The Influences of Addition of Sugar with or without *L. buchneri* on Fermentation and Aerobic Stability of Whole Crop Maize Silage Ensiled under Anaerobic Silos

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ABSTRACT : The whole plant of crop maize was chopped and ensiled in airtight 1-L capacity glass jars to determine the influence of residual sugar on anaerobic yeast growth and on the fermentation of lactic acid by *L. buchneri* in whole crop maize silage. There were a total of six treatments used in this experiment as follow: added 25 g de-mineralised water per kg chopped maize serving as control (con), 37.5 g glucose solution containing 12.5 g glucose (g_1), 75 g glucose solution containing 25 g glucose (g_2), 25 g *L. buchneri* suspension intended for 10^6 cfu g^{-1} (L.b.), g_1+L.b. and g_2+L.b. All silos were stored in the dark at 20°C until end of experiment. Jars were opened on duplicates at day 2, 7, 14, 28, 56 or triplicates at day 91 after ensiling for measuring the pH, microbiological enumeration and fermentative products. Results indicated that acidification rates for all silages were very fast, no difference occurred among treatments before day 28. After day 28 the pH values for silages inoculated by *L. buchneri.* with or without sugar tended to increase especially for treated only with *L. buchneri*, resulting in higher ($p<0.01$) finial pH than uninoculated silages. Compared with control silage, the added sugar significantly ($p<0.01$) increased dry matter (DM) loss, *L. buchneri* enhanced ($p<0.01$) DM loss further at different sugar existence. Silages inoculated by *L. buchneri* only or in combination with sugar addition contained less ($p<0.01$) lactic acid than the correspondent silages without inoculation with *L. buchneri*. In comparison with control, ethanol production is about 3 or 6 fold higher due to addition 12.5 or 25 g glucose per kg chopped maize at ensiling. The added sugar resulted in less acetic acid concentration ($p<0.01$) than control, but inoculation with *L. buchneri* increased ($p<0.01$) acetic acid than correspondent uninoculated silages at different sugar levels. No butyric acid and propionic acid were found in uninoculted silages, silages inoculated with *L. buchneri*. produced more propionic acid, 1-propanol and butyric acid. *Lactobacilli* counts were not influenced by added sugar, but increased ($p<0.01$) with inoculation of *L. buchneri*. The added sugar increased significantly ($p<0.01$) the yeast count, whereas *L. buchneri* showed the contrary effect. No differences were found in the aerobic stability among all treatments. In conclusions, 1) the added sugars encourage the growth of yeast and yeasts convert extra sugar into ethanol in maize silages. 2) The added sugars and *L. buchneri* do not influence the aerobic stability of silages stored in anaerobic silos. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 8 : 1128-1133)***

Key Words : Maize Silage, *L. buchneri*, Sugar, Fermentation, Aerobic Stability

INTRODUCTION

Previous studies have shown that inoculation of whole maize with a strain *L.buchneri* reduce the yeast count and improve the aerobic stability (Driehuis et al., 1996). Inoculation with *L. buchneri* does not influence the primary fermentation (conversion of sugars into acids), but induce a secondary fermentation in which part of lactic acid present in silage is converted to acetic acid and 1,2-propanediol (Driehuis et al., 1999; Oude Elferink et al., 1998). There are indications that 1,2 propanediol can be further converted to 1-propanol and propionic acid (Driehuis et al., 1998). The increase in acetic (and propionic) acid lead to a reduced survival of yeasts during the anaerobic ensilage phase and growth inhibition of yeasts and moulds during the aerobic phase (Driehuis et al., 1998,1999; Oude Elferink et al., 1998). However, extra sugar can enhance growth of yeast under anaerobic condition in high DM grass silage (Guan et al., 2001) and sweet sorghum silage (Schmidt et al., 1997), there is still no data available in respect to the effects of extra sugar influencing the fermentation characteristics of whole maize crop silage with *L. buchneri*. Therefore the objectives of this study were to determine the influence of residual sugar on anaerobic yeast growth and on the fermentation of lactic acid by *L. buchneri* in whole crop maize silage.

