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NEW SOLUTIONS TO REDUCE NEGATIVE IMPACTS OF AEROBIC DETERIORATION OF CORN SILAGE

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NEW SOLUTIONS TO REDUCE NEGATIVE IMPACTS OF AEROBIC DETERIORATION OF CORN SILAGE

Francesco Ferrero

PhD thesis

New solutions to reduce negative impacts of aerobic deterioration of corn silage

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Table of contents

Preface

This PhD thesis is the result of work which was carried out at the Department of Agricultural, Forest and Food Science (DISAFA) of the University of Torino (Italy), at the Agriculture and Agri-Food Canada, Lethbridge Research Center (Lethbridge, Alberta, Canada) and at the Forage Center of Excellence of Lallemand Animal Nutrition, in partnership with the William H. Miner Agricultural Research Institute, (Chazy, NY,USA).

The PhD project was conducted under the supervision of Professor Giorgio Borreani and Mr. Ernesto Tabacco. The research period at the Agriculture and Agri-Food Canada, Lethbridge Research Center and at the Forage Center of Excellence has been possible thanks to the collaboration with Dr. T.A. McAllister and Dr. P. Drouin, respectively.

The main aim of the thesis has been to extend the current understanding on the negative impact of aerobic deterioration of corn silage and to analyze different solutions to help avoid it. The specific aims of the thesis have been:

i. to analyze the effects of aerobic deterioration on the microbial, fermentative and nutritional qualities of corn silage, with particular focus on the microbial and hygiene aspects;

ii. to evaluate the effect of the fermentative profile on inhibiting the causal agents of aerobic deterioration;

iii. to evaluate the effect of chemical and biological additives on modifying the fermentative profile in order to avoid or reduce the aerobic deterioration of silages;

iv. to analyze new technologies and tools to improve knowledge on the microbial dynamics involved in the fermentation and aerobic deterioration of corn silage.

Aerobic microorganisms, such as yeast, mold and acetic acid bacteria, are spoiling agents that also represent a toxigenic risk to a silage. Thus, the starting hypothesis of this work has been that the inhibition of the growth of spoiling microorganism, through the metabolic activity of lactic acid bacteria inocula, through the addition of chemical additives, and/or through the adoption of correct silage management practices, can reduce aerobic deterioration and its negative impact in microbial, fermentative, nutritional and hygienic terms.

The first part of the thesis is devoted to an introduction in which the latest scientific advances about aerobic deterioration are reviewed. This chapter is structured in 4 sections: the first section describes the ensiling process, focusing on the microbial populations that are active in the process and on the

fermentative profile of silages. The second section analyzes the effect of aerobic deterioration on the nutritional, fermentative, microbial and hygienic qualities of silage. The third section describes the additives used in silage to avoid aerobic deterioration. The fourth part highlights the importance of the microbial analysis of silage and shows the different traditional and innovative methods that can be used to analyze the microbial communities involved in the silage process.

After the introduction, which presents the main topics of the thesis, the experimental part describes the research activities carried out during the PhD period and it is divided into 5 chapters, which correspond to original papers published in different International Peer-Reviewed Journals.

This thesis is based on the following five papers, which will be referred to in the text by their Roman numerals:

- Chapter 6 "**Paper I**": **Ferrero F.**, Piano S., Tabacco E., Borreani G., 2019. Effects of time of conservation and new *Lactobacillus hilgardii* inoculum on fermentation profile and aerobic stability of whole corn and sorghum silage. J. Sci. Food Agric. 99:2530-2540. https://doi.org/10.1002/jsfa.9463.

- Chapter 7 "**Paper II**": **Ferrero F.**, Tabacco E., Borreani G., 2019. Effects of a mixture of monopropionine and monobutyrin on the fermentation quality and aerobic stability of whole crop maize silage. Anim. Feed Sci. Technol. In press. https://doi.org/10.1016/j.anifeedsci.2019.114319.

- Chapter 8 "**Paper III**": **Ferrero F.**, Prencipe S., Spadaro D., Gullino M.L., Cavallarin L., Piano S., Tabacco E., Borreani G., 2019. Increase in aflatoxins due to *Aspergillus* section *Flavi* multiplication during the aerobic deterioration of corn silage treated with different bacteria inocula. J. Dairy Sci. 102:1176-1193. https://doi.org/10.3168/jds.2018-15468.

- Chapter 9 "**Paper IV**": Spadaro D., Matic S., Prencipe S., **Ferrero F.**, Borreani G., Gisi U., Gullino M.L., 2019. *Aspergillus fumigatus* population dynamics and sensitivity to demethylation inhibitor fungicides in whole-crop corn, high moisture corn and wet grain corn silages. Pest Manag. Sci. 76:685-694. https://doi.org/10.1002/ps.5566.

- Chapter 10 "**Paper V**": Borreani G., **Ferrero F.**, Nucera D., Casale M., Piano S., Tabacco E., 2019. Dairy farm management practices and the risk of contamination of tank milk from *Clostridium* spp. and *Paenibacillus* spp. spores in silage, total mixed ration, dairy cow feces and raw milk. J. Dairy Sci. 102:8273-8289. https://doi.org/10.3168/jds.2019-16462.

The conclusion summarizes the final remarks of the thesis and highlights future perspectives.

Summary

Ensiling is a forage preservation method that is based on spontaneous lactic acid fermentation under anaerobic conditions which allows the high nutritional value of fresh forage crops, such as corn, grasses, legumes and cereals, to be maintained. A good quality silage is the results of several factors, such as the crop characteristics, the harvesting technique and the management of the ensiling process. The microbial groups that develop during each phase of ensiling influence the end quality of the silage. The two most difficult silage problems are ensuring lactic fermentation against butyric fermentation and ensuring the aerobic stability of silages during feed-out.

The aim of the PhD dissertation is to examine different aspects related to the aerobic deterioration of corn silage, with particular focus on the different groups of microorganisms involved in the spoiling process, and on the way aerobic stability may be improved.

The first paper provides new insights into the effect of a new strain of *L. hilgardii* used as an inoculum to improve the aerobic stability of corn and sorghum silages after different conservation periods.

The second paper provides information about the addition of a chemical additive, composed of monoglycerides, on the aerobic stability of corn silage harvested at a high DM content.

The third paper analyzes the effect of aerobic deterioration on the multiplication of *Aspergillus flavus* and the *ex-novo* production of aflatoxins. The paper also provides information about the toxigenic potential of *A. flavus* strains isolated from corn silage.

The fourth paper determines the evolution of *Aspergillus fumigatus* during the ensiling process, from the harvest to air exposure of corn harvested as whole-crop, high moisture corn and wet grain, and monitors the sensitivity of isolates to one medical and one agricultural fungicide.

The fifth paper is aimed at on farm verifying the role of the aerobic deterioration of corn silage on the proliferation of *Clostridium* and *Paenibacillus* spores along the whole route from silage to milk, to identify, through 16S-DNA sequencing, the dominant anaerobic and facultative anaerobic sporeformer species along the milk chain and to evaluate the effect of management practices on mitigating the carry-over of anaerobic and facultative anaerobic spores in milk.

Introduction

1. The ensiling process

Ensiling is a conservation method for forages and other feeds based on a spontaneous lactic acid fermentation of plant carbohydrates under anaerobic conditions. The fermentation of water-soluble carbohydrates (WSC) into lactic and other organic acids by epiphytic population or added lactic acid bacteria (LAB) determine the decrease of pH with a consequent inhibition of spoilage microorganisms (Pahlow et al., 2003).

The widespread use of this technique is due to the possibility to rapidly stocking large amounts of wet product reducing the risks due to weather conditions (Grant and Adesogan, 2018). In addition, ensiling technique allow to preserve the majority of the original nutritional value and palatability of the forage at harvesting (Weinberg et al. 2004) throughout the year. Several fresh crop such as corn, grasses, alfalfa, wheat and legume grains (Wilkinson and Toivonen, 2003), TMR (Weinberg et al., 2011), as well as several crop residues such as corn stalk, and other by-products (Dinuccio et al., 2010; Menardo et al., 2015) are conserved as silage in many countries of the world.

The fermentation process is affected by many factors that in some conditions can lead to end result not always satisfactory. Moreover, the fermentation process is influenced by type and quality of forage crop, by several management factors such as silo packing speed, silage density, use of additive, chop length, and silo management during the conservation period (Pahlow et al., 2003; Kung, 2010a; Borreani et al., 2018; Kung et al., 2018a).

Throughout the world, whole-crop corn is the main crop conserved as silage (Wilkinson and Toivonen, 2003) because it presents the optimal characteristics for direct ensiling, such as adequate dry matter (DM) content, high content of water-soluble carbohydrates (WSC), and low buffering capacity (Bolsen et al., 1996). Furthermore, corn silage has high DM yields per hectare and supplies both fiber and starch in the animal ration (Allen et al., 2003). On the other hand, forage legume crops and other annual grasses which are considered to be low producing crops are more difficult to ensile than corn (Borreani et al., 2013; Peyraud et al., 2009) but can enhance the production efficiency and reduce the environmental impacts of dairy farming (Tabacco et al., 2018).

In many, but not all cases, the fermentation that a crop undergoes can be explained by how the microbial population in silage interact with factors such as oxygen, moisture content, buffering capacity, and sugar content of the crop (Kung, 2010a).

1.1. Phases of ensiling

The process of ensiling can be subdivided into four principal phases of different length and intensity (Barnett, 1954; Weinberg and Muck, 1996; Pahlow et al., 2003), which cannot be separated precisely from each other. Each of the four phases is characterized by different changes in the chemical and physical properties of the forage due to the activities of different groups of microorganisms (Figure 1).

Phase I, initial aerobic phase. The initial aerobic phase of ensiling starts with harvesting of forage in the field and ends with packing and sealing the silo. Oxygen trapped in the ensiling crop is rapidly consumed by respiration of aerobic and facultative aerobic microorganisms (such as fungi, yeasts and enterobacteria) and by plant enzymes which generate heat by degrading WSC into water and CO_2 (Woolford, 1990). As much oxygen as possible should be eliminated from the silage by good packing and sealing: in a silage finely chopped, well-compacted, and sealed without delay, this aerobic phase can be minimized to few hours (McDonald et al., 1991). Long aerobic phase results in high silage temperatures, high energy and dry matter (DM) losses, mold growth and browning reactions (Maillard reactions) (Pahlow et al., 2003) with reduction of nutritive value of the product, and the risk of increasing the probability of prevailing butyric fermentation over lactic fermentation (Ciotti et al., 1989; Borreani et al., 2008; Niyigena et al., 2019).

Phase II, main fermentation phase. This phase begins once the last trace of O_2 has been depleted and the silage becomes anaerobic. The phase can continue from few days to several week depending on crop properties and ensiling conditions. In the early days of fermentation, facultative and obligate anaerobic microorganisms such as enterobacteria, clostridia, certain bacilli, and yeasts can theoretically compete with the LAB flora for the nutrients. In successfully fermented silages, the LAB population became dominant whereas the other undesirable population, mainly clostridia, yeast and enterobacteria remained stable and/or progressively decrease in time when anaerobiosis is maintained. The dominance of LAB is favorite by high content of sugar, oxygen-free conditions, warm temperatures, and high silage moisture. The speed of disappearance of undesirable microorganism is closely correlated with the rate of pH decline and lactic acid production (Pahlow et al., 2003). In this regard, is more difficult to ferment legume herbages (or forages) compared to corn plant because they are naturally lower in sugar content and higher in acid-buffering capacity.

Phase III, stable phase. By the end of the Phase II (from 2 to 4 weeks), silage reach a final low pH that inhibits the growth of microorganisms and the silage is preserved for as long as air is prevented from entering the silo. During Phase III, mainly acid tolerant enzymes and some group of microorganisms

continue to be active, causing a slow acid hydrolysis of structural and storage carbohydrates that provides an important, continuous supply of water-soluble carbohydrates (WSC) (Woolford, 1984a; **paper I)**. The LAB population typically is reduced because of inhibition from the low pH and fermentation products and only few species (i.e. *Lactobacillus buchneri, L. diolivorans*) are able to maintain their activity (Krooneman et al., 2002; Muck et al., 2018; **paper I)**. Several highly acid tolerant yeast species survive this period in an almost inactive state, along with the bacilli and clostridia, which turn to dormancy as endospores (Pahlow et al., 2003).

Phase IV, feed-out phase. When the silo is opened for feed-out, oxygen has access to the silage. The Phase IV is divided in two sub-phases, the aerobic stability and the aerobic deterioration. The aerobic stability is the time during which the aerobic microorganisms are still inhibited by the low pH and the fermentative products. Later, the aerobic microorganisms start to multiply by consuming residual sugars, fermentation products (i.e., lactic and acetic acids) and other soluble nutrients in the silage resulting in heating, depleting inhibiting microbial molecules and consequently decrease the nutritional value of silage. The primary spoilage stage is the onset of deterioration due to the activity of yeasts and, occasionally, acetic acid bacteria (Spoelstra et al., 1988, Pahlow et al., 2003; **paper I**). This cause a rise in pH and respiration of organic acids, and thus the second spoilage stage starts, that includes the activity of many other (facultative) aerobic microorganisms, such as molds and enterobacteria (Oude Elferink et al., 1999). The main negative consequences of aerobic deterioration are DM and economical losses, losses of WSC and fermentation end products, reduction of the nutritive value of silage (Pahlow et al., 2003; Tabacco et al., 2011a; **paper II, III**) and worsening of hygienic quality of silages (Borreani and Tabacco, 2010; **paper III, IV, V**). During feed-out phase the presence of oxygen is unavoidable, but the aerobic deterioration can already start earlier due to damage to the silage covering (e.g. by rodents or birds) during phase II and III (Oude Elferink et al., 1999).

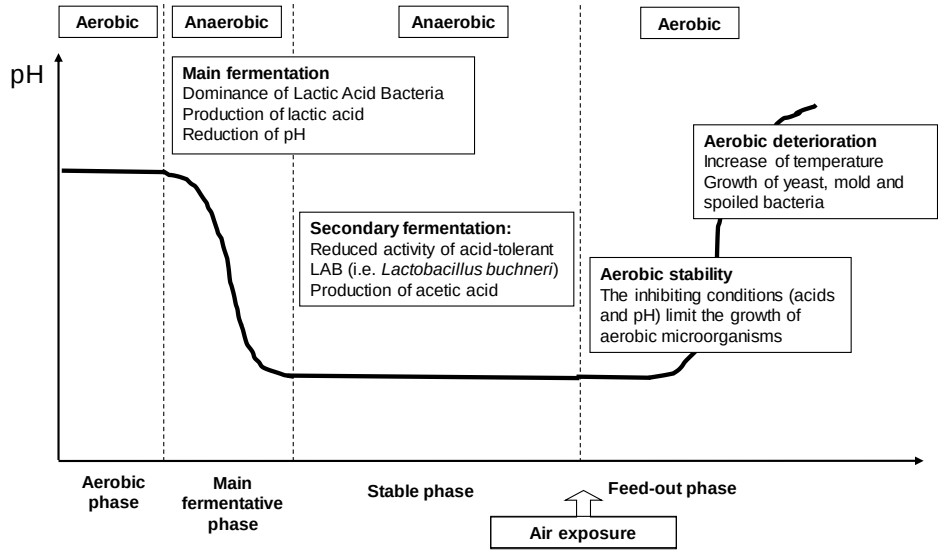

Figure 1. Evolution of pH and oxygen availability during the ensiling process when lactic acid fermentation dominates (or in well lactic acid fermented silage).

Reduction of silage nutritional, microbial and fermentative qualities can occur during each of the four phases (McDonald et al., 1991; Borreani et al., 2018a; Kung et al., 2018a). Thus, to avoid failures, it is important to control and optimize each phase of the ensiling process. In the past, much effort has been devoted to understanding and improving the efficiency in the second and third phases of ensiling (Kung et al., 2003; Muck et al., 2018). But it is now recognized that the changes which may occur during the fourth phase are equally as important as those in the first two phases from the viewpoint of preserving nutrients and maintaining good hygienic quality for silage consumption by animals (Wilkinson and Davies, 2013).

1.2. Microbial communities of silage

The several microorganisms present in silage play a key role in the successful outcome of the silage conservation. In each phase of ensiling many microorganisms can sequentially find optimal conditions for growth, and can be activated or inhibited with considerable impact on silage quality (McAllister et al., 2018). Basically, the groups of microorganisms involved in the process can be divided into two groups, the desirable and the undesirable microorganisms (Oude Elferink et al; 1999; Pahlow et al., 2003). Lactic acid bacteria are the desirable microorganisms to obtain a well fermented silage, whereas their undesirable competitors are those involved in anaerobic spoilage (e.g. clostridia and enterobacteria) and those involved in aerobic spoilage (e.g. yeasts, molds). Many of the spoilage microorganisms decrease the nutritional and hygienic value of the silage (Driehuis and Oude Elferink, 2000) thus representing a significant problem for farm profitability and feed quality (Tabacco et al., 2011a). Occurrence of changes in the microbial population depend mainly on crop composition, harvesting conditions (direct and wilted ensiling) and silo management, i.e., crop species, DM content, WSC content, buffering capacity, degree of anaerobiosis, and contamination of soil and manure (Pahlow et al., 2003).

1.2.1. Desirable microorganisms

Lactic acid bacteria. Lactic acid bacteria (LAB) are facultative aerobes, but some have a preference for anaerobic conditions (McDonald et al., 1991; Pahlow et al., 2003) and belong to the epiphytic microflora of plant material (Lin et al., 1992a). During silage making, an immediate increase in numbers of LAB during the harvesting has been observed as compared to standing crop (Lin et al., 1992b). The increase in LAB population it is presumed to be due to the presence of dormant and non-culturable cells since it can neither be the results of multiplication, considering the time scale, nor a contamination by the harvesting equipment (Pahlow, 1991). The competitiveness of LAB with other undesirable microorganisms during silage fermentation is influenced by several crop, management and environmental characteristics (e.g. sugar content, DM content, activity water, temperature, packing density) as well as by LAB properties such as acid- and osmo-tolerance, and substrate utilization (Woolford, 1984a; McDonald et al., 1991; Pahlow et al., 2003).

The main LAB genera associated with silage are *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Enterococcus*, *Lactococcus* and *Streptococcus*. The LAB population found in silage can be classified based on their sugar metabolism as reported in Table 1.

The main homofermentative LAB found in silage are *Lactobacillus acidophilus*, *L. plantarum*, *Pediococcus acidilactici*, *P. damnosus*, *P. pentosaceus*, *Lactococcus lactis* and *Streptococcus bovis* whereas between the obligate heterofermentative the main species in silage are genus *L. brevis*, *L. buchneri*, *L. reuteri* and *Leuconostoc mesenteroides* (Pahlow et al., 2003).

Lactic acid bacteria play an important role in silage process as they are responsible for the preservation. To control the ensiling process, maintain quality and to inhibit non-wanted microorganisms, LAB can be used as inocula in silage making. The LAB inocula have been selected to be used to improve silage fermentation (phase II) and/or to improve aerobic stability (phase IV) (Muck et al., 2018; **paper I**).

Table 1. Sugar metabolism of lactic acid bacteria found in silage (Woolford, 1984a; Kandler and Weiss, 1986; McDonald et al., 1991; Rooke and Hatfield, 2003)

Metabolism		Pathway of hexose Fermentation				Pathway of pentose Fermentation			
Traditional cluster	Kandler and Weiss (1986)	Sugar	End products	DM recovery (%)	Energy recovery (%)	Sugar	End products	DM recovery (%)	Energy recovery (%)
Homofermentative	**Obligate homofermentative**	Glucose or Fructose	Lactate	100	99.2	No fermentation	-	-	-
	Facultative heterofermentative	Glucose or Fructose	Lactate	100	99.2	Xylose or Arabinose	Lactate, Acetate	100	99.0
Heterofermentative	**Obligate heterofermentative**	Glucose	Lactate, Ethanol, CO_2	75.6	98.3	Xylose or Arabinose	Lactate, Acetate	100	99.0
		Fructose	Lactate, Acetate, Mannitol, CO_2	95.2	99.0				

1.2.2. Undesirable microorganisms

Enterobacteria. Enterobacteria are facultative anaerobic, Gram-negative, rod-shaped, often motile organisms. During silage fermentation, the growth of enterobacteria is undesirable because they compete with LAB for the available sugars and they can degrade the protein (Pahlow et al., 2003). The relevant members are considered nonpathogenic. However, as they contain an endotoxin in the outer membrane of the cell, some have been associated with feeding problems and cases of mastitis (Lindgren, 1991). Enterobacteria are able to reduce nitrate (NO_3) to nitrite (NO_2) with consequent formation of gaseous NO and NO_2, harmful to animal health (Woolford, 1984a). A little nitrite reduction is considered positive for silage quality, because of the inhibitor potential of nitrite against clostridia (Spoelstra, 1985). In spite of these potential positive effects, rapid suppression of enterobacteria is generally desirable (Pahlow et al., 2003). For the anaerobic growth, enterobacteria strictly depend on fermentable carbohydrates but they are not able to proliferate at pH lower than 4.5. Thus, ensiling conditions that induce a rapid and sufficient drop in silage pH contributes on inhibition of enterobacteria growth (McDonald et al., 1991). Enterobacteria in some conditions (i.e. short ensiling period or air infiltration during conservation) can survive the storage phase and can start growing again and reach numbers in excess of 10^8 cfu/g of fresh matter when silage pH increases during the aerobic spoilage phase (Lindgren, 1985; Oude Elferink et al., 1999). Epiphytic enterobacteria, including *Erwinia herbicola* and *Rahnella aquitilis*, often dominate fresh crops, but others supersede these during ensiling such as *Escherichia coli*, *Hafnia alvei*, and *Serratia fonticola* (Driehuis and Oude Elferink, 2000). Among these *Escherichia coli* O157:H7, a Shiga toxin producing bacterium, is the most notorious of the enterobacteria, but a rapid drop in pH has been shown to eliminate *E. coli* in silage (Chen et al., 2005; Dunière et al., 2011; Queiroz et al., 2018).

Clostridia. Clostridia are endospore-forming typically Gram-positive, anaerobic bacteria. Clostridia can compete with LAB when pH drop is delayed in forages with high a_w (>0.95) and/or no nitrate content (<100 mg/kg DM) since they are able to ferment carbohydrates as well as lactic acid. Some group of clostridia are able to ferment proteins, thus reducing the feeding value. The clostridial fermentation of hexoses sugars and lactic acid to butyric acid leads DM recovery below 50% due to the formation of gases (Pahlow et al., 2003). Furthermore, clostridia in silage reduce milk quality due to the fact that their spores be transferred to milk and can survive the pasteurization (Stadhousers and Jørgensen, 1990; Vissers et al., 2007b; **paper V**). The outgrowth of clostridial spores in raw milk causes butyric acid fermentation in cheese (late-blowing) resulting to commercial losses for the production of semi-hard cheeses (Stadhouders et al., 1993; Colombari et al., 2001; Julien et al., 2008). During silage fermentation, a rapid drop in silage pH prevent the development of

clostridia in silage, because clostridia are inhibited at pH below 4.2. The value of pH that inhibits the clostridia growth is related to the DM content of the forage (Wieringa, 1958) but commonly is indicated that with pH lower than 4.2 clostridia are not able to grow in any a_w conditions. For this reason, clostridia fermentation is uncommon in corn silages because it always reaches a pH lower than 4.2 (Kung et al., 2018a). Clostridia are more susceptible to low water activity than LAB (Pahlow et al., 2003), thus, in crops that don't present characteristic to an easy ensiling (e.g. legume crops, winter cereals) they can be inhibit by decreasing the a_w value of the crop by wilting to a higher DM content (Wieringa, 1958; Leibensperger and Pitt, 1987; Colombari et al., 1999; Muck, 2010). Finally, clostridia are also inhibited by nitrate (Spoelstra, 1985). An increase in the anaerobic spore content was found during aerobic deterioration of grass, corn and sorghum silages (Jonsson, 1991; Vissers et al., 2007a; Tabacco et al., 2009; **paper V**) as results of formation of micro-niches with less inhibitory activity in which the oxygen was depleted by aerobic microorganisms (Jonsson, 1989; Borreani et al., 2013).

Corrot (1986) reported a large heterogeneity in clostridia spore counts in whole-crop corn silage stored in horizontal silos with high contamination levels (>5.0 log MPN/g) in the peripheral zones compared to cores (2.0 log MPN/g). The presence of higher level of clostridia spores in the peripheral zones compared to cores as results of aerobic deterioration was emphasized by several studies (Vissers et al., 2007a; Borreani and Tabacco 2008; Tabacco et al., 2009; **paper V**).

Clostridia that ferment carbohydrates regularly isolated from silage include *C. tyrobutyricum*, *C. beijerinckii* and *C. butyricum*, species. Many clostridia are able to ferment both carbohydrates and proteins such as *C. sporogenes*. Proteolytic clostridia unable to ferment carbohydrates are not normally found in silage (Pahlow et al., 2003; **paper V**). The *C. botulinum*, responsible for botulism in animal, belongs to this group (Kehler and Scholz, 1996).

Acetic acid bacteria. Acetic acid bacteria are obligate aerobic, acid-tolerant bacteria, Gram-negative and catalase-positive rods. In silage, the main genus of acetic acid bacteria isolated is *Acetobacter* (Spoelstra et al., 1988; Dolci et al., 2011; **paper I**) whereas the other genus *Gluconobacter* plays a minor role and it was found in less amount (Pahlow et al., 2003). The undesirable activity of *Acetobacter* spp. is during feed-out phase as it was reported that it can initiate aerobic deterioration (Spoelstra et al., 1988). Even if oxygen is considered necessary for the growth of acetic acid bacteria a strain capable of surviving under anaerobic conditions has been identified (Du Toit et al., 2005). The oxidation of ethanol to acetic acid is the best-known metabolic pathway (Pahlow et al., 2003). However, oxidation of lactic and acetic acids to CO_2 and H_2O is also an important function of these bacteria. Furthermore, selective inhibition of yeast can also increase proliferation of acetic acid bacteria in silage (Driehuis and van Wikselaar, 1996). An interesting property of acetic acid

bacteria in ensiling is crop difference: acetic acid bacteria are found mainly in corn and other cereal silages (Oude Elferink et al., 2001), whereas they were seldom detected in aerobically stable or unstable grass silages even if extensive ethanol fermentation occurred (Nishino et al., 2011).

Bacilli. Bacilli are aerobe or facultative anaerobe, endospore-forming, Gram-positive, rod-shaped bacteria. The proliferation of bacilli in silage is generally considered undesirable. In the last few decades, studies have shown that many organisms formerly classified as the *Bacillus* genus actually represent several genera within the Bacilli class (Durak et al., 2006). At least 12 new genera of spore-forming bacteria have been defined (Ash et al., 1993). They ferment a wide range of carbohydrates to compounds such as organic acids (e.g. acetate, lactate and butyrate) or ethanol, 2,3-butanediol and glycerol (Pahlow et al., 2003) but are less efficient than LAB (McDonald et al., 1991). Bacilli are not regarded as initiators of spoiling during feed-out even if in later stages of aerobic deterioration their growth was detected (Lindgren et al. 1985). The number of bacilli during silo opening ranges from 10^1 to 10^5 cfu/g (Te Giffel et al., 2002). Soil is the main habitat of bacilli therefore, their numbers is influenced by contamination with soil or manure at harvest (McDonald et al., 1991; Nishino, 2011). The main species found in silage (e.g. *B. licheniformis*, *B. cereus*, *B. lentus*, *B. firmus*, and *B. sphaericus*) (McDonald et al., 1991) are usually found during aerobic deterioration or during ensiling because some of them belong to the aerobic bacilli, and do not grow at pH below 5.0 (Claus & Berkeley, 1986). In the last few decades, members of genus *Paenibacillus*, previously classified in the *Bacillus* genus (Durak et al., 2006), were found in silages. The most frequently reported are *P. polymyxa*, *P. pabuli*, *P. macerans* and *P. thermophylus* in silages (Borreani et al., 2013; Driehuis, 2013; Driehuis et al., 2016; **paper V**). They have been found able to survive pasteurization in spore form and to grow under refrigeration, thus resulting in product spoilage and limiting the shelf life of high-temperature short-time pasteurized fluid milk (Ivy et al., 2012).

Yeast. Yeasts are eukaryotic, facultative anaerobic, heterotrophic microorganisms. Those found in silage are generally divided into two groups according to substrate utilization and are either saccharolytic or lactate-fermenting (Jonsson and Pahlow, 1984; McDonald et al., 1991). The yeast population is involved both in anaerobic alteration and aerobic deterioration of silages. Under anaerobic silage conditions, yeasts compete with LAB to ferment sugars (to ethanol and CO_2) only in the first days of the second phase of ensiling, because of their need of small oxygen concentration for the synthesis of sterol during growing phase, with a DM recovery around 50% (Woolford, 1984). The alcoholic fermentation decreases the amount of sugar available for lactic acid fermentation, increases the losses of DM and could have negative effects on milk taste (Randby et al., 1999). The anaerobic and acidic environments of silages are in principle hostile for yeasts cells. Thus, the

survival of yeasts during fermentation and storage is affected by the degree of anaerobiosis and the concentrations of organic acids. Short-chain fatty acids such as propionic and acetic acids are known to inhibit yeasts at a low pH (Moon, 1983; **paper II**). If a complete anaerobiosis is kept the yeast count decrease during storage (**paper I**) but, if traces of oxygen still remain present, the survival of yeasts is ensured (Pahlow et al., 2003) until silo opening (conditions commonly found in peripheral areas of horizontal silos on farm). During the feed-out phase, many yeast species degrade the lactic acid to CO_2 and H_2O. The yeast growth during aerobic exposure results in heating, DM, nutrient and energy losses and cause the rise of pH allowing spoilage molds and bacteria to grow.

Mold. Molds are eukaryotic microorganisms. In silage, the presence of molds is linked to the presence of trace of oxygen. During storage, this is usually observed only in the surface layers of the silage, but during aerobic spoilage (Phase 4) the whole silage can become moldy. Mold species that regularly have been isolated from silage belong to the genera *Penicillium*, *Fusarium*, *Aspergillus*, *Mucor*, *Geotrichum*, *Monascus* and *Trichoderma* (Pelhate, 1977; Pahlow et al., 2003; O'Brien et al., 2007; Spadaro et al., 2015; Schenck et al., 2019; **paper III, IV**). During aerobic deterioration, the mold activity follow the resurgence of yeast activity that had consumed some inhibiting factors such as organic acid, increasing the pH of the silage. Molds cause a reduction in feed value and palatability of the silage, and can also have a negative effect on human and animal health as they are associated with allergenic reactions and mycotoxins production (Oldenburg, 1991; **paper III**). There is still uncertainty concerning the conditions under which mycotoxins are formed in silage. A mold-infested silage is usually easily identified by the large filamentous structures and colored spores that many species produce. On the other hand, a moldy silage does not necessarily contain high levels of mycotoxins, and not all types of mycotoxins that a mold species can produce are necessarily present. Ensiling methods that minimized air ingress and allow to produce fermentative end product with antifungal proprieties (i.e. acetic and propionic acid) limits mold growth (Oude Elferink et al., 1999).

The mold population in silage come from the field. Several fungi are able to grow on corn plant and produce mycotoxins during preharvest stage but only a few are adapted to ensiling conditions (Garon et al., 2006). Thus, fungal and mycotoxins contamination in silage could be derived from infection in the field and/or during silage storage. The use of foliar fungicides to increase corn yield may cause changes that may be beneficial to plant nutritive quality (Venancio et al., 2009; Haerr et al., 2015) as fungal contamination may increase NDF and ADF concentrations, thus decreasing *in vitro* NDF digestibility when ensiled as corn silage (Yates et al., 1997; Queiroz et al., 2012). Recently, several work pointed out the capability of some fungi to develop resistance to fungicides applied during crop cultivation. The resistance to fungicides in addition to the

ability to adapt to the hostile environment of silage could determine the dominance of few species which will increase of the risks for fungal and mycotoxins contamination. The main species that were resistant to fungicides were found in strains of *Fusarium* (Müllenborn et al., 2008), *Penicillium* (Kinay et al., 2007) and *Aspergillus* (Santoro et al., 2017; **paper V**).

1.2.3. Microbial dynamics in silage

The microbial dynamics of the main groups of microorganism involved in silage process in three different conditions: well fermented silage with the prevalence of lactic fermentation; prevalence of butyric fermentation acted by clostridia; and in case of air infiltration during conservation (phases II and III) are reported in Figure 2, 3 and 4, respectively.

Lactic fermentation. In well fermented silages lactic acid bacteria dominate the fermentation determining a pH drop. The anaerobic environment inhibits the aerobic microorganisms (acetic acid bacteria and mold) whereas the decrease in pH value does not allow the growth of facultative anaerobe (except lactic acid bacteria) and strictly anaerobe groups of microorganisms. During air exposure the silages remain stable until some aerobic or facultative anaerobe microorganisms start to grow leading to the aerobic deterioration. During aerobic deterioration molds and clostridia appears after a first activity of yeast (and sometimes acetic acid bacteria) (**paper I, III, IV, V**).

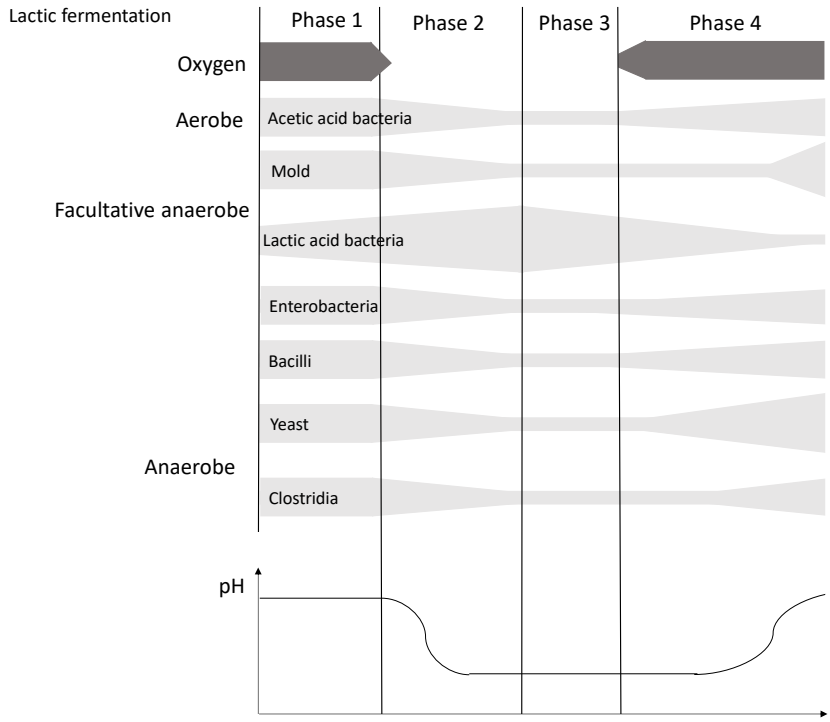

Figure 2. Microbial dynamics of the main groups of microorganism involved in silage process and pH evolution in case of dominance of lactic fermentation.

Butyric acid fermentation. When lactic acid bacteria are not able to dominate the fermentation and, in absence of nitrate, with pH higher than 4.2 and a_w higher than 0.95, clostridia became the dominant groups leading to butyric fermentation. The butyric fermentation is uncommon in corn silage but can be found in crops with low WSC, high a_w due to inadequate wilting period (e.g. legume, winter cereal). In case of clostridial activity, saccharolytic clostridia start the fermentation determining high DM losses and not allowing a pH drop. The following proteolytic activity lead to the decrease of the nutritional value of silages.

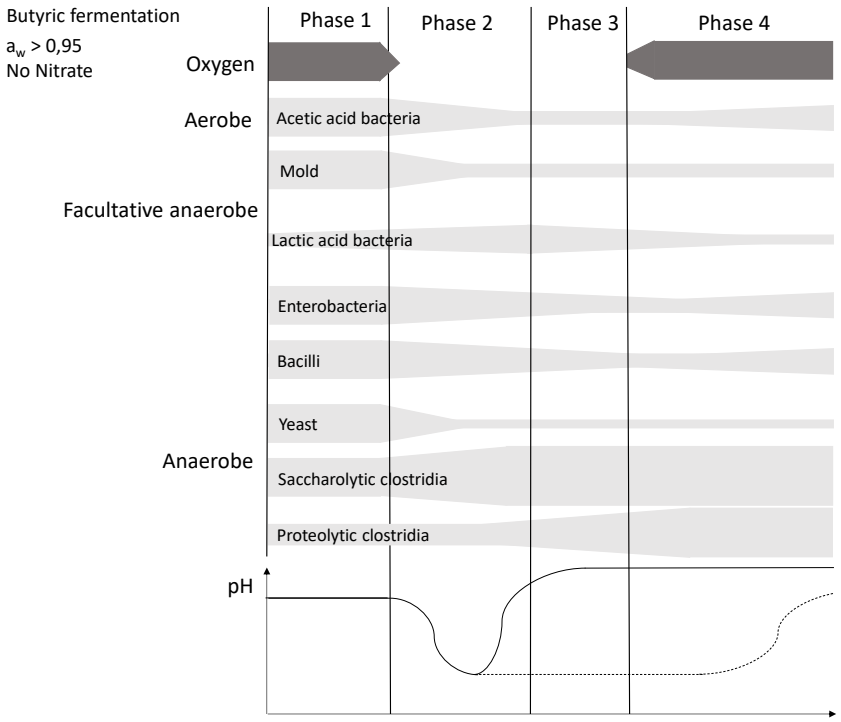

Figure 3. Microbial dynamics of the main groups of microorganism involved in silage process in case of dominance of butyric acid fermentation.

Air infiltration during conservation. During the second and third phases, air can penetrate the silo due to an ineffective seal (Borreani et al., 2018). The presence of traces of air allows different groups of microorganisms to compete with LAB. Thus, the pH does not drop and the spoiling microorganism (mainly yeast and acetic acid bacteria) are able to remain active. At opening, the spoiling microorganisms, that are present in high level, start to quickly multiply leading to a fast deterioration of the silage.

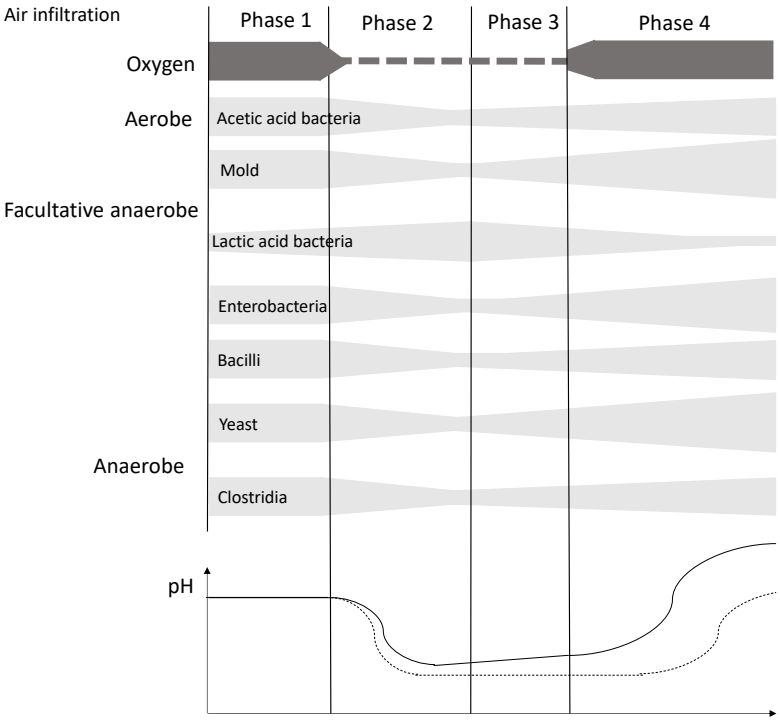

Figure 4. Microbial dynamics of the main groups of microorganism involved in silage process in case of air infiltration during conservation.

