a relationship between the produced acetic acid and DM losses on maize silage treated with a mixture of *L. buchneri*, *L. plantarum*, and *Enterococcus faecium*. In the present study, the DM recovery after silo conservation increased with increasing maturity at harvest (*i.e.* with DM content at ensiling) and that of untreated silages was higher than that observed for treated silages, when inoculum activity was effective (harvest stages I, II, and III). These higher DM losses are consistent with observations made by other authors on maize silage treated with *L. buchneri* (Kleinschmit and Kung, 2006).

Besides improving silage fermentation and aerobic stability, the inoculant used in the current study was tested to verify whether it could improve the digestibility of the silage, through the production of FE, which has the potential of increasing the digestibility of fibre by breaking the linkages between hemicelluloses and lignin. Regardless of the inoculation, ensiling promoted a great cell-wall hydrolysis and this could be affirmed considering the great differences between the pre-ensiled and ensiled hemicellulose and aNDF concentrations, especially in the maize harvested at a late stage of maturity (average reduction of 34 and 71 g/kg of DM in aNDF for harvest stages III and IV, respectively). Reduced fibre concentrations after ensiling, compared to pre-ensiled material, have been reported for maize silages by other authors (Filya, 2003; Kang et al., 2009).

Inoculation affected the starch concentrations of silages, which were lower in the treated than in the untreated silages (23 g/kg DM on average over the studied stages of maturity). This finding was unexpected and no explanation can be given as to why the inoculation of maize silage with a mixture of *L. buchneri* and *L. casei* resulted in silages with a lower starch concentration than the untreated silages.

After ensiling, the in vitro digestibility of the aNDF (both after 24 and 48 h of incubation) of the maize silages resulted to be lower than that observed in the pre-ensiled maize; these results are in agreement with previous findings of Tabacco et al. (2011a) and Opsi et al. (2013). This might be due to the solubilization of hemicelluloses during ensiling, which may improve DM digestibility, but not change or even decrease the digestibility of the residual NDF, as hypothesized by Weinberg et al. (2007) who worked on maize silage treated with 10 different sources of lactic acid bacteria.

Unexpectedly and regardless of the DM content and stage of maturity at harvest, inoculation did not affect the digestibility of NDF of the maize silages in the present experiment. Similar results have been reported by Filya (2003) and Tabacco et al. (2011b), who found that inoculation with *L. buchneri*, alone or combined with a homofermentative LAB, did not affect the NDF degradability of maize silages, but these authors used a strain of *L. buchneri* (40788) for their inoculation experiment that is not known to produce FE. Other studies on maize silage, treated with the same inoculum used in the current study, have instead reported improved NDF digestibility after 48 h by 68 g/kg of NDF (Nsereko et al., 2008). Contrasting results have been reported by Kang et al. (2009) who found that inoculation, with the same LAB mixture as the current study, *in situ* increased aNDF-D 48-h by 57 g/kg of aNDF, in one of the two studied maize hybrids. Reich and Kung (2010) have compared the effectiveness of combining *L. buchneri* 40788 with three lactic acid bacteria (*L. plantarum*, *Pediococcus acidilactici*, or *P. pentosaceus*) on the fermentation, aerobic stability and nutritive value of maize silage and found that *L. buchneri* paired with *L. plantarum* or *P. acidilactici* improved aNDF-D 48-h (27 and 41 g/kg of aNDF, respectively) for unknown reasons, whereas a combination with *P. pentosaceus* did not. These authors concluded that more research was necessary to study potential interactions among lactic acid bacteria to improve NDF-D.

When the present results were analysed separately for each harvest stage, the stage I and III treated maize silage showed higher aNDF-D 48-h than those of the untreated silages, with increased values of 43 and 49 g/kg of aNDF for stage I and III, respectively. These values are comparable with differences in NDF-D 48-h reported by other authors (Nsereko et al., 2008; Kang et al., 2009; Reich and Kung, 2010). The higher aNDF-D of the treated silages in these two stages of maturity did not led to a higher potential milk yield, when estimated with MILK2006, because of the decreased starch concentration and the higher DM losses that occurred during ensiling, which were likely due to the *L. buchneri* activity. The animal responses to the feeding of maize silage, in terms of milk yield estimated by means of MILK2006, were within the range reported by other authors using the same model in the same environment (Tabacco et al., 2011a; Opsi et al., 2013). It is interesting to observe that ensiling slightly reduced the potential milk yield per Mg of harvested DM, mainly due to the DM losses that occurred during fermentation, and that a drop in the potential milk yield per Mg of harvested DM was only observed when the maize was harvested at the BL stage of maturity, thus confirming the potential of maize silage to be harvested at more advanced stages, till 2/3 ML, without losing its milk yield potential.

5. Conclusion

Inoculation with *L. buchneri*, which produces FE, has increased aerobic stability by shifting the fermentation of silage towards a heterolactic pathway, but did not affect the aNDF digestibility of the silage after 260 days of conservation. Furthermore, there was clear evidence that the effect of inoculation decreased with increasing DM content at ensiling, and that the inoculum was ineffective at higher DM contents than 400 g/kg, probably due to adverse conditions that might have negatively influenced the inoculation outcome. Finally, the potential milk production estimated with MILK2006 model showed that the greater aNDF digestibility of the treated silage, which was observed in two trials, could not counterbalance the lower starch content after ensiling and the higher DM losses attributable to the *L. buchneri* activity during ensiling in terms of milk per Mg of ensiled DM.

Conflict of interest

Authors declare that there is no conflict of interests.

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