MATERIAL AND METHODS

Experimental procedure

The whole crop maize (variety LG2181) was harvested through a precision-chop forage harvester. The yield of maize was c. 18 ton/ha. The intended inoculation level for *L. buchneri* was 10^6 cfu g^{-1}, the suspension were applied using a pressure sprayer while mixing in a concrete mixer. Laboratory silos were airtight 1-L capacity glass jars (*c.* 0.5 kg per jar), which were stored in the dark at 20°C. There were 14 jars per treatment and two jars on day 2, 7, 14, 28, 56 or three jars each treatment on day 91 post ensiling were opened, one 30 g silage sample per jar was taken and add

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270 g demineralized water, and then blend 5 min with stomacher for measuring the pH, microbiological enumeration and fermentative products.

There were a total of six treatments used in this experiment as follow: added 25 g de-mineralised water per kg chopped maize serving as control (con), 37.5 g glucose solution containing 12.5 g glucose (g_1), 75 g glucose solution containing 25 g glucose (g_2), 25 g *L. buchneri* suspension intended for 10^6 cfu *L. buchneri* per g maize (L.b.), 12.5 g glucose kg^{-1}+*L. buchneri* 10^6 cfu g^{-1} (g_1+L.b), and 25.0 glucose g kg^{-1}+*L. buchneri* 10^6 cfu g^{-1}.

Analytical procedure

The maize samples taken from control and treated only with *L. buchneri* at day o were analyzed for pH, *lactobacilli*, total lactic acid bacteria (LAB), yeast and mould, but only sample from the control also for measuring DM, crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF) and water soluble carbohydrate (WSC). All silage samples were subject to analyze for pH, DM, ethanol and volatile fatty acids (VFA), lactic acid, sugar and the numbers of LAB, *lactobacilli*, yeast and mould. Dry matter, CP, NDF, ADF, WSC and ash were determined as described by van Vuuren et al. (1993). Bacteria counts, pH and concentrations of lactic acid, VFA and ethanol were determined in extracts of samples of maize or silage, prepared as described by Spoelstra (1983). Lactic acid was determined as described by Spoelstra (1983). VFA and ethanol were determined by gas chromatography, using Hewlett Packard 5730A equipment, a 25 m medium bore capillary column (Chrompack CP-Sil-5CB) and helium as carrier gas. *Lactobacilli* were enumerated on double layered poured plates of Rogosa SL Agar (Difco) acidified with glacial acetic acid to pH 5.4, incubated 3 days at 30°C. Lactic acid bacteria (LAB) were enumerated on doubled layered pour plates of Rogosa SL Agar (Difco) adjusted with sodium hydroxide to pH 6.2 containing 100 mg L^{-1} cycloheximide, incubated 3 days at 30°C. Yeast and mould were enumerated on double layered pour plates of Malt Extract Agar (MEA) acidified by lactic acid to pH 3.5, incubated 3 days at 30°C. Aerobic stability of silage were determined by incubation at 20°C of 0.3 kg lots in insulated containers with holes lids and bottoms to allow air to enter and carbon dioxide to escape. Temperature was measured continually by a thermocouple placed in the center of material, coupled to data taker. Aerobic stability was defined as the time needed to increase the temperature 1°C above ambient temperature.

Statistical analysis

The statistical analysis included one-way analysis of variance and Duncan's multiple range test; these were performed by ANOVA using the GLM procedure of the Statistix as a randomized complete block design.

RESULTS

Composition of maize before ensiling

The chemical composition and the epiphytic bacteria number of LAB, *Lactobacilli*, yeast and mould of the pre-ensiled maize crop are given in table 1. The treatment with inoculant showed a slight higher the LAB and *lactobacilli*, lower yeast than control maize.