An example of microbial dynamics of whole-crop corn silage (41% DM) conserved for 250 days are reported in Figure 5 (Ferrero et al., unpublished data). In the first 60 days of conservation the majority of microbial changes appeared: lactic acid bacteria dominated the fermentation and reached 9.0 log cfu/g in the first days of fermentation; enterobacteria and molds quickly decreased in their number whereas yeast and acetic acid bacteria showed a slower decrease. Interestingly, the data showed an effect of DM content of corn silage on the microbial dynamics: the LAB activity is limited by the reduced a_w, thus the production of acids is low (Comino et al., 2014) and the reduction of the yeast (Kung, 2009) and other undesirable microorganism is lower than wetter silages.

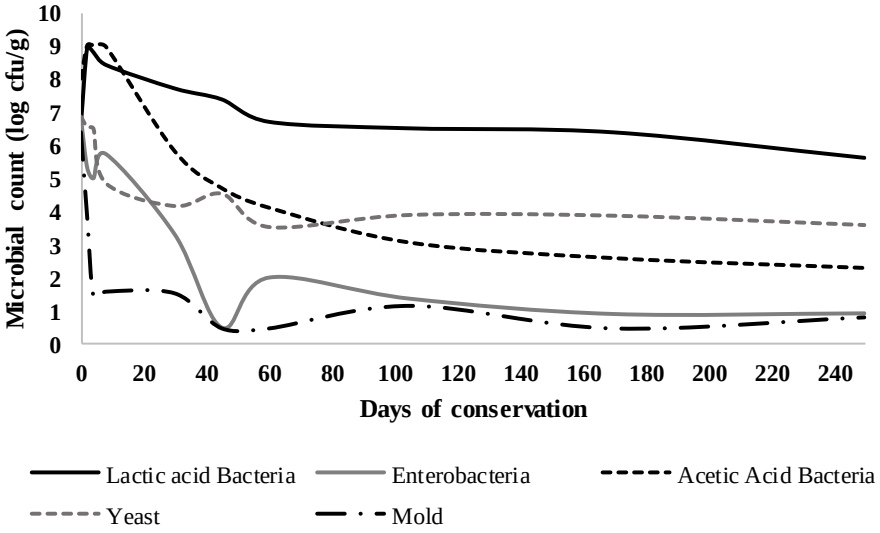

Figure 5. Microbial dynamics during fermentation of whole-crop corn silage at 41% DM (Ferrero et al., unpublished data).

The microbial dynamics of that silage during aerobic exposure are reported in Figure 6 (Ferrero et al., unpublished data). The high number of yeast and other spoiling microorganisms led to low stability of silages. Furthermore, data confirmed a first activity of yeast and acetic acid bacteria followed by molds activity. Enterobacteria was not found to be very active during aerobic exposure of air.

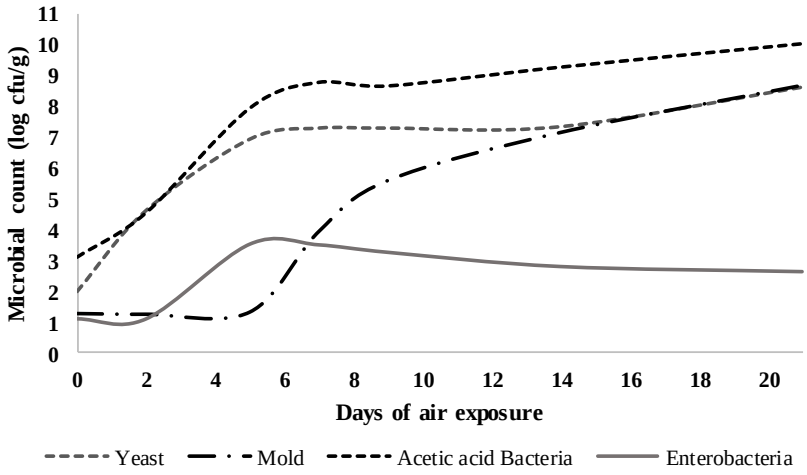

Figure 6. Microbial dynamics during aerobic exposure of whole-crop corn silage at 41% DM after 60 d of conservation (Ferrero et al., unpublished data).

1.3. Fermentative profile of silage

The goal of forage conservation as silage is to preserve as much as the nutritive value of the fresh crop (Kung et al., 2010) avoiding undesirable compounds that could negatively affect animal performance (Kung et al., 2018a). The fermentative profile of silage is determined by the microbial fermentation that take place. The type of fermentation is affected by many crop characteristics (i.e. buffering capacity, DM content, WSC content) as well as management factors such as the speed of packing, pack density, use of additives, chop length, covering management, and silo management during feed-out (Kung et al., 2018a). Measuring the pH and quantifying the fermentative products (i.e. organic acids and alcohols) are the main basis of evaluating silage fermentation quality. Table 2 reported the typical suggested concentrations of common fermentation end products in various silages.

Table 2. Concentrations of common fermentation end products in well fermented silages (adapted from Borreani and Tabacco, 2010; Mogodiniyai Kasmaei et al., 2013; Kung et al., 2018a; **paper IV**; Tabacco et al., unpublished data)

	Legume silage (25-40% DM)	**Legume silage (40-45% DM)**	**Grass silage (25-35% DM)**	**Corn silage*** **(30-45% DM)**	**High moisture corn (55-70% DM)**	**Wet grain (70-75% DM)**
pH	4.3-4.5	4.7-5.0	4.3-4.7	3.5-4.1	3.7-4.2	3.8-4.3
Lactic acid (g/kg DM)	60-80	20-40	60-100	11-94	10-33	5-20
Acetic acid (g/kg DM)	20-30	5-20	10-30	10-56	3-12	<5.0
Propionic acid (g/kg DM)	<5.0	<1.0	<1.0	1-12	<1.0	<1.0
Butyric acid (g/kg DM)	<5.0	<0.1	<10	<0.1	<0.1	<0.1
Ethanol (g/kg DM)	5-10	5	5-10	4-30	4-9	2-20
NH_3-N (% of total N)	10-15	<12	8-12	5-7	<10	<10

* Corn silage: ensiling of the whole chopped plant; High moisture corn: ensiling of the chopped ear (cob and grain); Wet grain: ensiling of the whole chopped wet grain.

1.3.1. pH

The final pH of silage is a rapid indication of the type of fermentation that took place. Two samples may have the same pH, but different concentrations of acids as it is affected by several factors, mainly type of crop and DM content. In general in presence of high values of a_w (between 0.98 to 1.00) pH lower than 4.2 is important for silage conservation as it inhibits the growth of several undesirable microorganisms. Some authors suggested that the pH should be less than 0.0257 × DM% + 3.71 even if a higher pH (0.0028 × DM (g/kg) + 4.209) might be allowed if silage is preserved in bales and not in bunkers (Field and Wilman, 1996; Weissbach, 1996). Several factors can be responsible for silages that present a pH that is higher than 4.2. First, the pH value of silage is influenced by the type of crops, its buffering capacity and DM content. Commonly legume silages have a higher pH than corn or other grass silages at the same DM content due to lower WSC content and higher buffering capacity. The DM content influenced a_w, especially of wilted silages, that can cause a higher than expected pH as the growth of LAB is curtailed and the rate and extent of fermentation is reduced (Kung et al., 2010; **paper II**). Third, low amount of WSC due to crop characteristics (Pahlow et al., 2003) or poor field management practices (Borreani et al., 2018a) resulted in high pH. Corn silage usually has a pH lower than 4 (Borreani and Tabacco, 2010; **paper IV**) even if factors can determine high pH such as restricted fermentation due to cold/hot climatic conditions (Bernardes et al., 2018; Kim and Adesogan, 2006; Zhou et al., 2016), suboptimal management factors such as slow silo filling, inadequate packing densities, air penetration (Kleinschmit et al., 2005; Borreani et al., 2018a; Brüning et al., 2018), inoculation with *Lactobacillus buchneri* (Kleinschmit and Kung, 2006a; **paper I, III**) or other undesirable fermentation (McDonald et al., 1991).

1.3.2. Lactic acid

Lactic acid is produced by LAB and should be the main acid detected in well fermented silages. Lactic acid is the most contributor to the decline in pH during fermentation because its pKa (3.86) makes it about 10 to 12 times stronger than any of the other major acids [e.g., acetic acid (pKa of 4.75) and propionic acid (pKa of 4.87)] found in silages (Kung et al., 2018a). Similarly to what reported for high pH, low amount of lactic acid is due to high DM content (Kung et al., 2010; **paper II**), low amount of WSC (Pahlow et al., 2003), cold/hot climatic conditions (Bernardes et al., 2018) or undesirable fermentation (McDonald et al., 1991). Wet grass silage with high amount of WSC can reach up to 100 g/kg DM of lactic acid. In corn silage the amount of lactic acid range between 30 to 80 g/kg DM. Higher amount can be found in strictly homolactic fermented silages with low DM content (less than 300 g/kg) whereas lower

amount can be found in those silages having higher DM content than 350 g/kg or having undergone a heterolactic fermentation due to the activity of *L. buchneri* (Driehuis et al., 1999; Krooneman et al., 2002).

1.3.3. Acetic acid

Acetic acid, based on concentration, is the second acid found in silage. In the past, the presence of acetic acid was considered an indication of poorly fermented silage as result of the activity of other microorganism than lactic acid-producing bacteria (mainly clostridia). However, these undesirable fermentations are correlated with concentrations of acetic acid (>50 g/kg DM) coupled with other organic acid such as butyric and propionic, and are frequently found in extremely wet (DM content less than 200 g/kg) silages (McDonald et al., 1991). On the other hand, moderate concentrations of acetic acid in silage are beneficial because they inhibit yeasts, resulting in improved aerobic stability when silage is exposed to air (Danner et al., 2003; **paper I**). Moderately higher than normal concentrations of acetic acid (20 to 40 g/kg DM) are often found in silages treated with *L. buchneri* and conserved for more than 60 days, because of the partial conversion of lactic to acetic acid during the Phase III (Driehuis et al., 1999; Krooneman et al., 2002). However, production of acetic acid from this organism should not be misinterpreted as a poor fermentation as happens in the fermentation Flieg– Zimmer's score (Zimmer, 1966). Moreover, the use of silages with a high concentration of acetic acid does not appear to have a negative effect on animal intake (Kung et al., 2018a).

1.3.4. Lactic-to-acetic ratio

The ratio of lactic acid to acetic acid is commonly used as a qualitative indicator of fermentation. Good silage fermentations usually have a ratio of these acids of about 2.5 to 3.0. However, this value can be modified by several factors. Treated silage with a homofermentative LAB results in a slightly higher ratio of lactic acid to acetic acid because of the solely production of lactic acid by these bacteria. On the other hand, silages treated with *L. buchneri* or other heterofermentative LAB can result in higher concentrations of acetic acid and a lower lactic acid to acetic acid ratio than untreated silages (Kleinschmit and Kung, 2006a; **paper I, III**). The lactic-to-acetic ratio is commonly utilized to verify the lactic fermentation that takes place (Kung et al., 2018a): a lactic-to-acetic ratio around or higher than 3 indicates the prevalence of homolactic fermentation, whereas the prevalence of heterolactic fermentation is represented by lower ratios. Kleinschmit and Kung (2006a) reported ratio of 2.3 and 1.3 in corn silages treated with increasing application rate of *L. buchneri*.

1.3.5. Propionic acid

Propionic acid is usually undetectable in dry silages whereas traces (1 to 2 g/kg DM) were found in silages with DM lower than 250 g/kg. Propionic acid higher than 5.0 g/kg DM are usually associated with clostridial fermentation even if silages treated with *L. buchneri* sometimes have higher concentrations of propionic acid because 1,2-propanediol produced by *L. buchneri* can be converted to propionic acid by *Lactobacillus diolivorans* (Krooneman et al., 2002). The presence of propionic acid in silage is considered positive thanks to its antifungal activity and improvement in aerobic stability (Moon, 1983; Woolford, 1975; **paper II**). As a consequence, several efforts were dedicated in the past to select Propionibacteria as silage inocula, but most of the published works report difficult growing of these bacteria in wheat, sorghum and corn silages (Weinberg et al., 1995; Higginbotham et al., 1998).

1.3.6. Butyric acid

Butyric acid should not be detectable in well-fermented silages because it is an indication that the silage has undergone clostridial fermentation, which leads to large losses of DM and poor recovery of energy (Woolford, 1984a). Corn silage usually doesn't present any traces of butyric acid. For the other crops, a fermentation problem can be defined as one where butyric acid is greater than 2.5 g/kg of DM even if traces lower than 5 g/kg DM of this acid are considered acceptable in legume and grass silage with DM content lower than 350 g/kg. In aerobic spoiled corn silage the presence of butyric acid can be due to the multiplication of sporeformer bacteria, especially in peripheral areas of the bunker (**paper VI**). Paradoxically, silages containing butyric acid presented high aerobic stability when exposed to air because this acid has strong antifungal effect (Kung et al., 2018a). Nonetheless the presence of butyric acid is always to be considered negative.

1.3.7. Ethanol

Ethanol is the alcohol most commonly found in silages. It can be produced by a variety of microorganisms (heterofermentative LAB, enterobacteria, and yeasts) but is usually recognized as an indicator of yeast activity. In whole-plant corn and legume silages it range from 5 to 10 g/kg DM whereas it can be higher in other crops such as sorghum silage (**paper I**) or sugarcane silage overpassing in same conditions 200 g/kg of DM (Daniel et al., 2013). High concentrations of ethanol in silages are often associated with high numbers of yeasts and high DM losses (Pahlow et al., 2003; **paper II**), and such silages usually spoil readily when exposed to air because some yeasts can assimilate lactic acid under these

conditions (Kung et al., 2018a).

1.3.8. 1,2-propanediol (1,2PD)

Even if a variety of microorganisms are able to produce 1,2PD, including some species of clostridia and yeasts, in well fermented silages, 1,2PD represent a typical end-product of the metabolism of *L. buchneri* (Oude Elferink et al., 2001; **paper I**, **III**). Sometimes, 1,2PD can be not detected in silages treated with *L. buchneri* because the naturally occurring microorganism, *L. diolivorans* can metabolize this compound producing 1-propanol and propionic acid (Krooneman et al., 2002).

1.3.9. Volatile organic compounds (VOC)

A variety of other volatile organic compounds (VOC) can be found in silages, as reported in Figure 7 (Hafner et al., 2013; Weiss, 2017). Even if VOCs present in the silage when consumed by animals usually does not represent a problem, emissions of VOCs from silage have been identified as a contributor to poor air quality because the emitted VOC are precursors to tropospheric ozone (Bonifacio et al., 2017). The VOCs emission is sensitive to environmental conditions, with the greatest emission occurring under hot and windy conditions (Hafner et al., 2010). The VOCs emission mainly occur during feed-out phase thus changes in storage and feeding silage management can substantially reduce VOC emission (Hafner et al., 2010). Alcohols compose the largest mass of VOC, with ethanol being the predominant alcohol. Furthermore, the alcohols may react with acids in the silage, producing esters that have been found to have negative effect on intake (Gerlach et al., 2013). In particular, limited research available indicates a correlation of strong odors with the levels of ethyl and propyl esters of lactate and acetate (Kung et al., 2018a). The majority of VOC are likely produced directly or indirectly by undesirable microbes such as enterobacteria, clostridia, or yeasts and in less amount by heterofermentative LAB (Hafner et al., 2013), thus a correct silage management acted to reduce aerobic and anaerobic deterioration can reduce the formation and emission of VOCs (Hafner et al., 2013; **paper II**).

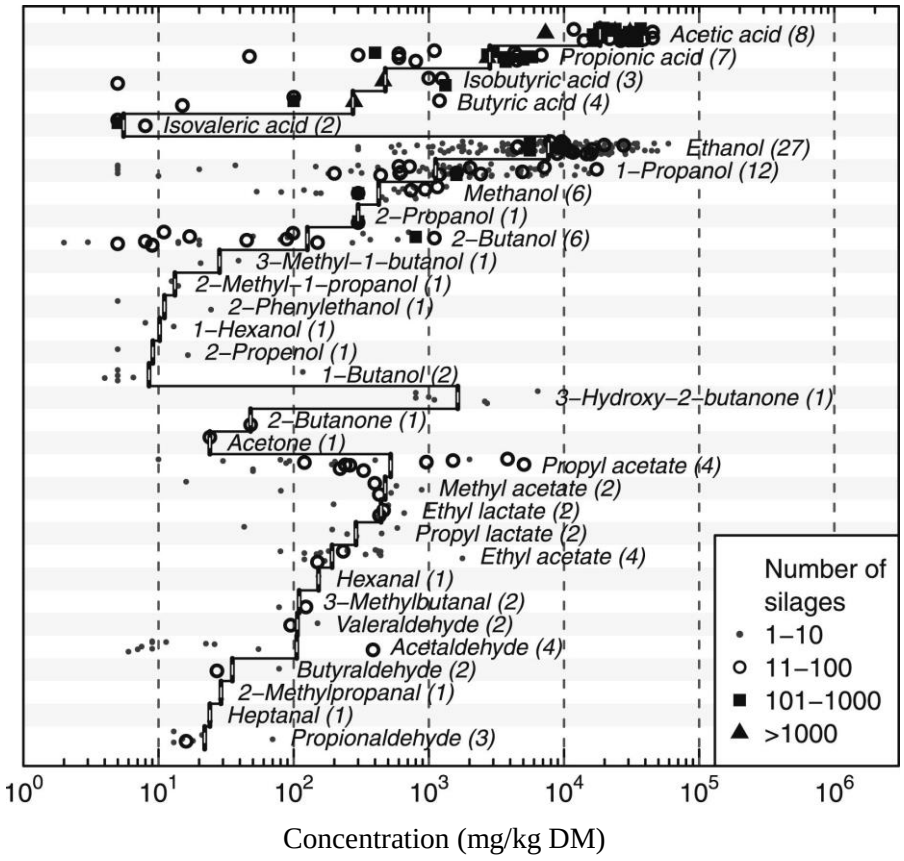

Figure 7. Concentrations of VOCs (mg/kg DM) found in corn silage, numbers in brackets are the number of studies from which data for each compound were taken (Hafner et al., 2013).

1.3.10. Ammonia-nitrogen (NH_3-N)

The nitrogenous compounds of silages change during fermentation are due to plant and microbial proteolytic processes. The main change that occur are an increase in soluble N (55-60% of total N) and NH_3-N (usually less than 10–15% of total N). Higher concentrations of ammonia than normal are a result of excessive protein breakdown in the silo caused by enterobacteria growth in Phase I and II (commonly associated with slow packing and filling of the silo, delayed sealing, slow drop in pH), or by clostridial fermentation. Moreover, high concentration of soluble N and NH_3-N are usually found in wet silages (<30% DM) and in wet legume silages as result of proteolytic activity from clostridia (Kung et al., 2018a).

2. The Aerobic Deterioration of corn silage

During feed-out silages are inevitably exposed to air (Driehuis et al., 1999) and oxygen has free access to the silo face to approximately 1 m behind the face (Honig, 1991). Because of the presence of air, the growth of undesirable aerobic microorganisms such as yeast, molds and acetic acid bacteria, most often responsible for the onset of aerobic instability, is unavoidable (Spoelstra et al., 1988; Pahlow et al., 2003; **paper I**). Woolford (1986) described the presence of air as the 'Achilles heel' of the ensiling process with its inextricable link to aerobic deterioration and the losses of silage dry matter and of silage chemical and hygienic quality.

Aerobic deterioration is common to many silages that are opened and exposed to air even if the rate of spoilage is highly dependent on the numbers and activity of the spoilage organisms in the silage (Muck et al., 2018; **paper I, II, III, V**). During feed-out phase, after a first time defined as aerobic stability of silages during which the aerobic microorganisms are inhibited by the low pH and the fermentative products, these microorganisms start to multiply in the presence of air and contribute to heating and major chemical changes of the silage, with a substantial decrease in nutritional value (Borreani et al., 2018a).

After silo opening three successive sub-phases are observed (Pahlow et al., 2003; Borreani et al., 2018a; Okatsu et al., 2019):

- *Aerobic stability*. This phase starts after the silo is opened; during aerobic stability the aerobic microorganisms are inhibited by the low pH and the fermentative end products and no changes occur in silage quality. The length of aerobic stability is influenced by crop characteristics, fermentative and microbial characteristics, management practices, length of conservation period, use of additive; environmental factors (Wilkinson and Davis, 2013; Borreani et al., 2018a; Muck et al., 2018; **paper I, II**).
- *Invisible aerobic deterioration*. Aerobic deterioration occurs because the end fermentative products of silage become themselves substrates for aerobic microorganisms growth (Pahlow et al., 2003). In the first phase of aerobically deteriorating silages, there is an increase in silage temperature above the ambient one as a result of the microbial oxidation of acids and water-soluble carbohydrate (WSC) to carbon dioxide and water (Ranjit and Kung, 2000). A laboratory standardized test defines a silage to be unstable when its temperature increase 2°C (Ranjit and Kung, 2000) or 3°C (EFSA, 2012) above the ambient temperature. Unfortunately, most microbial deterioration is invisible initially and the occurrence of deterioration may only be detected by a

temperature rise in the forage (Muck and Holmes, 2000; Borreani and Tabacco, 2010). During aerobic deterioration different microbial populations develop subsequently. However, it is generally accepted that yeasts play the major role in initiating the aerobic spoilage of silage causing a rise in temperature (Woolford et al., 1982; Jonsson and Pahlow, 1984; Wilkinson and Davis, 2013; **paper III**), even if other authors found acetic acid bacteria to be the starter or co-responsible with yeast of aerobic deterioration (Spoelstra et al., 1988; Dolci et al., 2011; **paper I**). Moreover, as a consequence of degradation of acids the proliferation of a wider range of other bacteria (i.e. Bacillaceae, Enterobacteriaceae) can take place.

- *Visible aerobic deterioration*. When sufficient acid is metabolized, strictly aerobic molds proliferate by oxidizing residual sugars and acids thereby causing further increases in temperature and pH. Once the temperature is above 45°C, the amount of yeast present declines, and other microbial organisms, such as molds, bacilli, *Listeria*, clostridia, and Enterobacteriaceae, begin to accumulate (Lindgren et al., 1985; Vissers et al., 2007; Borreani and Tabacco, 2008). When mold count increases over 10^5 log cfu/g of silage, deterioration becomes apparent giving an indication that spoilage has reached an 'advanced stage' (Muck, 2010; Borreani and Tabacco, 2010; Okatsu et al., 2019).

In 2018 we conducted an experiment (Tabacco et al., unpublished data) to verify the effect of acetic acid addition (around 30 g/kg of DM) after silo opening (170 d of conservation) in 39% DM corn silage. The additive was applied using a hand sprayer, by spraying uniformly onto the forage, which was constantly hand mixed. The temperature profile, pH, yeast and acetic acid bacteria of corn untreated (A) or treated with acetic acid (B) during aerobic stability is reported in Figure 8. The control silage showed a typical temperature profile with a first peak due to yeast activity and a following second peak due to further microbial activity. The pH and temperature increased as result of yeast activity. Silages treated with acetic acid showed an extension of the aerobic stability (from 50 h for control to 150 h for treated silage) and the rise of temperature when aerobic stability was broken was very slow compared to control. The microbial count suggested that the delayed peak of temperature for treated silage was due to the activity of acetic acid bacteria as their number increased to a very high level (10^8 cfu/g) compared to yeasts (that were around detection limit). Similar results were observed in some samples of **paper I**.

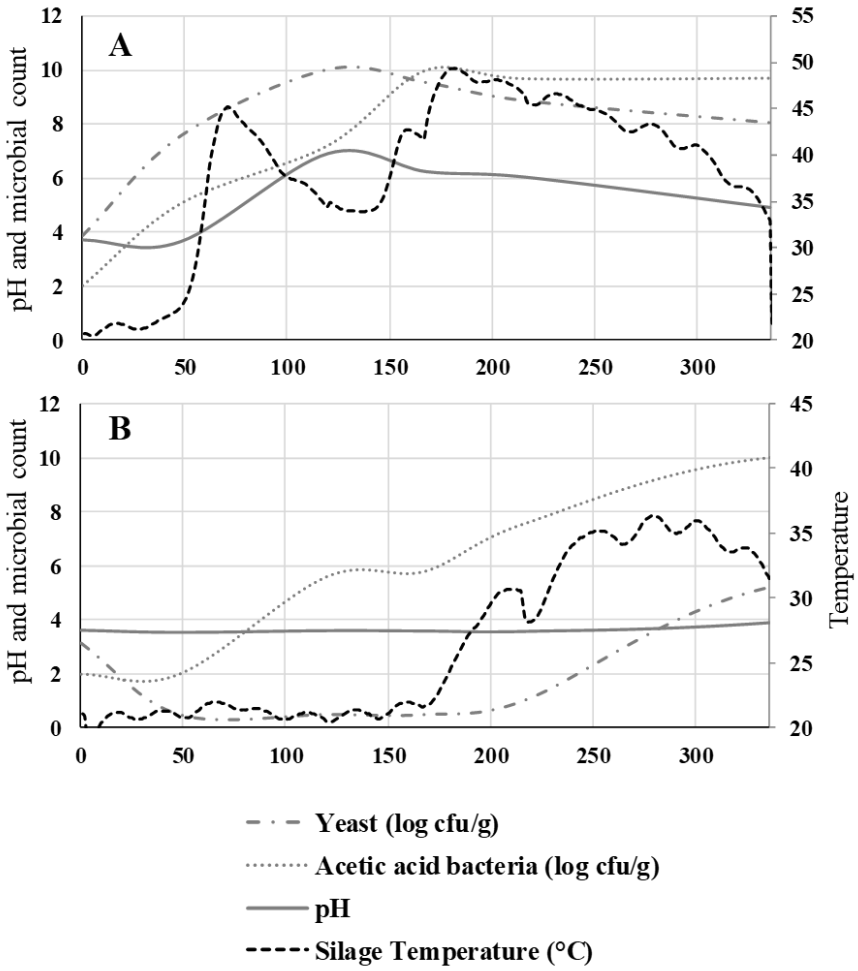

Figure 8. The temperature profile, pH, yeast and acetic acid bacteria of corn silage untreated (A) or treated with acetic acid (B) after silo opening (170 d of conservation) (Tabacco et al., unpublished data).

The activity of spoilage microorganisms such as mold and aerobic bacteria (Driehuis and Oude Elferink, 2000) determining losses of silage DM, reduction of silage chemical and hygienic quality (Oldenburg, 1991; Pahlow et al., 2003; Borreani et al., 2018a; **paper III, IV, V**), which are connected to the proliferation of potentially pathogenic or otherwise undesirable microorganisms (Lindgren et al., 2002) and mycotoxin synthesis (Richard et al., 2009; Cavallarin et al., 2011; **paper III**).

2.1. Effect of fermentative profile on aerobic spoilage microorganisms

Ohyama et al. (1980) negatively correlated the hours of aerobic stability with DM content, pH and yeast, whereas the same authors indicated as factor positively correlated to aerobic stability the amount of fermentative end products (except lactic acid). The type and the amount of fermentation end products can influence the stability, and consequently the deterioration of silages (Muck and O'Kiely, 1992). The aerobic stability of corn silage was found to be strictly correlated to the number of yeasts (Tabacco et al., 2011b; **paper I**) thus a fermentative profile with antifungal compound can be beneficial to avoid aerobic deterioration (O'Kiely and Muck, 1992, Muck et al., 2018). As reported above, the main organic acids found in corn silages are lactic, acetic and to a lesser extent propionic acid (Kung et al., 2018a). These acids inhibit the growth of most bacteria and fungi that cause aerobic deterioration. The inhibition is mainly dependent to pH, with greatest inhibition observed at or below the pKa, of the acids. Furthermore, the degree of inhibition is also affected positively by increasing the chain length of fatty acids (Moon, 1983). Moon (1983) reported that propionic acids was more effective in yeast inhibition than acetic acid and lactic acid. Since the pKa, of acetic acid and propionic acid is similar (4.73 and 4.87) the growth rate inhibition should be similar at the same pH. However, this is not the case. Moreover, as the pKa of lactic acid is 3.86 the amount of dissociated acid should be much less than acetate and propionate and require a considerably higher concentration for the same concentration of associated acid. This suggests that the growth inhibition is not due entirely to the associated acid concentration and that the three acids may not act in the same manner on the cell (Moon, 1983). Acetic acid was reported to be effective in improving aerobic stability by yeast reduction in several work (Kleinschmit and Kung, 2006a; Comino et al., 2014; **paper I**). Figure 9 reported the scatterplot of acetic acid and yeast count of corn silage ranging from 26 to 42% DM, the higher the amount of acetic acid is, the lower the yeast count. Propionic acid is not found in high amount in silages thus, due to its strongly antifungal effect, usually it is added as chemical additive in silages (Kung et al., 2018a; Muck et al., 2018; **paper III**). Butyric acid has paradoxically, strong antifungal characteristics (Kung et al., 2018a) but its presence is always to be considered negative as result of clostridia fermentation. Nevertheless, the addition as chemical of small amount of butyric acid has shown positive effect on aerobic stability (**paper II**).

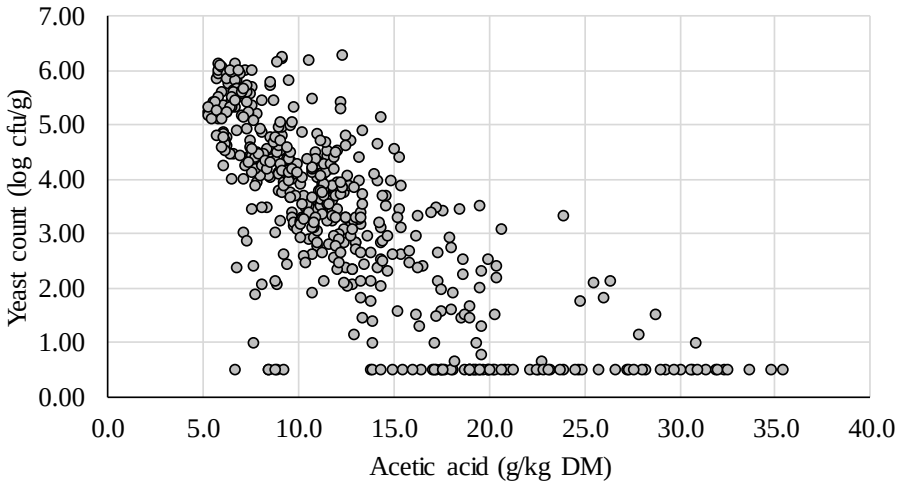

Figure 9. Scatterplot of acetic acid and yeast count from corn silages with DM content ranging from 26 to 42% (Tabacco et al., unpublished data).

2.2. Implications of aerobic deterioration on silage quality

The main objectives of making silage are to preserve forage for use in those seasons when fresh forage is not available, to preserve forage when there is a surplus of it during the growing season, and to preserve forage that cannot be grazed (Driehuis and Oude Elferink, 2000). Clearly, silages provide the forage base for many beef and dairy feeding systems. Silages supply energy, protein, and digestible fiber to ruminant diets and the ensiling process will have substantial effects on the nutritive value of the silage (Grant and Adesogan, 2018). Forage provides the animal with dietary fiber, which is essential for the normal functioning of the rumen.

Silages may have changes in the nutritive value due to the procedures during crop harvesting, conservation and feed-out phase management due to biochemical and microbiology phenomena. Aerobic deterioration usually results in losses of DM up to 20% (Tabacco et al., 2011a; Borreani et al., 2018a), loss of important nutritional components such as fermentative products, WSC and starch (**paper III**). Furthermore, the accumulation of degradation products can also affect palatability and produce metabolic disorders in dairy cows (Wilkinson, 1999; Driehuis and Oude Elferink, 2000). The feeding value of silage is mainly determined by intake, digestibility (Huhtanen et al., 2002) and quality and availability of nutrients that influence animal performance. The intake of silage is generally lower than the intake of fresh forage even if proper management in the ensilage can result in well-preserved silage and result in a similar intake of fresh forage (Oliveira et al., 2016).

2.2.1 Dry Matter losses and reduction of nutritional value

During each phase of ensiling, minimum value of the DM losses are unavoidable (Borreani et al., 2018a). Good management practices can reduce these losses to provide the quality forage needed for each animal group (Rotz, 2003). The DM losses that occur during deterioration process can reach up to 20% of the total stored DM of farm silages (Köhler et al., 2019), both in temperate (Vissers et al., 2007a; Borreani and Tabacco, 2010; Schmidt and Kung, 2010) and warm climates (Huisden et al., 2009; Kang et al., 2009; Bernardes et al., 2012). The losses could reach 70% of the stored DM in the peripheral areas and near the sidewalls of the bunkers and are related to the depletion of the digestible carbohydrate and organic acid fractions (Borreani et al., 2018a), with the most digestible materials being used up first.

Borreani et al. (2018a) reviewed and summarized the relationship between

DM losses and mold count in spoiling farm silages in the last decade. These authors found that when mold level in the silage was greater than 5.0 log cfu/g (i.e., mold becomes visible on the silage), the DM losses are greater than 20%; when mold counts exceeded 6.0 log cfu/g of silage, losses could exceed 40% of the original ensiled DM. Furthermore, mold higher than 5.0 log cfu/g of silage, determine substantial changes in nutritional quality occurred, with starch content beginning to decrease (**paper III**). Unfortunately, most microbial deterioration is invisible initially and may only be detected by a temperature rise in the forage (Borreani et al., 2018a). Also this initial phase of deterioration is involved in DM losses with negative consequence for farm profitability. In a controlled laboratory experiment on corn and sorghum silages, Tabacco et al. (2011a) observed an average loss of 10% of the estimated milk yield when aerobic spoilage computed as the sum of hourly differences between silage and air temperature reached approximately 1,000°C·h.

In addition to the direct economic loss of DM and nutrients, spoiling or spoiled silage can also cause indirect losses, due to the lower nutritive value, reduced palatability, and the risk of negative effects on animal performance and health (Hoffman and Ocker, 1997; Kung et al., 1998; Gerlach et al., 2014).

2.2.2. Microbial hazard and health risk

Unlike properly made and managed silage, poorly made or contaminated silages can harbor pathogens (Queiroz et al., 2018) that reduce animal performance (Driehuis, 2013), cause diseases of cattle (Pedroso et al., 2010), reduce the safety of dairy product (Colombari et al., 1999; Queiroz et al., 2018) and constitute a threat to human health (Ogunade et al., 2016; Driehuis et al., 2018). Contamination of food products with pathogenic bacteria or mycotoxins can cause huge economic losses due to recalls of unsafe foods and decreases in the shelf life of dairy products (Cogan et al., 2017; Queiroz et al., 2018).

Undesirable microorganisms and their toxins can be present in silage when it is consumed by the animal due to contamination of the crop pre-harvest, during storage and during feed-out with aerobic deterioration being the main source of microorganisms. Potential hazards to human, animal and food safety from silage have been reviewed by Driehuis (2013) and Driehuis et al. (2018). These authors classified the risk for animal and human health as microbial hazard, plant toxins and ergot alkaloids (e.g. phytoestrogens) and chemical hazard (e.g. nitrate, nitrite, oxide gases of nitrogen, butyric acid, biogenic amines). Most of the risk are associated to aerobic or anaerobic spoiled silages, because properly made and managed silage is an excellent feed that poses no health risks to humans or livestock (Driehuis et al., 2018). About microbial hazard, poor silage fermentation conditions or elevated pH level are generally associated with aerobic deterioration and can promote the growth of clostridia (Vissers 2007a; **paper V**), also pathogen as *C. botulinum* (Driehuis et al., 2018),

Listeria monocytogenes (Fenlon et al., 1989; Nucera et al., 2016), *Escherichia coli* (Pedroso et al., 2010; Driehuis et al., 2018; Queiroz et al., 2018), *Bacillus cereus*, *Salmonella* spp. (Queiroz et al., 2018), mold and mycotoxins.

Mycotoxins are secondary metabolites of molds but they are not essential for the mold to survive. Therefore, a high mold count does not necessarily correlate to a high mycotoxin load and vice-versa. Forages can be contaminated with several mycotoxigenic mold in the field pre-harvest, during storage, or after ensiling during feed-out, therefore problems associated with mycotoxins in silage can be minimized by preventing fungal growth before and after ensiling (Ogunade et al., 2018).

Mycotoxins that are frequently present in ensiled forages are summarized in Table 3 (Driehuis, 2013; Ogunade et al., 2018; **paper III**).

Table 3. Major mycotoxins and mycotoxigenic mold in silage (mod. Driehuis, 2013; Ogunade et al., 2018)

Mycotoxin group	**Toxins**	**Mold species**
Aflatoxins	Aflatoxin B_1, B_2, G_1, G_2	*Aspergillus flavus, A. parasiticus*
Trichothecenes	T2, diacetoxyscirpenol	*Fusarium langsethiae, F. poae, F. sporotrichioides*
	Deoxynivalenol (DON), nivalenol	*F. graminearum, F. culmorum*
Fumonisins	Fumonisins B_1, B_2	*F. verticillioides, F. proliferatum*
Resorcylic acid lactones	Zearalenone	*F. graminearum, F. culmorum*
Ochratoxins	Ochratoxin A	*A. ochraceus, Penicillium verrucosum*
P. roqueforti toxins	Roquefortine C	*P. roqueforti, P. paneum*
A. fumigatus toxins	Gliotoxin	*A. fumigatus*
M. ruber toxins	Monacolin K, citrinin	*Monascus ruber*

Fungi can invade, colonize and produce mycotoxins during either pre-harvest or post-harvest stages. The *Aspergillus*, *Penicillium* and *Fusarium* genera are considered to be the most important colonizing fungi and producers of mycotoxins, even if other genera (e.g. *Alternaria*, *Mucor*, *Geotrichum*, *Monascus*) have been found in silages (Cheli et al., 2013). Temperature, water activity, and relative humidity can influence the growth of different genera: *Aspergillus* and *Penicillium* grow at lower a_w and at higher temperatures than

Fusarium. The post-harvest contamination of mycotoxins can be due to the possible increase in concentration of mycotoxins due to DM losses, the possible *ex-novo* production due to the fungal growth during aerobic deterioration (Garon et al., 2006; Cavallarin et al., 2011; **paper VI, V**). Rumen microflora can degrade and inactivate mycotoxins. As a result, ruminants are among the least susceptible animal species (Cheli et al., 2013). For dairy cows, the main problem about mycotoxins is the aflatoxin B_1 ingestion since its carry-over of to milk as aflatoxin M_1 (Veldman et al., 1992).

Figure 10 shows the dominant molds isolated in corn silage at opening, after 60 d of conservation and after 14 days of aerobic exposure (Ferrero et al., unpublished data). The mold count differ during each phase (10^5, 10^2 and 10^7 cfu/g at harvest, opening and during aerobic deterioration, respectively). At harvest the corn crop is mainly contaminated by *Fusarium* spp., with possible contamination of DON, zearalenone or fumonisins. This genus of molds disappears during the conservation process. During aerobic deterioration the molds count quickly increased and the major risk of mycotoxin contamination is linked to the growth of *Aspergillus* spp. (Spadaro et al., 2015; **paper III, IV**), with *A. fumigatus* being the dominant species.

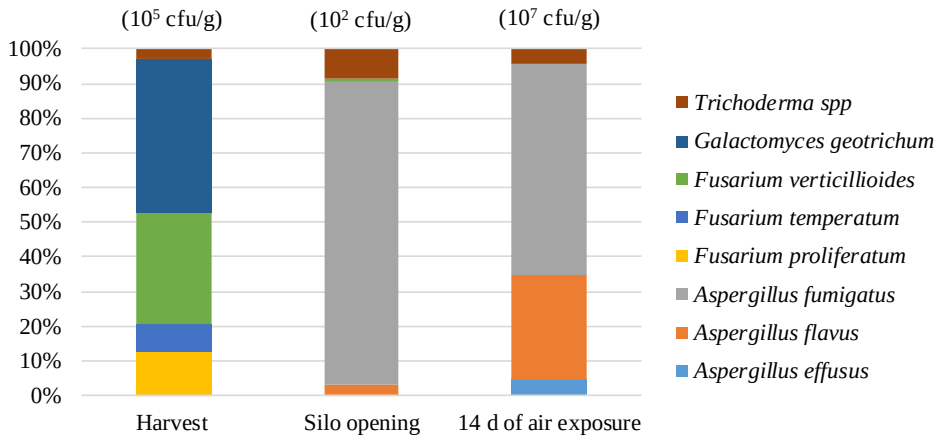

Figure 10. Dominant molds isolated in corn before ensiling and in silage at opening, after 60 d of conservation and after 14 days of aerobic exposure (Ferrero et al., unpublished data).

