



# Metagenome analyses reveal the influence of the inoculant *Lactobacillus buchneri* CD034 on the microbial community involved in grass ensiling



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## ABSTRACT

Silage is green fodder conserved by lactic acid fermentation performed by epiphytic lactic acid bacteria under anaerobic conditions. To improve the ensiling process and the quality of the resulting silage, starter cultures are added to the fresh forage. A detailed analysis of the microbial community playing a role in grass ensiling has been carried out by high throughput sequencing technologies. Moreover, the influence of the inoculant *Lactobacillus buchneri* CD034 on the microbial community composition was studied. For this purpose, grass was ensiled untreated or inoculated with *L. buchneri* CD034.

The fresh forage as well as silages after 14 and 58 days of fermentation were characterized physico-chemically. Characteristic silage conditions such as increased titers of lactic acid bacteria and higher concentrations of acetic acid were observed in the inoculated silage in comparison to the untreated samples. Taxonomic community profiles deduced from 16S rDNA amplicon sequences indicated that the relative abundance of *Lactococci* diminished in the course of fermentations and that the proportion of bacteria belonging to the phyla *Proteobacteria* and *Bacteroidetes* increased during the fermentation of untreated silage. In the inoculated silage, members of these phyla were repressed due to an increased abundance of *Lactobacilli*. In addition, metagenome analyses of silage samples confirmed taxonomic profiles based on 16S rDNA amplicons. Moreover, *Lactobacillus plantarum*, *Lactobacillus brevis* and *Lactococcus lactis* were found to be dominant species within silages as analyzed by means of fragment recruitments of metagenomic sequence reads on complete reference genome sequences. Fragment recruitments also provided clear evidence for the competitiveness of the inoculant strain *L. buchneri* CD034 during the fermentation of the inoculated silage.

The inoculation strain was able to outcompete other community members and also affected physico-chemical characteristics of the silage.

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## 1. Introduction

Ensiling is a method for conservation of green fodder such as grass or corn by means of lactic acid fermentation by lactic acid bacteria. The resulting silage can afterwards be used as fodder for ruminants or as substrate for biogas production in anaerobic

digesters. Since lactic acid bacteria are present on the green fodder as epiphytic bacteria, ensiling is a spontaneous and natural process as soon as anaerobic conditions prevail. Anaerobiosis and the subsequent formation of lactic and other volatile acids prevent growth of contaminating microorganisms such as yeasts, molds and other bacteria. Known members of microbial ensiling communities involved in lactic acid fermentation belong to the phylum *Firmicutes* and to the genera *Lactobacillus*, *Lactococcus*, *Weissella* and *Leuconostoc* (Woolford and Pahlow, 1998) comprising species such as *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactococcus*

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**Table 1**

Physico-chemical parameters of the raw material as well as of untreated and inoculated silage samples after 14 and 58 days of fermentation.

|                                            | Raw material | Untreated silage, 14 d | Untreated silage, 58 d | Inoculated silage, 14 d | Inoculated silage, 58 d |
|--------------------------------------------|--------------|------------------------|------------------------|-------------------------|-------------------------|
| Dry matter [%]                             | 36.82        | 30.91                  | 31.55                  | 29.51                   | 28.97                   |
| LAB <sup>b</sup> [log cfu <sup>a</sup> /g] | 5.86         | 8.72                   | 8.43                   | 9.95                    | 9.79                    |
| Yeast [log cfu <sup>a</sup> /g]            | 5.82         | 6.49                   | 5.58                   | 4.78                    | 2.60                    |
| pH                                         | 6.28         | 4.59                   | 4.41                   | 4.81                    | 4.94                    |
| Lactic acid [% of FM <sup>c</sup> ]        | 0.08         | 6.02                   | 7.77                   | 4.35                    | 2.63                    |
| Acetic acid [% of FM <sup>c</sup> ]        | 0.12         | 1.37                   | 1.88                   | 2.46                    | 5.17                    |
| Ethanol [% of FM <sup>c</sup> ]            | 0.00         | 0.56                   | 0.49                   | 0.58                    | 0.98                    |
| 1,2 Propanediol [% of FM <sup>c</sup> ]    | 0.00         | 0.00                   | 0.00                   | 0.44                    | 1.77                    |

<sup>a</sup> Colony forming units.<sup>b</sup> Lactic acid bacteria.<sup>c</sup> Fresh matter.