Developments of pH and WSC consumption in course of ensilage

Figure 1 and figure 2 present the development of pH and WSC consumption during the ensiling of maize respectively. Rates of pH-drop were very fast for all silages possessing pH value *c.* 4.3 or below 4 at day 2 and 7 respectively, no difference occurred among treatments before day 28. After day 28 the pH for silages inoculated by *L. buchneri* with or without sugar tended to increase especially for treated only with *L. buchneri*, resulting in higher ($p<0.01$) finial pH than uninoculated silages. Compared with control silage, supplementing only sugar at ensiling didn't significantly affect ($p>0.05$) pH of silages. Inoculation by *L. buchneri*. increased the final pH,

Table 1. Chemical analyses and epiphytic bacteria of the fresh maize at ensiling

Items	Con	g_1	g_2	L.b	L.b+g_1	L.b+g_2
DM, g kg^{-1} DM	290	299.5	299.8	291.7	303.3	302.2
WSC, g kg^{-1} DM	85.7	116.1	138.8	88	113.2	132.9
N, g kg^{-1} DM	10.3	-	-	10.1	-	-
NDF, g kg^{-1} DM	462.9	-	-	456.5	-	-
ADF, g kg^{-1} DM	222.1	-	-	243.3	-	-
LAB, log_{10} cfu g^{-1}	5.99	-	-	6.2	-	-
Lactobacilli, log_{10} cfu g^{-1}	5.64	-	-	6.2	-	-
Yeast, log_{10} cfu g^{-1}	4.7	-	-	4.3	-	-
Mould, log_{10} cfu g^{-1}	3.7	-	-	3.6	-	-

-: Not determinated.

Inoculant containing *L. buchneri* supply: 9.5×10^7 cfu on medium of Rogosa 6.2.

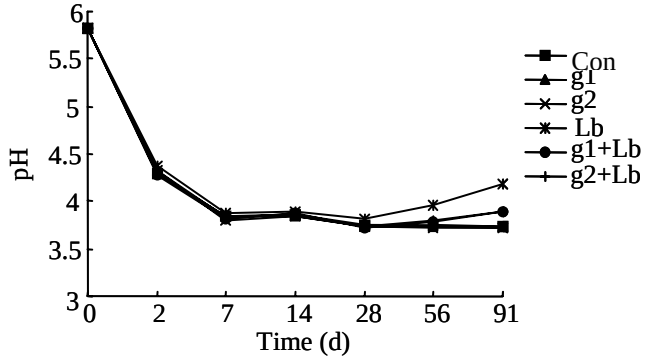

Figure 1. Development of pH in maize silages with different treatments during the whole ensiling.

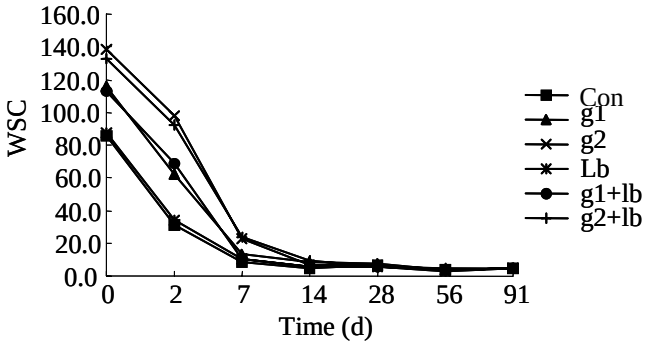

Figure 2. Water soluble carbohydrate (WSC, g kg^{-1} DM) changes in maize silages with different treatments during the whole ensiling.