2.3. Prevention of aerobic deterioration

The preservation of the nutritive, fermentative, microbiological and hygienic qualities of silages during the feed-out phase of ensiling, by increasing the aerobic stability of silages, is a challenge for any livestock producer throughout the world (Borreani et al., 2018a). The most important factors influencing the quality and the preservation efficiency of forage during ensiling are the degree of anaerobiosis reached in the silo and its maintenance over the entire conservation period (Woolford, 1990; Borreani et al., 2007). The prevention of aerobic deterioration begins from the first phase of ensiling and lasts for all the others. Different factors can influence the presence and the magnitude of aerobic deterioration such as crop characteristics (DM content at ensiling, particle size), management factors and environmental factors (e.g. temperature, rain).

To prevent the formation of butyric acid during fermentation and to ensure the aerobic stability of the silage during the feed-out phase avoiding aerobic deterioration are two of the main practical issues of this technique (Pahlow et al., 2003). Several biochemical, microbiological, physical and management practices have been studied by many authors to ensure lactic acid fermentation and to improve aerobic stability (Wilkinson and Davies, 2013; Borreani et al., 2018a). The harvest of the crop with not adequate DM, inadequate silo filling techniques, packing densities, air penetration through silo, slow feed-out rate (Kleinschmit et al., 2005; Borreani et al., 2018a; Brüning et al., 2018), short conservation period (Weinberg and Chen, 2013; **paper I**) and high ambient temperature (Bernardes et al., 2018) can lead to undesirable fermentation or to aerobic deterioration of fed silages.

Figure 11 reports the potential DM losses during silage-making stages in case of adoption of good management practices, non-optimal management practices and environmental factor (rain) (Borreani et al., 2018a). It appears that a good management of ensiling process should be applied in each making-stage to keep losses as low as possible. Furthermore, the highest losses occur during feed-out phase even if good management practices are adopted and the presence of aerobic deterioration is minimized.

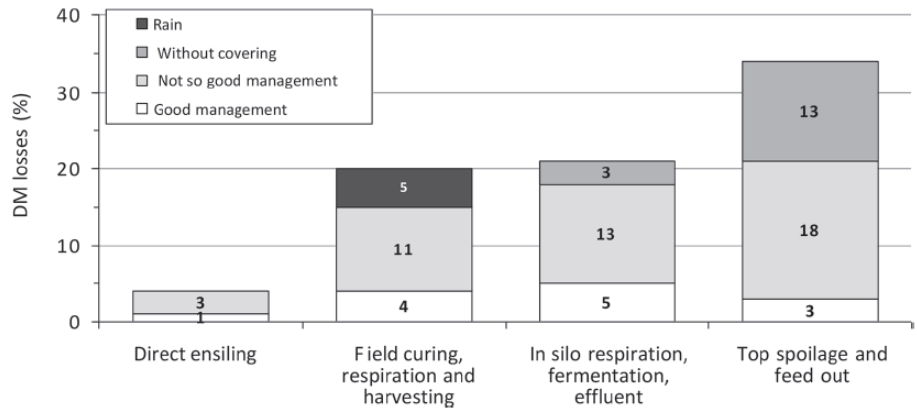

Figure 11. Potential DM losses during silage-making stages. The white portion of bar graph indicates when good management practices are used; the light gray portion is the range of additional losses associated with non-optimal management practices; and the dark gray portion is the additional losses when no covering is applied (Borreani et al., 2018a).

2.3.1. Management factors

In order to obtain a high quality silage several management factors should be adopted. Choosing a correct DM at harvest (in particular in wilted crops) and the accuracy in chopping herbage can ensure good fermentation. Good quality silage throughout the whole silo mass can be obtained by fast filling ensuring high silage density through proper compaction (Muck and Holmes, 2000). Others factor influencing the exclusion of oxygen throughout the conservation period are properly sealing and covering the silo (Borreani et al., 2007; Bernardes et al., 2012), weighing down the cover effectively, lining bunker walls (Lima et al., 2017) and reducing the risk of mechanical damage to the cover by protecting it with a net or tarpaulin (Wilkinson and Davies, 2013). Another factor that can avoid undesirable fermentation or increase aerobic stability is the use of silage additive (Muck et al., 2018). Furthermore, a crucial point is to plan the silo size to achieve the correct daily feed-out rate, which depends on the season and latitude as previously reported by Borreani and Tabacco (2012).

2.3.2. Additives

Additive can influence the fermentation, the aerobic stability or the nutritional value of silages and can be classified as: **chemical additives** (additive that contain components to improve silage quality); and **biological additives** (additive that generate components to improve silages quality) (Da Silva et al., 2015; Muck et al., 2018) with biological inoculation being the predominant technology employed to influence the silage fermentation (Wilkinson and Muck, 2019). Table 4 reported a classification of additives used in silage (modified from. McDonald et al., 1991 Kung et al., 2003; Muck et al., 2018).

Table 4. Classification of additive used in silage (modified McDonald et al., 1991 Kung et al., 2003; Muck et al., 2018)

Type	**Additive**	
	Biological	**Chemical**
Fermentation stimulants	Homofermentative LAB (hoLAB)	Nutrient
	Enzymes produced by bacteria	Enzymes
Fermentation inhibitors		Inorganic acid
		Organic acid
		Salts
Aerobic deterioration inhibitors	Heterofermentative LAB (heLAB)	Inorganic acid
	Propionibacteria	Organic acid
		Nutrient
Fermentation stimulant + aerobic deterioration inhibitor	Combination of hoLAB and heLAB	
Nutritional value enhancer	Enzymes produced by bacteria	Nutrient
		Enzymes
Reducers of effluent		Absorbents

Over the years, silage effective strains of homofermentative LAB have been screened to stimulate the fermentation. These microorganisms are able to produce large amount of lactic acid and rapidly drop of pH and inhibition of

undesirable fermentation. Others additives to stimulate the fermentation are represented by enzymes (directly added or produced by inoculated bacteria) that release nutrients available for the fermentation. Nutrient available for fermentation can be also directly added, the main are glucose, sucrose, molasses, whey, beet pulp (McDonald et al., 1991).

To avoid undesirable fermentation (i.e. clostridial fermentation) several acids can be added. These determine a rapid drop of pH by direct acidification determining restricted fermentation of WSC due to low microbial activity. The main additive used is represented by formic acid. However, those silages had low aerobic stability, due to the high yeast count and the high contents of residual WSC due to restricted fermentation (Bolsen et al., 1996).

Some additives have been developed to improve the nutritional value of silages. These additive works by means of enzymatic activity of inoculated microorganism or by direct addition of nutrient (e.g. minerals, N-based compounds or energy compounds).

The use of substance to avoid the losses by effluent and to increase the DM content of silage are used in no wilted or low wilted forages and are represented by straw, barley and others (McDonald et al., 1991).

The additive used to improve aerobic stability will be treated below, for the others additive past (McDonald et al., 1991; Kung et al., 2003) and recent sources (Oliveira et al., 2017; Muck et al., 2018) are available to well summarize their characteristics and their effects.

3. Evaluation of additive to reduce the aerobic deterioration

Lactate-assimilating yeasts are usually thought to initiate aerobic spoilage, thus additives containing (chemical additives) or generating (biological additives) antifungal components (i.e. acetic and propionic acids) have been developed to slow or to inhibit the activity of yeast and consequently to improve the aerobic stability of silages for decades (Da Silva et al., 2015; Muck et al., 2018).

3.1. Microbial inocula

Over time, different silage inoculants have been developed (Weinberg and Muck, 1996; Muck et al., 2018). A recent paper on the effect of different inocula (Xu et al., 2019a) reported a description of four generations of inocula differing in their mode of action (Table 5).

Silage inoculants were traditionally developed to dominate the epiphytic bacterial population and direct the fermentation along the homolactic pathway. Among the homofermentative and facultative heterofermentative LABs, the most frequently used are *Enterococcus faecium*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Lactobacillus plantarum* and *Lactobacillus acidophilus* (Weinberg et al. 2004; Blajman et al., 2018). However, the resulting silage was sometimes more susceptible to aerobic deterioration during feed-out due to the low production of antifungal compounds (i.e. acetic acid) (Weinberg et al., 1993). This led to the development of inoculants that were aimed solely at improving the aerobic stability of silages (Driehuis et al., 1996). At that time, critics of this approach to improving the aerobic stability were raised about the ability of microbial inoculation to alter the process of fermentation and improve aerobic stability (Kung et al., 2003), high concentrations of acetic acid in reduction of DM intake (Anil et al., 1993) and animal performance and about less efficient pathways that can lead to large losses in DM (Pahlow et al., 2003; Kleinschmit and Kung, 2006a).

To overcome this, ‘dual purpose’ inoculants containing a mixture of homofermentative and heterofermentative LAB have been developed with the aim of improving the efficiency of the anaerobic fermentation and aerobic feed-out phases of silage production, respectively (Adesogan et al., 2004; Kang et al., 2009; Huisden et al., 2009; Arriola et al., 2011). The inocula of third generation have been developed with the purpose of improving the nutritional quality of

silages (Nsereko et al., 2008) even if digestion of NDF was not been consistently improved among the studies (Hofherr et al., 2008; Kang et al., 2009; Comino et al., 2014). The fourth generation have been developed to be probiotic for animals by combining lactic acid bacteria (i.e. *L. buchneri*) with probiotic yeasts (*Saccharomyces cerevisiae*). This combination allows to ensure the fermentation characteristics and aerobic stability, and to enhance nutritive value and microbial communities of corn silage. Furthermore, during aerobic exposure *S. cerevisiae* could potentially increase with probiotic properties in corn silage just prior to feeding (Xu et al., 2019a).

Table 5. The four generation of microbial inoculants developed over the year.

Generation (Period)	Microbial group	Action	Reference
First (from late '10)	Homofermentative LAB	Preservation of silage nutrients by production of lactic acid and decline of pH	Gorini (1907); Fred et al. (1921)
Second (from 1996)	Heterofermentative LAB	Improvement of aerobic stability by inhibition of yeasts and molds through production of acetic acid	Driehuis et al. (1996); Muck (1996); Weinberg and Muck, 1996
Dual purpose inocula (from 2004)	Mixture of homofermentative and heterofermentative LAB		Adesogan et al. (2004)
Third (from 2008)	Heterofermentative LAB with feruloyl esterases	Improve the digestibility of fiber by hydrolyzation of feruloylated polysaccharides	Nsereko et al. (2008)
Fourth (from 2019)	Mixture of yeasts and heterofermentative LAB	Probiotic effect to the animal by promotion of rumen health, improvement of feed efficiency, and mitigation of methane emissions	Xu et al. (2019)

The variable effectiveness from year to year is one of the main issues of using microbial inoculants since they are dependent to the environmental conditions and the forage characteristics (Pitt and Leibensperger, 1987; Muck, 2004; Muck, 2013). Several factors can influence the viability and/or effect of microbial inocula:

- Application rate: to have an impact on fermentation the inoculated species must be able to dominate the fermentation being at least 2-fold

higher than the epiphytic population (Pahlow, 1991; Kleinschmit and Kung, 2006a).

- DM and water activity for growth (Kung et al., 2018a; **paper II**). High DM content and low moisture can affect the activity of the inoculants. However, very wet plant material can promote native bacteria to grow, also affecting the impact of the inoculants on fermentation.

- Amount of WSC available to the growth of inoculum (Kung et al., 2003). Water-soluble carbohydrates are the main food source for LAB. Crops with low sugars, like alfalfa and warm-season grasses, are more challenging for LAB to reduce pH and achieve good fermentation. It has been suggested that in crops like alfalfa, sugar content may limit the effect of inoculants (Muck et al., 2018).

- Temperature. Different species of microorganisms used as inocula has different tolerance at low or high temperature (Kim and Adesogan, 2006; Zhou et al., 2016; Bernardes et al., 2018). High temperatures reduced LAB populations, and increased clostridial bacteria because they often require higher temperature for optimal growth than LAB. Therefore, warmer conditions should be more favorable for clostridial fermentation (Bernardes et al., 2018). The time that inoculants are exposed to high temperatures also has an effect on their viability.

- Length of period in anaerobiosis. Some LAB inocula (i.e. *Lactobacillus* buchneri) need longer ensiling period than 90 d to be efficacious (Driehuis et al., 1999; Kleinschmit and Kung, 2006b; **paper I**).

- Inhibition by fermentative end products (McDonald, 1991). Production of compounds with no effect on the desirable results (i.e. the production of acetic acid inhibit yeasts but not acetic acid bacteria in initiating the aerobic deterioration) (Kung, 2009).

- Quality of the solution used to distribute the inoculum and times the microorganisms remain in the solution (Kung, 2009).

- Temperature of the tank during distribution: storage is an important aspect of a high quality inoculant that contains live microorganisms. Each inoculants can require refrigeration, freezing, room temperature for optimum storage. Moisture, oxygen and sunlight can decrease the stability of inoculants resulting in lower viable counts and a product that does not meet label guarantees (Kung, 2011).

It is important to note that not all inoculants necessarily react the same to a given stressor. The ability of specific bacterial strains to withstand a multitude of stressors can be a key determinant for a successful inoculant (Kung, 2009).

The most commonly recommended inoculation rate for LAB based-inoculant range from 10^5 to 10^6 colony forming units (cfu) of the organism per gram of wet forage (Pahlow et al., 2003). Additions of 1,000,000 (1×10^6) cfu/g

of forage are probably not cost effective in North America for heterolactic bacteria (e.g. *L. buchneri*) due to their difficult in multiplication in industrial batch.

Heterofermentative LAB inocula have been developed since the end of '90s to improve aerobic stability. Contrary to past thinking, heterofermentative LAB has achieved the most widespread use in improving aerobic stability. Muck (1996) was the first to suggest that inoculation with *Lactobacillus buchneri* (LB) might improve the aerobic stability of silages. The effect of LB was analyzed by Driehuis et al. (1996) that suggested that improved aerobic stability was due to the ability of LB to ferment lactic acid to acetic acid and 1,2-propanediol (Oude-Elferink et al., 2001). Treatment with LB inoculants has improved the aerobic stability of corn silage (Driehuis et al., 1999; Ranjit and Kung, 2000; Schmidt and Kung, 2010; **paper I, III**), grass silage (Driehuis et al., 2001; Li and Nishino, 2011), wheat (Weinberg et al., 2002; Adesogan et al., 2003), sorghum silage (Tabacco et al., 2011a; **paper I**) in laboratory silo experiments and of corn silage at farm level (Mari et al., 2009; Tabacco et al., 2011b). Figure 12 reported the boxplot of yeast count, acetic acid and aerobic stability of silages untreated or treated with *L. buchneri* for data published in this thesis and data obtained from the literature review.

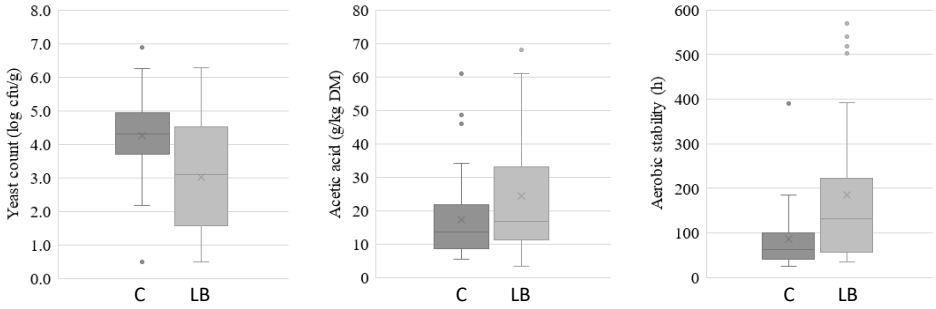

Figure 12. Yeast count, acetic acid and aerobic stability of silages untreated (C) or treated (LB) with *L. buchneri* (data from Kleinschmit and Kung, 2006a; Hu et al., 2009; Arriola et al., 2011; Teller et al., 2012; Basso et al., 2012; Queiroz et al., 2013; Ogunade et al., 2017; Rabelo et al., 2017; Basso et al., 2018; Kung et al., 2018b; Rabelo et al., 2018; Xu et al., 2019a; Xu et al., 2019b; **paper I, III**).

Silage treated with *L. buchneri* showed higher aerobic stability than untreated. The higher aerobic stability is due to a lower yeast count in treated silages due to the production of acetic acid by the inoculum. The reduction of yeast allow to improve the aerobic stability of corn silage of on average 94 hours. However, the inoculation of *L. buchneri* had no consistent effect in corn silages (Schmidt et al., 2007; Schmidt and Kung, 2010; **paper I**) in particular

after short period of conservation (<60 d) (Driehuis et al., 1999; Kleinschmit and Kung, 2006b; **paper I**) and in high DM silages (>38%) (Hu et al., 2009; Comino et al., 2014; Xu et al., 2019a; **paper II**) as reported in Figure 13.

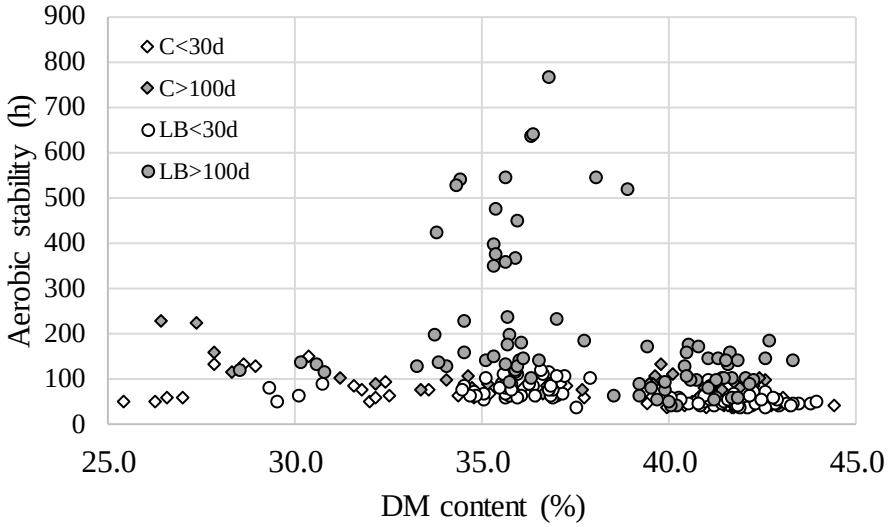

Figure 13. Scatter plot of aerobic stability and DM content of corn silages untreated (C) or treated with *L. buchneri* (LB) after different conservation period (**paper I, II, III,** unpublished data).

The need of a longer ensiling period than 90 d to be efficacious is a criticism of using *L. buchneri* as silage inoculant because there is a need in some farm situations to open silages early (**paper I**). Thus, other species from *L. buchneri* group have been evaluated for their effects on silage fermentation and aerobic stability, including primarily *L. brevis*, *L. diolivorans*, *L. hilgardii*, *L. kefiri*, and *L. parafarraginis* (Muck et al., 2018). Daniel et al. (2015) found that the inoculation of *L. brevis* and *L. kefiri* alone or in combination altered the fermentation of sugarcane silages but did not improve the aerobic stability. The inoculation of *L. brevis* and *L. parafarraginis* influenced the fermentation of corn stover silage (Xu et al., 2017) even if no data on aerobic stability were reported. Liu et al. (2014; 2018) reported that *L. parafarraginis* improved aerobic stability in a commercial oat silage. *L. diolivorans* was recently proposed as silage inoculum as it improved the aerobic stability alone (Schein et al., 2018; Moyer et al., 2019) or in combination with *L. buchneri* and *L. rhamnosus* (Huenting et al., 2018). *Lactobacillus hilgardii* isolated from sugarcane silage (Avila et al., 2014) improved the aerobic stability in sugarcane (Carvalho et al., 2014) and in corn silage (Assis et al., 2014; **paper I**). *L. hilgardii* was selected to be effective after short conservation period but among

the trials its effect is not consistent (Assis et al., 2014; Polukis et al., 2016; Reis et al., 2018; **paper I**). Figure 14 reported the aerobic stability of silages untreated or treated with *L. hilgardii* or *L. buchneri* after different conservation periods. It appears that *L. hilgardii* (LH) was able to increase the aerobic stability already after 30 days of conservation. At 30 and 100 days of ensiling (short to medium conservation) the effect of LH was higher than LB. Furthermore, it was confirmed that after long conservation period (e.g. more than 200 days) *L. buchneri* is able to greatly increase the aerobic stability.

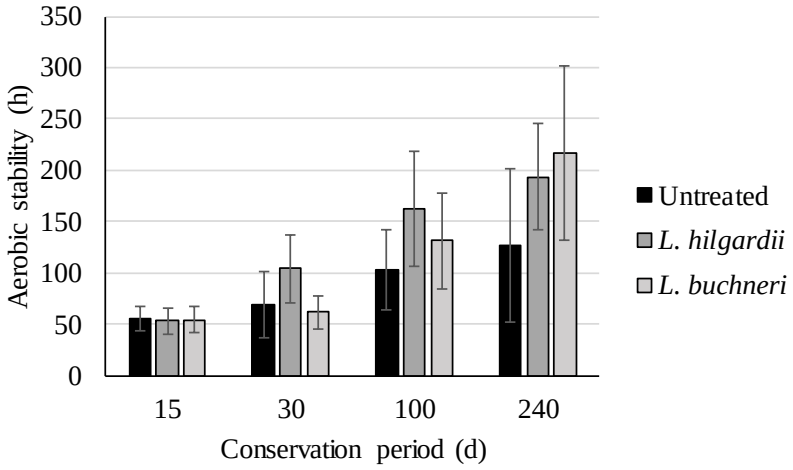

Figure 14. Aerobic stability of silages untreated or treated with *L. hilgardii* or *L. buchneri* after different conservation periods (**paper I, III,** unpublished data).

Further advancement in silage inocula development came from the combination of *L. buchneri* and *L. hilgardii* in order to obtain the positive effect after short and long conservation period (**paper I**). Nevertheless, the effect of the new combined inoculum was not consistent among the different experiments, in particular after short conservation period (e.g. less than 30 d) (**paper I, III**, Drouin and Chaucheyras, 2018b; Borreani et al., 2018b; Gomes et al., 2018; Szucs et al., 2018; Costa et al., 2019).

Other species of microorganisms, such as Propionic acid bacteria and *Bacillus* species, have been studied as inocula to improve the aerobic stability (Weinberg et al., 1995; Higginbotham et al., 1998; Lara et al., 2016; Muck et al., 2018). However their effect was not consistent among the experiments.

3.1.1. Fermentation dominance of inocula

The advancement in silage microbiology allows us to understand why inoculants are often successful in the silo and how they may alter silage quality. As LAB inoculant strains have been selected for rapid grow under non-optimal conditions (i.e. high DM, low temperature) we expect their dominance during fermentation. Often, inocula affect the fermentation even if applied at less than 10% of the epiphytic LAB population (Muck, 2013). During the first phase of fermentation a strong change in LAB composition have been detected (Drouin and Chaucheyras, 2018a) and many of inoculant strains are not faster in dominating the fermentation (e.g. *Lactobacillus buchneri* need at least 60 d to be effective). This suggest that many inoculant strains have other competitive advantages to be competitive with epiphytic population (Muck, 2013).

Some inoculant LAB strains produce bacteriocins antimicrobial compounds (Muck, 2013). Vazquez et al. (2005) studied the effects of bacteriocins from 6 strains of LAB (*L. brevis, L. casei, L. helveticus, Lactococcus lactis, Leuconostoc mesenteroides, P. acidilactici*) on the growth of those strains. They found that the bacteriocin from a particular strain generally reduced the growth of other strains even if in some cases the growth increased, by the presence of bacteriocin from another strain. There is also evidence that some LAB produce antifungal compounds (Muck, 2013). Two strains of *L. plantarum* have been found to produce the antifungal 3-phenyllactic acid and 3-hydroxydecanoic acid (Broberg et al., 2007; Prema et al., 2010). These studies show that some LAB strains are capable of inhibiting a considerable spectrum of bacteria and fungi. These indication, coupled with the analysis of fermentation profile, could help us to understand the not consistent effect of inocula under some conditions.

3.2. Chemical additives

The variable effectiveness from year to year is one of the main issues of using microbial inoculants since they are dependent to the environmental conditions and the forage characteristics (Muck, 2004; Muck, 2013). Therefore, chemical additives, such as organic or inorganic acids and their salts, may be more robust than biological additives to improve aerobic stability especially for high DM content silages and for early opening (Kung et al., 2003; Da Silva et al., 2015).

The most common active ingredients as chemical additives are acids and their salts (Muck et al., 2018). As previously described for microbial inocula, chemical additives are used in silage production to stimulate the fermentation, to inhibit the fermentation or to reduce the aerobic deterioration (McDonald et al., 1991; Muck et al., 2018). Morais et al. (2016) reported a list of chemical additive commonly used in silage process and their application rate (Table 6).

The first use of chemical additives (e.g. formic and sulfuric acids) was to stimulate a rapid acidification (Virtanen, 1933; Henderson, 1993) to suppress clostridia and other undesired bacteria and improving protein preservation during ensiling. However, those silages had low aerobic stability, due to the high yeast count and the high contents of residual water-soluble carbohydrates (WSC) caused by restricted fermentation (Bolsen et al., 1996). Thus, a second group of acids, able to improve the aerobic stability during feed out through direct inhibition of yeasts and molds, has been used (McDonald et al., 1991; Auerbach et al., 2012). The main acids belonging to the group of aerobic stability enhancers are sorbic, benzoic, propionic, and acetic acids, or their mixtures (Knick ́and Spörndly, 2009; Bernardes et al., 2015; Weiss et al., 2016) with the strongest effect showed by propionic acid and its mixture (Woolford, 1975). One problem on the use of propionic acidic is represented by its corrosive nature. In the recent years many additives contain other antifungal compounds such as sorbic acid and benzoate have been developed. Recently, free fatty acids and monoglycerides of fatty acids (i.e. monopropionine and monobutyrin) are reported to be potent antimicrobial/microbicidal agents, obtaining higher aerobic stability (**paper II**).

Table 6. Chemical additives commonly used in silage process and their application rate (Morais et al., 2016)

Additive	Application rate (on fresh matter)
Ammonia	1.1 to 2.3%
Ammonium isobutyrate	2%
Diammonium phosphate	4,6%
Formic acid	3 to 4 L/t
Sulfur dioxide	1.3% to 1.7%
Urea	0.4 to 2%
Urea solution	50 L/t
Acetic acid, isobutyric acid	8 L/t
Ammonium formate, propionate, ethyl benzoate and benzoate	4 L/t
Ammonium propionate, sodium propionate, acetic acid, benzoic acid and sorbic acid	0.1to 0.2%
Formic acid, ammonium formate, propionic acid, benzoic acid	4 L/t
Formic acid, ammonium formate, propionic acid, benzoic acid and ethylbenzoate	6 L/t
Formic acid (42.5%), formic ammonia (30.3%) and propionic acid (10%)	6 L/t
Formic acid (55%), propionic acid (20%), ammonium formate (4.3%) and potassium sorbate (2.5%)	4 L/t
Formic acid (55%), propionic acid (5%) and ammonium formate (24%)	4 to 4.5 L/t
Formic acid (55%), propionic acid (5%) and ammonium formate (24%) plus ammonium propionate	4.5 L/t
Propionic acid (80%) and acetic acid (20%)	1.5%
Propionic acid and formic acid	3.5 kg/t
Propionic acid-based additive: ammonium and sodium propionate, ethoxyquin, BHA, and BHT	0.1 to 0.2%
Propionic acid (50%) and formic acid (50%)	3 L/t
Propionic acid (90%), ammonium propionate (4%) and 1,2-propandiol (4%)	3 L/t
Propionic acid, ammonium propionate, sodium benzoate, potassium sorbate	1.5 to 3 L/t
Propionic, acetic, benzoic and sorbic acids, sodium and ammonium hydroxide, methylparaben and propylparaben (Liquid mold inhibitor, 82% acid content)	0.10%
Propionic acid, formic acid, benzoic acid and calcium formate	3.4 kg/t
Propionic acid (37%), sodium benzoate (14%) and sodium propionate (11%)	5 L/t
Sodium benzoate (22.9%) and sodium propionate (8.3%)	3 to 6 L/t
Sodium benzoate (5 to 50%), potassium sorbate (5 to 35%) and sodium nitrite <5%	2 to 6 L/t
Sodium benzoate, sodium azide and calcium formate	3.5 kg/t
Potassium sorbate, sodium benzoate, ammonium propionate	1 to 2 L/t

To achieve the maximum effect against spoilage bacteria and fungi, commercial additives often contain mixtures of different acids at various concentrations (Muck et al., 2018). Furthermore, higher levels of acids could be required in high DM silages than in wetter ones to reduce the yeast count (Kung, 2009) because of the lactic acid bacteria activity could be limited by the lack of moisture and consequently the production of acids is low. Therefore, chemical additives, such as organic or inorganic acids, may be more robust than biological additives (Pitt et al., 1991; Kung et al., 2003).

Several factors can influence the effect of chemical additive:

- Application rate (Moon, 1983; Pitt et al., 1991; Morais et al., 2016);
- Chemical state: better action of salts of different organic acids than the corresponding free acid (Woolford, 1984b);
- pH: because these acids are more effective when the pH is below their pKa values and they are mostly undissociated (Moon, 1983);
- Environmental temperature (Bernardes et al., 2015; Weiss et al., 2016);
- Resistance or degradation of some products by microorganisms (Kung, 2009);
- Type and calibration of applicator machineries (Kung, 2009).

Various chemical additives with antifungal properties have been used to enhance the aerobic stability of silages (McDonald et al., 1991). The undissociated acid functions by staying active on the surface of microorganisms and competing with amino acids for space on active sites of enzymes and by altering the cell permeability of microbes. Undissociated propionic acid has strong antifungal properties, and the fraction of propionic acid that is undissociated is dependent on pH. Sorbate, benzoate and acetic acid are commonly found as components of many antifungal formulations but are too expensive to be used alone in high concentrations. Knicky and Spörndly (2011) tested an additive comprising sodium benzoate, potassium sorbate, and sodium nitrite on the quality of silages fermented from various forage crops. The application of the tested additive mixture reduced the growth of undesirable microflora and thereby reduced silage losses and prolonged the aerobic stability of the silages. A mixture of formic, propionic, formate, benzoic, and sorbic acids and glycerol applied at 4 L/t was tested in corn silage by Nadeau et al. (2011). These authors found improved aerobic stability after 28 and 110 d (average of 283 h compared with 137 h in the untreated silages). Buffered propionic acid based additives and ammonium hydroxide improved aerobic stability of corn silage in a dose-dependent manner from 1 to 3 g/kg of fresh weight (Kung et al., 2000). Potassium sorbate is more effective against yeast than sodium benzoate (Auerbach and Nadeau, 2013; Bernardes et al., 2014). To compare products differing in application rates of these active substances, Auerbach and Nadeau (2013) used sodium benzoate equivalents, assuming

potassium sorbate possessed twice the antifungal activity of sodium benzoate. Nevertheless, when potassium sorbate and sodium benzoate were applied separately at higher rates, their effects on aerobic stability in corn silage were similar, although potassium sorbate was more effective against yeast and mold activity during air exposure (Bernardes et al., 2015). The effects of potassium sorbate and sodium benzoate on yeast counts, ethanol content, and aerobic stability of corn silage are dose-dependent (Auerbach and Nadeau, 2013; Bernardes et al., 2015; Hafner et al., 2014; Nadeau et al., 2015a). Application of a low level of potassium sorbate to corn silage (91 mg/kg of fresh matter) may decrease aerobic stability, as it has been shown to increase yeast count and ethanol content compared with untreated silage (Hafner et al., 2014). In contrast, addition of 1.0 g of potassium sorbate/kg of fresh matter has inhibited yeast growth, resulting in decreased ethanol content (Hafner et al., 2014) and improved aerobic stability of corn silage (Teller et al., 2012; Bernardes et al., 2015). Recent research with this product applied at 2 L/t found improvements in aerobic stability of corn silage as soon as 1 d of ensiling, even though yeast counts were similar between the treated and untreated silages (Da Silva et al., 2017).

Figure 15 reported aerobic stability of corn silage opened after 120 d of conservation at 32 and 38% DM untreated (Control) or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 1.5 L (Mixture 1.5) or 2 L (Mixture 2.0) or an additive containing buffered propionic acid and citric acid (Kung et al., 2018b). Treatment with buffered propionic acid improved the aerobic stability of silages compared with untreated silage, but the effect from a mixture of sodium benzoate, potassium sorbate, and sodium nitrite was markedly greater. The effect of the additive was influenced by the DM content of silage with higher effect in lower DM content silages.

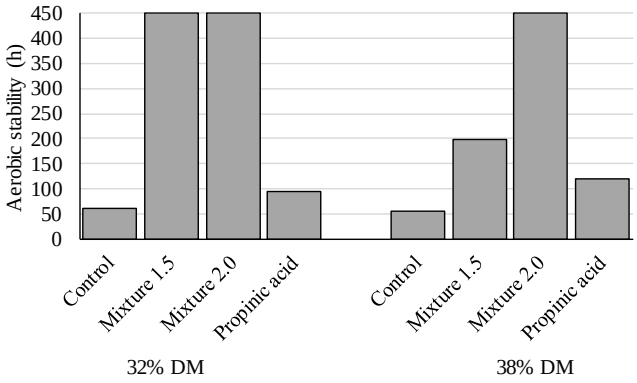

Figure 15. Aerobic stability of corn silages at 32 and 38% DM untreated (Control) or treated with an additive containing sodium benzoate, potassium sorbate, and sodium nitrite at 1.5 L (Mixture 1.5) or 2 L (Mixture 2.0) or an additive containing buffered propionic acid and citric acid (Kung et al., 2018b).

In 2018, we conducted a trial to verify the effect of acetic acid added at ensiling and at silo opening on aerobic stability of corn silage. Corn silage was harvested at 39% DM and untreated (C) or treated with 30 g/kg DM of acetic acid (AA). After 170 d of conservation silages were opened and half of them were untreated (C and AA) whereas the others were treated with 30 g/kg DM of acetic acid (C+AA and AA+AA) by spraying uniformly onto the forage, which was constantly hand mixed. Figure 16 reports the temperature during aerobic stability test. The control silage showed the low aerobic stability (around 50 h). The treatments AA and C+AA received the same doses of acetic acid (30 g/kg DM) but the application at silo opening instead at ensiling allow to reach higher stabilities (+50 h). This difference could be due to a reduction in acetic acid content during fermentation as a consequence of microbial activity that utilized the acid as substrate. The AA+AA silages, with a theoretical acetic acid content of 60 g/kg DM, showed more than 300 h of aerobic stability.

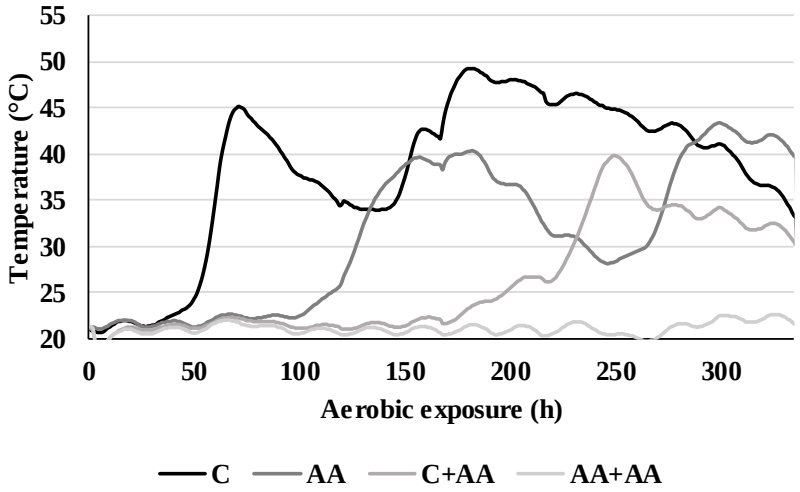

Figure 16. Temperature during aerobic stability test of corn silage conserved 170 d (Tabacco et al., unpublished data). C = untreated; AA = treated with 30 g/kg DM of acetic acid before ensiling; C+AA = C treated with 30 g/kg DM of acetic acid at silo opening; AA+AA = AA treated with 30 g/kg DM of acetic acid at silo opening. Room temperature 21°C.

Wilkinson and Muck (2019) reported as the future goals in silage production the development of additives that consistently improve fiber and nutrient availability to livestock and silage additives that reduce or eliminate mycotoxins and other microbial toxins that enter the silo on the harvested crop.

4. New technologies and tools to improve knowledge in aerobic deterioration

As described above, in many cases the fermentation that a crop undergoes can be explained by how microbial population interacts with crop and environmental characteristics (Kung, 2010a). Thus, the quantitative and qualitative analyses and the understanding of the microbial population play a key role to obtain a high quality silage.

4.1. The importance of microbial analysis

The microbial population in the fresh forage is composed by a complex mixture of bacteria, yeasts and molds with colony-forming units ranging from 10^4 to 10^9 cfu/g (Pahlow et al., 2003). Once the ensiling process begins, the diversity of the community decreases as a consequence of oxygen exclusion and low pH. Under these conditions many microorganisms (such as epiphytic LAB, enterobacteria, yeasts, and molds) can enter a viable but unculturable state precluding their complete characterization through traditional methods. Due to the importance of microbial populations associated with the ensiling process over the years, many efforts to characterize members in detail based on morphological, physiological, and biochemical traits have been made (McAllister et al., 2018).

Until about 10-15 years ago, our knowledge of what occurred in the silo was limited by what would grow on various selective media, and identification of species from those agar plates was tedious work. With a rapid development of new techniques it was possible to analyze in depth the microbial population of silages and the results has also been useful to study how various factors affect fermentation (Muck, 2013).

4.2. Technique to analyze the microbial composition of silages

To assess the composition of bacterial communities associated with plants, a wide variety of techniques, both culture-based (e.g., most probable number, selective media) as well as culture-independent analyses of microbial communities using techniques such as denaturing gradient gel electrophoresis (DGGE), quantitative real time polymerase chain reaction (qPCR), terminal restriction fragment length polymorphism (T-RFLP), or length heterogeneity PCR have been employed for the past decade (McAllister et al., 2018).

The ability to extract microbial DNA from silages, the technological advances in DNA sequencing and bioinformatics analysis (Escobar-Zepeda et al., 2015), called next generation sequencing (NGS) has been at the core of the changes that have occurred recently in silage microbiology (McAllister et al., 2018). These techniques allowed to the identification and quantification of specific species/strains and to study the whole microbial community of silages.

Quantitative PCR was used to specifically quantify and document the persistence of specific species, or even strains of bacteria and fungi during both fermentation and aerobic deterioration of silages (Schmidt et al., 2008; Xu et al., 2019a). Using these techniques to identify mycotoxin-producing molds or potential pathogens (e.g. *Listeria* spp.) in silage could provide new approaches to exclude these undesirable microorganisms from silage. Presently, most studies have been based on the study of variable 16S rRNA, 18S rRNA/internal transcribed spacer (ITS) regions for the identification of bacterial and fungal populations, respectively. These developments allowed to identify and quantify strains not able to grow on agar and reveal new species in silages. However these studies provide limited information on the function of the microbial species involved in the ensiling process.