*lactis*, *Leuconostoc mesenteroides* and *Weissella cibaria* (Langston and Bouma, 1960a,b; Pang et al., 2011). Species of the genera *Enterococcus* and *Pediococcus* are also described as members of microbial ensiling communities (Pahlow et al., 2003). To improve the ensiling process (e.g. acid formation rate, aerobic stability), silage additives such as chemical substances or starter cultures can be added. Homofermentative lactic acid bacteria were preferably used for this purpose as they allow for a rapid pH decrease. In 1996 Weinberg and Muck proposed that the usage of heterofermentative lactic acid bacteria could be beneficial as they also produce other volatile fatty acids (Weinberg and Muck, 1996) inhibiting growth of yeast and fungi after aeration. The production of acetic acid was supposed to be the biggest advantage because it features enhanced antifungal properties (Danner et al., 2003; Holzer et al., 2003). Since then, several studies have shown that the application of the heterofermentative lactic acid bacterium *Lactobacillus buchneri* for inoculation of grass, alfalfa, corn and small-grain silages indeed is beneficial regarding acid formation and aerobic stability (Holzer et al., 2003; Kleinschmit and Kung, 2006; Kung et al., 2003; Mari et al., 2009; Reich and Kung, 2010; Schmidt and Kung, 2010). *L. buchneri* CD034 has been isolated from stable ensiling and is supposed to positively influence the ensiling process and the silage quality (Heinl et al., 2012).

Within microbial communities (e.g. ensiling communities) bacteria interact with each other and with their environment. Microbial communities often contain a huge amount of bacteria which cannot be cultivated under laboratory conditions. Moreover, bacteria involved in ensiling such as *Lactobacillus* and *Lactococcus* strains are known to enter into the viable but non-culturable state (Oliver, 2005). Hence, such bacteria will be missed in analyses based on cultivation experiments (Singh et al., 2009; Warnecke and Hess, 2009). However, cultivation, identification and enumeration of lactic acid bacteria on MRS agar plates (Man et al., 1960) often are applied in ensiling experiments. Metagenomic approaches by means of direct DNA extraction and subsequent analyses are therefore necessary to comprise the non-cultivable part of microbial communities. By applying denaturing gradient gel electrophoresis (DGGE) (Nishino and Touno, 2005) or sequencing of 16S rDNA fragments (McGarvey et al., 2013; Pang et al., 2011) of microbial ensiling communities it was possible to track changes within these communities and to identify dominant species. Moreover, specific primers have been developed to analyze the abundance of selected species (Klocke et al., 2006; Schmidt et al., 2008; Stevenson et al., 2006). However, both methods do not give detailed information on the composition of the complete microbial community. Hence, direct total community DNA or 16S rDNA amplicon sequencing and subsequent data analysis should provide more comprehensive insights into the composition of the whole community of interest. Metagenome analyses were already applied to analyze microbial communities of agricultural importance in anaerobic digesters fermenting maize silage as substrate (Jaenicke et al., 2011; Krause et al., 2008; Schlüter et al., 2008; Zakrzewski et al., 2012a,b).

Better understanding of processes underlying silage formation and the effects of applied starter cultures, especially *L. buchneri* strains, might help to improve this process. Hence, we analyzed an ensiling process and corresponding microbial ensiling communities by means of a metagenomic approach (including metagenomic DNA analyses as well as 16S rDNA amplicon sequencing) to (1) follow up changes in taxonomic community profiles in the course of the ensiling process, (2) identify microorganisms that are dominantly involved in ensiling and to (3) elucidate the competitiveness of the inoculation strain *L. buchneri* CD034 within the epiphytic bacterial community.

## 2. Materials & methods

### 2.1. Ensiling of grass samples

Silage was prepared from freshly cut grass (third cut) obtained from local farmers (Styria, Austria) in autumn 2010. The grass had a dry matter content of 36.82% (see Table 1). For inoculation  $1 \times 10^6$  cells of *L. buchneri* CD034 per gram of raw material were sprayed onto the grass and thoroughly mixed. *L. buchneri* CD034 had previously been isolated from stable silage (Heinl et al., 2012). Two aliquots of approximately 4 kg of untreated and inoculated grass were compressed in plastic bags kept in plastic buckets. Afterwards, the bags were sealed and the buckets were closed and left at room temperature for 14 and 58 days respectively.

### 2.2. Physico-chemical analysis of grass silages

Before the inoculation as well as after 14 and 58 days of fermentation physico-chemical properties of silages were determined. The pH values, concentrations of lactic acid bacteria (LAB) and yeasts as well as concentrations of lactic acid, acetic acid, ethanol and 1,2 propanediol were determined in an extract. The extract was prepared by homogenization of 30 g ensiled material with 100 ml of sterile physiological NaCl solution in a Stomacher (Seward Ltd., United Kingdom) and subsequent filtration. The pH was then determined directly in the extract (WTW inoLab 720, WTW GmbH, Germany). For enumeration of LAB and yeast the extract was used for decimal dilution series which were plated out onto MRS agar (Merck KGaA, Germany) for LAB enumeration (Man et al., 1960) as well as on YGC agar (Merck KGaA, Germany) for yeast and mold enumeration. The main products of lactic acid fermentation and pyruvate metabolism (lactic and acetic acid, ethanol, 1,2 propanediol) were determined by high-performance liquid chromatography (HPLC) (Agilent 1100 HPLC system, Agilent Technologies Deutschland GmbH, Germany) applying a cleared extract after Carrez precipitation. The dry matter content (DM) was determined by drying 30 g of fresh grass (105 °C, 24 h) and subsequent calculation of the mass loss.

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