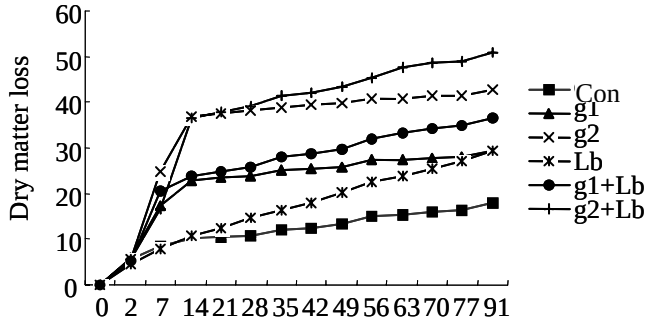

Figure 3. Dry matter loss (g kg^{-1} DM) in maize silages with different treatments during the whole ensiling.

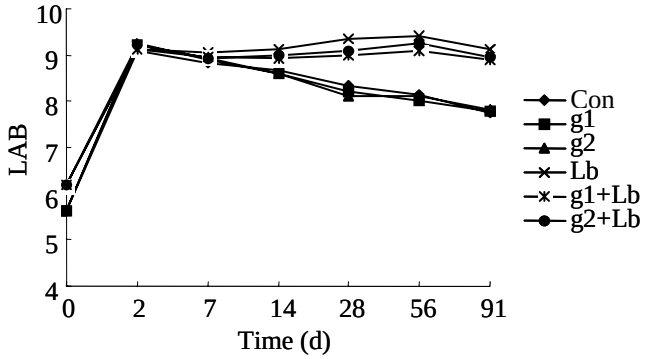

Figure 4. Lactic acid bacteria (LAB, log_{10} cfu g^{-1}) in maize silages with different treatments during the whole ensiling.

simultaneously in combination with added sugar had lower ($p<0.01$) pH than that of inoculated silage. Additionally more effect was observed with high sugar addition, indicating the added sugar inhibiting the activity of *L. buchneri* which make the slight rise of pH. Water soluble sugar change shows that nearly the same sugar amount left in silages at 14 days until the end of ensilage (figure 2), indicating added extra sugars were fermented into ethanol instead of lactic acid, confirmed by fermentation products (data not shown).

Dry matter loss

As shown in figure 3, the added sugar significantly ($p<0.01$) increased DM loss, and the more intensive effect was observed with increasing sugar inclusion. *L. buchneri* enhanced ($p<0.01$) DM loss further at different sugar existence. Data from sugar consumption suggested that all added sugars were nearly consumed that resulted in the similar amount of sugar left in all the final silages, in line with the changes of DM loss.

Changes in microbiological composition during ensilage

Figure 4, figure5 and figure6 show the changes in the bacteria numbers during the ensiling. *Lactobacilli* count increased sharply and reached the peaks at day 2 post-ensiling for all silages, then tended to reduce slowly for the control and silage treated only with sugar. However, for the silages inoculated with *L. buchneri* kept the same count and even though somewhat increase after 2 weeks until the end of trial. *Lactobacilli* counts in silages of 91 days varied from 7.76 to 9.23 (log cfu g^{-1}), they were not influenced by added sugar. Inoculation with *L. buchneri* increased ($p<0.01$) *Lactobacilli* counts, but added sugar impaired the function of *L. buchneri*. The count of yeast increased rapidly after ensiling and reached the peaks at day 7, then tended to decrease. Yeast counts for all silages after day 56 were below 2 until the end of trial. Yeast altering showed that added sugar increased significantly ($p<0.01$) the yeast counts, whereas *L. buchneri* reduced the yeast count, and added sugar nearly eliminating the inhibition of *L. buchneri* on yeast. Mould reduced significantly after ensiling and kept very low throughout the whole ensilage.

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Chemical composition of silages after 91 days

Table 2 presents the fermentative products, LAB, yeast, mould and aerobic stability measured at 91 days ensilage.