At least four techniques have been used to study microbial communities in silages: length heterogeneity PCR (LH-PCR), terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE) and automated ribosomal intergenic spacer analysis (ARISA). All four techniques use PCR to amplify a portion of the microbial DNA and then use various methods to separate the amplified DNA (McAllister et al., 2018). In recent years, as a microbiological approach, next-generation sequencing (NGS) has begun to be applied to microbiota analyses of silage (Ni et al., 2017).

4.3. Next Generation Sequencing

The Next Generation Sequencing or parallel sequencing is a technology that allows sequencing of large genomes in a short time. The main use is the sequencing of DNA (metagenomics), RNA (metatranscriptomics), proteins (metaproteomics) or metabolites (metabolomics).

Metagenomics is defined as the direct analysis of purified DNA from environmental samples and enables taxonomic identification of the microbial communities present within them (Tennant et al., 2017). Metagenome analysis have been used to analyze microbial community in freshly harvested forage (Eikmeyer et al., 2013), during ensiling (Bao et al., 2016), and aerobic exposure (Dunière et al., 2017). These techniques have generated new insight into the complexity of the microbial ecology of ensiling, characterizing the role of epiphytic populations in silage quality and how silage additives can generate microbiomes more conducive to the production of high-quality silage (Mogodiniyai Kasmaei et al., 2016; McAllister et al., 2018). Ni et al. (2017) analyzed the microbiota of pre-ensiled crop and silage using DGGE and next-generation sequencing (NGS) in three different crops (wilted Italian ryegrass, whole crop corn, and wilted alfalfa). Across the three crop species, DGGE detected 36 and 28 bands, and NGS identified 253 and 259 genera in the pre-ensiled crops and silages, respectively.

Although molecular techniques have been developed to revolutionize our understanding of the role of microorganisms in ensiling, the scientific integrity of the information generated depends on the representativeness and purity of the nucleic acids that are extracted from the forage. Furthermore, several factors (such as the nature of the primers selected for amplification, the presence of PCR inhibitors, the sequencing platform) can influence the nature of the results generated. Moreover, the breadth and depth of the bioinformatics pipelines and limits of the gene databases used in the interpretation of data play a key role to the final results (Escobar-Zepeda et al., 2015; McAllister et al., 2018).

There are two main approaches to metagenome analysis. The first, mostly used, is a sequencing of a specific variable DNA region (e.g. 16S, ITS). This process is sometimes called metaprofiling. The second approach is the sequencing of the entire genome of the sample, this is called shotgun metagenomic. The differences between the two approaches are reported in Table 7.

Regardless of the methods adopted, a key point for the study of bacterial communities using techniques based on NGS (and all the others techniques), is the study of microbiological dynamics during silage process, avoiding a simple "photograph" of specific moment.

Table 7. Differences between the two approaches of metagenome analysis (Escobar-Zepeda et al., 2015; Tennant et al., 2017).

	Metaprofiling	**Shotgun metagenomic**
Taxonomic analysis	+*	+++
Functional analysis	-	+
Identification of new species	-/+	+
Sequencing of whole genome	-	+
Bias during sequencing	+/-	-/+
Cost	+	+++
Difficulties in bioinformatics analysis	+	+++

* often is not possible to reach the species level.

4.4. Microbial dynamics during aerobic deterioration

When compared to fermentation process, the microbial dynamics during aerobic deterioration have been studied by few authors. Lindgren et al. (1985) reported the microbial dynamics during aerobic deterioration of surface and 30 cm depth samples of 90% grass and 10% red clover silage stored in a tower silo. The authors characterized the dominant yeasts at silo opening and during aerobic deterioration. The results showed that the dynamics of bacteria, bacilli and enterobacteria depend on the survival of aerobic microorganisms during fermentation and storage period. Several factors as oxygen diffusion, temperature, length of conservation can affect their survival. Dolci et al. (2011) studied microbial dynamics by means of DGGE during aerobic exposure of corn silage inoculated with *L. buchneri*, *L. plantarum* and *E. faecium*) stored in polyethylene or oxygen barrier film bags for 110 d. At opening, bands identified as *L. buchneri* were dominant in both treatments indicating the ability of this microorganisms to be active after long conservation period (**paper I, III**). During aerobic exposure, from the silage began to heat (around 5 d of air exposure) and continuing through the air exposure, the dominant band in LB treated silages belonged to *Acetobacter pasteurianus.* The presence of large amount of acetic acid (i.e. produced by *L. buchneri*) could have indirectly stimulated the activity of *A. pasteurianus* (Spoelstra et al., 1988; Nishino et al., 2009; **paper I**). To regard to fungal community at silo opening unusual species for silages were found, whereas during aerobic deterioration *Pichia kudriavzevii* and *Aspergillus fumigatus* appeared (**paper IV**).

Eikmeyer et al. (2013) were the first to study the bacterial microbiome of silage during ensiling, however, these authors focused only on bacterial communities and did not describe the nature of the fungal microbiome. Several authors utilized next generation sequencing to analyze the bacterial change during fermentation (Eikmeyer et al., 2013; Bao et al., 2016; Gharechahi et al., 2017; Keshri et al., 2019) but few work are available on metagenome analysis during aerobic deterioration. Romero et al. (2018) reported the change of bacterial and fungal microbiome during fermentation of 4 corn silage hybrids, whereas Keshri et al. (2018) reported the microbiome dynamics (bacterial and fungal) during ensiling of corn with and without *Lactobacillus plantarum* inoculant. The first use of NGS technologies during aerobic deterioration is by Dunière et al. (2017) that characterized the bacterial and fungal core microbiomes associated with small grain cereals (barley, oats, triticale) during ensiling and upon aerobic exposure. Their paper highlighted the dominance of yeasts belonging to the Saccharomycetales order in the core microbiome of small grain silages after ensiling. Aerobic exposure was characterized by an increase of OTUs belonging to the Hypocreales, frequently associated with saprophytic fungi, indicating a decaying process in silage, or with plant pathogenic fungi.

Recently, Peng et al. (2018) evaluated the addition of condensed tannins as an additive to control unfavorable microbial development and improve aerobic stability of purple prairie clover. Taxonomic bacterial community profiles were dominated by Lactobacillales after fermentation, with a notable increase in *Bacillus* spp. as a result of aerobic exposure. The addition of condensed tannins decreased bacterial diversity during both ensiling and aerobic exposure but increased fungal diversity during aerobic exposure only.

Nair et al. (2019) studied the effect of fungal resistance traits in barley combined with fungicide application on aerobic stability and bacterial and fungal communities. Aerobic stability was not affected by fungicide application. Although the application of the fungicide changed the chemical and microbial composition of barley, it appeared to have little impact on the fungal community responsible for the aerobic deterioration of barley silage.

5. Reference

Adesogan A.T., Krueger N., Salawu M.B., Dean D.B., Staples C.R., 2004. The influence of treatment with dual-purpose bacterial inoculants or soluble carbohydrates on the fermentation and aerobic stability of bermudagrass. J. Dairy Sci. 87:3407-3416. https://doi.org/10.3168/jds.2008-1788.

Adesogan A.T., Salawu M.B., Ross A.B., Davies D.R., Brooks A.E., 2003. Effect of *Lactobacillus buchneri*, *Lactobacillus fermentum*, *Leuconostoc mesenteroides* inoculants, or a chemical additive on the fermentation, aerobic stability and nutritive value of crimped wheat grains. J. Dairy Sci. 86:1789-1796. https://doi.org/10.3168/jds.S0022-0302(03)73764-3.

Allen M.S., Coors J.G., Roth G.W., 2003. Corn silage. Pages 547-608. In Silage Science and Technology (Agronomy Series No. 42). Buxton D.R., Muck R.E., Harrison H.J. Ed. American Society of Agronomy, Madison, WI.

Anil M.H., Mbanya J.N., Symonds H.W., Forbes J.M., 1993. Responses in the voluntary intake of hay or silage by lactating cows to intraruminal infusions of sodium acetate or sodium propionate, the tonicity of rumen fluid or rumen distension. Brit. J. Nutr. 69:699-712. https://doi.org/10.1079/BJN19930071.

Arriola K.G., Kim S.C., Adesogan A.T., 2011. Effect of applying inoculants with heterolactic or homolactic and heterolactic bacteria on the fermentation and quality of corn silage. J. Dairy Sci. 94:1511-1516. https://doi.org/10.3168/jds.2010-3807.

Assis F.G.D., Avila C.L.D., Pinto J.C., Schwan R.F., 2014. New inoculants on maize silage fermentation. Rev. Bras. Zoot. 43:395-403. http://dx.doi.org/10.1590/s1516-35982014000800001.

Auerbach H., Nadeau E., 2013. Effects of chemical additives on whole crop maize silage traits. Pages 736-737. In Proc. 22nd Int. Grassl. Congr. Sept. 15-19, Sydney, New South Wales, Australia. Michalk D.L., Millar G.D., Badgery W.B., Broadfoot K.M. Ed. New South Wales Department of Primary Industry, Orange, New South Wales, Australia.

Auerbach H., Weiss K., Nadeau E., 2012. Benefits of using silage additives. Pages 75-144. In Proc. 1st Int. Silage Summit, Leipzig, Saxony, Germany. Auerbach H., Luckstadt C., Weissbach F. Ed. Anytime Publishing Services, Worthington, UK.

Ávila C.L.S., Carvalho B.F., Pinto J.C., Duarte W.F., Schwan R.F., 2014. The use of Lactobacillus species as starter cultures for enhancing the quality of sugar cane silage. J. Dairy Sci. 97:940-951. https://doi.org/10.3168/jds.2013-6987.

Bao W., Mi Z., Xu H., Zheng Y., Kwok L.Y., Zhang H., Zhang W.Y., 2016. Assessing quality of *Medicago sativa* silage by monitoring bacterial composition with single molecule, real-time sequencing technology and various physiological parameters Sci. Rep. 6:28358. https://doi.org/10.1038/srep28358.

Barnett A.J.G., 1954. Silage fermentation. Butterworths Sci. Pub. London.

Basso F.C., Bernardes T.F., Roth A.P.T.P., Lodo B.N., Berchielli T.T., Reis R.A., 2012. Fermentation and aerobic stability of corn silage inoculated with *Lactobacillus buchneri*. R. Bras. Zootec. 41:1789-1794. https://doi.org/10.1590/S1516-35982012000700032.

Basso F.C., Rabelo C.H.S., Lara E.C., Siqueira G.R., Reis R.A., 2018. Effects of *Lactobacillus buchneri* NCIMB 40788 and forage: Concentrate ratio on the growth performance of finishing feedlot lambs fed maize silage. Anim. Feed Sci. Technol. 244:104-115. https://doi.org/10.1016/j.anifeedsci.2018.08.008

Bernardes T. F., Nussio L.G., Amaral R.C. 2012. Top spoilage losses in maize silage sealed with plastic films with different permeabilities to oxygen. Grass Forage Sci. 67:34–42. https:// doi .org/10 .1111/ j .1365 -2494 .2011 .00823 .x.

Bernardes T.F., Daniel L.P., Adesogan A.T, McAllister T.A., Drouin P., Nussio L., Huhtanen P., Tremblay G.F., Bélanger G., Cai Y., 2018. Silage review: Unique challenges of silages made in hot and cold regions. J. Dairy Sci. 101:4001-4019. https://doi.org/10.3168/jds.2017-13703.

Bernardes T.F., De Oliveira I.L., Lara M.A.S., Casagrande D.R., Avila C.L.S., Pereira O.G., 2015. Effects of potassium sorbate and sodium benzoate at two application rates on fermentation and aerobic stability of maize silage. Grass Forage Sci. 70:491-498. http://dx.doi.org/10.1111/gfs.12133.

Blajman J.E., Pàez R.B., Vinderola C.G., Lingua M.S., Signorini M.L. 2018. A meta-analysis on the effectiveness of homofermentative and heterofermentative lactic acid bacteria for corn silage. J. Appl. Microbiol. 125:1655-1669. https://doi.org/10.1111/jam.14084.

Bolsen K.K., Ashbell G., Weinberg Z.G., 1996. Silage fermentation and silage additives. Review. Asian-Austral. J. Anim. 9:483-493. https://doi.org/10.5713/ajas.1996.483.

Bonifacio H. F., Rotz C.A., Hafner S.D., Montes F., Cohen M., Mitloehner F.M., 2017. A process-based emission model of volatile organic compounds from silage sources on farms. Atmos. Environ. 152:85-97. https://doi.org/10.1016/j.atmosenv.2016.12.024.

Borreani G. Tabacco E., 2010. The relationship of silage temperature with the microbiological status of the face of corn silage bunkers. J. Dairy Sci. 93:2620-2629. http://doi.org/10.3168/jds.2009-2919.

Borreani G., Coppa M., Revello-Chion A., Comino L., Giaccone D., Ferlay A., Tabacco E., 2013. Effect of different feeding strategies in intensive dairy farming systems on milk fatty acid profiles, and implications on feeding costs in Italy. J. Dairy Sci. 96:6840-6855. https://doi.org/10.3168/jds.2013-6710.

Borreani G., Dolci P., Tabacco E., Cocolin L., 2013. Aerobic deterioration stimulates outgrowth of spore-forming *Paenibacillus* in corn silage stored under oxygen-barrier or polyethylene films. J. Dairy Sci. 96:5206-5216. https://doi.org/10.3168/jds.2013-6649.

Borreani G., Ferrero F., Coppa M., Demey V., Tabacco E., 2018b. Effect of different inocula on aerobic stability of corn silage. Pages 238-239. In: Proceedings, XVIII International Silage Conference Ed: Gerlach K., Südecum K.H. Bonn, DE, 24-26 July.

Borreani G., Tabacco E., 2008. Low permeability to oxygen of a new barrier film prevents butyric acid bacteria spore formation in farm corn silage. J. Dairy Sci. 91:4272-4281. http://doi.org/10.3168/jds.2008-1151

Borreani G., Tabacco E., 2008. Low permeability to oxygen of a new barrier film prevents butyric acid bacteria spore formation in farm corn silage. J. Dairy Sci. 91:4272-4281. https://doi.org/10.3168/jds.2008-1151.

Borreani G., Tabacco E., 2012. Effect of silo management factors on aerobic stability and extent of spoilage in farm maize silages. Pages 71-72. In Proc. 16th Int. Silage Conf. Kuoppala K., Rinne M., Vanhatalo A., Ed. MTT Agrifood

Research Finland, Hämeenlinna, Finland.
Borreani G., Tabacco E., Cavallarin L. 2007. A new oxygen barrier film reduces aerobic deterioration in farm-scale corn silage. J. Dairy Sci. 90:4701–4706. https://doi.org/10.3168/jds.2007-0310.
Borreani G., Tabacco E., Schmidt R.J., Holmes B.J., Muck R.E., 2018a. Silage review: Factors affecting dry matter and quality losses in silages. J. Dairy Sci. 101: 3952-3979. https://doi.org/10.3168/jds.2017-13837.
Broberg A., Jacobsson K., Strom K., Schnurer J., 2007. Metabolite profiles of lactic acid bacteria in grass silage. Appl. Environ. Microbiol. 73:5547-5552. https://doi.org/10.1128/AEM.02939-06.
Brüning D., Gerlach K., Weiß K., Südekum K.H., 2018. Effect of compaction, delayed sealing and aerobic exposure on maize silage quality and on formation of volatile organic compounds. Grass Forage Sci. 73:53-66. https://doi.org/10.1111/gfs.12288
Carvalho B.F., Ávila C.L.S., Miguel M.G.C.P., Pinto J.C., Santos M.C., Schwan R.F., 2014. Aerobic stability of sugar-cane silage inoculated with tropical strains of lactic acid bacteria. Grass Forage Sci. 70:308-323. http://dx.doi.org/10.1111/gfs.12117.
Cavallarin L., Tabacco E., Antoniazzi S., Borreani G., 2011. Aflatoxin accumulation in whole crop maize silage as a result of aerobic exposure. J. Sci. Food Agric. 91:2419-2425. https://doi.org/10.1002/jsfa.4481.
Cavallarin L., Tabacco E., Antoniazzi S., Borreani G., 2011. Aflatoxin accumulation in whole crop maize silage as a result of aerobic exposure. J. Sci. Food Agric. 91: 2419-2425. https://doi.org/10.1002/jsfa.4481.
Cheli F., Campagnoli A., Dell'Orto V., 2013. Fungal populations and mycotoxins in silages: from occurrence to analysis. Anim. Feed Sci. Tech. 183:1-16. https:// doi.org/10.1016/j.anifeedsci.2013.01.013.
Chen Y., Sela S., Gamburg M., Pinto R., Weinberg Z.G., 2005. Fate of *Escherichia coli* during ensiling of wheat and corn. Appl. Environ. Microbiol. 71:5163-5170. https://doi.org/10.1128/AEM.71.9.5163-5170.2005.
Ciotti A., Canale A., Valente M.E., Peiretti P.G., 1989. Direct and delayed sealing of round bales of lucerne at low degrees of wilting. In: Proc. 16th International Grassland Congress. Nice, France, pp. 971-972.
Claus D., Berkeley R.C.W., 1986. Genus *Bacillus*. Pages 1105-1139. In: Bergey's manual of systematic bacteriology. Vol. 2. Ed: Sneath et al. Williams and Wilkins, Baltimore, MD.
Cogan T., Hawkey R., Higgie E., Lee M.R.F., Mee E., Parfitt D., Wilkinson J.M., 2017. Silage and total mixed ration hygienic quality on commercial farms: Implications for animal production. Grass Forage Sci. 72:601-613. https://doi.org/10.1111/gfs.12265.
Colombari G., Borreani G., Crovetto G.M., 1999. Comparison of lucerne silage and ventilated hay in maize silage-based rations for dairy cows for the production of milk destined for Grana cheese. Grass Forage Sci. 54:184-194. https://doi.org/10.1046/j.1365-2494.1999.00168.x.
Colombari G., Borreani G., Crovetto G.M., 2001. Effect of ensiling alfalfa at low and high dry matter on production of milk used to make Grana cheese. J. Dairy Sci. 84: 2494-2502. https://doi: 10.3168/jds.S0022-0302(01)74700-5.
Comino L., Tabacco E., Righi F., Borreani G., 2014. 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. Anim. Feed Sci. Technol. 198:94-106. https://doi.org/10.1016/j.anifeedsci.2014.10.001.

Costa D.M., Moyer K.Z., Hellings E.A., Santos E.M.,. Moyer N.A, Kung Jr. L., 2019. An evaluation of *Lactobacillus hilgardii* 4785 to improve the aerobic stability of corn silage. J. Dairy Sci. 102(suppl.1):332.

Da Silva E.B., Savage R.M., Polukis S.A., Smith M.L., Gray A.M., Mester R.N., Kung Jr L., 2017. Effectiveness of a chemical additive on improving the aerobic stability of corn silage after short periods of ensiling. J. Dairy Sci. 100(Suppl. 2):263. (Abstr.).

Daniel J.L.P., Checolli M., Zwielehner J., Junges D., Fernandes J., Nussio L.G., 2015. The effects of *Lactobacillus kefiri* and *L. brevis* on the fermentation and aerobic stability of sugarcane silage. Anim. Feed Sci. Technol. 205:69-74. https://doi.org/10.1016/j.anifeedsci.2015.04.015.

Daniel J.L.P., Weiss K., Custodio L., Sa Neto A., Santos M.C., Zopollatto M., Nussio L.G., 2013. Occurrence of volatile organic compounds in sugarcane silages. Anim. Feed Sci. Technol. 185:101-105. https://doi.org/10.1016/j.anifeedsci.2013.06.011.

Dinuccio E., Balsari P., Gioelli F., Menardo S., 2010. Evaluation of the biogas productivity potential of some Italian agro-industrial biomasses Biores. Technol. 101:3780-3783. https://doi.org/10.1016/j.biortech.2009.12.113.

Dolci P., Tabacco E., Cocolin L., Borreani G., 2011. Microbial dynamics during aerobic exposure of corn silage stored under oxygen barrier or polyethylene films. Appl. Environ. Microbiol. 77:7499-7507. https://doi.org/10.1128/AEM.05050-11.

Dreihuis F., Spoelstra S.F., Cole S.C.J., Morgan R., 1996. Improving aerobic stability by inoculation with *Lactobacillus buchneri*. Pages 103-107. In: Jones D.I.H, Jones R., Dewhurst R., Merry R., Haigh P.M., Ed: Proc. 11th Int. Silage Conf.. Aberystwyth, IGER.

Driehuis F. 2013. Silage and the safety and quality of dairy foods: a review. Agric. Food Sci. 22:16-34. https://doi.org/10.23986/afsci.6699.

Driehuis F., Hoolwerf J., Rademaker J.L.W., 2016. Concurrence of spores of *Clostridium tyrobutyricum*, *Clostridium beijerinckii* and *Paenibacillus polymyxa* in silage, dairy cow faeces and raw milk. Int. Dairy J. 63:70-77. https://doi.org/10.1016/j.idairyj.2016.08.004.

Driehuis F., Oude Elferink S.J.W.H., 2000. The impact of the quality of silage on animal health and food safety: a review. Veterinary Quarterly 22:212-216. https://doi.org/10.1080/01652176.2000.9695061.

Driehuis F., Oude Elferink S.J.W.H., Spoelstra S.F., 1999. Anaerobic lactic acid degradation during ensilage of whole-crop maize inoculated with *Lactobacillus buchneri* inhibits yeast growth and improves aerobic stability. J. Appl. Microbiol. 87:583-594. https://doi.org/10.1046/j.1365-2672.1999.00856.x.

Driehuis F., Oude Elferink S.J.W.H., Van Wikselaar P.G., 2001. Fermentation characteristics and aerobic stability of grass silage inoculated with *Lactobacillus buchneri*, with or without homofermentative lactic acid bacteria. Grass Forage Sci. 56:330-343. https://doi.org/10.1046/j.1365-2494.2001.00282.x.

Driehuis F., van Wikselaar P.G., 1996. Effects of addition of formic, acetic or propionic acid to maize silage and low dry matter grass silage on the microbial flora and aerobic stability. Pages 256-257. In: Jones D.I.H, Jones R., Dewhurst R., Merry R., Haigh P.M., Ed: Proc. 11th Int. Silage Conf.. Aberystwyth, IGER.

Driehuis F., Wilkinson J.M., Jiang Y., Ogunade I., Adesogan A.T., 2018. Animal and human health risks from silage. J. Dairy Sci. 101:4093-4110. https://doi.org/10.3168/jds.2017-13836.

Drouin P., Chaucheyras F. 2018a. How do time of fermentation and lactic acid bacteria inoculation influence microbial succession during ensiling? Pages 32-33. In: Proc. XVIII Int. Silage Conf. Ed: Gerlach K., Südecum K.H. Bonn, DE, 24-26 July.

Drouin P., Chaucheyras F. 2018b. Impact of inoculation with *Lactobacillus buchneri* and *Lactobacillus hilgardii* on bacterial and fungal communities during feed-out period of corn silage. Pages 142-143. In: Proc. XVIII Int. Silage Conf. Ed: Gerlach K., Südecum K.H. Bonn, DE, 24-26 July.

Du Toit W.J, Pretorius I.S., Lonvaud-Funel A.., 2005. The effect of sulphur dioxide and oxygen on the viability and culturability of s strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces bruxellensis* isolated from wine. J. Appl. Microbiol. 98:862-871. https://doi.org/10.1111/j.1365-2672.2004.02549.x.

Dunière L., Gleizal A., Chaucheyras-Durand F., Chevallier I., Thevenot-Sergentet D., 2011. Fate of *Escherichia coli* O26 in corn silage experimentally contaminated at ensiling, at opening or after aerobic exposure and protective effect of various bacterial inoculants. Appl. Environ. Microbiol. https://doi.org/77:8696-8704. 10.1128/AEM.06320-11.

EFSA, 2012. EFSA Panel on additives and products or substances used in animal feed (FEEDAP); Guidance for the preparation of dossiers for technological additives. EFSA J. 10:2528. https://doi.org/10.2903/j.efsa.2012.2528.

Eikmeyer F.G., Köfinger P., Poschenel A., Jünemann S., Zakrzewski M., Heini S., Mayrhubere E., Grabherrc R., Pühlera A., Schwabb, Schlütera A., 2013. Metagenome analyses reveal the influence of the inoculant *Lactobacillus buchneri* CD034 on the microbial community involved in grass ensiling. J Biotechnol. 167:334-43. https://doi.org/10.1016/j.jbiotec.2013.07.021.

Escobar-Zepeda A., Poncede León A.V., Sanchez-Flores A., 2015. The road to metagenomics: from microbiology to DNA sequencing technologies and bioinformatics. Front. Genetic. 6:1-15. https://doi.org/10.3389/fgene.2015.00348.

Fenlon D.R., Wilson J., Weddell J.R., 1989. The relationship between spoilage and *Listeria monocytogenes* contamination in bagged and wrapped big bale silages. Grass Forage Sci. 44:97-100. https://doi.org/10.1111/j.1365-2494.1989.tb01915.x.

Field M., Wilman D., 1996. pH in relation to dry matter content in clamped and baled grass silages harvested in England and Wales. Proceedings of the XIth International Silage Conference, 8-11th September, 1996, Aberystwyth, Wales, UK pp. 126-127.

Fred E.B., Peterson W.H., Anderson J.A., 1921. The relation of lactic acid bacteria to corn silage. J. Biol. Chem. 41:319-327.

Garon D, Richard E., Sage L., Bouchart V., Pottier D., Lebailly P., 2006. Mycoflora and multimycotoxin detection in corn silages: experimental study. J. Agric. Food Chem. 54:3479-3484. https://doi.org/10.1021/jf060179i.

Gerlach K., Weiss K., Ross F., Buscher W., Sudekum K.H., 2013. Changes in maize silage fermentation products during aerobic deterioration and its impact on feed intake by goats. Agric. Food Sci. 22:168-181. https://doi.org/10.23986/afsci.6739.

Gerlach, K., Weiss, K., Ross, F., Buscher, W., Sudekum, K. H., 2014. Aerobic exposure

of grass silages and its impact on dry matter intake and preference by goats. Small Rumin. Res. 117:131-141. https://doi.org/10.1016/j.smallrumres.2013.12.033.

Gharechahi J., Kharazian Z.Z., Sarikhan S., Jouzani G.S., Aghdasi M., Salekdeh G.H., 2017. The dynamics of the bacterial communities developed in maize silage. Microbial. Biotechnol. 10:1663-1676. https://doi.org/10.1111/1751-7915.12751.

Gomes A.L.M., Osmari M.P., Machado J., Poppi A.C.O., Mari L.J., Chevaux E., Nussio L.G., Jobim C.C., Daniel J.L.P., 2018. Effects of three strains of heterofermentative bacteria on the conservation of sugarcane silage. Pages 276-277. In: Proceedings, XVIII International Silage Conference Ed: Gerlach K., Südecum K.H. Bonn, DE, 24-26 July.

Gorini C., 1907. Ricerche batteriologiche sui foraggi conservati nei silo. Annuario dell'Istituto agraria dott. Andrea Polti, Volumi V, VI, VII, VIII, IX, X, XI, 1904-1914. Milano, Tipografia Agraria.

Grant R.J., Adesogan A.T., 2018. Journal of Dairy Science Silage Special Issue: Introduction. J. Dairy Sci. 101:3935-3936. https://doi.org/10.3168/jds.2018-14630.

Haerr K.J., Lopes N.M., Pereira M.N., Fellows G.M., Cardoso F.C., 2015. Corn silage from corn treated with foliar fungicide and performance of Holstein cows. J. Dairy Sci. 98:8962-8972. https://doi.org/10.3168/jds.2015-9887.

Hafner S.D., Franco R.B., Kung Jr L., Rotz C.A., Mitloehner F., 2014. Potassium sorbate reduces production of ethanol and 2 esters in corn silage. J. Dairy Sci. 97:7870–7878. https://doi.org/10.3168/jds.2014-8537.

Hafner S.D., Howard C.R., Muck R.E., Franco B., Montes F., Green P.G., Mitloehner F., Trabue S.L., Rotz C.A., 2013. Emission of volatile organic compounds from silage: Compounds, sources, and implications. Atmos. Environ. 77:827-839. https://doi.org/10.1016/j.atmosenv.2013.04.076

Hafner S.D., Montes F., Rotz C.A., Mitloehner F.M., 2010. A farm-level model of VOC emission from silage. In: International Symposium on Air Quality and Manure Management for Agriculture, Dallas, TX. 13-16 September. ASABE Publication No. 711P0510cd.

Henderson N., 1993. Silage additives. Anim. Feed Sci. Technol. 45:35-56. http://dx.doi.org/10.1016/0377-8401(93)90070-Z.

Higginbotham G.E, Mueller S.C, Bolsen K.K, DePeters E.J., 1998. Effects of inoculants containing propionic acid bacteria on fermentation and aerobic stability of com silage. J. Dairy Sci. 81:2185-2192. https://doi.org/10.3168/jds.S0022-0302(98)75797-2.

Hoffman, P. C., Ocker, S. M., 1997. Quantification of milk yield losses associated with feeding aerobically unstable high moisture corn. J. Dairy Sci. 80 (E-Suppl. 1):234. (Abstr.)

Hofherr M.W., Reich L.J., Der Bedrosian M.C., Santos M.C., Hu W., Kung Jr L., 2008. Effect of a microbial inoculant producing ferulic acid esterase on the fermentation and NDF digestibility of normal and BMR corn silages. J. Dairy Sci. 91:(E-Suppl. 1)1-32.

Honig H., 1991. Reducing losses during storage and unloading of silage. In: Forage conservation towards 2000. Pages 116-128. Ed: Pahlow G., Honig H. Proc. Eur. Grassl. Fed., Braunschweig, Germany. 23-25 January.

Hu W., Schmidt R.J., McDonell E.E., Klingerman C.M., Kung Jr L., 2009. The effect of *Lactobacillus buchneri* 40788 or *Lactobacillus plantarum* MTD-1 on the

fermentation and aerobic stability of corn silages ensiled at two dry matter contents. J. Dairy Sci. 92:3907-3914. https://doi.org/10.3168/jds.2008-1788.
Huenting K., Aymanns T., Pries M., 2018. Effects of storage time and silage additives on aerobic stability of maize silages. Pages 252-253. In: Proc. XVIII Int. Silage Conf. Ed: Gerlach K., Südecum K.H. Bonn, DE, 24-26 July.
Huhtanen P., Khalili H., Nousiainen J.I., Rinne M., Jaakkola S., Heikkila T., Nousiainen J., 2002. Prediction of the relative intake potential of grass silage by dairy cows. Livest. Product. Sci. 73:111-130. https://doi.org/10.1016/S0301-6226(01)00279-2.
Huisden C.M., Adesogan A.T., Kim S.C., Ososanya T., 2009. Effect of applying molasses or inoculants containing homofermentative or heterofermentative bacteria at two rates on the fermentation and aerobic stability of corn silage. J. Dairy Sci. 92:690-697. https://doi.org/10.3168/jds.2008-1546.
Jonsson A. 1989. The role of yeasts and clostridia in silage deterioration. PhD Dissertation, Swedish University of Agricultural Science. Report 42. Uppsala, Sweden.
Jonsson A. 1991. Growth of *Clostridium tyrobutyricum* during fermentation and aerobic deterioration of grass silage. J. Sci. Food Agric. 54:557-568. https://doi.org/10.1002/jsfa.2740540407
Jonsson, A., Pahlow G., 1984. Systematic classification and biochemical characterisation of yeasts growing in grass silage inoculated with *Lactobacillus* cultures. Anim. Res. Dev. 20:7-22.
Julien M.C., Dion P., Lafrenière C., Antoun H., Drouin P., 2008. Sources of clostridia in raw milk on farms. Appl. Environ. Microbiol. 74:6348-6357. https://doi:10.1128/AEM.00913-08.
Kandler O., Weiss N., 1986. Genus *Lactobacillus Beijerinck* 1901, 212AL. Pages 1209-1234. In: Bergey's manual of systematic bacteriology. Vol. 2. Ed: Sneath et al. Williams and Wilkins, Baltimore, MD.
Kang T.W., Adesogan A.T., Kim S.C., Lee S.S., 2009. Effects of an esterase-producing inoculant on fermentation, aerobic stability, and neutral detergent fiber digestibility of corn silage. J. Dairy Sci. 92:732-738. https://doi.org/10.3168/jds.2007-0780.
Kehler W., Scholz H., 1996. Botulismus des Rindes. Übersichten zur Tierernährung, 24: 83-91.
Keshri J., Chen Y., Pinto R., Kroupitski Y., Weinberg Z.G., Saldinger S.S., 2019. Bacterial dynamics of wheat silage. Front. Microbiol. 10:1532. https://doi.org/10.3389/fmicb.2019.01532.
Keshri J., Chen Y., Pinto R., Kroupitski Y., Weinberg Z.G., Saldinger S.S., 2018. Microbiome dynamics during ensiling of corn with and without *Lactobacillus plantarum* inoculant. Appl. Microbiol. Biotechnol. 102:4025-4037. https://doi.org/10.1007/s00253-018-8903-y.
Kim S.C., Adesogan A.T., 2006. Influence of ensiling temperature, simulated rainfall, and delayed sealing on fermentation characteristics and aerobic stability of corn silage. J. Dairy Sci. 89:3122-3132. https://doi.org/10.3168/jds.S0022-0302(06)72586-3.
Kinay P., Mansour M.F., Gabler F.M., Margosan D.A., Smilanick J.L., 2007. Characterization of fungicide-resistant isolates of *Penicillium digitatum* collected in California. Crop Prot. 26:647-656. https://doi.org/10.1016/j.cropro.2006.06.002.
Kleinschmit D.H., Kung Jr L., 2006a. A meta-analysis of the effects of *Lactobacillus*

buchneri on the fermentation and aerobic stability of corn and grass and small-grain silages. J. Dairy Sci. 89:4005-4013. https://doi.org/10.3168/jds.S0022-0302(06)72444-4.

Kleinschmit D.H., Kung Jr L., 2006b. The Effects of *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* R1094 on the fermentation of corn silage. J. Dairy Sci. 89:3999-4004. https://doi.org/10.3168/jds.S0022-0302(06)72443-2

Kleinschmit D.H., Schmidt R.J., Kung Jr L., 2005. The effects of various antifungal additives on the fermentation and aerobic stability of corn silage. J. Dairy Sci. 88:2130-2139. https://doi.org/10.3168/jds.S0022-0302(05)72889-7.

Knick´M., Spörndly R., 2009. Sodium benzoate, potassium sorbateand sodium nitrite as silage additives. J. Sci. Food Agric. 89:2659-2667. https://doi.org/10.1002/jsfa.3771.

Knick´M., Spörndly R., 2011. The ensiling capability of a mixture of sodium benzoate, potassium sorbate, and sodium nitrite. J. Dairy Sci. 94 :824-831. https://doi.org/10.3168/jds.2010-3364.

Köhler B., Taube F., Ostertag J., Thurner S., Kluß C., Spiekers H., 2019. Dry-matter losses and changes in nutrient concentrations in grass and maize silages stored in bunker silos. Grass Forage Sci. 74:274-283. https://doi.org/10.1111/gfs.12430.

Krooneman J., Faber F., Alderkamp A.C., Oude Elfering S.J.H.W., Driehuis F., Cleenwerck L., Swings J., Gottschal J.C., Vancanneyt M., 2002. *Lactobacillus diolivorans* sp. nov., a 1,2-propanediol-degrading bacterium isolated from aerobically stable maize silage. Int. J. Syst. Evol. Microbiol. 52:639-646. https://doi.org/10.1099/00207713-52-2-639.

Kung Jr L., 2009. Potential factors that may limit the effectiveness of silage additives. Pages 37-45. In: Proc. XV Int. Silage Conf., Madison, WI. July 27-29, 2009. Ed: Broderick G.A., Adesogan A.T., Bocher L.W., Bolsen K.K., Contreras-Govea F.E., Harrison J.H., Muck R.E. U.S. Dairy Forage Research Center, Madison, WI.

Kung Jr L., 2010a. Understanding the biology of silage preservation to maximize quality and protect the environment. In: Proc. 2010 California Alfalfa and Forage Symposium and Corn/Cereal Silage Conference, Visalia, CA, USA.

Kung Jr L., 2010b. Aerobic stability of silage. In: Proc. 2010 California Alfalfa and Forage Symposium and Crop/cereal Conference, Visalia, CA, USA.

Kung Jr L., 2011. Silage fermentation and additives. Available online: http://www1.foragebeef.ca/$Foragebeef/frgebeef.nsf/all/frg69/$FILE/silagefermentationadditives.pdf . Accessed Aug. 25, 2019.

Kung Jr L., Robinson J.R., Ranjit N.K., Chen J.H., Golt C.M., Pesek J.D., 2000. Microbial populations, fermentation end-products, and aerobic stability of corn silage treated with ammonia or a propionic acid-based preservative. J. Dairy Sci. 83:1479-1486. https://doi.org/10.3168/jds.S0022-0302(00)75020-X.

Kung Jr L., Shaver D.R., Grant R.J., Schmidt R.J., 2018a. Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. J. Dairy Sci. 101:4020-4033. https://doi.org/10.3168/jds.2017-13909.

Kung Jr L., Smith M.L., da Silva B.E., Windle M.C., da Silva T.C., Polukis S.A., 2018b. An evaluation of the effectiveness of a chemical additive based on sodium benzoate, potassium sorbate, and sodium nitrite on the fermentation and aerobic stability of corn silage. J. Dairy Sci. 101:5949-5960. https://doi.org/10.3168/jds.2017-14006.

Kung Jr L., Stokes M.R., Lin C.J., 2003. Silage additives. Pages 305-360. In Silage

Science and Technology (Agronomy Series No. 42). Buxton D.R., Muck R.E., Harrison H.J. Ed. American Society of Agronomy, Madison, WI.

Lara E.C., Basso F.C., de Assis F.B., Souza F.A., Berchielli T.T., Reis R.A., 2016. Changes in the nutritive value and aerobic stability of corn silages inoculated with *Bacillus subtilis* alone or combined with *Lactobacillus plantarum*. Anim. Prod. Sci. 56:1867–1874. httpd://doi.org/ 10.1071/AN14686.

Leibensperger R.Y., Pitt R.E., 1987. A model of clostridial dominance in ensilage. Grass Forage Sci. 42:297-317. https://doi.org/10.1111/j.1365-2494.1987.tb02118.x.

Li Y., Nishino N., 2011. Bacterial and fungal communities of wilted Italian ryegrass silage inoculated with and without *Lactobacillus rhamnosus* or *Lactobacillus buchneri*. Lett. Appl. Microbiol. 52:314-321. https://doi.org/10.1111/j.1472-765X.2010.03000.x.

Lima L.M., Dos Santos J.P., Casagrande D.R., Ávila C.L.S., Lara M.S., Bernardes T.F. 2017. Lining bunker walls with oxygen barrier film reduces nutrient losses in corn silages. J. Dairy Sci. 100:4565–4573. https://doi.org/10.3168/jds.2016-12129.

Lin C., BoIsen K.K., Brent B.E., Fung D.Y.C., 1992a. Epiphytic lactic acid bacteria succession during the pre-ensiling and ensiling periods of alfalfa and maize. J. Appl. Bact. 73:373-387. https://doi.org/10.1111/j.1365-2672.1992.tb04992.x.

Lin C., Bolsen K.K., Brent B.E., Hart R.A., Dickerson J.T., Feyerherm A.M., Aimutis W.R., 1992b. Epiphytic microflora on alfalfa and whole-plant corn. J. Dairy Sci. 75:2484-2493. https://doi.org/10.3168/jds.S0022-0302(92)78010-2.

Lindgren S. 1991. Hygienic problems in conserved forage. p. 177-190. In G. Pahlow and H. Honig (ed.) Forage conservation towards 2000. Proc. Eur. Grassl. Fed., Braunschweig, Germany. 23-25 Jan. 1991. Landbauforschung Volkenrode Sonderheft 123. Bundesforschungsanstalt flir Landwirtschaft Braunschweig-Volkenrode, Braunschweig, Germany.