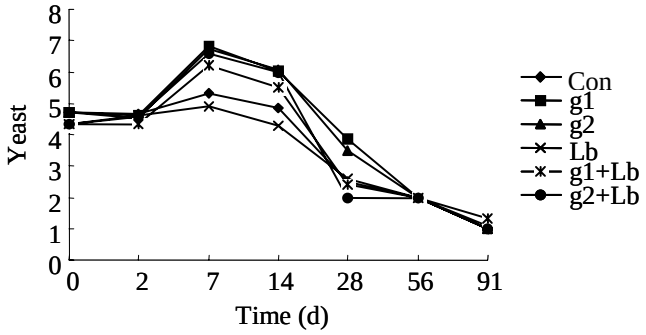

Figure 5. Yeast (log_{10} cfu g^{-1}) count in maize silages with different treatments during the whole ensiling.

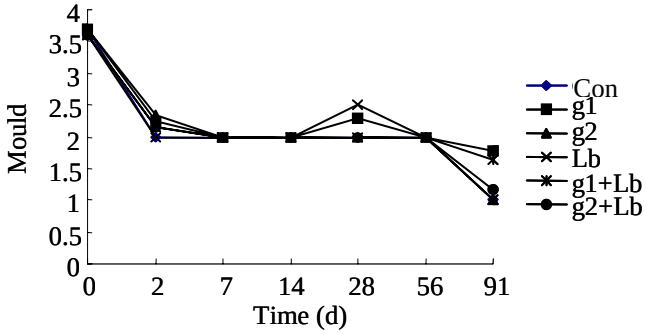

Figure 6. Mould (log_{10} cfu g^{-1}) count in maize silages with different treatments during the whole ensiling.

The concentration of lactic acid varied from 34.85 to 95.9 g (kg DM)$^{-1}$ and increased ($p<0.01$) with addition of extra sugar at ensiling. Silages inoculated by *L. buchneri* only or in combination with sugar addition contained less ($p<0.01$) lactic acid than the correspondent silages without inoculation with *L. buchneri*, contributing the higher finial pH due to partial lactic acid was fermented into acetic acid and other products. In comparison with control, ethanol production is about 3 or 6 fold higher due to addition 12.5 or 25 g glucose at ensiling, showing that ethanol concentration doubled if added double amount of glucose. Similar results in inoculated silages were found, which seems that ethanol formation was independently on *L.buchneri*. The added sugar resulted in less acetic acid concentration ($p<0.01$) than control, but inoculation with *L.buchneri* increased ($p<0.01$) acetic acid than correspondent uninoculated silages at different sugar levels. No butyric acid and propionic acid were found in uninoculted silages, however silages inoculated with *L.buchneri* were observed in propionic acid, 1-propanol and butyric acid. On the basis of inoculation of *L. buchneri*, added extra sugar reduced the production of propionic acid, butyric acid and 1-propanol ($p<0.01$), indicating that extra sugar suppressed the activity of *L. buchneri*.

Lactobacilli counts in silages of 91 days varied from 7.76 to 9.23 (log cfu g^{-1}), they were not influenced by added sugar. Inoculation with *L. buchneri* increased ($p<0.01$) *Lactobacilli* counts, but added sugar impaired the function of *L.buchneri*. The count of both yeast and mould were less than 2 (log cfu g^{-1}), indicated that they were not observed in silages at day 91 post-ensiling.

Aerobic stability

All the silages ensiled under anaerobic silos were stable upon exposure to air, longer than 280 h (table 2), showing that aerobic stability was not influenced by the added extra sugar and inoculation of *L.buchner* under anaerobic condition.