Lindgren S., Pahlow G., Oldenburg E., 2002. Influence of microbes and their metabolites on feed and food quality. Pages 503-511. In Proc. 19th Gen. Meet. EGF, La Rochelle, France. Br. Grassl. Soc., Reading, UK

Lindgren S., Petterson K., Kaspersson A., Jonsson A., Lingvall P., 1985. Microbial dynamics during aerobic deterioration of silages. J. Sci. Food Agric. 36:765-774. https://doi.org/10.1002/jsfa.2740360902.

Liu Q.H., Lindow S.E., Zhang J.G., 2018. *Lactobacillus parafarraginis* ZH1 producing anti-yeast substances to improve the aerobic stability of silage. Anim. Sci. J. 89:1302-1309. https://doi.org/ 10.1111/asj.13063.

Liu Q.H., Yang F.Y., Zhang J.G., Shao T., 2014. Characteristics of *Lactobacillus parafarraginis* ZH1 and its role in improving the aerobic stability of silages. J. Appl. Microbiol. 117:405-416. https://doi.org/10.1111/jam.12530.

Mari L.J., Schmidt R.J., Nussio L.G., Hallada C.M., Kung L Jr., 2009. An evaluation of the effectiveness of *Lactobacillus buchneri* 40788 to alter fermentation and improve the aerobic stability of corn silage in farm silos. J. Dairy Sci. 92:1174-1176. https://doi.org/10.3168/jds.2008-1700.

McAllister T.A., Dunière L., Drouin P., Xu S., Wang Y., Munns K., Zaheer R., 2018. Silage review: Using molecular approaches to define the microbial ecology of silage. J. Dairy Sci. 101:4060-4074. https://doi.org/10.3168/jds.2017-13704.

Menardo S., Balsari P., Tabacco E., Borreani G., 2015. Effect of conservation time and the addition of lactic acid bacteria on the biogas and methane production of corn stalk silage. BioEnerg. Res. 8:1810-1823. https://doi.org/10.1007/s12155-

015-9637-7.
Mogodiniyai Kasmaei K., Rustas B.O., Spörndly R. Udén P., 2013. Prediction models of silage fermentation products on crop composition under strict anaerobic conditions: a meta-analysis. J. Dairy Sci. 96:6644-6649. https://doi.org/10.1111/gfs.12238.
Mogodiniyai Kasmaei K., Rustas B.O., Spörndly R. Udén P., 2016. Separating the effects of forage source and field microbiota on silage fermentation quality and aerobic stability. Grass Forage Sci. 72:281-289. https://doi.org/10.1111/gfs012238.
Moon N.J., 1983. Inhibition of the growth of acid-tolerant yeasts by acetate, lactate and propionate and their synergistic mixtures. J. Appl. Bacteriol., 55:454-460. https://doi.org/10.1111/j.1365-2672.1983.tb01685.x.
Morais G., Daniel J.L.P., Siqueira G.R., Silva N.C., Schonell E.P., Nussio L.G.., 2016. A review on additives for grain silages. National Agricultural and Food Centre (NPPC), Lužianky. 54-70.
Moyer K.Z., Hellings E.A., Costa D.M., Santos E.M., Moyer N.A., Kung Jr L., 2019. The effects of *Lactobacillus diolivorans* on fermentation characteristics and aerobic stability of corn silage. J. Dairy Sci. 102(suppl.1):205-206.
Muck R.E., 1996. A lactic acid bacteria strain to improve aerobic stability of silages. Pages 42-43. In: Research Summaries. U.S. Dairy Forage Res. Center, Madison, WI.
Muck R.E., 2004. Effects of corn silage inoculants on aerobic stability. T. ASAE. 47:1011-1016. https://doi.org/10.13031/2013.16571.
Muck R.E., 2010. Silage microbiology and its control through additives. R. Bras. Zootec. 39:183-191. https://dx.doi.org/10.1590/S1516-35982010001300021.
Muck R.E., 2013. Recent advances in silage microbiology. Agric. Food Sci. 22:3-15. https://doi.org/10.23986/afsci.6718.
Muck R.E., Holmes B.J., 2000. Factors affecting bunker silo densities. Appl. Eng. Agric. 16:613-619. https://doi.org/10.13031/2013.5374.
Muck R.E., Nadeau E.M.G., McAllister T.A., Contreras-Govea F.E., Santos M.C., Kung Jr L., 2018. Silage review: Recent advances and future uses of silage additives. J. Dairy Sci. 101:3980-4000. https://doi.org/10.3168/jds.2017-13839.
Muck R.E., O'Kiely P., 1992. Aerobic deterioration of lucerne (*Medicago sativa*) and Maize (*Zea mais*) Silages - Effects of fermentation products. J. Sci. Food Agric. 59:145-149. https://doi.org/10.1002/jsfa.2740590203.
Müllenborn C., Steiner U., Ludwig M., Oerke E.C., 2008. Effect of fungicides on the complex of Fusarium species and saprophytic fungi colonizing wheat kernels. Eur. J. Plant Pathol. 120:157-166. https://doi.org /10.1007/s10658-007-9204-y.
Nadeau E., Arnesson A., Jakobsson J., Auerbach H., 2015. Chemical additives reduce yeast count and enhance aerobic stability in high dry matter corn silage. Pages 354-355. In Proc. XVII Int. Silage Conf., Piracicaba, Sao Paulo, Brazil. Daniel J.L.P., Morais G., Junges D.,. Nussio L.G. Ed. University of Sao Paulo, Piracicaba, SP, Brazil.
Nadeau E., Svensson E., Zaralis K., Helander C., Pauly T., Arnesson A., 2011. Effects of additive on aerobic stability and nutritive value of maize silage stored during different time periods when harvested at advancing maturity stages. Page 393. In Advances in Animal Biosciences, vol. 2, Proc. 8th Int. Symp. Nutr. Herbiv., Aberythwyth, Wales. Cambridge University Press, Cambridge, UK.
Nair J. Turkington T.K., Blackshaw R., Geddes C.M., Lupwayi N.Z., Xu S., Yang J. Yang H.E., Wang Y., McAllister T.A., 2019. Impact of field fungal

contamination of barley on ensiling properties, nutritional quality and the microbiome of barley silage. Grass Forage Sci. 74:231-243. https://doi.org/10.1111/gfs.12419.

Ni K., Minh T.T., Tu T.T.M., Tsuruta T., Pang H., Nishino N., 2017. Comparative microbiota assessment of wilted Italian rye-grass, whole crop corn, and wilted alfalfa silage using denaturing gradient gel electrophoresis and next-generation sequencing. Appl. Microbiol. Biotechnol. 101:1385-1394. https://doi.org/10.3168/jds.2017-12876.

Nishino N., 2011. Aerobic stability and instability of silages caused by bacteria. In: Proc. 2nd International Symposium on Forage Quality and Conservation. Pages 127-141. Ed. Daniel J.L.P., Zopollatto M. Nussio L.G. Piracicaba, Fundação de Estudos Agrários Luiz de Queiroz.

Nishino N., Wang C., Li Y., Parvin S., Kan K., 2009. Occurrence and survival in whole crop corn silage of *Acetobacter pasteurianus*. Pages: 165-166. In: Broderick G.A. et al. (ed.), Proc. 15th International Silage Conference. U.S. Dairy Forage Research Center, USDA-ARS, Madison, WI.

Niyigena V., Coffey K.P., Coblentz W.K., Philipp D., Rhein R.T., Young A.N., Caldwell J.D., Shanks B.C., 2019. Intake and digestibility by gestating sheep of alfalfa silage wrapped with or without an enhanced oxygen barrier plastic after time delays up to three days. Anim. Feed Sci. Technol. 254:114193. https://doi.org/10.1016/j.anifeedsci.2019.05.016.

Nsereko V.L., Smiley B.K., Rutherford W.M., Spielbauer A., Forrester K.J., Hettinger G.H., Harman E.K., Harman B.R., 2008. Influence of inoculating forage with lactic acid bacterial strains that produce ferulate esterase on ensilage and ruminal degradation of fiber. Anim. Feed Sci. Technol. 145:122-135. https://doi.org/10.1016/j.anifeedsci.2007.06.039.

Nucera D.M., Grassi M.A., Morra P., Piano S., Tabacco E., Borreani G., 2016. Detection, identification, and typing of *Listeria* species from baled silages fed to dairy cows. J. Dairy Sci. 99:6121-6133. https://doi.org/10.3168/jds.2016-10928.

O'Brien M., Padraig O'Kiely P., Forristal P.D., Fuller H.T., 2007. Quantification and identification of fungal propagules in well-managed baled grass silage and in normal on-farm produced bales. Anim. Feed Sci. Technol. 132:283-297. https://doi.org/10.1016/j.anifeedsci.2006.04.013.

O'Kiely P., Muck R.E., 1992. Aerobic deterioration of lucerne (*Medicago sativa*) and maize (*Zea mais*) Silages - Effects of Yeasts. J. Sci. Food Agric. 59:139-144. https://doi.org/10.1002/jsfa.2740590202.

Ogunade I.M., Jiang Y., Kim D.H., Pech Cervantes A.A., Arriola K.G., Vyas D., Weinberg Z.G., Jeong K.C., Adesogan A.T., 2017. Fate of *Escherichia coli* O157:H7 and bacterial diversity in corn silage contaminated with the pathogen and treated with chemical or microbial additives. J. Dairy Sci. 100:1780-1794. https://doi.org/10.3168/jds.2016-11745.

Ogunade I.M., Kim D.H., Jiang Y., Weinberg Z.G., Jeong K.C., Adesogan A.T., 2016. Control of *Escherichia coli* O157:H7 in contaminated alfalfa silage: Effects of silage additives. J. Dairy Sci. 99:4427-4436. https://doi.org/10.3168/jds.2015-10766.

Ohyama Y., Hara S.H., Masaki S.H., 1980. Analysis of the factors affecting aerobic deterioration of grass silages. Proc. Forage Conservation in the 80's. Occasional Symposium. British Grassland Society, 11:257-263.

Okatsu Y., Swanepoel N., Maga E.A., Robinson P.H., 2019. Impacts of some factors

that effect spoilage of silage at the periphery of the exposed face of corn silage piles. Anim. Feed Sci. Technol. 247:234-247. https://doi.org/10.1016/j.anifeedsci.2018.11.018.

Oldenberg E., 1991. Mycotoxins in conserved forage. Pages 191-205. In: Forage conservation towards 2000. Proc. European Grassland Federation Conference. Bundesforschungsanstalt für Landwirtschaft Braunschweig-Volkenrode. Landbauforschung Volkenrode Sonderheft, No.123.

Oliveira A.S., Weinberg Z.G., Ogunade I.M., Cervantes A.A.P., Arriola K.G., Jiang Y., Kim D., Li X., Gonçalves M.C.M., Vyas D., Adesogan A.T., 2017. Meta-analysis of effects of inoculation with homofermentative and facultative heterofermentative lactic acid bacteria on silage fermentation, aerobic stability, and the performance of dairy cows. J. Dairy Sci. 100:4587-4603. https://doi.org/10.3168/jds.2016-11815.

Oliveira J.S., Santos E.M., dos Santos A.P.M., 2016. Intake and digestibility of silages, advances in silage production and utilization. Available online: https://www.intechopen.com/books/advances-in-silage-production-and-utilization/intake-and-digestibility-of-silages. https://doi.org/10.5772/65280.

Oude Elferink S.J.W.H., Driehuis F., Becker P.M., Gottschal J.C., Faber F., Spoelstra S.F., 2001. The presence of *Acetobacter* sp. in ensiled forage crops and ensiled industrial byproducts. Med. Fac. Landbouww. Univ. Gent. 66:427-430.

Oude Elferink S.J.W.H., Driehuis F., Gottschal J.C., Spoelstra S.F., 1999. Silage fermentation and their manipulation. Proc. FAO Electronic Conf. on Tropical Silage, Rome, IT, 1 September -15 December 1999.

Oude Elferink S.J.W.H., Krooneman J., Gottschal J.C., Spoelstra S.F., Faber F., Driehuis F., 2001. Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. Appl. Environ. Microbiol. 67:125-132. https://doi.org/10.1128/AEM.67.1.125-132.2001.

Pahlow G., 1991. Role of microflora in forage conservation. Proc. Conference on Forage Conservation Towards 2000. Pages 26-36. Ed: Pahlow G,. Honig H. Braunschweig, Germany.

Pahlow G., Muck R.E., Driehuis F., Oude Elferink S.J.W.H., Spoelstra S.F., 2003. Microbiology of ensiling. Pages 31-93. In Silage Science and Technology (Agronomy Series No. 42). Buxton D.R., Muck R.E., Harrison H.J. Ed. American Society of Agronomy, Madison, WI.

Pedroso A.F., Adesogan A.T., Queiroz O.C.M., Williams S., 2010. Control of *Escherichia coli* O157:H7 in corn silage with or without various inoculants: Efficacy and mode of action. J. Dairy Sci. 93:1098-1104. https://doi.org/10.3168/jds.2009-2433.

Pelhate L., 1977. Maize silage: incidence of moulds during conservation. Folia Vet. Lat. 7:1-16.

Peng K., Jin L., Niu Y.D., Huang Q., McAllister T.A., Yang H.E., Denise H., Xu Z., Acharya S., Wang S., Wang Y., 2018. Condensed tannins affect bacterial and fungal microbiomes and mycotoxin production during ensiling and upon aerobic exposure. Appl. Environ. Microbiol. 84:e02274-17. https://doi.org/10.1128/AEM.02274-17.

Peyraud J.L., Taboada M., Delaby L., 2014. Integrated crop and livestock systems in Western Europe and South America: a review. Eur. J. Agron. 57:31-42. https://doi.org/10.1016/j.eja.2014.02.005.

Pitt R.E., Leibensperger R.Y., 1987. The effectiveness of silage inoculants: a systems approach. Agric. Syst. 25:27-49. https://doi.org/10.1016/0308-521X(87)90097-

7.
Pitt R.E., Liu Y., Muck R.E., 1991. Simulation of the effect of additives on aerobic stability of alfalfa and corn silages. T. ASAE 34:1633-1641. https://doi.org/10.13031/2013.31781.
Polukis S.A., Smith M.L., Savage R.M., Silva B.E., Laubach A., Gray A., Kung Jr L., 2016. The effect of microbial inoculants on the aerobic stability of high moisture corn. J. Animal Sci. 99(E-Suppl. 1):678. (Abstr.). https://doi.org/10.2527/jam2016-0678.
Prema P., Smila D., Palavesam A., Immanuel G., 2010. Production and characterization of an antifungal compound (3-phenyl-lactic acid) produced by *Lactobacillus plantarum* strain. Food Bioproc. Technol. 3:379-386. https://doi.org/10.1007/s11947-008-0127-1.
Queiroz O.C.M., Arriola K.G., Daniel J.L.P., Adesogan A.T., 2013. Effects of 8 chemical and bacterial additives on the quality of corn silage. J. Dairy Sci. 96:5836-5843. https://doi.org/10.3168/jds.2013-6691.
Queiroz O.C.M., Kim S., Adesogan A.T., 2012. Effect of treatment with a mixture of bacteria and fibrolytic enzymes on the quality and safety of corn silage infested with different levels of rust. J. Dairy Sci. 95:5285-5291. https://doi.org/10.3168/jds.2012-5431.
Queiroz O.C.M., Ogunade I.M., Weinberg Z., Adesogan A.T., 2018. *Silage review*: Foodborne pathogens in silage and their mitigation by silage additives. J. Dairy Sci. 101:4132-4142. https://doi.org/10.3168/jds.2017-13901.
Rabelo C.H.S., Basso F.C, Lara E.C., Jorge L.G.O., Härter C.J., Mesquita L.G., Silva L.F.P., Reis R.A., 2018. Effects of *Lactobacillus buchneri* as a silage inoculant and as a probiotic on feed intake, apparent digestibility and ruminal fermentation and microbiology in wethers fed low-dry-matter whole-crop maize silage. Grass Forage Sci. 73:67-77. https://doi.org/10.1111/gfs.12303.
Rabelo C.H.S., Basso F.C., Lara E.C., Jorge L.G.O., Härter C.J., Mari L.J., Reis R.A., 2017. Effects of *Lactobacillus buchneri* as a silage inoculant or probiotic on in vitro organic matter digestibility, gas production and volatile fatty acids of low dry-matter whole-crop maize silage. Grass Forage Sci. 72:534-544. https://doi.org/10.1111/gfs.12273.
Randby A.T., Selmer-Olsen I., Baevre L., 1999. Effect of ethanol in feed on milk flavour and chemical composition. J. Dairy. Sci. 82:420-428. https://doi.org/10.3168/jds.S0022-0302(99)75248-3.
Ranjit N.K., Kung Jr L., 2000. The effect of *Lactobacillus buchneri*, *Lactobacillus plantarum*, or a chemical preservative on the fermentation and aerobic stability of corn silage. J. Dairy Sci. 83:526-535. https://doi.org/10.3168/jds.S0022-0302(00)74912-5.
Ranjit N.K., Kung L. Jr., 2000. The effect of *Lactobacillus buchneri*, *Lactobacillus plantarum*, or a chemical preservative on the fermentation and aerobic stability of corn silage. J. Dairy Sci. 83:526-535. https://doi.org/10.3168/jds.S0022-0302(00)74912-5.
Reis C.B., Oliveira S.A., Carvalho B.F., Schwan R. F., Ávila C.L.S., 2018. Wild *Lactobacillus hilgardii* (CCMA 0170) strain modifies the fermentation profile and aerobic stability of corn silage. J. Appl. Anim. Res. 46:632-638. https://doi.org/10.1080/09712119.2017.1371609.
Richard E., Heutte N., Bouchart V., Garon D., 2009. Evaluation of fungal contamination and mycotoxin production in maize silage. Anim. Feed Sci. Technol. 148:309-320. https://doi.org/10.1016/j.anifeedsci.2008.02.004.

Romero J.J., Joo Y., Park J., Tiezzi F., Gutierrez-Rodriguez E., Castillo M.S., 2018. Bacterial and fungal communities, fermentation, and aerobic stability of conventional hybrids and brown midrib hybrids ensiled at low moisture with or without a homo- and heterofermentative inoculant. J. Dairy Sci. 101:3057-3076. https://doi.org/10.3168/jds.2017-13754.

Rooke, J. A., and R. D. Hatfield. 2003. Biochemistry of ensiling. Pages 95-139. In Silage Science and Technology (Agronomy Series No. 42). Buxton D.R., Muck R.E., Harrison H.J. Ed. American Society of Agronomy, Madison, WI.

Rotz C.A. 2003. How to maintain forage quality during harvest and storage. Pages 227–239 in Proc. of 2003 Western Canadian Dairy Seminar, Advances in Dairy Technology, vol. 15.

Santoro K, Matić S, Gisi U, Spadaro D, Pugliese M, Gullino M.L. 2018. Abundance, genetic diversity and sensitivity to demethylation inhibitor fungicides of *Aspergillus fumigatu*s isolates from organic substrates with special emphasis on compost. Pest Manag. Sci. 73:2481-2494. https://doi.org/10.1002/ps.4642.

Schein H., Hirz M., Buchebner M., Kramer W., 2018. The use of *Lactobacillus diolivorans* as silage inoculant. Pages 166-167. In: Proc. XVIII Int. Silage Conf. Ed: Gerlach K., Südecum K.H. Bonn, DE, 24-26 July.

Schenck J., Djurle A., Jensen D.F., Müller M., O'Brien M., Spörndly R., 2019. Filamentous fungi in wrapped forages determined with different sampling and culturing methods. Grass Forage Sci. 74:29-41. https://doi.org/10.1111/gfs.12399.

Schmidt R.J., Emara M.G., Kung Jr L., 2008. The use of a quantitative real-time polymerase chain reaction assay for identification and enumeration of *Lactobacillus buchneri* in silage. J. Appl. Microbiol. 105:920-929. https://doi.org/10.1111/j.1365-2672.2008.03834.x.

Schmidt R.J., Hu W., Klingerman C.M., McDonell E.E., Kung, Jr L., 2007. The effect of *Lactobacillus buchneri* 40788 with or without *Pediococcus pentosaceus* on the fermentation and aerobic stability of corn silage made at different locations. J. Dairy Sci. 90(Suppl. 1):181.

Schmidt R.J., Kung Jr L., 2010. The effects of *Lactobacillus buchneri* with or without a homolactic bacterium on the fermentation and aerobic stability of corn silages made at different locations. J. Dairy Sci. 93:1616-1624. https://doi.org/10.3168/jds.2009-2555.

Spadaro D., Bustos-Lopez M. del Pilar, Gullino M.L., Piano S., Tabacco E., Borreani G., 2015. Evolution of fungal populations in corn silage conserved under polyethylene or biodegradable films. J. Appl. Microbiol. 119:510-520.

Spoelstra S.F. 1985., Nitrate in silage. A review. Grass For. Sci. 40:1-11. https://doi.org/10.1111/j.1365-2494.1985.tb01714.x.

Stadhouders J., Hup G., Nieuwenhof F.F.J., 1983. Silage and cheese quality. NIZO mededeling M19A. Netherlands Institute for Dairy Research, Ede, The Netherlands.

Stadhousers J., Jørgensen K., 1990. Prevention of the contamination of raw milk by a hygienic milk production. Bull. Int. Dairy Fed. 251:31-35.

Szucs J.P., Suli A., Demey V., 2018. The effect of two heterofermentative bacteria (*L. hilgardii* CNCM I-4785 and *L. buchneri* NCIMB 40788) and their combination on fermentation and aerobic stability of corn silage at different opening times. Pages 341-342. In: Proc. XVIII International Silage Conference Ed: Gerlach K., Südecum K.H. Bonn, DE, 24-26 July.

Tabacco E., Comino L., Borreani G., 2018. Production efficiency, costs and

environmental impacts of conventional and dynamic forage systems for dairy farms in Italy. Eur. J. Agron. 99:1-12. https://doi.org/10.1016/j.eja.2018.06.004.

Tabacco E., Piano S., Cavallarin L.,. Bernardes T.F, Borreani G.. 2009. Clostridia spore formation during aerobic deterioration of maize and sorghum silages as influenced by *Lactobacillus buchneri* and *Lactobacillus plantarum* inoculants. J. Appl. Microbiol. 107:1632-1641. https://doi.org/10.1111/j.1365-2672.2009.04344.x.

Tabacco E., Piano S., Revello-Chion A., Borreani G., 2011b. Effect of *Lactobacillus buchneri* LN4637 and *Lactobacillus buchneri* LN40177 on the aerobic stability, fermentation products, and microbial populations of corn silage under farm conditions. J. Dairy Sci. 94:5589-5598. https://doi.org/10.3168/jds.2011-4286.

Tabacco E., Righi F., Quarantelli A., Borreani G., 2011a. Dry matter and nutritional losses during aerobic deterioration of corn and sorghum silages as influenced by different lactic acid bacteria inocula. J. Dairy Sci. 94:1409-1419. https://doi.org/10.3168/jds.2010-3538.

te Giffel M.C., Wagendorp A., Herrewegh A., Driehuis F., 2002. Bacterial spores in silage and raw milk. Antonie van Leeuwenhoek 81:625-630.

Teller R.S., Schmidt R.J., Whitlow L.W., Kung Jr L., 2012. Effect of physical damage to ears of corn before harvest and treatment with various additives on the concentration of mycotoxins, silage fermentation, and aerobic stability of corn silage. J. Dairy Sci. 95:1428-1436. https://doi.org/10.3168/jds.2011-4610.

Tennant R.K., Sambles C.M., Diffey G.E., Moore K.A., Love J., 2017. Metagenomic Analysis of Silage. J. Visual. Experim. 119:e54936. https://doi.org/10.3791/54936.

Vazquez J.A., Gonzalez M.P., Murado M.A., 2005. Stimulation of bacteriocin production by dialyzed culture media from different lactic acid bacteria. Current Microbiol. 50:208-211. https://doi.org/10.1007/s00284-004-4415-3.

Veldman A., Meijst J.A.C., Borggreve G.J., Heeres-van der Tol J.J., 1992. Carry-over of aflatoxin from cows' food to milk. Anim. Prod. 55:163-168. https://doi.org/10.1017/S0003356100037417.

Venancio W.S., Rodrigues M.A.T., Begliomini E., de Souza N.L., 2009. Physiological effects of strobilurin fungicides on plants. Cienc. Exatas Terra Cienc. Agrar. Eng. 9:59-68.

Virtanen A.I., 1933. The A-I-V-method of preserving fresh fodder. Empire J. Experiment. Agric. 1:143-155.

Vissers M.M.M., Driehuis F., te Giffel M.C., Jong P.D., Lankveld J.M.G., 2007b. Quantification of the transmission of microorganisms to milk via dirt attached to the exterior of teats. J. Dairy Sci. 90:3579-3582. https://doi:10.3168/jds.2006-633.

Vissers MM.M., Driehuis F., te Giffel M.C., Jong P.D., Lankveld J.M.G.. 2007a. Concentrations of butyric acid bacteria spores in silage and relationships with aerobic deterioration. J. Dairy Sci. 90:928-936. https://doi.org/10.3168/jds.S0022-0302(07)71576-X.

Weimberg Z.G., Chen Y., Miron D., Raviv Y., Nahim E., Bloch A., Yosef E., Nikbahat M., Miron J., 2011. Preservation of total mixed rations for dairy cows in bales wrapped with polyethylene stretch film – A commercial scale experiment. Anim. Feed Sci. Technol. 164:125-129. https://doi.org/10.1016/j.anifeedsci.2010.11.016.

Weinberg Z.G, Ashbell G., Hen Y. Azrieli A., 1995. The effect of a propionic acid bacterial inoculant applied at ensiling on the aerobic stability of wheat and sorghum silages. J. Ind. Microbiol. 15:493–497. https://doi.org/10.1007/BF01570020.

Weinberg Z.G., Ashbell G., Hen Y., Azrieli A., Szakacs G., Filya I., 2002. Ensiling whole-crop wheat and corn in large containers with *Lactobacillus plantarum* and *Lactobacillus buchneri*. J. Ind. Microbiol. Biotechnol. 28:7-11. https://doi.org/10.1038/sj/jim/7000207.

Weinberg Z.G., Ashbell G., Hen Y., Azrieli A.,1993. The effect of applying lactic acid bacteria at ensiling on the aerobic stability of silages. J. Appl. Microbiol. 75:512-518. https://doi.org/10.1111/j.1365-2672.1993.tb01588.x

Weinberg Z.G., Muck R.E., 1996. New trends and opportunities in the development and use of inoculants for silage. FEMS Microbiol. Rev. 19:53-68. https://doi.org/10.1016/0168-6445(96)00025-3.

Weinberg Z.G., Muck R.E., Weimer P.J., Chen Y., Gamburg M., 2004. Lactic acid bacteria used in inoculants for silage as probiotics for ruminants. Appl. Biochem. Biotechnol. 118:1-9. https://doi.org/10.1385/ABAB:118:1-3:001.

Weiss K., 2017. Volatile organic compounds in silages-Effects of management factors on their formation: A review. Slovak J. Anim. Sci. 50:55-67.

Weiss K., Kroschewski B., Auerbach H., 2016. Effects of air exposure, temperature and additives on fermentation characteristics, yeast count, aerobic stability and volatile organic compounds in corn silage. J. Dairy Sci. 99:8053-8069. https://doi.org/10.3168/jds.2015-10323.

Weissbach F., 1996. New Developments in Crop Conservation. Proceedings of the Xlth International Silage Conference 8th - 11th September 1996, University of Wales, Aberystwyth, pp. 1-25

Wieringa G.W., 1958. The effect of wilting on butyric acid fermentation in silage. Neth. J. Agric. Sci. 6:204-210.

Wilkinson J.M., 1999. Silage and animal health. Natural Toxins. 7:221-232.

Wilkinson J.M., Davies D.R., 2013. The aerobic stability of silage: key findings and recent developments. Grass Forage Sci. 68:1-19. https://doi.org/10.1111/j.1365-2494.2012.00891.x.

Wilkinson J.M., Muck R.E., 2019. Ensiling in 2050: Some challenges and opportunities. Grass Forage Sci. 74:178-187. https://doi.org/10.1111/gfs.12418.

Wilkinson J.M., Toivonen M.I.. 2003. World Silage: A survey of forage conservation around the world. Chalcombe Publications, Lincoln.

Woolford M.K. 1990. The detrimental effect of air on silage. J. Appl. Bacteriol. 68:101–116. https://doi.org/10.1111/j.1365-2672.1990.tb02554.x.

Woolford M.K., 1975. Microbiological screening of the straight chain fatty acids (C1-C12) as potential silage additives. J. Sci. Food Agric. 26:219-228. https://doi.org/10.1002/jsfa.2740260213.

Woolford M.K., 1984a. The Silage Fermentation. [Microbiological Series, No.14] New York, NY, and Basle: Marcel Dekker.

Woolford M.K., 1984b. The antimicrobial spectra of some salts of organic acids and glutaraldehyde in respect to their potential as silage additives. Grass Forage Sci. 39:53-57. https://doi.org/10.1111/j.1365-2494.1984.tb01664.x.

Woolford M.K., K.K. Bolsen, Peart L.S., 1982. Studies on the aerobic deterioration of whole-crop cereal silages. J. Agric. Sci. 98:529-535. https://doi.org/10.1017/S0021859600054290.

Xu D., Ding W., Ke W., Li F., Zhang P., Guo X., 2019b. Modulation of metabolome

and bacterial community in whole crop corn silage by inoculating homofermentative *Lactobacillus plantarum* and heterofermentative *Lactobacillus buchneri.* Front Microbiol. 23:3299. https://doi.org/10.3389/fmicb.2018.03299.

Xu S., Yang J., Qi M., Smiley B., Rutherford W., Wang Y., McAllister T.A., 2019a. Impact of *Saccharomyces cerevisiae* and *Lactobacillus buchneri* on microbial communities during ensiling and aerobic spoilage of corn silage. Anim. Sci. J. 97:1273-1285. https://doi.org/10.1093/jas/skz021.

Xu Z., He H., Zhang S., Kong J., 2017. Effects of inoculants *Lactobacillus brevis* and *Lactobacillus parafarraginis* on the fermentation characteristics and microbial communities of corn stover silage. Scientific Reports 7:13614. https://doi.org/10.1038/s41598-017-14052-1.

Yates I.E., Bacon C.W., Hinton D.M., 1997. Effects of endophytic infection by *Fusarium moniliforme* on corn growth and cellular morphology. Plant Dis. 81:723-728. https://doi.org/10.1094/PDIS.1997.81.7.723.

Zhou Y., Drouin P., Lafrenière C., 2016. Effect of temperature (5-25°C) on epiphytic lactic acid bacteria populations and fermentation of whole-plant corn silage. J Appl. Microbiol. 121:657-71. https://doi.org/10.1111/jam.13198.

Zimmer E, 1966. A new appraisal of the silage after Flieg. Das Wirtschaftseigene Futter 12:229-303.

Experimental Part

6. Paper I: Effects of time of conservation and new *Lactobacillus hilgardii* inoculum on fermentation profile and aerobic stability of whole corn and sorghum silage

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Effects of conservation period and *Lactobacillus hilgardii* inoculum on the fermentation profile and aerobic stability of whole corn and sorghum silages

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6.1. Abstract

BACKGROUND. Lactic acid bacteria (LAB) inocula have been developed over the years to improve aerobic stability of silages. The aims of the study were to evaluate the effect of different conservation periods and the use of *Lactobacillus hilgardii* inoculum on aerobic stability, fermentative profile and microbial population of corn and sorghum silages. Trials were carried out on two corns and one sorghum crops. The crops were untreated (C) or treated with *L. buchneri* [LB, application rate 300,000 cfu g^{-1} FM), *L. hilgardii* (LH, application rate 300,000 cfu g^{-1} FM) and with a combination (LB+LH, application rate 150,000 cfu g^{-1} FM each one). Silos were opened after 15, 30, 100 and 250 d of conservation, and the silages were analysed for fermentative profile, microbial count and aerobic stability.

RESULTS. During conservation, the inocula influenced the fermentation profile. The use of LH increased the aerobic stability at 15 and 30 d in one over three trials, while after 100 and 250 d, the presence of LB alone or with LH determined greater stability. In all the trials the acetic acid content increased, the yeast count decreased and the aerobic stability increased during the conservation period.

CONCLUSION. This study has shown that a long period in complete anaerobiosis reduced yeast count and improved aerobic stability in all silages. The addition of LB confirmed to be a good option to increase aerobic stability of silages whereas the effect of LH alone or in combination with LB on aerobic stability was not consistent between trials.

Key words: aerobic stability, conservation period, corn silage, heterolactic inoculum, *Lactobacillus hilgardii*, sorghum silage.

6.2. Introduction

In forages conservation as silage, to prevent the formation of butyric acid during fermentation and to ensure the aerobic stability of the silage during the feed-out phase represent two of the main practical issues of this technique.[1] When silage is exposed to air during feed-out, it can be deteriorated by the activity of aerobic microorganisms, mainly yeasts and moulds.[2] Moreover, Spoelstra et al.,[3] found that aerobic deterioration of corn silage can sometimes be initiated by bacteria belonging to *Acetobacter* genus. In particular, *Acetobacter* bacteria are involved in aerobic deterioration when ethanol has been depleted, due to its oxidation to acetic and lactic acid.[3,4] Aerobic deterioration increases dry matter (DM) losses, reduces the nutritive value of silage[1] and increases risks to animal and human health.[5] Aerobic deterioration is initially manifested by an increase in temperature, and this is followed by visible moulds, mainly in peripheral areas of the silo.[6] This leads to decreases in farm profitability, as the DM losses can reach up to 20% in corn and sorghum silages.[7] Several biochemical, microbiological, physical and management practices have been studied by many authors to ensure aerobic stability,[8,9] and among these the use of lactic acid bacteria (LAB) inocula has become very popular.

In 1996 LAB inocula based on the heterolactic pathway by *L. buchneri*, were proposed.[10] The fermentative pathway of these obligate heterofermentative LAB, despite causes higher DM losses during the conservation period than the homolactic pathway, produces a moderate amount of acetic acid that inhibits yeasts and increases aerobic stability.[11] *L. buchneri* is able to transform lactic acid into acetic acid, and thus decrease the lactic-to-acetic acid ratio, which can be used as an indication of the prevalence of homolactic or heterolactic fermentation. A lactic-to-acetic acid ratio of less than 3 would be an indication of a more dominant heterolactic fermentation.[12] Acetic acid and 1,2-propanediol are the typical end products of the *L. buchneri* pathway.[13] Because of the great capability of *L. buchneri* to produce acetic acid from lactic acid during conservation and thus to improve aerobic stability,[11,14,15] it is currently one of the most commonly used commercially inocula, even if it showed contrasting results at the farm level.[7,16]

A further inoculation improvement has been achieved by mixing heterofermentative and homofermentative LAB to ensure both the anaerobic lactic fermentation and to improve aerobic stability.[17] Several studies[18,19] analyzed the effects of mixtures of heterofermentative and homofermentative LAB, and showed that the inocula did not consistently improve the aerobic stability of silage, but pH or DM losses were not increased, thus indicating that the inoculants did not adversely affect fermentation.

The need of a long conservation period (more than 90 d) to be efficacious is

a criticism of using *L. buchneri* as silage inoculant[19,20] because there is a need in some farm situations to have silages with high aerobic stability after only a short period of storage in the silo. Therefore, it is necessary to identify new strains that will be able to work early on the silage fermentation process in order to improve aerobic stability after short conservation period (e.g less than 30 d).

Ávila et al.,[21] isolated and selected 14 wild strains of LAB from sugarcane silages, and Carvalho et al.,[22] studied their fermentation profiles. These authors reported that using two isolated strains of *L. hilgardii* as silage inocula resulted in good fermentation profile, reduced yeast count and improve aerobic stability in sugarcane silage. The first indication on the use of these two strains to improve the aerobic stability of corn silages was given by Assis et al.,[23] who reported improvement in aerobic stability, but without any significant differences from a commercial strain of *L. buchneri* used as a positive control. More research is needed to evaluate if the use of *L. hilgardii* as silage inoculum is able to dominate the fermentation and to improve the aerobic stability after short conservation period.

Hence, the aims of this study were i) to evaluate the influence of different conservation periods; and ii) to evaluate the effect of *L. hilgardii* alone or in combination with *L. buchneri* on the aerobic stability, fermentation profile and microbial count of whole crop corn and sorghum silages.

6.3. Materials and methods

6.3.1. Crop and ensiling

Three trials (two on corn and one on sorghum) were carried out on a commercial farm located in Rocca de' Baldi (CN) in the western Po plain, northern Italy (44°27’18"N, 7°43’19"E, 408 m above sea level). Trial I and III were conducted in the 2014-2015 period, whereas Trial II was conducted in the 2015-2016 period in the same location. Two corn hybrids (PR32F73 in Trial I and P1517W in Trial II, Pioneer Hi-Bred Italia Srl, Gadesco Pieve Delmona, Cremona, Italy) and one sorghum (NICOL, *S. bicolor x S. sudanensis*, Pioneer Hi-Bred Italia Srl) were grown in three separate fields (2 ha each) and were harvested as chopped whole crops. Corn plants were harvested at two different DM contents (around 363 g kg^{-1} in Trial I, and 340 g kg^{-1} in Trial II) and the sorghum was harvested at a DM content of 254 g kg^{-1} (Trial III). The fresh forage of each trial was chopped, using a precision forage harvester (Claas Jaguar 950 equipped with an 8-row Orbis head, Claas, Harsewinkel, Germany), to a theoretical cut length of 12 mm. Each field was divided into five separate blocks to obtain five replications per treatment (one for each block). The fresh herbage of each block was divided into four 70-kg piles to obtain silos that were opened at four different conservation periods (15, 30,100 and 250 d). The piles were either untreated (**C**) or treated with different LAB strains and their combinations. The inoculants were applied to piles representing the respective conservation period for each block in the field. The LAB strains were *L. buchneri* (**LB**) NCIMB 40788 (Lallemand Animal Nutrition, BP 59, Cedex, France) (theoretical rate of 300,000 cfu g^{-1} FM), *L. hilgardii* (**LH**) CNCM I-4785 (Lallemand Animal Nutrition, theoretical rate of 300,000 cfu g^{-1} FM) and their combination (**LB+LH**) (theoretical rate of 150,000 cfu g^{-1} FM of each one). The microbial inoculants were diluted in sterilized water and applied using a hand sprayer, at a rate of 4 ml kg^{-1} of forage, by spraying uniformly onto the forage, which was constantly hand mixed. The same amount of water was added to the C treatment. To add the targeted amount of LABs, the inocula were plated on MRS agar (Merck, Whitehouse Station, NY), with added natamycin (0.25 g L^{-1}) and, based on the measured concentration of LABs, an appropriate amount was used to achieve the desired application rate.

The fresh forage was sampled prior to ensiling after the inoculum had been applied. The untreated and treated forages were then ensiled (about 10 to 14 kg of wet forage) in 20 L plastic silos equipped with a lid that only enabled gas release. The forage was packed by hand and the final packing densities, on a wet basis, were 591 ± 21 kg FM m^{-3}, 628 ± 26 kg FM m^{-3}, 580 ± 19 kg FM m^{-3}, for Trial I, II and III, respectively. All the laboratory silos were filled within three hours. The silos were weighed, conserved at ambient temperature (20 ±

1°C) and opened after 15, 30, 100 and 250 d. At opening, each silo was weighed to determine the weight losses: the content of each silo was mixed thoroughly and sub-sampled to determine the DM content, the chemical composition, the fermentation profile and the microbial counts. The losses due to fermentation were calculated as the difference between the weight of the forage placed in each plastic silo at ensiling and the weight of the silage at the end of the conservation period, expressed as percentages of the amount of DM ensiled in each plastic silo and used to determine the DM recovery. After sampling, the silages were subjected to an aerobic stability test, which involved monitoring the temperature increases due to the microbial activity of the samples exposed to air. An amount of about three kilograms from each silo was allowed to aerobically deteriorate at room temperature (20 ± 1°C) in 17 L polystyrene boxes (290 mm diameter and 260 mm height). A single layer of aluminium foil was placed over each box to prevent drying and dust contamination, but also to allow air penetration. The room and silage temperatures were measured hourly by a data logger. Aerobic stability was defined as the number of hours the silage remained stable before its temperature increased by 2°C above room temperature.[24] The silage was sampled after 7 d and 14 d of aerobic exposure in order to quantify the microbial changes that occurred in the silage during exposure to air.