DISCUSSION

As expected that inclusion of extra sugar at ensiling enhanced the growth of yeast and produced the much higher ethanol at the present study. What is the source of much high ethanol concentration in silages treated with sugar? Where is the added extra sugar? The answer is that extra ethanol production, in comparison with control, should derive from the extra added sugar converted into ethanol by yeast, this finding can be supported by three evidences as below. First of all, all the silages had the same pH drop rate under anaerobic conditions before 14 days during this time the normal acidification process took place in which sugars were converted into lactic acid by LAB, this process almost finished at day 14, that means the same amount sugars were fermented into lactic acid, but nearly the same sugar amount left in silages at 14 days until the end of ensilage (figure 2), indicating added extra sugars were not fermented into lactic acid. Secondly, the concentration of ethanol in silages treated only with sugar at day 91 amounted *c.* 3 or 6 fold than control silage, also double ethanol production was observed in high sugar addition than low sugar addition that is completely in agreement with amount of sugar addition at ensiling. This finding could be confirmed by the changes of moles of sugar and ethanol (data not shown). Thirdly, the micro-organism responsible for more ethanol production in present silages is yeast, because during the day 0 to day 14, there was a big difference only in count of yeast, moreover by day 14 ethanol production already reached their peaks (data not shown), and just during the same period the yeast counts showed the significant difference (see yeast altering), on the other hand, yeasts are capable of convert the sugar into ethanol. All those mentioned as above coincided with DM loss and development of pH in silages. All the inoculated silages with *L. buchneri* were characteristiced by increased concentration of acetic acid, 1-propanol and propionic acid, slightly increased pH and DM loss, reduced yeast counts and increased LAB counts, which confirmed

Table 2. Composition of the maize silages under anaerobic conditions in 1-L jars at day 91[1,2,3]

	Con	g_1	g_2	L.b	L.b+g_1	L.b+g_2	SEM
PH	$3.74^{d\text{-}D}$	$3.73^{d\text{-}D}$	$3.72^{d\text{-}D}$	$4.18^{a\text{-}A}$	$3.99^{b\text{-}B}$	$3.9^{c\text{-}C}$	0.01
DM, g kg^{-1}	$268.3^{ab\text{-}A}$	$279.7^{a\text{-}A}$	$273.4^{ab\text{-}A}$	267.4^{b}	$268.6^{ab\text{-}A}$	$275^{ab\text{-}A}$	5.59
Sugar, g kg^{-1} DM	5.02^{a}	4.95^{a}	5.07^{a}	4.59^{a}	4.81^{a}	4.92^{a}	0.24
DM loss, g kg^{-1} DM	$17.92^{e\text{-}E}$	$29.23^{d\text{-}D}$	$42.57^{b\text{-}B}$	$29.43^{d\text{-}D}$	$36.44^{c\text{-}C}$	$50.75^{a\text{-}A}$	0.76
Ethanol, g kg^{-1} DM	$6.02^{c\text{-}C}$	$19.51^{b\text{-}B}$	$37.5^{a\text{-}A}$	$7.29^{c\text{-}C}$	$22.26^{b\text{-}B}$	$36.18^{a\text{-}A}$	1.55
Acetic acid, g kg^{-1} DM	$32.32^{c\text{-}C}$	$27.96^{d\text{-}D}$	$26.88^{d\text{-}D}$	$53.98^{a\text{-}A}$	$41.96^{b\text{-}B}$	$43.42^{b\text{-}B}$	1.03
Lactic acid, g kg^{-1} DM	$93.46^{a\text{-}A}$	$95.9^{a\text{-}A}$	$95.9^{a\text{-}A}$	$34.85^{d\text{-}D}$	$69.5^{b\text{-}B}$	$62.7^{c\text{-}C}$	2.01
Propionic acid, g kg^{-1} DM	$0^{d\text{-}D}$	$0^{d\text{-}D}$	$0^{d\text{-}D}$	$5.52^{a\text{-}A}$	$1.26^{c\text{-}C}$	$2.95^{b\text{-}B}$	0.36
1,2-propandiol, g kg^{-1} DM	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0
1-propanol, g kg^{-1} DM	$6.72^{d\text{-}D}$	$3.69^{e\text{-}E}$	$3.57^{e\text{-}E}$	$16.7^{a\text{-}A}$	$11.29^{c\text{-}C}$	$12.88^{b\text{-}B}$	0.44
Butyric acid, g kg^{-1} DM	$0^{c\text{-}C}$	$0^{c\text{-}C}$	$0^{c\text{-}C}$	$1.02^{a\text{-}A}$	$0.75^{b\text{-}B}$	$0.76^{b\text{-}B}$	0.03
LA/AA	$2.89^{c\text{-}C}$	$3.42^{b\text{-}B}$	$3.57^{a\text{-}A}$	$0.65^{f\text{-}F}$	$1.66^{d\text{-}D}$	$1.45^{e\text{-}E}$	0.05
LAB, log cfu g^{-1}	$7.76^{c\text{-}C}$	$7.77^{c\text{-}C}$	$7.81^{c\text{-}C}$	$9.13^{a\text{-}A}$	$8.88^{b\text{-}B}$	$8.95^{b\text{-}B}$	0.05
Yeast, log cfu g^{-1}	$<2^{a}$	$<2^{a}$	$<2^{a}$	$<2^{a}$	$<2^{a}$	$<2^{a}$	
Mould, log cfu g^{-1}	$<2^{a}$	$<2^{a}$	$<2^{a}$	$<2^{a}$	$<2^{a}$	$<2^{a}$	
Aerobic stability (h)	$>280^{a}$	$>280^{a}$	$>280^{a}$	$>280^{a}$	$>280^{a}$	$>280^{a}$	