6.3.2. Sample preparation and analyses

All replicates of pre-ensiled material and the silage were split into five subsamples. One sub-sample was analysed immediately for the DM content by oven drying at 80°C for 24 h. Dry matter was corrected according to Porter and Murray,[25] in order to consider the losses of volatile compounds that can take place at 80°C. The second subsample was oven-dried at 65°C to constant weight and was air equilibrated, weighed and ground in a Cyclotec mill (Tecator, Herndon, VA, USA) to pass a 1 mm screen. The dried samples were analysed for total nitrogen (TN), according to the Dumas method (method number 992.23),[26] using a Nitrogen analyser Primacs SN (Skalar, Breda, The Netherlands), for crude protein (CP) (total N x 6.25), for ash by ignition (method number 942.05),[26] for water soluble carbohydrates (WSC), by the phenol sulphuric acid method, according to DuBois et al.,[27] and for ether extract (EE), using the Soxhlet method, according to AOAC (method number 920.39).[26] Neutral detergent fibre (aNDF) was analysed, using a Raw Fibre 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..[28] Acid detergent fibre (ADF) was analysed and expressed on a DM basis including residual ash.[29]

A third fresh sub-sample was used to determinate the water activity (a_w), pH,

nitrate (NO_3) contents and buffering capacity. The water activity was measured at 25°C on a fresh sample using an AquaLab Series 3TE (Decagon Devices Inc., Pullman, WA), which adopts the chilled-mirror dew point technique. The pH and concentration of NO_3 were determined on water extracts made from fresh forage mixed with distilled water (9:1 water-to-sample material ratio) and blended in a Stomacher blender (Seward Ltd, Worthing, UK) for 4 min. The total nitrate concentration was determined in the water extract, through semi-quantitative analysis, using Merckoquant test strips (Merck, Darmstadt, Germany; detection limit 100 mg NO_3 kg^{-1} DM). The pH was determined using a specific electrode. The buffering capacity (BC) was determined in the water extract, as described by Plaine and McDonald.[30]

A fourth sub-sample was extracted, using a Stomacher blender, for 4 min in H_2SO_4 0.05 mol L^{-1} at a 5:1 acid-to-sample material (fresh weight) ratio. An aliquot of 40 ml of silage acid extract was filtered with a 0.20-μm syringe filter and used for quantification of the fermentation products. The lactic and monocarboxylic acids (acetic, propionic and butyric acids) were determined, by means of high performance liquid chromatography (HPLC), in the acid extract.[31] Ethanol and 1,2-propanediol were determined by means of HPLC, coupled to a refractive index detector, on a Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA).

The fifth subsample was used for the microbial analyses. An aliquot of 30 g of silage was transferred to a sterile homogenisation bag, suspended 1:10 w/v in a peptone salt solution (1 g of bacteriological peptone and 9 g of sodium chloride per litre) and homogenized for 4 min in a laboratory Stomacher blender (Seward Ltd, London, UK). Serial dilutions were prepared, and the yeast and mould numbers were determined using the pour plate technique with 40.0 g L^{-1} of Yeast Extract Glucose Chloramphenicol agar (YGC agar, DIFCO, West Molesey, Surrey, UK) after incubation at 25 °C for 3 and 5 d for the yeasts and moulds, respectively. The yeasts and moulds colony forming units (cfu) were enumerated separately, according to their macromorphological features, on plates that yielded 1 to 100 cfu. The lactic acid bacteria were determined on MRS agar (Merck, Whitehouse Station, NY), with added natamycin (0.25 g L^{-1}), by incubating Petri plates at 30°C for 3 d under anaerobic conditions, according to Spoelstra et al..[3]

6.3.3. Statistical analysis

The NO_3 content was corrected for the dilution factor and expressed on DM basis. The microbial counts were $\log_{10}$ transformed and were presented on a wet weight basis. The values below the detection limit for yeasts and moulds (detection levels: 10 cfu g^{-1} of silage) were assigned a value corresponding to half of the detection limit in order to calculate the average value. The data were

analyzed by two-way analysis of variance for a 4 (treatments) x 4 (conservation periods) factorial arrangement in a completely randomized design with five replicates. The model included the fixed effects of treatments (TREAT), conservation period (PERIOD), and their interaction (T × P) and differences are reported as significant when $P < 0.05$. Data were analyzed using the General Linear Model of the Statistical Package for Social Science (v 24.0, SPSS Inc., Chicago, Illinois, USA). When the calculated values of F were significant, the Bonferroni post-hoc test ($P < 0.05$) was used to interpret any significant differences among the mean values. When the interaction was significant, means were separated considering TREAT effect inside each level of PERIOD effect. The aerobic stability was pooled together across trials and were regressed on the yeast count and acetic acid as the independent variable.

6.4. Results

The characteristics of the two corns and sorghum herbages, prior to ensiling, are reported in Table 1. The DM content was 363, 340 and 254 g kg^{-1} DM in Trials I, II and III, respectively. The DM content, CP, starch, and NDF contents were typical of whole corn forage harvested at a 50% kernel milk line, with slightly lower NDF, ADF, CP and ash and a greater LAB count, yeast count and starch in Trial I than in Trial II. The sorghum herbage was high in fibre (NDF >600 g kg^{-1} DM), high in WSC (around 122 g kg^{-1} DM) and low in starch (around 70 g kg^{-1} DM) compared to corn herbages.

In all the trials the treated and untreated silages were well fermented, with butyric acid always being under the detection limit (0.01 g kg^{-1} DM). The DM content, pH and fermentative products, after 15, 30, 100 and 250 d of conservation, are reported in Tables 2, 3 and 4 for Trial I, II and III, respectively.

In Trial I, no differences were observed for DM and ethanol for the different conservation periods. The inocula influenced the ethanol content that was higher in LB and LB+LH silages compared to other treatments. The lactic and acetic acids and the lactic-to-acetic acid ratio were affected by the interaction of treatment and conservation periods. At 100 and 250 d of conservation LB and LB+LH treatments showed lower lactic-to-acetic acid ratio than C and LH silages. This fermentative profile resulted in a higher pH value after 100 and 250 d for the former two treatments. At 250 d of conservation the presence of LB determined lower lactic acid and higher acetic acid than the C and LH silages. The 1,2-propanediol was found in the LB and LB+LH treatments after 100 d and after 250 d it reached the highest value in LB+LH silages.

The pH and the fermentative products in Trial II were affected by the interaction of treatments and conservation periods. The lactic-to-acetic acid ratio was lowest in LB+LH silages for all the openings. After at least 100 d of conservation LB+LH silages determined lower lactic acid and higher acetic acid than the other treatments. The 1,2-propanediol was found in all the treated silages after 100 d of conservation, with the highest value in LB+LH treatment. After 250 d of conservation, ethanol was higher in treated silages than in C.

The sorghum silages in Trial III showed different fermentative profiles from corn with a high concentration of ethanol (>60 g kg^{-1} DM), regardless of the conservation periods. The ethanol content was higher in the presence of LH than in C and LB silages. No differences were found in the lactic acid content between treatments and conservation periods. Acetic acid was higher in LB and LB+LH silages and it increased with conservation period. After 100 and 250 d of conservation the inocula containing LB determined higher content of 1,2-propanediol.

The LAB, yeast and mould counts, aerobic stability and DM recovery are reported in Tables 5, 6 and 7 for Trial I, II and III, respectively. In Trial I, the lowest DM recovery were found at 250 d of conservation in the LB+LH silages (96.5% of the total DM). The yeast count decreased with the conservation and was below the detection limit at 250 d, while the mould count was under the detection limit after 30 d of conservation, regardless of the treatment. The aerobic stability was greater in the treatments containing *L. buchneri* after longer conservation periods (greater than 200 and 400 h at 100 and 250 d, respectively). Until 100 d of conservation the LAB count was higher than 10^7 cfu g^{-1} of silages for all treatments while at 100 and 250 d it was lower in C and LH silages than LB and LB+LH silages.

The DM recovery in Trial II was similar to that of Trial I, with the lowest value in the LB+LH treatment. The yeast count was higher in C than in the inoculated silages after at least 30 d of conservation. At 15 and 30 d the highest aerobic stability was found in the LH and LB+LH treated silages (around + 20 h), while at 100 and 250 d of conservation it was higher in the LB+LH treatment. At each opening, the LAB count was lower in C than in treated silages.

Dry matter recovery lower than 92% were observed in the sorghum silage at 250 d of conservation. Yeast decreased during conservation and they were under the detection limit after 250 d of conservation. Aerobic stability increased with the conservation and it was higher in LB treated silages than the control. Overall, the aerobic stabilities in LH and LB+LH silages were similar to that observed in LB silages, but did not differ from that of C silages.

Figure 1 shows the scatterplot between the acetic acid content and aerobic stability, as affected by treatments, when all the data from the three trials were pooled together. Aerobic stability increased as the acetic acid increased, until 30 g kg^{-1} DM of acetic acid. Between 30 and 35 g kg^{-1} DM of acetic acid, the aerobic stability ranged between 100 and 600 h, whereas over 35 g kg^{-1} DM of acetic acid aerobic stability decreased. During aerobic stability test (data not shown), in all deteriorating silages (i.e. when silage temperature was above ambient temperature) yeast count increases above 10^7 cfu g^{-1} of silage, followed by an increase in mould count, with no differences among treatments. Figure 2 shows a scatterplot between the yeast count and aerobic stability, as affected by conservation periods, when all the data from the three trials were pooled together. The higher the yeast count at silo opening, the lower the aerobic stability.

6.5. Discussion

One sorghum silage and two corn silages, which are representative of common silages used in Italy,[6] were evaluated in this study. These silages are often affected by aerobic deterioration, which is a significant problem for farm profitability and feed quality.[7] In the present study, no evident difference in aerobic stability was found at 15 and 30 d of conservation between the LB silages and C silages, whereas at 100 d and 250 d of conservation, the LB inoculum, when used alone, improved aerobic stability in Trials I and III, with aerobic stability being higher than 500 hours in Trial I, after 250 d of conservation. Furthermore, the values of aerobic stabilities observed in Trial III were all very close among treatments, with an increase on average of 30 h, 20 h, and 19 h, for LB, LH and LB+LH treatments compared to the control. Our results are in agreement with those of several authors who found an increase in aerobic stability in different silages inoculated with *L. buchneri* conserved for 49,[32] 60,[33] 120 d,[34] and 135 d.[35] The different magnitude in the effect on aerobic stability of *L. buchneri* in the trials of our experiments is in agreement with the review by Wilkinson and Davies,[8] who found variable improvements (between 133 and 666 hours) in aerobic stability in silages treated with *L. buchneri*.

One of the aims of the present work was to verify the efficacy of *L. hilgardii* strains as inoculum for corn and sorghum silage on aerobic stability, when used alone or in combination with LB. In the present study, the addition of *L. hilgardii* alone increased the aerobic stability in one (Trial II) out of two corn silage trials, with higher aerobic stabilities observed after 15 and 30 d of conservation, whereas the combination of LH and LB resulted in higher aerobic stability for all the openings. In the other corn silage trial (Trial I) the *L. hilgardii* was probably unable to dominate the fermentation and this could explain the lack of a response in acetic acid production and thus improved aerobic stability. *L. hilgardii* has recently been proposed by Carvalho et al.,[22] and Carvalho et al.,[36] as an inoculum on sugar cane silage to increase aerobic stability. Assis et al.,[23] tested *L. hilgardii* inoculum on corn silage at 304 g kg^{-1} of DM. In contrast with our results, these authors found similar hours of aerobic stability in corn silages inoculated with *L. hilgardii* and *L. buchneri* at 30 d of conservation, while they found a small increase in aerobic stability at 90 d of conservation when using *L. hilgardii* alone. Recently, Reis et al.,[37] analysed the effect of *L. hilgardii* after three conservation duration. They found an increase of aerobic stability using the inoculum despite they did the aerobic stability test only after 103 d of conservation. Combinations of two or more species of lactic bacteria inocula have been developed over time to obtain the best benefits from each species but most of these combinations were homofermentative and facultative heterofermentative LAB.[35,38] To the best of our knowledge, this is the first time an inoculum, composed of two obligate heterofermentative LAB, has been used in corn and sorghum silages. We did this combination in order to

verify if the ascribed ability of *L. hilgardii* to increase aerobic stability after short conservation period could be beneficial for meeting the farmer' s needs for early opening of the silages. Another aim was to verify if *L. hilgardii* could exert a complementary action and further increase aerobic stability of silages when used in combination with LB, which is known to be effective, in most cases, after a conservation period longer than 90 d. In one of the trail (Trial II), when inoculated together LH and LB showed an aerobic stability that was greater than those observed for the control silages and for the silages treated with LH and LB alone, at all conservation period. This result could be explained by a synergistic role of LH and LB with LH able of dominating fermentation before LB was active.

The fermentative results obtained in this study indicate the presence of 1,2-propanediol in silages treated with *L. hilgardii,* according to the results of Assis et al.[23] and Reis et al..[37] These results confirm that *L. hilgardii* presumptively had fermentation pathway during conservation similar to that of *L. buchneri,* indicating a possible affinity to *L. buchneri* group, as previously reported by Kandler and Weiss,[39] Henil et al.,[40] and Pot et al..[41] In Trial I after 100 d of conservation and in Trial II after 100 and 250 d of conservation, the LB+LH silages had a higher amount of 1,2-propanediol than the *L. hilgardii* and *L. buchneri* treatments on their own; this presumptively represents an indication of a synergistic action of the two LAB.

Many studies have confirmed the relationship between acetic acid and aerobic stability, and have reported a linear correlation between both parameters.[17,20,24] In the present study, greater amounts of acetic acid were found in the LB and LB+LH treatments than in the other treatments, but aerobic stability has been found to decrease in samples with up to 35 g kg^{-1} DM of acetic acid (Figure 1). This could be explained by the presence of different microorganisms who started the aerobic deterioration. Furthermore, since most of those samples are from Trial II, this could also explain the lower hours of aerobic stability in the corn of Trial II than that of Trial I. Our main hypothesis is that acetic acid bacteria belonging to the *Acetobacter* genus may have started deteriorating these samples, as previously hypothesized by Kleinschmit and Kung[19] who, analyzed the effect of mixtures of heterofermentative and homofermentative LAB in silages and found inconsistent improvements in aerobic stability. This is in accordance with the results of Spoelstra et al.,[3] who found less than 100 hours of aerobic stability in corn inoculated with AAB. The growth of acetic acid bacteria during aerobic deterioration was observed by Dolci et al.,[4] in corn silage treated with a mixture of *L. buchneri*, *L. plantarum*, and *Enterococcus faecium*.

The length of conservation represents a critical point for the quality and stability of silage. A long conservation period in complete anaerobiosis determines a decrease in the yeast and mould counts and improves the aerobic stability of silages,[2] whatever the use of silage inocula or additives.[20] In the present study, the aerobic stability increased as the conservation period

increased, regardless of the plant material and treatments. At 250 d of conservation, the uninoculated silages reached more than 100 h of aerobic stability in Trial II, and just under 200 hours in Trials I and III. If the anaerobic environment is kept during conservation, some epiphytic heterofermentative LAB, mainly *L. buchneri*, transform part of the lactic acid into acetic acid.[1] Arriola et al.,[38] found no differences in aerobic stability in corn silages, whether untreated or treated with *L. buchneri*, opened after 575 d of conservation. In all the trials of the present experiment, regardless of the treatments, an increase in the conservation period determined a decrease in the yeast count and an increase in the aerobic stability (Figure 2), mainly due to a larger amount of acetic acid. However, there were some exceptions, as some samples showed low yeast levels and low aerobic stability (Figure 2). We speculated that this could be associated with the effect of AAB on aerobic deterioration. Weinberg and Chen,[42] analysed the fermentation profile and microbial count of corn and wheat silages opened at different conservation period (from 1 week to 12 months). They found an increase in acetic acid for each crop during conservation, that was from 8 to 47 g kg^{-1} DM for corn and from 3 to 20 g kg^{-1} DM for wheat. This resulted in a decrease in the yeast count and an improvement in aerobic stability.

In this study, data have shown that a longer period of conservation than 100 d in complete anaerobiosis reduced yeast count, regardless of LAB inoculum used, and it is responsible of improved aerobic stability of corn and sorghum silages. The addition of *L. buchneri* confirmed to be a good option to increase aerobic stability of silages. The addition of *L. hilgardii* alone or in combination with *L. buchneri* showed the ability of increasing the aerobic stability of the silage after short and long conservation period, and showed the opportunity of having a complementary and synergistic effect with *L. buchneri*, but results were not consistent between trials. Further in-depth knowledge is then required to better understand which are the conditions that favour LH to prevail during fermentation in order to increase the magnitude of success when this LAB strain is used alone or in combination with LB.

6.6. Acknowledgments

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information, and does not imply either recommendation or endorsement by the University of Turin, Italy.

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6.7. References

1. Pahlow G, Muck RE, Driehuis F, Oude Elferink SJWH, and Spoelstra SF, Microbiology of ensiling. In Buxton DR, Muck RE, and Harrison JH (Eds.), Silage science and technology (pp. 31–93). Madison, WI: American Society of Agronomy (2003).
2. McDonald P, Henderson AR, and Heron SJE, The Biochemistry of Silage (2nd ed.) Bucks UK: Chalcombe Publications (1991).
3. Spoelstra SF, Courtin MG, and Van Beers JAC, Acetic acid bacteria can initiate aerobic deterioration of whole crop maize silage. J Agric Sci 111: 127-132 (1988).
4. Dolci P, Tabacco E, Cocolin L, and Borreani G, Microbial dynamics during aerobic exposure of corn silage stored under oxygen barrier or polyethylene films. Appl Environ Microbiol 77: 7499–7507 (2011).
5. Driehuis F, and Oude Elferink SJWH, The impact of the quality of silage on animal health and food safety: A review. Vet Quart 22: 212-216 (2000).
6. Borreani G, and Tabacco E, The relationship of silage temperature with the microbiological status of the face of corn silage bunkers. J Dairy Sci 93: 2620-2629 (2010).
7. Tabacco E, Piano S, Revello-Chion A, and Borreani G, Effect of Lactobacillus buchneri LN4637 and Lactobacillus buchneri LN40177 on the aerobic stability, fermentation products, and microbial populations of corn silage under farm conditions. J Dairy Sci 94: 5589-5598 (2011).
8. Wilkinson JM, and Davies DR, The aerobic stability of silage: key findings and recent developments. Grass Forage Sci 68: 1-19 (2013).
9. Borreani G, Tabacco E, Schmidt RJ, Holmes BJ, and Muck RE, Factors affecting dry matter and quality losses in silages. J Dairy Sci 101: 3952–

3979.
10. Muck RE, A lactic acid bacterial strain to improve aerobic stability of silages. In Research Summaries (pp. 42-43). U.S. Dairy Forage Research Center (1996).
11. Driehuis F, Oude Elferink SJWH, and Spoelstra SF, Anaerobic lactic acid degradation during ensilage of whole crop maize inoculated with Lactobacillus buchneri inhibits yeast growth and improves aerobic stability. J Appl Microbiol 87: 583-594 (1999).
12. Kung Jr L, and Stokes MR, Analyzing silages for fermentation end products. Retrieved from http://ag.udel.edu/departments/anfs/faculty/kung/articles/analyzing_silages_for_fermentati.htm. (2001).
13. Oude Elferink SJWH, Krooneman J, Gottschal JC, Spoelstra SF, Faber F, and Driehuis F,. Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by Lactobacillus buchneri. Appl Environ Microbiol 67: 125-132 (2001).
14. Schmidt RJ, and Kung Jr L, The effects of Lactobacillus buchneri with or without a homolactic bacterium on the fermentation and aerobic stability of corn silages made at different locations. J Dairy Sci 93: 1616-1624 (2010).
15. Tabacco E, Righi F, Quarantelli A, and Borreani G, Dry matter and nutritional losses during aerobic deterioration of corn and sorghum silages as influenced by different lactic acid bacteria inocula. J Dairy Sci 94: 1409-1419 (2011).
16. Mari LJ, Schmidt RJ, Nussio LG, Hallada CM, and Kung Jr L, An evaluation of the effectiveness of Lactobacillus buchneri 40788 to alter fermentation and improve the aerobic stability of corn silage in farm silos. J Dairy Sci 92: 1174-1176 (2009).
17. Kleinschmit DH, and Kung Jr L, A meta-analysis of the effects of Lactobacillus buchneri on the fermentation and aerobic stability of corn and grass and small-grain silages. J Dairy Sci 89: 4005–4013 (2006a).
18. Adesogan AT, Recent advances in bacterial silage inoculant technology. Retrieved from http://dairy.ifas.ufl.edu/rns/2008/adesogan.pdf. (2008).
19. Kleinschmit DH, and Kung Jr L, The effects of Lactobacillus buchneri 40788 and Pediococcus pentosaceus R1094 on the fermentation of corn silage. J Dairy Sci 89: 3999-4004 (2006b).
20. Comino L, Tabacco E, Righi F, Revello-Chion A, Quarantelli A, and Borreani G, 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. Anim Feed Sci Technol 198: 94-106 (2014).
21. Ávila CLS, Carvalho BF, Pinto JC, Duarte WF, and Schwan RF, The use of Lactobacillus species as starter cultures for enhancing the quality of sugar cane silage. J Dairy Sci 97: 940-951 (2014).
22. Carvalho BF, Ávila CLS, Miguel MGCP, Pinto JC, Santos MC, and Schwan RF, Aerobic stability of sugar-cane silage inoculated with tropical strains of lactic acid bacteria. Grass Forage Sci 70: 308-323 (2015).
23. Assis FGD, Ávila CLD, Pinto JC, and Schwan RF, New inoculants on maize silage fermentation. Rev Brasileira Zootec 43: 395-403 (2014).
24. Ranjit NK, and Kung Jr L, The effect of Lactobacillus buchneri, Lactobacillus plantarum, or a chemical preservative on the fermentation and aerobic

stability of corn silage. J Dairy Sci 83: 526-535 (2000).
25. Porter MG, and Murray RS, The volatility of components of grass silage on oven drying and the inter-relationship between dry-matter content estimated by different analytical methods. Grass Forage Sci 56: 405–411 (2001).
26. Association of Official Analytical Chemist (AOAC). Official Methods of Analysis. 18th ed. AOAC International, Gaithersburg, MD. (2005).
27. DuBois M, Gilles KA, Hamilton JK, Rebers PA, and Smith F, Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350-356 (1956).
28. Van Soest PJ, Robertson JB, and Lewis BA, Methods of dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 74: 3583-3597 (1991).
29. Robertson JB, and Van Soest PJ, The detergent system of analysis and its application to human foods. In James WPT, and Theander O (Eds). The Analysis of Dietary Fiber in Food. (pp. 123–158). New York, Marcel Dekker (1981).
30. Playne MJ, and McDonald P, The buffering constituents of herbage and of silage. J Sci Food Agric 17: 264–268 (1966).
31. Canale A, Valente ME, and Ciotti A, Determination of volatile carboxylic acids (C1-C5) and lactic acid in aqueous acid extracts of silage by high performance liquid chromatography. J Sci Food Agric 35: 1178-1182 (1984).
32. Herrmann C, Idler C, and Heiermann M, Improving aerobic stability and biogas production of maize silage using silage additives. Bioresour Technol 197: 393-403 (2015).
33. Weinberg ZG, Szakacs G, Ashbell G, and Hen Y, The effect of Lactobacillus buchneri and L. plantarum, applied at ensiling, on the ensiling fermentation and aerobic stability of wheat and sorghum silages. J Ind Microbiol Biotechnol 23: 218-222 (1999).
34. Huisden CM, Adesogan AT, Kim SC, and Ososanya T, Effect of applying molasses or inoculants containing homofermentative or heterofermentative bacteria at two rates on the fermentation and aerobic stability of corn silage. J Dairy Sci 92. 690-697 (2009).
35. Queiroz OCM, Arriola KG, Daniel JLP, and Adesogan AT, Effects of 8 chemical and bacterial additives on the quality of corn silage. J Dairy Sci 96: 5836-5843 (2013).
36. Carvalho BF, Ávila CLS, Pereira MN, and Schwan RF, Methylotrophic yeast, lactic acid bacteria and glycerine as additives for sugarcane silage. Grass Forage Sci, 72: 355-368 (2017).
37. Reis CB, Santos AO, Carvalho BF, Schwan RF, and Ávila CLS, Wild Lactobacillus hilgardii (CCMA 0170) strain modifies the fermentation profile and aerobic stability of corn silage. J Appl Anim Res 46: 632-638 (2018).
38. Arriola KG, Kim SC, and Adesogan AT, Effect of applying inoculants with heterolactic or homolactic and heterolactic bacteria on the fermentation and quality of corn silage. J Dairy Sci 9: 1511-1516 (2011).
39. Kandler O, and Weiss N, Genus Lactobacillus Beijerinck 1901, 212AL. In Sneath PHA, Mair NS, Sharpe ME, and Holt JG (Eds.) Bergey's manual of systematic bacteriology. Vol. 2 (pp. 1209-1234). Baltimore, MD: Williams and Wilkins (1986).

40. Heinl S, Wibberg D, Eikmeyer F, Szczepanowski R, Blom J, Linke B, Goesmann A, Grabherr R, Schwab H, Pühler A, and Schulter A, Insights into the completely annotated genome of Lactobacillus buchneri CD034, a strain isolated from stable grass silage. J Biotechnol 161: 153–166 (2012).
41. Pot B, Felis GE, De Bruyne K, Tsakalidou E, Papadimitriou K., Leisner J, and Vandamme P, The genus Lactobacillus. In Holzapfel WH and Wood BJB, Lactic acid bacteria: Biodiversity and Taxonomy. (pp. 249-354). Oxford, UK: John Wiley and Sons (2014).
42. Weinberg ZG, and Chen Y, Effects of storage period on the composition of whole crop wheat and corn silages. Anim Feed Sci Technol 185: 196-200 (2013).

6.8. Tables

Table 1. The chemical and microbial characteristics of the two corn (Trial I and II) and sorghum (Trial III) herbages prior to ensiling

Parameters†	Trial I	Trial II	Trial III
DM (g kg^{-1})	363.0 ± 14.03	339.9 ± 19.4	254.4 ± 13.3
pH	5.77 ± 0.80	5.80 ± 0.01	5.60 ± 0.06
Water activity (a_w)	0.981 ± 0.001	0.996 ± 0.002	0.991 ± 0.002
Nitrate (mg kg^{-1} DM)	631.7 ± 77.9	<100	<100
NDF (g kg^{-1} DM)	394.4 ± 26.6	425.4 ± 19.4	646.8 ± 25.9
ADF (g kg^{-1} DM)	188.0 ± 16.2	220.9 ± 14.3	367.9 ± 16.1
CP (g kg^{-1} DM)	67.8 ± 3.46	78.4 ± 2.01	66.5 ± 5.41
Ash (g kg^{-1} DM)	41.6 ± 5.69	55.4 ± 1.76	68.7 ± 4.12
Starch (g kg^{-1} DM)	353.5 ± 21.07	323.5 ± 23.5	69.4 ± 12.1
Buffering capacity (mEq kg^{-1} DM)	67.5 ± 23.0	56.0 ± 9.3	82.4 ± 7.4
WSC (g kg^{-1} DM)	89.8 ± 11.2	73.7 ± 3.24	121.6 ± 17.3
LAB (log_{10} cfu g^{-1})	8.18 ± 0.59	7.71 ± 0.56	7.51 ± 0.71
Yeasts (log_{10} cfu g^{-1})	6.71 ± 0.20	6.46 ± 0.29	6.31 ± 0.44
Moulds (log_{10} cfu g^{-1})	5.19 ± 0.30	5.26 ± 0.20	5.79 ± 0.17

† Values represent the average of replications and the standard deviation. ADF = acid detergent fiber; cfu = colony forming unit; CP = crude protein; DM = dry matter; LAB = lactic acid bacteria; NDF = neutral detergent fiber; WSC = water soluble carbohydrates.

Table 2. DM, pH and fermentative parameters of corn silage of Trial I at different conservation periods

Time	Treat[†]	DM[‡]	pH	Lactic acid	Acetic acid	Lactic-to-acetic ratio	Propionic acid	Ethanol	1.2-propanediol
(d)		(g kg^{-1})		(g kg^{-1} DM)	(g kg^{-1} DM)		(g kg^{-1} DM)	(g kg^{-1} DM)	(g kg^{-1} DM)
15	C	354.7	3.86^{ab}	43.8	9.6	4.6	0.0	11.9	0.0
	LB	367.3	3.90^{a}	39.4	9.5	4.2	0.0	16.3	0.0
	LH	355.1	3.85^{b}	41.3	9.2	4.5	0.0	12.7	0.0
	LB+LH	362.5	3.85^{b}	41.4	9.1	4.5	0.0	14.4	0.0
30	C	361.2	3.87	44.9	11.2	4.0	0.0	11.8	0.0
	LB	366.1	3.91	46.2	11.2	4.1	0.0	16.3	0.0
	LH	357.3	3.87	44.2	11.0	4.0	0.0	13.4	0.0
	LB+LH	368.6	3.89	47.1	10.9	4.4	0.0	14.4	0.0
100	C	356.1	3.77^{b}	51.8^{a}	13.1	4.0^{a}	0.0	10.5	0.0^{c}
	LB	361.0	3.90^{a}	37.5^{b}	26.8	2.0^{b}	0.0	15.6	3.4^{b}
	LH	354.0	3.81^{b}	50.2^{ab}	14.1	3.6^{a}	0.0	12.3	0.0^{c}
	LB+LH	359.8	3.92^{a}	42.1^{ab}	18.6	2.3^{b}	0.0	13.6	4.3^{a}
250	C	354.8	3.72^{c}	62.7^{a}	16.1^{b}	4.0^{a}	0.0	10.5	1.0^{b}
	LB	358.4	4.07^{a}	37.2^{b}	29.7^{a}	1.3^{b}	2.5	15.1	8.5^{a}
	LH	346.7	3.78^{b}	62.3^{a}	18.5^{b}	3.4^{a}	0.0	11.8	1.9^{b}
	LB+LH	357.8	4.04^{a}	41.3^{b}	30.5^{a}	1.4^{b}	2.7	13.2	9.7^{a}
General means of treatments									
C		35.7	3.81	50.8	12.5	4.1	0.0	11.2^{c}	0.2
LB		36.3	3.95	40.1	19.3	2.9	0.6	15.8^{a}	3.0
LH		35.3	3.83	49.5	13.2	3.9	0.0	12.5^{bc}	0.5
LB+LH		36.2	3.93	43.0	17.3	3.1	0.7	13.9^{ab}	3.5
General means of conservation periods									
15		36.0	3.87	41.4	9.4	4.4	0.0	13.8	0.0
30		36.3	3.89	45.6	11.1	4.1	0.0	14.0	0.0
100		35.8	3.85	45.4	18.1	3.0	0.0	13.0	1.9
250		35.4	3.90	50.9	23.7	2.5	1.3	12.7	5.3
Standard error of the mean									
TREAT		3.52	0.006	1.279	1.233	0.120	0.211	0.664	0.146
PERIOD		3.52	0.006	1.279	1.233	0.120	0.211	0.664	0.146
T x P		7.04	0.012	2.558	2.466	0.240	0.422	1.328	0.292
Level of significance									
TREAT		NS	***	***	**	***	*	***	***
PERIOD		NS	***	***	***	***	***	NS	***
T x P		NS	***	***	**	***	**	NS	***

[†] C = control; LB = *Lactobacillus buchneri*; LH = *L. hilgardii*; [‡]DM = dry matter.

*** P <0.001; ** P <0.01; * P <0.05.

Table 3. DM, pH and fermentative parameters of corn silage of Trial II at different conservation periods

Time	Treat†	DM‡	pH	Lactic acid	Acetic acid	Lactic-to-acetic ratio	Propionic acid	Ethanol	1.2-propanediol
(d)		(g kg^{-1})		(g kg^{-1} DM)	(g kg^{-1} DM)		(g kg^{-1} DM)	(g kg^{-1} DM)	(g kg^{-1} DM)
15	C	327.7	3.87	45.7^{a}	10.7	4.3^{a}	0.0	10.3	0.0^{c}
	LB	340.1	3.88	45.6^{a}	10.9	4.2^{ab}	0.0	10.0	0.0^{c}
	LH	347.0	3.88	41.1^{b}	10.7	3.9^{bc}	0.0	9.1	0.1^{b}
	LB+LH	346.3	3.88	41.5^{ab}	11.1	3.8^{b}	0.0	8.9	0.8^{a}
30	C	348.2	3.77	43.5	11.7	3.7^{a}	0.0	9.6	0.0^{c}
	LB	328.8	3.79	46.5	13.0	3.6^{ab}	0.0	10.8	0.3^{c}
	LH	337.0	3.79	45.6	14.0	3.3^{ab}	0.0	10.8	1.1^{b}
	LB+LH	353.7	3.79	41.5	13.8	3.0^{b}	0.0	11.0	1.5^{a}
100	C	334.2	3.63^{b}	58.2^{a}	15.0^{c}	3.9^{a}	1.5	10.6	0.0^{c}
	LB	348.0	3.69^{b}	50.9^{a}	18.0^{b}	2.9^{b}	1.2	9.7	4.4^{b}
	LH	340.5	3.68^{b}	52.4^{a}	18.4^{b}	2.9^{b}	0.1	9.6	4.2^{b}
	LB+LH	328.4	3.82^{a}	39.6^{b}	25.7^{a}	1.6^{c}	0.3	10.9	10.4^{a}
250	C	363.6	3.57^{d}	60.7^{a}	13.4^{c}	4.5^{a}	0.0	8.9^{b}	0.0^{d}
	LB	328.1	3.78^{b}	47.9^{b}	29.5^{ab}	1.6^{b}	0.0	13.5^{a}	9.9^{b}
	LH	341.3	3.69^{c}	52.5^{ab}	26.0^{b}	2.0^{b}	0.0	11.3^{ab}	7.1^{c}
	LB+LH	344.4	3.88^{a}	35.4^{c}	33.4^{a}	1.1^{c}	1.3	14.1^{a}	12.9^{a}
General means of treatments									
C		34.2	3.72	51.6	12.7	4.1	0.4	9.9	0.0
LB		33.7	3.78	47.7	17.2	3.2	0.3	10.9	3.3
LH		34.1	3.76	47.6	16.8	3.1	0.0	10.1	2.9
LB+LH		34.3	3.84	39.7	20.3	2.4	0.3	11.0	6.1
General means of conservation period									
15		34.0	3.88	43.5	10.8	4.0	0.0	9.6	0.2
30		34.2	3.78	44.3	13.1	3.4	0.0	10.5	0.7
100		33.8	3.70	50.3	19.3	2.8	0.8	10.2	4.7
250		34.4	3.73	49.1	25.6	2.3	0.3	11.9	7.5
Standard error of the mean									
TREAT		4.13	0.007	1.046	0.385	0.081	0.132	0.322	0.266
PERIOD		4.13	0.007	1.046	0.385	0.081	0.132	0.322	0.266
T x P		8.26	0.014	2.092	0.770	0.162	0.264	0.644	0.532
Level of significance									
TREAT		NS	***	***	***	***	NS	*	***
PERIOD		NS	***	***	***	***	***	***	***
T x P		NS	***	***	***	***	**	**	***

† C = control; LB = *Lactobacillus buchneri*; LH = *L. hilgardii*; ‡DM = dry matter.

*** P <0.001; ** P <0.01; * P <0.05.

Table 4. DM, pH and fermentative parameters of sorghum silage of Trial III at different conservation periods

Time	Treat[†]	DM[‡]	pH	Lactic acid	Acetic acid	Lactic-to-acetic ratio	Propionic acid	Ethanol	1.2-propanediol
(d)		(g kg^{-1})		(g kg^{-1} DM)	(g kg^{-1} DM)		(g kg^{-1} DM)	(g kg^{-1} DM)	(g kg^{-1} DM)
15	C	231.7	3.84	63.8	14.1	4.5	0.0	62.0	0.0
	LB	228.9	3.85	68.6	14.5	4.7	0.0	60.4	0.0
	LH	231.1	3.83	69.0	14.1	4.9	0.0	68.1	0.0
	LB+LH	232.3	3.84	69.2	14.4	4.8	0.0	66.2	0.0
30	C	229.8	3.85	69.3	14.7	4.7	0.0	61.3	0.0
	LB	226.9	3.85	69.5	14.8	4.7	0.0	59.1	0.0
	LH	229.2	3.84	69.3	13.9	5.0	0.0	63.8	0.0
	LB+LH	230.6	3.84	68.3	14.3	4.8	0.0	63.3	0.0
100	C	232.8	3.85^{c}	69.0	20.2	3.5	0.0	59.8	0.0^{c}
	LB	232.0	3.89^{a}	70.5	23.2	3.1	2.9	61.2	1.2^{a}
	LH	234.2	3.87^{b}	71.5	19.4	3.7	0.0	69.2	0.0^{c}
	LB+LH	233.1	3.85^{c}	68.7	24.1	3.0	0.0	65.8	0.6^{b}
250	C	245.2^{a}	3.86^{bc}	70.1	28.4	2.5	0.0	63.2	0.7^{b}
	LB	223.5^{b}	4.02^{a}	58.5	38.8	1.6	4.8	58.4	5.3^{a}
	LH	232.7^{b}	3.84^{c}	81.5	28.9	2.9	0.0	66.1	1.3^{b}
	LB+LH	225.9^{b}	3.97^{ab}	68.2	36.4	2.1	2.6	63.1	4.7^{a}
General means of treatments									
C		23.5	3.85	68.0	19.4^{bc}	3.8^{ab}	0.0^{b}	61.6^{b}	0.2
LB		22.8	3.90	66.8	22.8^{a}	3.5^{b}	1.9^{a}	59.8^{b}	1.6
LH		23.2	3.84	72.8	19.1^{c}	4.1^{a}	0.0^{b}	66.8^{a}	0.3
LB+LH		23.0	3.87	68.6	22.3^{ab}	3.7^{ab}	0.6^{ab}	64.6^{ab}	1.3
General means of conservation period									
15		23.1	3.84	67.7	14.3^{c}	4.7^{a}	0.0^{b}	64.2	0.0
30		22.9	3.84	69.1	14.5^{c}	4.8^{a}	0.0^{b}	61.9	0.0
100		23.3	3.86	69.9	21.7^{b}	3.3^{b}	0.7^{ab}	64.0	0.5
250		23.2	3.92	69.6	33.1^{a}	2.3^{c}	1.8^{a}	62.7	3.0
Standard error of the mean									
TREAT		1.15	0.009	1.818	0.923	1.27	0.497	1.395	0.123
PERIOD		1.15	0.009	1.818	0.923	1.27	0.497	1.395	0.123
T x P		2.293	0.018	3.636	1.846	0.254	0.994	2.790	0.246
Level of significance									
TREAT		**	***	NS	**	**	*	**	***
PERIOD		NS	***	NS	***	***	*	NS	***
T x P		**	***	NS	NS	NS	NS	NS	***

[†] C = control; LB = *Lactobacillus buchneri*; LH = *L. hilgardii*; [‡]DM = dry matter.

*** P <0.001; ** P <0.01; * P <0.05.

Table 5. Microbial count, aerobic stability and weight losses of corn silage of Trial I at different conservation periods

Time (d)	Treat[†]	LAB[‡] (log_{10} cfu g^{-1})	Yeasts (log_{10} cfu g^{-1})	Moulds (log_{10} cfu g^{-1})	Aerobic stability (h)	DM recovery (%)
15	C	8.13	4.26	<1.00	69	98.5
	LB	8.21	4.45	<1.00	64	98.1
	LH	8.00	3.96	1.57	66	98.3
	LB+LH	8.22	4.16	<1.00	62	98.2
30	C	9.30^{a}	3.50	<1.00	83	98.4^{b}
	LB	9.25^{a}	3.14	<1.00	89	97.9
	LH	8.72^{b}	3.22	<1.00	97	98.2^{ab}
	LB+LH	9.44^{a}	3.35	<1.00	104	98.1^{ab}
100	C	7.43^{b}	3.71^{a}	<1.00	76^{b}	98.0^{b}
	LB	9.06^{a}	2.22^{b}	<1.00	204^{a}	97.2^{a}
	LH	7.72^{b}	3.78^{a}	<1.00	77^{b}	97.6^{ab}
	LB+LH	9.66^{a}	2.56^{b}	<1.00	254^{a}	97.3^{a}
250	C	6.54^{b}	<1.00	<1.00	184^{b}	97.6^{b}
	LB	8.23^{a}	<1.00	<1.00	539^{a}	96.3^{a}
	LH	6.90^{b}	1.02	<1.00	200^{b}	97.3^{b}
	LB+LH	8.12^{a}	<1.00	<1.00	444^{a}	96.5^{a}
General means of treatments						
C		7.85	3.09	<1.00	103	98.1
LB		8.69	2.58	<1.00	224	97.4
LH		7.83	3.00	<1.00	90	97.9
LB+LH		8.86	2.67	<1.00	216	97.5
General means of conservation period						
15		8.14	4.21	<1.00	65	98.3
30		9.18	3.30	<1.00	93	98.2
100		8.47	3.07	<1.00	153	97.5
250		7.45	<1.00	<1.00	321	96.9
Standard error of the mean						
TREAT		0.067	0.109	-	14.8	0.043
PERIOD		0.067	0.109	-	14.8	0.043
T x P		0.134	0.218	-	29.6	0.086
Level of significance						
TREAT		***	**	-	***	***
PERIOD		***	***	-	***	***
T x P		***	**	-	***	*

[†] C = Control; cfu = colony forming unit; LB = *Lactobacillus buchneri*; LH = *L. hilgardii*; [‡]LAB = lactic acid bacteria.
*** P <0.001; ** P <0.01; * P <0.05.

Table 6. Microbial count, aerobic stability and weight losses of corn silage of Trial II at different conservation periods

Time (d)	Treat[†]	LAB[‡] (log_{10} cfu g^{-1})	Yeasts (log_{10} cfu g^{-1})	Moulds (log_{10} cfu g^{-1})	Aerobic stability (h)	DM recovery (%)
15	C	8.16^{c}	3.90	1.32	65^{b}	98.3
	LB	8.24^{bc}	3.87	1.37	62^{b}	98.3
	LH	8.28^{b}	3.57	1.29	81^{a}	98.3
	LB+LH	8.50^{a}	3.72	1.51	83^{a}	98.3
30	C	7.63^{c}	3.72^{a}	<1.00	72^{b}	98.1
	LB	7.85^{bc}	3.52^{a}	<1.00	70^{b}	98.1
	LH	8.05^{ab}	2.56^{b}	<1.00	96^{a}	98.1
	LB+LH	8.24^{a}	2.64^{b}	<1.00	96^{a}	98.0
100	C	6.33^{c}	3.17^{a}	1.19	97^{b}	97.8^{c}
	LB	8.09^{b}	1.85^{b}	<1.00	123^{ab}	97.6^{b}
	LH	7.85^{b}	1.16^{b}	<1.00	119^{ab}	97.4^{b}
	LB+LH	8.51^{a}	<1.00^{b}	1.04	147^{a}	97.1^{a}
250	C	6.18^{c}	2.17^{a}	<1.00	102^{b}	97.7^{c}
	LB	8.43^{a}	<1.00^{b}	<1.00	138^{b}	97.0^{b}
	LH	7.44^{b}	1.41^{ab}	<1.00	124^{b}	97.0^{b}
	LB+LH	8.58^{a}	<1.00^{b}	1.05	365^{a}	96.4^{a}
General means of treatments						
C		7.12	3.30	<1.00	83	98.0
LB		8.14	2.54	<1.00	96	97.5
LH		7.93	2.22	<1.00	104	97.7
LB+LH		8.45	1.98	1.13	163	97.5
General means of conservation period						
15		8.30	3.77	1.37^{a}	73	98.3
30		7.94	3.11	<1.00^{b}	84	98.1
100		7.70	1.73	<1.00^{b}	122	97.5
250		7.66	1.15	<1.00^{b}	182	97.0
Standard error of the mean						
TREAT		0.035	0.116	0.092	10.4	0.028
PERIOD		0.035	0.116	0.092	10.4	0.028
T x P		0.070	0.232	0.184	20.8	0.056
Level of significance						
TREAT		***	***	NS	***	***
PERIOD		***	***	***	***	***
T x P		***	***	NS	***	***

[†] C = control; cfu = colony forming unit; LB = *Lactobacillus buchneri*; LH = *L. hilgardii*; [‡]LAB = lactic acid bacteria.
*** P <0.001; ** P <0.01; * P <0.05.

Table 7. Microbial count, aerobic stability and weight losses of sorghum silage of Trial III at different conservation periods

Time (d)	Treat†	LAB‡ (log_{10} cfu g^{-1})	Yeasts (log_{10} cfu g^{-1})	Moulds (log_{10} cfu g^{-1})	Aerobic stability (h)	DM recovery (%)
15	C	8.70^{ab}	5.08	1.95^{a}	111	92.9
	LB	8.93^{a}	5.00	1.13^{b}	107	93.5
	LH	8.78^{ab}	4.47	<1.00^{b}	105	92.5
	LB+LH	8.63^{b}	4.49	1.89^{a}	101	92.8
30	C	8.49	2.57	1.11^{b}	148	92.6
	LB	8.79	2.59	1.78^{a}	143	93.4
	LH	8.74	2.70	1.39^{ab}	152	92.3
	LB+LH	8.73	2.73	1.75^{a}	154	92.7
100	C	6.56^{b}	2.64	<1.00	124	92.1
	LB	8.64^{a}	1.33	<1.00	176	92.8
	LH	6.51^{b}	1.89	<1.00	145	91.7
	LB+LH	8.03^{a}	1.75	<1.00	160	92.2
250	C	6.80	1.15	<1.00	192	91.0
	LB	7.36	<1.00	1.21	270	91.7
	LH	7.31	<1.00	<1.00	255	91.4
	LB+LH	7.52	<1.00	1.20	237	91.1
General means of treatments						
C		7.64	2.86^{a}	1.07	144^{b}	92.2^{a}
LB		8.43	2.36^{b}	1.21	174^{a}	92.9^{b}
LH		7.84	2.39^{b}	<1.00	164^{ab}	92.0^{a}
LB+LH		8.22	2.37^{b}	1.34	163^{ab}	92.3^{a}
General means of conservation period						
15		8.76	4.76^{a}	1.44	106^{c}	92.9^{c}
30		8.69	2.65^{b}	1.51	149^{b}	92.7^{c}
100		7.44	1.90^{c}	<1.00	151^{b}	92.2^{b}
250		7.25	<1.00^{d}	<1.00	239^{a}	91.3^{a}
Standard error of the mean						
TREAT		0.087	0.127	0.080	6.80	0.095
PERIOD		0.087	0.127	0.080	6.80	0.095
T x P		0.174	0.254	0.161	13.6	0.190
Level of significance						
TREAT		***	*	**	*	***
PERIOD		***	***	***	***	***
T x P		***	NS	***	NS	NS

† C = control; cfu = colony forming unit; LB = *Lactobacillus buchneri*; LH = *L. hilgardii*; ‡LAB = lactic acid bacteria
*** P <0.001; ** P <0.01; * P <0.05.

6.9. Figures

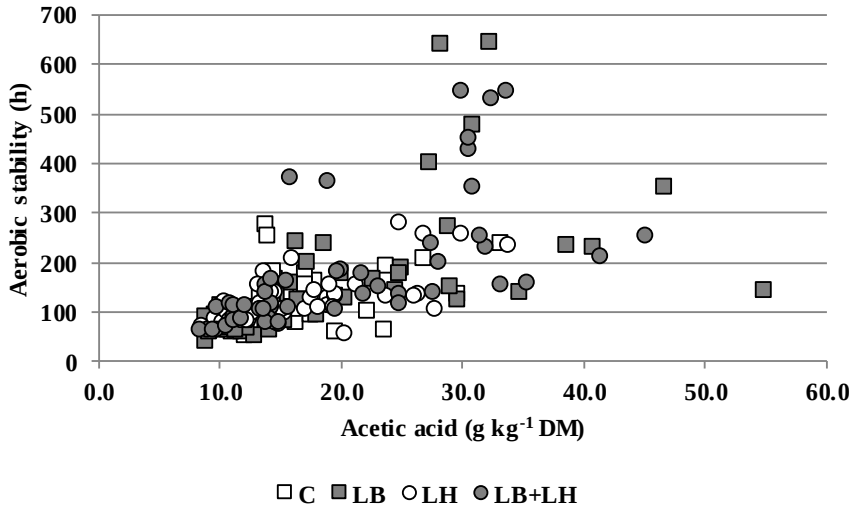

Figure 1. Scatterplot between acetic acid and aerobic stability of the three trials as affected by treatments (C = control; LB = *Lactobacillus buchneri*; LH = *Lactobacillus hilgardii*).

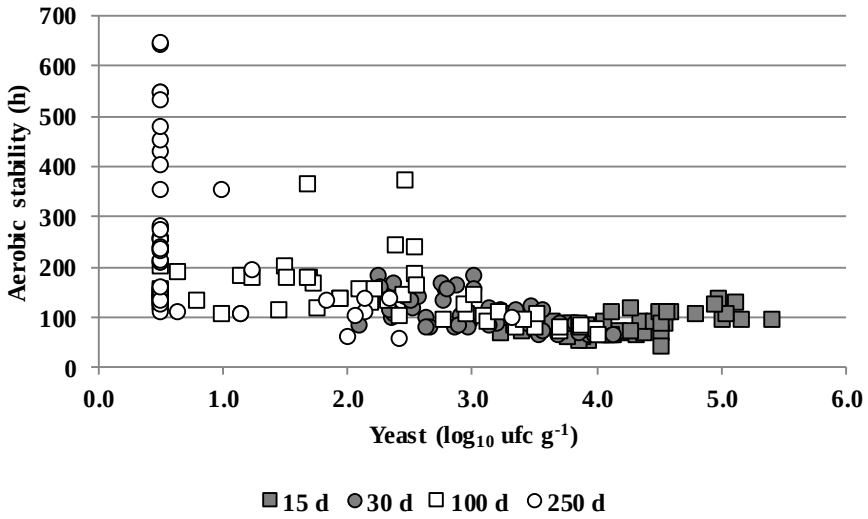

Figure 2. Scatterplot between yeast count and aerobic stability of the three trials as affected by conservation periods.

7. Paper II: Effects of a mixture of monopropionine and monobutyrin on the fermentation quality and aerobic stability of whole crop maize silage

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Effects of a mixture of monopropionine and monobutyrin on the fermentation quality and aerobic stability of whole crop maize silage

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7.1. Abstract

The ensiling of high DM content maize, with the aim of achieving a higher proportion of starch in the silage, leads to difficulties in containing aerobic spoilage, due to its low lactic acid bacteria (LAB) activity during fermentation. The aim of the present study has been to evaluate the effects of a new chemical additive, containing a mixture of monopropionine and monobutyrin, on the aerobic stability of whole crop maize silages in two trials in which the conservation period differed. Maize was harvested at a DM of around 410 g/kg and was then untreated (C) or treated with *L. buchneri* (LB) NCIMB 40788, a new additive, which was applied at a rate of 5 g/kg fresh matter (FM) (A0.5), at rate of 10 g/kg FM (A1.0) and at rate of 15 g/kg FM (A1.5). The obtained silages were conserved for 100 d and 240 d for Trials I and II, respectively. At opening, the DM losses, chemical composition, fermentation profile, microbial counts and aerobic stability were determined for each silo. In both trials, the addition of the chemical additive influenced the fermentation profile, and a lower level of acetic acid and ethanol was observed than for the C and LB silages. The propionic and butyric acids were only found in the silages treated with the chemical additive at concentrations that were linearly related to the calculated amount derived from the acid hydrolysis of the additive, thus indicating no clostridia activity had occurred. The application of the new additive reduced the yeast count, and aerobic stability was thus improved. The aerobic stability increased in an added dose dependent manner, with a mean increase of 120 h in A1.5, compared to the C silages. The DM losses were higher in the C silages than in the A1.5 ones. The chemical additive did not affect the nutritional parameters. The addition of LB was not very efficient, probably due to the low water activity of the high DM content maize and the low temperature at harvesting, which could have negatively influenced its activity. The addition of monopropionine and monobutyrin showed a positive effect, as it improved the aerobic stability, even in less than optimal conditions (high DM maize content and low temperature). The effect of the additive was related to its application rate, with the best results being observed when it was applied at 1.5% of fresh matter.

Key words: aerobic stability, maize silage, chemical additive, monopropionine, monobutyrin, fermentative profile.

7.2. Introduction

The preservation of the nutritive value and the microbiological safety of maize silages during the feed-out phase of ensiling, by reducing or avoiding aerobic deterioration, is a challenge for any livestock producer (Borreani et al., 2018). Several sub-optimal management factors, such as slow silo filling, inadequate packing densities, air penetration through the silo face or under a plastic cover, inappropriate unloading techniques (Kleinschmit et al., 2005; Borreani et al., 2018; Brüning et al., 2018) and high ambient temperature (Bernardes et al., 2018) can lead to the aerobic instability of silages. These factors determine dry matter (DM) and economic losses, decreases of the nutritional value and a worsening of the hygienic quality of maize silages (Borreani and Tabacco, 2010). Furthermore, a delay in harvesting maize for silage to an advanced milk line of over 2/3 to close the black layer stage, with the aim of achieving a higher proportion of starch in the silage, could result in a progressive deterioration of the ensilability characteristics. A higher DM content, a lower water activity (a_w) and a lower concentration of fermentable sugars all make lactic acid bacteria (LAB) activity more difficult (Comino et al., 2014). Thus, the high DM content of whole crop maize silages ferment less, these silages are more difficult to pack into the silo and it is more challenging to contain aerobic spoilage during consumption (Wilkinson and Davies, 2013).

Since lactate-assimilating yeasts are usually believed to initiate aerobic spoilage, additives containing (chemical additives) or generating (biological additives) antifungal components are available on the market to improve the aerobic stability of silages (Da Silva et al., 2015; Muck et al., 2018). The variable effectiveness from year to year is one of the main issues of using microbial inoculants, since they are dependent on the environmental conditions and the forage characteristics (Muck, 2004; Muck, 2013). Furthermore, high DM silages are more prone to aerobic spoilage, and thus theoretically require higher levels of stabilizing fermentative products (e.g. acetic acid) to reduce the yeast count (Kung, 2009) than wetter silages, but the LAB activity is limited by the reduced a_w and, consequently, the production of acids is low (Comino et al., 2014). *Lactobacillus buchneri*-based inocula, due to the conversion of lactic acid to acetic acid, is known to improve the aerobic stability of maize silages, but it often requires about 50 to 60 d (or longer) of conservation to be effective in maize silage (Driehuis et al. 1999; Ferrero et al., 2019). Furthermore, some experimental evidence has reported that *L. buchneri*-based inocula are less effective in improving the aerobic stability of maize silages with a higher DM content (Comino et al., 2014; Xu et al, 2019). Therefore, chemical additives, such as organic or inorganic acids, may be more robust than biological additives in improving aerobic stability, especially for high DM content silages and for early opening (Kung et al., 2003; Da Silva et al., 2015). The most common active ingredients in chemical additives are acids and their salts (Muck et al.,

2018). The main acids belonging to the group of aerobic stability enhancers are sorbic, benzoic, propionic and acetic acids, or their mixtures (Bernardes et al., 2015; Weiss et al., 2016). Commercial additives often contain mixtures of different acids at various concentrations to achieve the maximum effect against spoilage bacteria and fungi (Muck et al., 2018). Free fatty acids and monoglycerides of fatty acids are known to be potent antimicrobial agents that are able to kill Gram-positive and Gram-negative bacteria and fungi on contact (Thormar and Hilmarsson, 2007). Over the last few decades, the 1-monoglycerides of short and medium chain fatty acids have been proposed as antibacterial molecules against food-borne pathogens and spoilage bacteria (Bergsson et al., 2002; Thormar and Hilmarsson, 2007). These antimicrobial lipids, which are mainly derived from natural sources, present a relative lack of toxicity and have recently been investigated for animal feeding and disease prevention as an alternative to the use of antibiotic substances (Thormar and Hilmarsson, 2007). In 2010, mixtures of C1 to C7 organic acid monoglycerides and glycerol were patented as antibacterial and anti-mould agents for animal feeds (Cantini, 2010). To the best of our knowledge, no previous investigations have been made on the application of these carboxylic acid esters to improve the aerobic stability of maize silage.

Hence, the aim of the present study has been to evaluate the effects of the application of three different rates of a new chemical additive, containing a monopropionine and monobutyrin mixture, on the aerobic stability of whole crop maize silages in two trials in which the conservation period differed.

7.3. Material and methods

7.3.1. Crop and ensiling

Two experiments were carried out at the experimental farm of the University of Turin in the western Po plain, northern Italy (44°53' N, 7°41' E, altitude 232 m a.s.l.) on maize (*Zea mays* L.) harvested for silage. The whole maize crop (Kasimens, KWS Italia S.p.A., Monselice (PD), Italy) was harvested at around the 2/3 milk line stage and was chopped using a precision forage harvester (Claas Jaguar 950 equipped with an 8-row Orbis head, Claas, Harsewinkel, Germany) to a theoretical cut length of 12 mm and a roll clearance of 2 mm. The field was divided into four separate blocks to obtain four replications per treatment (one in each block). The fresh herbage of each block was divided into five 40-kg piles for each experiment . Five treatments were applied to each pile: untreated negative control (**C**); *L. buchneri* **(LB)** NCIMB 40788 (Lallemand Animal Nutrition, France) at a theoretical application rate of 300,000 cfu/g of fresh matter (FM)); the new additive composed of a mixture of monoglycerides (85%) and diglycerides (15%) of propionic and butyric acid (SILO S.p.A., Firenze, Italy) applied at a rate of 5 g/kg FM **(A0.5)**, at a rate of 10 g/kg FM (**A1.0)** and at a rate of 15 g/kg FM (**A1.5)**. The microbial inoculant was diluted in sterilized water and applied using a hand sprayer, at a rate of 4 mL/kg of forage, by spraying uniformly onto the forage, which was constantly hand mixed. To add the targeted amount of LAB, the inoculum was plated onto MRS agar (Merck, Whitehouse Station, NY), natamycin (0.25 g/L) was added and, on the basis of the measured concentration of LAB, an appropriate amount was used to achieve the desired application rate. The chemical additive was applied in a 1:3 w/w water-to-additive solution, using a hand sprayer, by spraying uniformly onto the forage, which was constantly hand mixed. The same amount of water was added to the C treatment.

The fresh forage was sampled before ensiling after the additives had been applied. The untreated and treated forages were then ensiled (about 10 to 12 kg of wet forage) in 20 L plastic silos equipped with a lid that only enabled gas release. The forage was packed by hand, and the final packing densities, on a wet basis, were 545 ± 28 kg FM/m^3 and 552 ± 25 kg FM/m^3 for trial I and trial II, respectively. All the laboratory silos were filled within three hours. The silos were weighed and opened after 100 d and 240 d for Trial I and II, respectively. At opening, each silo was weighed to determine the DM losses, and the content was then mixed thoroughly and sub-sampled to determine the DM content, the chemical composition, the fermentation profile and the microbial counts. The weight 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, and they were expressed as percentages of the

amount of DM ensiled in each plastic silos. After sampling, the silages were subjected to an aerobic stability test, which involved monitoring the temperature increases due to the microbial activity of the samples exposed to air. About three kilograms from each silo was allowed to aerobically deteriorate in 17 L polystyrene boxes (290 mm diameter and 260 mm height). A single layer of aluminum foil was placed over each box to prevent drying and dust contamination, but also to allow air penetration. The ambient and silage temperatures were measured hourly by means of a data logger. Aerobic stability was defined as the number of hours the silage remained stable before its temperature increased by 2°C above the ambient temperature. The silages were sampled after 7 d of aerobic exposure in order to quantify the microbial, fermentative and nutritional changes in the silages during air exposure. The DM losses during air exposure were calculated using the ash content, as reported by Borreani et al. (2018). Small increases in the ash content of deteriorated silage represented large percentage unit increases in DM loss, as can be seen when the equation used to calculate DM losses according to the ash content is used: DM loss (%) = [1 – (ash silage at opening/ash silage after 7 d of air exposure)] × 100.

7.3.2. Sample preparation and analyses

The pre-ensiled material and the silages were split into five subsamples. One sub-sample was analyzed immediately for the DM content by oven drying at 80°C for 24 h. Dry matter was corrected according to Porter and Murray (2001), in order to consider the volatile compound losses that can take place at 80°C. The second subsample was oven-dried at 65°C to constant weight and was air equilibrated, weighed and ground in a Cyclotec mill (Tecator, Herndon, VA, USA) to pass a 1 mm screen. The dried samples were analyzed for total nitrogen (TN), according to the Dumas method (method number 992.23, AOAC, 2005), using a Nitrogen analyzer Primacs SN (Skalar, Breda, The Netherlands), for crude protein (CP) (total N x 6.25) and for ash by ignition (method number 942.05, AOAC, 2005). Neutral detergent fiber (aNDF) was analyzed, using a Raw Fiber Extractor (FIWE, VELP Scientifica, Usmate Velate, IT), with the addition of heat-stable amylase (A3306, Sigma Chemical Co., St. Louis, MO, USA) and expressed on a DM basis, including residual ash, as described by Van Soest et al. (1991). Acid detergent fiber (ADF) was analyzed and expressed on a DM basis, including residual ash (Robertson and Van Soest, 1981).

A third fresh sub-sample was used to determinate the water activity (a_w), pH, nitrate (NO_3), the ammonia nitrogen (NH_3-N) contents and the buffering capacity (BC). The water activity was measured at 25°C on a fresh sample using an AquaLab Series 3TE (Decagon Devices Inc., Pullman, WA), which adopts the chilled-mirror dew point technique. The fresh forage was extracted for pH, NO_3 and NH_3-N determination, using a Stomacher blender (Seward Ltd,

Worthing, UK), for 4 min in distilled water at a 9:1 water-to-sample material (fresh weight) ratio. The total nitrate concentration was determined in the water extract, through semi-quantitative analysis, using Merckoquant test strips (Merck, Darmstadt, Germany; detection limit 100 mg NO_3/kg). The ammonia nitrogen content and pH were determined using specific electrodes. The buffering capacity was determined in the water extract, as described by Plaine and McDonald (1966).

A fourth sub-sample was extracted, using a Stomacher blender, for 4 min in H_2SO_4 0.05 mol/L at a 5:1 acid-to-sample material (fresh weight) ratio. An aliquot of 40 ml of silage acid extract was filtered with a 0.20-µm syringe filter and used for quantification of the fermentation products. The lactic and monocarboxylic acids (acetic, propionic and butyric acids) were determined, by means of high performance liquid chromatography (HPLC), in the acid extract (Canale et al., 1984). Ethanol, 1 propanol and 1,2-propanediol were determined by means of HPLC, coupled to a refractive index detector, on a Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA, USA).

The fifth subsample was used for the microbial analyses. For the microbial counts, a sample of 30 g was transferred to a sterile homogenization bag, suspended 1:10 w/v in a peptone salt solution (1 g of bacteriological peptone and 9 g of sodium chloride per liter) and homogenized for 4 min in a laboratory Stomacher blender (Seward Ltd, London, UK). Serial dilutions were prepared, and the yeast and mold 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 the yeasts and molds, respectively. The yeast and mold colony forming units (cfu) were enumerated separately, according to their macromorphological features, on plates that yielded 1 to 100 cfu. The LAB were determined on MRS agar (Merck, Whitehouse Station, NY) to which natamycin was added (0.25 g/L), by incubating Petri plates at 30°C for 3 d under anaerobic conditions. Since LAB are facultative anaerobes, the choice of anaerobic incubation was made to improve the selectivity of the media against *Bacillus* spp.

7.3.3. Statistical analysis

The microbial counts were $\log_{10}$ transformed and presented on a wet weight basis. The values below the yeast and mold detection limits (detection levels: 10 cfu/g of silage) were assigned a value corresponding to half of the detection limit in order to calculate the average. The NO_3 content was corrected for the dilution factor and expressed on a DM basis. The fermentative characteristics, microbial counts, DM losses and aerobic stability indices were analyzed for their statistical significance via analysis of variance, with their significance

reported at a 0.05 probability level, using the General Linear Model of the Statistical Package for Social Science (v 24.0, SPSS Inc., Chicago, Illinois, USA). The data were analyzed utilizing the treatment as the fixed factor, with four replicates. When the calculated values of F were significant, the REGWF test (P <0.05) was used to interpret any significant differences between the mean values. The yeast count and the aerobic stability were pooled together across the Trials and were regressed on the propionic acid content as the independent variable, the ethanol content was pooled together across the Trials and was regressed on the yeast count as the independent variable, and the weight losses on a DM basis were pooled together across the Trials and were regressed on the ethanol content as the independent variable.

7.4. Results

The chemical and microbial characteristics of the forage at harvest, before applying the additive, are reported in Table 1. The DM content was 415 and 417 g/kg for Trials I and II, respectively. The other parameters were typical of whole maize forage harvested at a 2/3 kernel milk line. On average, the NDF was 30.0 g/kg DM and the starch content was 38.3 g/kg DM. The LAB, yeast and mold counts were around or higher than 6.0 log cfu/g in both Trials. The DM content, pH and fermentative end products of the Trial I and II silages are summarized in Table 2. Higher pH, acetic acid and ethanol contents were found in the C and LB silages than in those treated with the chemical additive. The ethanol content decreased as the additive application rate increased till 4.8 g/kg DM was reached in the A1.5 silages. The propionic and butyric acids increased linearly with the application rate in the silages treated with the chemical additive, whereas they were not found in the C and LB silages. In Trial II, a greater amount of ethanol was found in C and LB than in the silages treated with the chemical additive, in which it decreased at a dose dependent manner. Figure 1 reports the relationship between the average amount of propionic and butyric acids applied through the additive and the propionic and butyric acid contents determined by means of HPLC at harvesting (mean values of Trials I and II) and at opening in Trials I and II. The propionic and butyric acid concentrations, measured by means of HPLC, were related linearly to the calculated amount derived from the acid hydrolysis of the additive ($R^2 = 0.99$). Traces of 1,2-propanediol were only found in the LB silages in both Trials. The microbial count and the aerobic stability of the silages of Trials I and II are reported in Table 3. In Trial I, the LAB and yeast counts were higher in the C and LB silages than for the other treatments. For all the silages, except for the LB silages of Trial I, the mold count was under the detection limit. In both Trials, the highest value of aerobic stability was reached in the A1.5 silages (with a mean increase of 120 h in A1.5 compared to the C silages). In Trial II, the LAB count was higher in the LB silages (6.70 $\log_{10}$ cfu/g) than for the others treatments and it was lower in the A1.5 silages (3.39 $\log_{10}$ cfu/g). The weight losses for Trials I and II are reported in Figure 2 on a DM basis. The silages treated with the chemical additive had lower DM losses than the C and LB silages and, in particular, the losses decreased when the dose of the chemical additive applied was increased. The nutritional parameters of the Trial I and II silages are summarized in Table 4. The treatments did not affect the nutritional parameters in either Trial. The fermentative parameters, microbial counts and DM losses of the Trial I and II silages, after 7 d of air exposure, are reported in Table 5. After 7 d of air exposure, the pH was higher than 5 in the C and LB silages and below 4 in the A0.5, A1.0 and A1.5 ones of Trial I. The lactic acid content was higher in the silages treated with the chemical additive than in the C and LB silages. The yeast count was higher than 6.0 log cfu/g in

all the treatments, except for the A1.5 silages, in which it was below 4.0 log cfu/g. The mold count was below 3.0 log cfu/g in all the silages. The A1.0 and A1.5 silages showed the highest values of lactic acid and ethanol in the air exposed silages of Trial II, whereas they were under the detection limit in the C, LB and A0.5 silages. Only the C and LB silages showed the presence of molds after 7 d of air exposure. The nutritional parameters of the silages after 7 d of air exposure in Trials I and II are reported in Table 6. The control silages showed higher aNDF and hemicellulose contents than the treated silages in Trial I. The relationships between the propionic acid and yeast count, between the propionic acid and aerobic stability, between the yeast count and ethanol content, and between the ethanol content and weight losses when the data are pooled together across the two trials are reported in Figure 3. The higher the propionic acid is, the lower the yeast count (*P*-value <0.001) and the higher the aerobic stability (*P*-value <0.001). The higher the yeast count is, the higher the ethanol content (*P*-value <0.001), and the higher the ethanol content is, the higher the weight losses (*P*-value <0.001).

7.5. Discussion

The main practical issue in maize silage conservation is to ensure the best aerobic stability during the feed-out phase (Borreani et al., 2018). Several solutions have been developed to ensure aerobic stability during the feed-out phase, which represents a crucial point for the nutritional and microbial quality of silages (Muck et al., 2018). This study has been aimed at evaluating the effect of a mixture of short chain fatty acids (C3 and C4) and monoglyceride as a chemical additive to improve the aerobic stability of maize silage. The monoglycerides of fatty acids have recently been proposed for their antimicrobial activities against bacteria, fungi and yeasts in aquaculture (Parini et al., 2016) and in infant formulas (Al-Holy et al., 2010) for their very low toxicity for humans and mammals. These molecules showed inhibitory activities against several Enterobacteriaceae (*Escherichia*, *Yersinia*) and some animal pathogens (i.e. *Bacillus cereus*, *Staphylococcus aureus*, *Candida albicans*), but no inhibitory action against homofermentative LAB, such as *L. plantarum* and *L. acidophilus*, were observed (Parini et al., 2016). Pure glycerol fatty acid esters have an amphiphilic structure, which gives these biomolecules a broad range of physico-chemical and biological properties. The monoglycerides of propionic and butyric acids are apolar molecules that behave like polar molecules, which are able to pass the hydrophilic membrane of pathogenic bacteria (Nikaido, 2003; Schlievert and Peterson, 2012). Single cell organisms, e.g. *E. coli*, need glycerol to build membranes and to grow and reproduce. Monoglycerides are transported into the bacterial cell by the Glycerol Uptake Facilitator (GUF), and they thus slow down or block the glycerol uptake. As a result, the bacterial cell is deprived of energy and the cell wall is disrupted, which leads to bacterial cell death. Some researchers have shown that the monoglycerides of organic acids are 10- to 100- fold more effective against bacteria than their original acids at a pH of around 7 (Makkink, 2013; Parini at al., 2016).

An increase in the aerobic stability of silages, related to the reduction in the yeast count, has been reported by several authors (Kleinschmit and Kung, 2006; Tabacco et al., 2011). The use of *L. buchneri*-based inocula to improve aerobic stability has proved valuable, due to its production of acetic acid (Muck et al., 2018). In the present experiment, the addition of *L. buchneri* resulted in aerobic stability close to that of the untreated control. The ineffective activity of *L. buchneri* was confirmed by a high yeast count, a low amount of acetic acid and only traces of 1,2-propanediol in the LB treatment, even though the conservation period was longer than 60 d. The ineffective activity of *L. buchneri* in the present study could be explained by considering the high DM content of the maize silage (higher than 40%), which determined, through a low a_w, an unfavorable environment for the LAB activity, and/or a low temperature at ensiling due to the late harvesting in autumn (Zhou et al., 2016). Previous

studies in temperate environments reported variability in the effectiveness of *L. buchneri*-based inocula on maize silage, with a higher DM content than 40%, even after long conservation periods (Comino et al., 2014; Borreani et al., 2018). Hu et al. (2009) found an improvement in aerobic stability in maize silage treated with *L. buchneri*, compared with untreated silage, after 260 d of conservation, regardless of the different DM contents of the silages (33% and 39%).

In the present study, the addition of the new chemical additive has resulted in a higher aerobic stability, especially when it was applied at doses of 1.0% and 1.5% of FM in both Trials I and II. The high aerobic stabilities in the silages treated with the chemical additive were consistent with the reduction in the yeast count. This reduction could be attributable to the mixture of monoglycerides, and not to the effect of acetic acid produced by LAB during the fermentation process (below 10 g/kg of DM). The amount of ethanol was higher in C and LB than in the silages treated with the chemical additive. After fermentation, the main source of ethanol in silage is mainly attributable to the alcoholic fermentation of glucose by yeasts or to heterolactic fermentation by LAB (Woolford, 1984). However, the pathway of obligate heterofermentative LAB also determines a large amount of acetic acid (i.e more than 20 g/kg DM), especially after long conservation periods. Thus, the lower content of acetic acid and higher lactic-to-acetic ratio found in the present study, compared to a typical heterofermentative profile of maize silages (Kleinschmit and Kung, 2006), led us to hypothesize that most of the ethanol was derived from the yeast pathways. Furthermore, the decrease in the ethanol content was consistent with the decrease in the yeast count. A reduction in the ethanol content has a positive effect on the environmental sustainability of silage, because ethanol makes the largest contribution to the VOC emissions from maize silage, in terms of mass emitted and potential ozone formation (Hafner et al., 2013). The heat of combustion of ethanol (29.7 MJ/kg) is higher than that of either acetic acid (15.5 MJ/kg) or carbohydrates (17.6 MJ/kg); therefore, animals fed ethanol could be energetically more efficient (Daniel et al., 2013). However, most of the ingested ethanol is partially oxidized to acetate by rumen microorganisms with concomitant increases in methane production (Durix et al., 1991), which might decrease energy efficiency.

Butyric acid is a typical fermentative end product of clostridia that is a undesirable microorganism in silages, which causes high DM losses during fermentation and a reduction of the nutritional value. Moreover, it affects the hygienic quality of silage by creating optimal conditions for the proliferation of spoilage microorganisms and the accumulation of undesirable products (Pahlow et al., 2003; Flythe and Russell, 2004). In a present experiment, the fermentative profile of all the treated silages showed the presence of butyric acid ranging from 0.6 to 3.0 g/kg DM. The absence of clostridia growth can be excluded as a result of the absence of butyric acid in the untreated control silage and of the increase in butyric acid at ensiling after the application of the additive. Furthermore, after fermentation, the butyric acid content of the treated silages

did not show any increase, compared to their corresponding samples before ensiling. The manufacturer of the additive stated that the mixture of monoglycerides maintains its molecular structure up to pH 2. Therefore, the presence of propionic and butyric acid, originating from the additive addition, was due to monoglyceride hydrolysis during sample extraction for the HPLC analysis, which was made under very acidic conditions (pH around 1.5).

In the present experiment, the different application rates of the additive affected several parameters. The DM losses were higher in C and LB and lower in the A1.5 silages. Similar results were found in silages treated with chemical additives based on acids, or their salts, by several authors (Muck and Kung, 1997; Bernardes et al., 2015). In the present experiment, the inoculation with the microbial inoculant based on *L. buchneri* determined the highest values of the LAB count, whereas the addition of the chemical additive led to a reduction in the LAB count. Nevertheless, the amount of lactic acid in all the silages is consistent with values found in high DM maize silages (Comino et al., 2014; Herrmann et al., 2015) thus, it could be hypothesized that the chemical additive partially inhibited the LAB after the first phase of the fermentation, and it did not influence the fermentation quality of the silages. Further studies are needed to improve the knowledge about this new chemical additive for short conservation periods.

A longer conservation period of silages has been found to be useful to improve aerobic stability, as it causes a notable reduction of the yeast count (Ferrero et al., 2019). In the present experiment, even though the conservation time was higher in Trial II than in Trial I (240 d vs 100 d), the aerobic stability was lower in all the treatments in Trial II than in Trial I. The fermentative end product with an antifungal effect (acetic and propionic acid) and the yeast count were similar between the two Trials, therefore the differences in aerobic stability could be due to different factors. The main parameter considered useful to explain the different aerobic stability was the temperature during air exposure (21°C and 27°C for Trials I and II, respectively). The influence of a high temperature on the reduction of aerobic stability has already been reported by Ashbell et al. (2002) and by Borreani and Tabacco (2010) at a farm level. The reason for this reduction was an increase in the growth of spoilage microorganisms under a high temperature, which led to increased silage deterioration (Bernardes et al., 2018). In this experiment, the negative effect of temperature was more detectable in C, LB and A0.5 than in the A1.0 and A1.5 silages. This indicates that the addition of the chemical additive, when added at doses of at least 1.0% of FM, is able to determine an inhibitory activity against yeast, even under unfavorable conditions (i.e. high environment temperature).

Chemical additives may be costly but, although their cost is somewhat higher than microbial-based additives, the magnitude of the final result is not dependent on the growth of microorganisms to produce active end products (Kung et al., 2018). Furthermore, other acids, such as benzoic and sorbic acid, are also effective mold and yeast inhibitors but, due to their high cost, they are

often sold in mixtures with propionic acid (Adesogan, 2014). Sorbates (sorbic acid, sodium sorbate, potassium sorbate), benzoates (benzoic acid, sodium benzoate, potassium benzoate), propionates, sodium nitrate and sodium nitrite are the most widely used food preservatives (Piper and Piper, 2017). They have been tested as potential silage additives since 1970 by Wolford (1975), and sodium and potassium salts are preferred over the acid form because they are more soluble in water. Their effect has resulted to be selective among different groups of microorganisms with a pH dependent inhibitory activity (Woolford et al., 1975). Sodium sorbate and sodium benzoate have shown strong inhibitory activity against yeasts and molds, especially for pH values below 5. These additives act at a concentration of 55 to 110 mmol/l (mean application rate from 7 to 14.5 kg/t of fresh forage, Wolfoord, 1984). Piper and Piper (2017) reported that sodium sorbate and sodium benzoate presented potential hazards for human health. This led to the necessity of finding new additives to improve aerobic stability, especially for early opening and for conditions where stabilizing inocula have not always worked as expected, with molecules that are suspected of having detrimental effects on animal and human health.

The cost of the new additive is unknown thus, despite its good efficiency, an economic convenience analysis is needed. However, it would be possible to only apply the additive only to the surface of a silage mass (top), where the risk of deterioration is greater and losses are generally observed in field conditions (Da Silva et al., 2014), thus reducing its cost.

7.6. Conclusion

A high quality silage can be obtained with relative easiness if appropriate weather, substrate availability and good management conditions are ensured, otherwise the use of additives may aid the success of the process. In the present study, it has been confirmed that the reduction of the yeast count is the main factor that can guarantee high aerobic stability. The addition of monopropionine and monobutyrin has shown a positive effect on improving the aerobic stability as a result of a reduction of the yeast count, especially when a natural decrease in the yeast count is not easy (i.e. not very long conservation period, high DM content of the silages, high environmental temperature). The effect of the additive has been found to be related to its application rate, with the best results being obtained when the additive was applied at 1.5% of fresh matter. However, further investigation are still needed to evaluate the cost of the product and its effect after a short conservation period.

7.7. Acknowledgements

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information, and does not imply either recommendation or endorsement by the University of Turin, Italy.

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7.8. References

Adesogan, A.T., 2014. Avoiding the two greatest silage problems. Proc. 50th Florida Dairy Production Conference. Florida, Gainesville. pp. 9-18.

Al-Holy, M.A., Castro, L.F., Al-Qadiri, H.M., 2010. Inactivation of *Cronobacter* spp. (*Enterobacter sakazakii*) in infant formula using lactic acid, copper sulfate and

monolaurin. Letters Appl. Microbiol. 50, 246-251. https://doi.org/10.1111/j.1472-765X.2009.02782.x.

AOAC International., 2005. Official Methods of Analysis. 18th ed. AOAC International, Gaithersburg, MD.