[1,2,3] Con: Control; g_1 (add 12.5 g glucose per kg maize); g_2 (add 25 g glucose per kg maize); L.b.: *L. buchner*; DM: Dry matter.
[a,b,c,d] Means within a row with no common superscripts differ ($p<0.05$).
[A,B,C,D] Mean within a row with no common superscripts differ ($p<0.01$).

the description of typical *L. buchneri* treated silages (Driehuis et al., 1996; Driehuis et al., 1998,1999; Oude Elferink et al., 1998). Previous studies with pure cultures of *L.buchneri* have shown that *L. buchneri* is capable of converting lactic acid into acetic acid and 1,2-propandiol (Oude Elferink et al., 1998), however, sometimes propanol and propionic acid instead of 1,2-propanediol were detected (Driehuis et al., 1996,1998,1999). The developments of different fermentative products concentration during the whole ensilage suggested that there are two degradation pathways of lactic acid by *L. buchneri*, both of them begin to function after 14 days at the same time (data not shown). The first metabolism way of lactic acid is that, after 2 weeks (this experiment), *L. buchneri* started to ferment lactic acid into acetic acid and carbon dioxide, slight more DM loss in inoculated silage with *L. buchneri* attribute to loss of carbon dioxide. The second pathway is that lactic acid was converted into 1,2-propandiol by *L. buchneri*, Surprisingly, 1-propanol and propionic acid not 1,2-propanediol was measures as a dominant fermentation products in the *L. bucheneri* treated maize silage, which is in line with those described by Driehuis et al. (1999). This suggests that 1,2-propanediol is further degraded into 1-propanol and (or) propionic acid within silages. The fact that *L. bucheneri* is unable to degrade 1,2-propanediol in pure cultures or in the heterogeneous silages habitant (Oude Elferink et al., 2001) strongly indicates that other bacteria are involved in the further degradation of 1,2-propanediol..But how about the partition of lactic acid for two degradation ways that exist at the same time maybe should be studied further. Another finding in this study is that there is an antagonism between sugar content and function of *L. buchneri*, confirmed by the fermentative product concentration in final silages under the anaerobic conditions. *L. buchneri* shows a trace influence on the ethanol production, explanation is that the process of the conversion of sugar into ethanol by yeast took place at the initial stage and finished before day 14, and *L. buchneri* started to function at 2 weeks post-ensiling. As respects to aerobic stability, all silages ensiled under anaerobic silos are stable upon the exposure to air. In conclusion, the added sugars encourage the growth of yeast that convert all the extra sugar into ethanol. There is an antagonism between added sugar and *L. buchneri.*

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