Ashbell, G., Weinberg, Z. G., Hen, Y., Filya, I., 2002. The effects of temperature on the aerobic stability of wheat and corn silages. J. Ind. Microbiol. Biot. 28, 261-263. https://doi.org/10.1038/sj.jim.7000237.

Bergsson, G, Steingrímsson, Ó, Thormar, H., 2002. Bactericidal effects of fatty acids and monoglycerides on *Helicobacter pylori*. Int. J. Antimicrob. Agents. 20, 258-262. https://doi.org/10.1016/S0924-8579(02)00205-4.

Bernardes, T.F., De Oliveira, I. L., Lara, M.A.S., Casagrande, D.R., Avila, C.L.S., Pereira, O.G., 2015. Effects of potassium sorbate and sodium benzoate at two application rates on fermentation and aerobic stability of maize silage. Grass Forage Sci. 70, 491-498. https://doi.org/10.1111/gfs.12133.

Bernardes, T.F., Daniel, L.P., Adesogan, A.T., McAllister, T.A., Drouin, P., Nussio, L.G., Huhtanen, P., Tremblay, G.F., Bélanger, G., Cai, Y., 2018. *Silage review*: Unique challenges of silages made in hot and cold regions. J. Dairy Sci. 101, 4001-4019. https://doi.org/10.3168/jds.2017-13703.

Bolsen, K.K., Ashbell, G., Weinberg, Z.G., 1996. Silage fermentation and silage additives. Review. Asian-Austral. J. Anim. 9, 483-493. https://doi.org/10.5713/ajas.1996.483.

Borreani, G., Ferrero F., Coppa M., Demey V., Tabacco E., 2018. Effect of different inocula on aerobic stability of corn silage. In: Proc. 18th International Silage Conference, Gerlach, K., Südecum, K.H. (Eds.), Bonn, Germany, pp. 238-239.

Borreani, G., Tabacco, E., 2010. The relationship of silage temperature with the microbiological status of the face of corn silage bunkers. J. Dairy Sci. 93, 2620-2629. https://doi.org/10.3168/jds.2009-2919.

Borreani, G., Tabacco, E., Schmidt, R.J., Holmes, B.J., Muck, R.E., 2018. *Silage review*: Factors affecting dry matter and quality losses in silages. J. Dairy Sci. 101, 3952-3979. https://doi.org/10.3168/jds.2017-13837.

Brüning, D., Gerlach, K., Weiß, K., Südekum, K.H., 2018. Effect of compaction, delayed sealing and aerobic exposure on maize silage quality and on formation of volatile organic compounds. Grass Forage Sci. 73, 53-66. https://doi.org/10.1111/gfs.12288.

Canale, A., Valente, M.E., Ciotti, A., 1984. Determination of volatile carboxylic acids (C1-C5) and lactic acid in aqueous acid extracts of silage by high performance liquid chromatography. J. Sci. Food Agric. 35, 1178-1182. https://doi.org/10.1002/jsfa.2740351106.

Cantini, F., 2010. Compositions containing C1 to C7 organic acid monoglycerides and glycerol, their preparation and use as antibacterials and anti-mould agents. European Patent, EP 2 410 871 B1.

Comino, L., Tabacco, E., Righi, F., Revello-Chion, A., Quarantelli, A., Borreani, G., 2014. 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. Anim. Feed Sci. Technol. 198, 94-106. https://doi.org/10.1016/j.anifeedsci.2014.10.001.

Daniel, J.L.P., Amaral, R.C., Sá Neto, A., Cabezas-Garcia, E.H., Bispo, A.W., Zopollatto, M., Cardoso, T.L., Spoto, M.H.F., Santos, F.A.P., Nussio, L.G., 2013. Performance of dairy cows fed high levels of acetic acid or ethanol. J.

Dairy Sci. 96, 398-406. https://doi.org/10.3168/jds.2012-5451.
Da Silva, T.C., Smith, M.L., Barnard, A.M., Kung Jr., L., 2015. The effect of a chemical additive on the fermentation and aerobic stability of high-moisture corn. J. Dairy Sci. 98, 8904-8912. https://doi.org/10.3168/jds.2015-9640.
Driehuis, F., Oude Elferink, S.J.W.H., Spoelstra, S.F., 1999. Anaerobic lactic acid degradation during ensilage of whole-crop maize inoculated with *Lactobacillus buchneri* inhibits yeast growth and improves aerobic stability. J. Appl. Microbiol. 87, 583-594. https://doi.org/10.1046/j.1365-2672.1999.00856.x.
Durix, A., Jean-Blain, C., Sallmann, H.P., Jouany J.P., 1991. Use of a semicontinuous culture system (RUSITEC) to study the metabolism of ethanol in the rumen and its effects on ruminal digestion. Can. J. Anim. Sci. 71, 115-123. https://doi.org/10.4141/cjas91-013.
Ferrero F., Piano, S., Tabacco, E., Borreani, G., 2019. Effects of conservation period and *Lactobacillus hilgardii* inoculum on the fermentation profile and aerobic stability of whole corn and sorghum silages. J. Food Sci. Agric. 99, 2530-2540. https://doi.org/10.1002/jsfa.9463.
Flythe, M.D., Russell, J.B., 2004. The effect of pH and a bacteriocin (bovicin HC5) on *Clostridium sporogenes* MD1, a bacterium that has the ability to degrade amino acids in ensiled plant materials. FEMS Microbiol. Ecol. 47, 215-222. https://doi.org/10.1016/S0168-6496(03)00259-9.
Goering, H.K., Van Soest, P.J., 1970. Forage fiber analyses (apparatus, reagents, procedures, and some applications). Agriculture Handbook No. 379, Agric. Res. Serv., USDA, Washington, DC.
Grant, R.J., Ferraretto, L.F., 2018. *Silage review*: Silage feeding management: silage characteristics and dairy cow feeding behavior. J. Dairy Sci. 101, 4111-4121. https://doi.org/10.3168/jds.2017-13729.
Hafner, S.D., Howard, C., Muck, R., Franco, R.B., Montes, F., Green, P.G., Mitloehner, F., Trabue, L., Rotz. C.A., 2013. Emission of volatile organic compounds from silage: Compounds, sources, and implications. Atmosph. Environ. 77, 827-839. https://doi.org/10.1016/j.atmosenv.2013.04.076.
Herrmann, C., Idler C., Heiermann, M., 2015. Improving aerobic stability and biogas production of maize silage using silage additives. Biores. Technol. 197, 393-403. https://doi.org/10.1016/j.biortech.2015.08.114.
Hu, W., Schmidt, R.J., McDonell, E.E., Klingerman, C.M., Kung Jr., L., 2009. The effect of *Lactobacillus buchneri* 40788 or *Lactobacillus plantarum* MTD-1 on the fermentation and aerobic stability of corn silages ensiled at two dry matter contents. J. Dairy Sci. 92, 3907-3914. https://doi.org/10.3168/jds.2008-1788.
Kleinschmit, D.H., Kung Jr., L., 2006. A meta-analysis of the effects of Lactobacillus buchneri on the fermentation and aerobic stability of corn and grass and small-grain silages. J. Dairy Sci. 89, 4005-4013. https://doi.org/10.3168/jds.S0022-0302(06)72444-4.
Kung Jr., L., Sheperd, A.C., Smagala, A.M., Endres, K.M., Bessett, C.A., Ranjit, N.K., Glancey, J.L., 1998. The effect of preservatives based on propionic acid on the fermentation and aerobic stability of corn silage and a total mixed ration. J. Dairy Sci. 81, 1322-1330. https://doi.org/10.3168/jds.S0022-0302(98)75695-4.
Kung Jr., L., Myers, C.L., Neylon, J.M., Taylor, C.C., Lazartic, J., Mills, J.A., Whiter, A.G., 2004. The effects of buffered propionic acid-based additives alone or combined with microbial inoculation on the fermentation of high moisture corn and whole-crop barley. J. Dairy Sci. 87, 1310-1316. https://doi.org/10.3168/jds.S0022-0302(04)73280-4.

Kung Jr., L., Robinson, J.R., Ranjit, N.K., Chen, J.H., Golt, C.M., Pesek, J.D., 2000. Microbial populations, fermentation end-products, and aerobic stability of corn silage treated with ammonia or a propionic acid-based preservative. J. Dairy Sci. 83, 1479-1486. https://doi.org/10.3168/jds.S0022-0302(00)75020-X.

Kung Jr., L., Smith, M.L., da Silva, E.B., Windle, M.C., da Silva, T.C., Polukis, A., 2018. An evaluation of the effectiveness of a chemical additive based on sodium benzoate, potassium sorbate, and sodium nitrite on the fermentation and aerobic stability of corn silage. J. Dairy Sci. 101, 5949-5960. https://doi.org/10.3168/jds.2017-14006.

Kung Jr., L., 2009. Potential factors that may limit the effectiveness of silage additives. In: Proc. XV International Silage Conference. U.S. Dairy Forage Research Center, Madison, WI. pp: 37-45.

Kung Jr., L., Stokes, M.R., Lin, C.J., 2003. Silage additives. In: Buxton, D.R., Muck, R.E., Harrison, H.J. (Eds.) Silage Science and Technology (Agronomy Series No. 42). American Society of Agronomy, Madison, WI. pp: 305-360.

Leaver, J.D., 1975. The use of propionic acid as an additive for maize silage. Grass Foragse Sci. 30, 17-21. https://doi.org/10.1111/j.1365-2494.1975.tb01349.x.

Makkink, C., 2013. Piglets benefit from monoglycerides in sow's milk. Int. Pig Topics 28:13.

McDonald, P., Henderson, A.R., Heron, S.J.E., 1991. The Biochemistry of Silage. 2nd ed. Chalcombe Publications, Bucks, UK.

Moon, N.J., 1983. Inhibition of the growth of acid tolerant yeasts by acetate, lactate and their synergistic mixtures. J. Appl. Bacteriol. 55, 453-460. https://doi.org/10.1111/j.1365-2672.1983.tb01685.x.

Muck, R.E., 2013. Recent advances in silage microbiology. Agric. Food Sci. 22, 3-15. https://doi.org/10.23986/afsci.6718.

Muck, R.E., Nadeau, E.M.G., McAllister, T.A., Contreras-Govea, F.E., Santos, M.C., Kung Jr., L., 2018. *Silage review*: Recent advances and future uses of silage additives. J. Dairy Sci. 101, 3980-4000. https://doi.org/10.3168/jds.2017-13839.

Nikaido, H., 2003. Molecular basis of bacterial outer membrane permeability - revisited. Microbiol. Molecular Biol. Rev. 67, 593-656. DOI: 10.1128/MMBR.67.4.593–656.2003.

Ogunade, I.M., Kim, D.H., Jiang, Y., Weinberg Z.G., Jeong, K.C., Adesogan, A.T., 2016. Control of *Escherichia coli* O157:H7 in contaminated alfalfa silage: Effects of silage additives. J. Dairy Sci. 99, 4427-4436. https://doi.org/10.3168/jds.2015-10766.

Pahlow, G., Muck, R.E., Driehuis, F., Oude Elferink, S.J.W.H., Spoelstra, S.F., 2003. Microbiology of ensiling. In: Buxton, D.R., Muck, R.E., Harrison, H.J. (Eds.) Silage Science and Technology (Agronomy Series No. 42). American Society of Agronomy, Madison, WI. pp: 31-93.

Parini, M., Yu, Y., Paoli, A., Gaddipati, R., 2016. Selective antibacterial action of a specific composition of short- and medium-chain 1-monoglycerides. Aquaculture Asia Pacific, 12, 48-50.

Piper, J.D., Piper, P.W., 2017. Benzoate and sorbate salts: A systematic review of the potential hazards of these invaluable preservatives and the expanding spectrum of clinical uses for sodium benzoate. Comprehensive Rev. Food Sci. Food Safety, 16, 868-880. https://doi.org/10.1111/1541-4337.12284.

Playne, M.J., McDonald, P., 1966. The buffering constituents of herbage and of silage. J. Sci. Food Agric. 17, 264-268. https://doi.org/10.1002/jsfa.2740170609.

Porter, M.G., Murray, R.S., 2001. The volatility of components of grass silage on oven drying and the inter-relationship between dry-matter content estimated by different analytical methods. Grass Forage Sci. 56, 405-411. https://doi.org/10.1046/j.1365-2494.2001.00292.x.

Queiroz, O.C. M., Arriola, K.G., Daniel, J.L.P., Adesogan, A.T., 2013. Effects of 8 chemical and bacterial additives on the quality of corn silage. J. Dairy Sci. 96, 5836-5843. https://doi.org/10.3168/jds.2013-6691.

Ranjit, N.K. Kung Jr., L., 2000. The effect of *Lactobacillus buchneri*, *Lactobacillus plantarum*, or a chemical preservative on the fermentation and aerobic stability of corn silage. J. Dairy Sci. 83, 526-535. https://doi.org/10.3168/jds.S0022-0302(00)74912-5.

Robertson, J.B., Van Soest, P.J., 1981. The detergent system of analysis and its application to human foods. In: James, W.P.T., Theander, O. (Eds.) The Analysis of Dietary Fiber in Food., Marcel Dekker, New York, NY. pp: 123-158.

Schlievert, P.M., Peterson, M.L., 2012. Glycerol monolaurate antibacterial activity in broth and biofilm cultures. PLoS ONE 7(7): e40350. https://doi.org/10.1371/journal.pone.0040350.

Tabacco, E., Righi, F., Quarantelli, A., Borreani, G., 2011. Dry matter and nutritional losses during aerobic deterioration of corn and sorghum silages as influenced by different lactic acid bacteria inocula. J. Dairy Sci. 94, 1409-1419. https://doi.org/10.3168/jds.2010-3538.

Thormar, H., Hilmarsson, H., 2007. Review: The role of microbicidal lipids in host defense against pathogens and their potential as therapeutic agents. Chemistry Physics Lipids, 150, 1-11. https://doi.org/10.1016/j.chemphyslip.2007.06.220.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods of dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583-3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2.

Weiss, K., Kroschewski, B., Auerbach, H., 2016. Effects of air exposure, temperature and additives on fermentation characteristics, yeast count, aerobic stability and volatile organic compounds in corn silage. J. Dairy Sci. 99, 8053-8069. https://doi.org/10.3168/jds.2015-10323.

Wilkinson, J.M., Davies, D.R., 2013. The aerobic stability of silage: key findings and recent developments. Grass Forage Sci. 68, 1-19. https://doi.org/10.1111/j.1365-2494.2012.00891.x.

Woolford, M.K., 1975. Microbiological screening of food preservatives, cold sterilants and specific antimicrobial agents as potential silage additives. J. Sci. Food Agric. 26, 229-237. https://doi.org/10.1002/jsfa.2740260214.

Woolford, M.K., 1984. The silage fermentation. Marcel Dekker, Inc., New York and Basel.

Xu, S., Yang, J., Qi, M., Smiley, B., Rutherford, W., Wang, Y., McAllister, T.A. 2019. Impact of *Saccharomyces cerevisiae* and *Lactobacillus buchneri* on microbial communities during ensiling and aerobic spoilage of corn silage. J. Anim. Sci. 97, 1273-1285. https://doi.org/10.1093/jas/skz021.

Zhou, Y., Drouin, P. Lafrenière, C., 2016. Effect of temperature (5-25°C) on epiphytic lactic acid bacteria populations and fermentation of whole-plant corn silage. J. Appl. Microbiol. 121, 657-671. https://doi.org/10.1111/jam.13198.

7.9. Tables

Table 1. Chemical and microbial characteristics of the forage at harvest

Parameters[1]	Trial I		Trial II	
	Value	**SD**	**Value**	**SD**
DM (g/kg)	415.3	4.81	417.2	3.34
pH	5.97	0.02	6.03	0.02
NH_4 (g/kg DM)	1.04	0.13	1.01	0.11
NH_3-N (% of total N)	0.10	0.004	0.12	0.011
NO_3 (mg/kg FM)	<100	-	<100	-
Buffering capacity (mEq/kg DM)	34	0.6	36	2.4
aNDF (g/kg DM)	30.8	2.9	29.0	1.2
ADF (g/kg DM)	15.9	1.6	15.6	0.6
ADL (g/kg DM)	3.3	0.3	3.1	0.4
Hemicelluloses (g/kg DM)	14.9	1.4	13.4	0.7
CP (g/kg DM)	7.0	0.3	6.8	0.3
Ash (g/kg DM)	2.9	0.1	3.1	0.2
Starch (g/kg DM)	37.9	1.5	38.8	1.6
Yeast (log_{10} cfu/g)	6.71	0.18	7.16	0.28
Mold (log_{10} cfu/g)	6.34	0.26	6.19	0.41
LAB (log_{10} cfu/g)	5.92	0.28	6.32	0.16

[1] ADF = acid detergent fiber; ADL = lignin; cfu = colony forming unit; CP = crude protein; DM = dry matter; LAB = lactic acid bacteria; NDF = neutral detergent fiber; SD = standard deviation.

Table 2. DM, pH and fermentative products of the silages at opening in Trial I and II.

Parameters[1]	C	LB	A0.5	A1.0	A1.5	SE	*P*-value
	Trial I						
DM corrected (g/kg)	413.2	416.4	419.3	430.7	424.4	2.29	0.102
pH	3.74[a]	3.75[a]	3.72[b]	3.71[b]	3.71[b]	0.004	<0.001
Lactic acid (g/kg DM)	49.2	47.9	42.1	44.2	43.9	1.041	0.144
Acetic acid (g/kg DM)	11.2[a]	12.6[a]	7.5[b]	7.5[b]	7.2[b]	0.626	<0.001
Lactic-to-acetic ratio	4.4[b]	3.8[b]	5.6[a]	5.9[a]	6.1[a]	0.249	<0.001
Propionic acid (g/kg DM)	<0.1[d]	<0.1[d]	1.5[c]	3.0[b]	4.2[a]	0.457	<0.001
Butyric acid (g/kg DM)	<0.1[d]	<0.1[d]	0.9[c]	1.8[b]	3.0[a]	0.309	<0.001
1,2-propanediol (g/kg DM)	<0.1	0.1	<0.1	<0.1	<0.1	-	-
Ethanol (g/kg DM)	16.9[a]	15.0[a]	9.9[b]	7.4[bc]	4.8[c]	1.245	<0.001
	Trial II						
DM corrected (g/kg)	415.2	423.8	424.3	429.1	422.4	1.92	0.317
pH	3.73	3.73	3.69	3.70	3.71	0.006	0.053
Lactic acid (g/kg DM)	49.9	49.8	48.6	45.1	45.7	0.876	0.255
Acetic acid (g/kg DM)	10.6[ab]	11.1[a]	9.7[b]	8.8[c]	8.7[c]	0.272	<0.001
Lactic-to-acetic-ratio	4.7[ab]	4.5[b]	5.0[ab]	5.1[ab]	5.3[a]	0.098	0.036
Propionic acid (g/kg DM)	<0.1[d]	<0.1[d]	1.2[c]	3.0[b]	4.2[a]	0.461	<0.001
Butyric acid (g/kg DM)	<0.1[d]	<0.1[d]	0.6[c]	1.5[b]	2.4[a]	0.252	<0.001
1,2-propanediol (g/kg DM)	<0.1	0.2	<0.1	<0.1	<0.1	-	-
Ethanol (g/kg DM)	26.8[a]	29.7[a]	18.4[b]	8.8[c]	5.7[c]	2.587	<0.001

[1] DM = dry matter; C = untreated control; A0.5 = treatment with chemical additive at 0.5% FM; A1.0 = treatment with chemical additive at 1.0% FM; A1.5 = treatment with chemical additive at 1.5% FM; SE = standard error of the mean;

[2] Means with different letters (a–d) in a row differ significantly (P <0.05).

Table 3. Microbial count and aerobic stability of the silages at opening in Trial I and II

Parameters[1]	C	LB	A0.5	A1.0	A1.5	SE	*P*-value
Trial I							
LAB (log_{10} cfu/g)	6.29^{ab}	7.13^{a}	5.66^{bc}	5.03^{c}	5.16^{bc}	0.269	0.006
Yeast (log_{10} cfu/g)	4.29^{a}	4.40^{a}	1.83^{b}	2.81^{ab}	1.93^{b}	0.341	0.04
Mold (log_{10} cfu/g)	<1.00	1.56	<1.00	<1.00	<1.00	-	-
Aerobic stability 2°C (h)	89^{b}	87^{b}	141^{ab}	147^{ab}	207^{a}	13.95	0.009
Trial II							
LAB (log_{10} cfu/g)	5.16^{b}	6.70^{a}	4.71^{b}	3.55^{c}	3.39^{c}	0.335	<0.001
Yeast (log_{10} cfu/g)	4.72^{a}	4.15^{ab}	2.77^{b}	<1.00^{c}	1.05^{c}	0.465	<0.001
Mold (log_{10} cfu/g)	<1.00	<1.00	<1.00	<1.00	<1.00	-	-
Aerobic stability 2°C (h)	52^{b}	52^{b}	67^{b}	131^{ab}	173^{a}	15.78	0.015

[1] cfu = colony forming unit; LAB = lactic acid bacteria; C = untreated control; A0.5 = treatment with chemical additive at 0.5% FM; A1.0 = treatment with chemical additive at 1.0% FM; A1.5 = treatment with chemical additive at 1.5% FM; SE = standard error of the mean;

[2] Means with different letters (a–c) in a row differ significantly (P <0.05).

Table 4. Nutritional parameters of the silages at opening in Trial I and II

Parameters[1]	C	LB	A0.5	A1.0	A1.5	SE	*P*-value
Trial I							
aNDF (g/kg DM)	29.6	32.8	28.8	32.2	29.7	0.619	0.144
ADF (g/kg DM)	15.2	17.1	14.6	17.2	15.6	0.365	0.051
Hemicellulose (g/kg DM)	14.4	15.7	14.2	15.0	14.1	0.279	0.358
CP (g/kg DM)	7.1	7.3	7.3	6.9	6.8	0.075	0.285
Ash (g/kg DM)	3.1	3.1	2.9	3.1	3.0	0.038	0.409
Starch (g/kg DM)	38.6	37.3	38.0	35.6	37.4	0.411	0.208
Trial II							
aNDF (g/kg DM)	29.1	28.6	29.6	31.7	28.5	0.542	0.298
ADF (g/kg DM)	15.6	15.7	16.1	16.8	15.0	0.281	0.369
Hemicellulose (g/kg DM)	13.5	12.9	13.5	15.0	13.5	0.287	0.178
CP (g/kg DM)	7.3	7.5	7.3	7.0	6.7	0.115	0.204
Ash (g/kg DM)	3.1	3.1	3.1	3.3	3.0	0.036	0.201
Starch (g/kg DM)	40.0	40.0	38.9	37.3	39.7	0.518	0.412

[1] ADF = acid detergent fiber; CP = crude protein; DM = dry matter; aNDF = neutral detergent fiber; C = untreated control; A0.5 = treatment with chemical additive at 0.5% FM; A1.0 = treatment with chemical additive at 1.0% FM; A1.5 = treatment with chemical additive at 1.5% FM; SE = standard error of the mean;

[2] Means with different letters (a–c) in a row differ significantly ($P < 0.05$).

Table 5. Fermentative and microbial characteristics of the silages after 7 d of air exposure in Trial I and II

Parameters[1]	C	LB	A0.5	A1.0	A1.5	SE	*P*-value
	Trial I						
DM corrected (g/kg)	403.2	409.8	419.2	426.4	431.7	0.389	0.093
PH	5.32^{b}	6.48^{a}	3.95^{c}	3.80^{c}	3.71^{c}	0.293	<0.001
Lactic acid (g/kg DM)	11.5^{b}	4.0^{b}	33.4^{a}	39.1^{a}	43.7^{a}	4.434	<0.001
Acetic acid (g/kg DM)	4.3	0.7	4.3	5.7	7.6	0.911	0.164
Ethanol (g/kg DM)	0.5	<0.1	2.7	3.5	4.1	0.675	0.216
Propionic acid (g/kg DM)	<0.1^{c}	<0.1^{c}	1.8^{b}	3.6^{a}	4.9^{a}	0.535	<0.001
Butyric acid (g/kg DM)	<0.1^{c}	<0.1^{c}	1.3^{b}	2.5^{a}	3.3^{a}	0.362	<0.001
Yeast (log_{10} cfu/g)	8.76^{a}	8.81^{a}	7.67^{a}	6.02^{ab}	3.30^{b}	0.619	0.002
Mold (log_{10} cfu/g)	1.83	2.67	<1.00	<1.00	1.60	0.495	0.654
DM losses (%)	9.2	5.9	5.6	4.3	0.3	1.52	0.519
	Trial II						
DM corrected (g/kg)	413.4	434.3	426.2	417.8	415.9	0.316	0.215
pH	3.95	4.03	4.05	3.71	3.71	0.059	0.125
Lactic acid (g/kg DM)	36.3^{abc}	32.0^{bc}	30.6^{c}	47.3^{a}	45.2^{ab}	2.180	0.012
Acetic acid (g/kg DM)	7.3	4.3	2.2	10.2	10.2	1.363	0.249
Ethanol (g/kg DM)	4.3	<0.1	<0.1	2.6	1.9	0.787	0.406
Propionic acid (g/kg DM)	0.4^{d}	0.0^{d}	2.0^{c}	3.6^{b}	5.1^{a}	0.592	<0.001
Butyric acid (g/kg DM)	<0.1^{c}	<0.1^{c}	1.1^{b}	2.8^{a}	3.0^{a}	0.384	<0.001
Yeast (log_{10} cfu/g)	7.13^{a}	7.35^{a}	6.67^{a}	4.12^{b}	3.38^{b}	0.491	0.001
Mold (log_{10} cfu/g)	3.28^{ab}	4.79^{a}	<1.00^{c}	<1.00^{c}	<1.00^{c}	0.552	0.006
DM losses (%)	9.3	8.7	8.2	7.0	6.9	1.15	0.969

[1] DM = dry matter; cfu = colony forming unit; C = untreated control; A0.5 = treatment with chemical additive at 0.5% FM; A1.0 = treatment with chemical additive at 1.0% FM; A1.5 = treatment with chemical additive at 1.5% FM; SE = standard error of the mean;

[2] Means with different letters (a–d) in a row differ significantly (P <0.05).

Table 6. Chemical parameters of the silages after 7 d of air exposure in Trial I and II

Parameters[1]	C	LB	A0.5	A1.0	A1.5	SE	*P*-value
	Trial I						
aNDF (g/kg DM)	35.3^{ab}	36.7^{a}	30.1^{c}	30.5^{bc}	31.6^{abc}	0.836	0.017
ADF (g/kg DM)	18.6	19.0	16.0	16.5	17.1	0.405	0.055
Hemicellulose (g/kg DM)	16.8^{a}	17.7^{a}	14.2^{b}	14.1^{b}	14.5^{b}	0.443	0.005
CP (g/kg DM)	7.1	7.7	7.4	7.0	7.0	0.136	0.357
Ash (g/kg DM)	3.4	3.2	3.2	3.2	3.0	0.063	0.491
Starch (g/kg DM)	37.3	37.8	37.0	37.2	35.9	0.414	0.757
	Trial II						
aNDF (g/kg DM)	33.1	32.7	31.1	31.3	32.5	0.561	0.792
ADF (g/kg DM)	18.4	18.2	17.2	16.9	17.9	0.310	0.480
Hemicellulose (g/kg DM)	14.6	14.5	13.9	14.5	14.6	0.271	0.941
CP (g/kg DM)	7.5	7.4	7.2	7.1	7.2	0.888	0.666
Ash (g/kg DM)	3.4	3.4	3.4	3.4	3.4	0.046	0.982
Starch (g/kg DM)	37.3	38.7	38.3	37.6	36.0	0.377	0.197

[1] ADF = acid detergent fiber; CP = crude protein; NDF = neutral detergent fiber; C = untreated control; 0.5% = treatment with chemical additive at 0.5% FM; 1.0% = treatment with chemical additive at 1.0% FM; 1.5% = treatment with chemical additive at 1.5% FM; SE = standard error of the mean;

[2] Means with different letters (a–c) in a row differ significantly (P <0.05). **P* <0.05; ***P* <0.01; ****P* <0.001

NS = not significant.

7.10. Figures

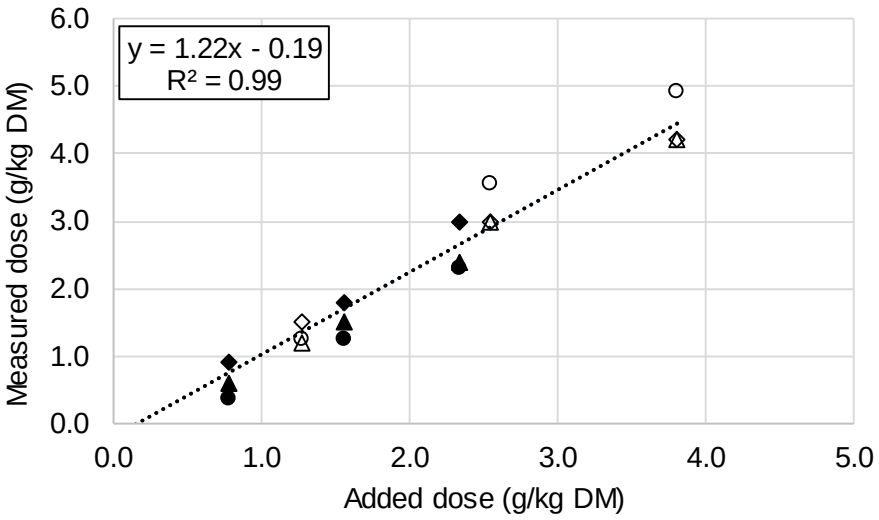

Figure 1. Relationship between the average amount of propionic and butyric acids applied with the additive and the propionic and butyric acid contents determined by means of HPLC at harvesting (mean values of Trials I and II) and at opening in Trials I and II. Butyric acid in black; propionic acid in white. The shapes represent: circle, harvest; rhombus, opening of Trial I; triangle, opening of Trial II.

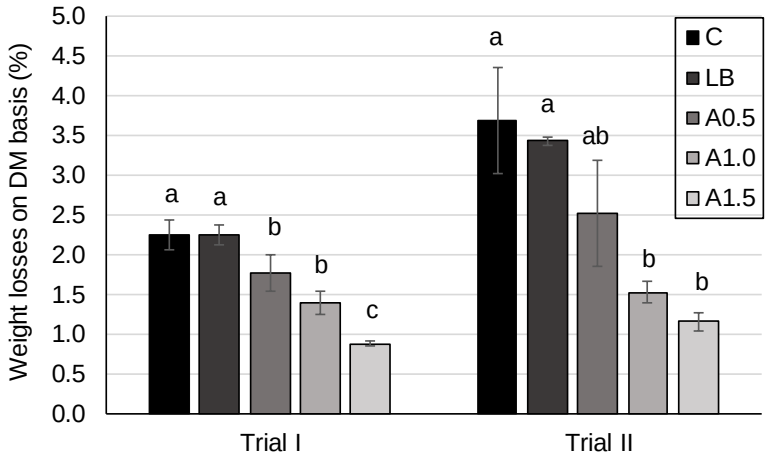

Figure 2. The weight losses on a DM basis in Trials I and II.

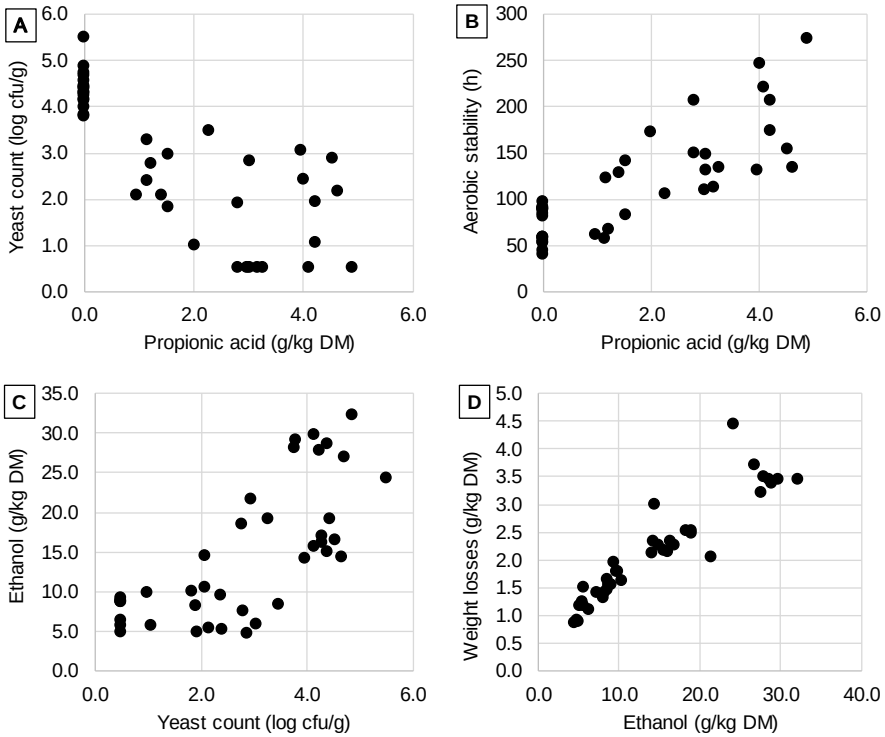

Figure 3. Relationship between propionic acid and the yeast count (A) (R^2 = 0.61, *P*-value <0.001, SE = 0.968), relationship between propionic acid and aerobic stability (B) (R^2 = 0.67, *P*-value <0.001, SE = 33.7), relationship between yeast count and ethanol content (C) (R^2 = 0.52, *P*-value <0.001, SE = 5.96) and relationship between ethanol content and weight losses on a DM basis (D) (R^2 = 0.87, *P*-value <0.001, SE = 0.340).

8. Paper III: Increase in aflatoxins due to *Aspergillus* section *Flavi* multiplication during the aerobic deterioration of corn silage treated with different bacteria inocula

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Increase in aflatoxins due to *Aspergillus* section *Flavi* multiplication during the aerobic deterioration of corn silage treated with different bacteria inocula

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8.1. Abstract

The growth of *Aspergillus flavus* and the production of aflatoxins (AF) during the aerobic deterioration of corn silage represent a problem for animal and human health. This experiment was conducted to evaluate whether the presence of *A. flavus* and AF production originate from the field or additional AF are produced during the fermentation phase or during aerobic deterioration of corn silage. The trial was carried out in Northern Italy on corn at a dry matter (DM) level of 34%. The fresh herbage was either not treated (C) or treated with a *L. buchneri* (LB) NCIMB 40788 [(at $3*10^5$ cfu/g fresh matter (FM)], *L. hilgardii* (LH) CNCM I-4785 (at $3*10^5$ cfu/g FM) or their combination (LB+LH) (at $1.5*10^5$ cfu/g FM of each one), ensiled in 20 L silos and opened after 250 d of ensiling. After silo opening, the aerobic stability was evaluated and samples were taken after 7 and 14 d of air exposure. The pre-ensiled material, the silages at silo opening and the aerobically exposed silages were analyzed for DM content, fermentative profiles, microbial count, nutritive characteristics, DM losses and aflatoxin B_1, B_2, G_1 and G_2 contents. Furthermore, a subsample of colonies with macromorphological features of *A.* section *Flavi* was selected for AF gene pattern characterization and in vitro AF production. The presence of *A. flavus* was below the detection limit (<1.00 $\log_{10}$ cfu/g) in the fresh forage prior to ensiling, whereas it was found in 1 out of 16 silage samples at silo opening at a level of 1.24 $\log_{10}$ cfu/g. The AF were found in both the fresh forage and at opening in all the samples, with a predominance of AFB_2 (mean value of 1.71 μg/kg DM). The inoculation of lactic acid bacteria (LAB) determined a reduction in the lactic-to-acetic ratio compared to the control. A larger amount of acetic acid resulted in a lower yeast count in the LB containing silages and higher aerobic stability in the treated silages than in the control ones. At the beginning of aerobic deterioration, the yeasts increased to over 5 $\log_{10}$ cfu/g, whereas the molds were close to the value observed at silo opening. When the inhibiting conditions were depleted (pH and temperature higher than 5 and 35°C, respectively), both the total molds and *A. flavus* reached higher values than 8.00 and 4.00 $\log_{10}$ cfu/g, respectively, thus determining the *ex-novo* production of AFB_1 during aerobic deterioration, regardless of treatments. The analysis of gene pattern showed that 64% of the selected colonies of *A. flavus* showed the presence of all four AF gene patterns, and 43% of the selected colonies were able to produce aflatoxins in vitro. During air exposure, after 1000°C h have been cumulated, starch content decreased (below 10% DM) and concentration of NDF, ADF, hemicelluloses, CP and ash increased. The inoculation with LB and LB+LH increased the aerobic stability of the silages and delayed the onset of aerobic microbial degradation, which in turn indirectly reduced the risk of *A. flavus* outgrowth and AFB_1 production after silage opening.

Key words: aerobic deterioration; aflatoxin, mycotoxins, silage, fermentation quality.

8.2. Introduction

Producing high-quality and microbiologically safe silage, while avoiding DM losses as much as possible, is a challenge for any livestock producer (Borreani et al., 2018). Aerobic deterioration increases dry matter (DM) losses, reduces the nutritive value of silage and increases risks to animal and human health, due to the growth of pathogenic microorganisms and production of endotoxins and mycotoxins (Pahlow et al., 2003). Mycotoxins are secondary metabolites with low molecular weight which are mainly produced by fungi belonging to the *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* genera (Keller et al., 2013). Mold and mycotoxin contamination of several feed and forages represents an important problem for human and animal health (Driehuis, 2013; Spadaro et al., 2015). Furthermore, this contamination causes the rise of food production costs as the result of the increased need for testing, the lower prices received for contaminated loads, the potential lawsuits from consumers, and the decreased livestock performance (Mitchell et al., 2016). Only a few of the detected mycotoxins in the milk supply chain are responsible for significant changes in food safety, and among these, the most harmful are aflatoxins (**AF**) (Murphy et al., 2006). Aflatoxins can be produced by such species of *Aspergillus* section *Flavi* as *A. flavus* and *A. parasiticus* (Varga et al., 2011). The potential ability of *A. flavus* strains to produce AF has been analyzed in several studies, for example, through the analysis of the production of sclerotia and the presence of aflatoxin biosynthesis gene pathways and the *in vitro* production of AF (Cotty, 1989; Abbas et al., 2005; Criseo et al., 2008). The incidence of toxigenic *A. flavus* from different matrices has been found to range from 50% to 70%. (Nesci and Etcheverry, 2002; Mauro et al., 2013; Prencipe et al., 2018).

The AF that occur naturally are aflatoxin B_1 (**AFB_1**), G_1 (**AFG_1**) and their dihydro derivatives B_2 (**AFB_2**) and G_2 (**AFG_2**). AFB_1 is the most toxic and carcinogenic (IRAC, 2012) and, when it is ingested through contaminated rations to lactating animals, it is in part hydroxylated in the liver to aflatoxin M_1 (**AFM_1**). Aflatoxin M_1 appears in milk within 12 h from ingestion, with a mean carry-over of around 3.5% (Veldman et al., 1992). The World Health Organization (WHO, 2002) evaluated the two maximum concentrations of aflatoxin M_1 that had been proposed by the Codex Committee on Food Additives and Contaminants, 50 and 500 ppt (for EU and USA, respectively), concluding that based on worst-case assumptions, the projected risk of liver cancer attributable to aflatoxin M_1 would be very small if either of these maximum levels were implemented. In order to reduce the risk of AFM_1 contamination, the daily ingestion of AFB_1 should be limited to 40 µg and 400 µg per cow per day for the EU and the USA, respectively (Veldman et al., 1992). Corn silage is the main source of AF in warm regions (i.e. Southwestern US) whereas in temperate ones it is generally less contaminated than other feeds

(e.g. corn grain, peanuts and cottonseed). However, the large use of corn silage on dairy farms through the world imposes the need for a careful management of the factors that could increase AFB_1 contamination in the field or during silage conservation.

Aflatoxins in corn silage can be derived from both the field and produced *ex-novo* during aerobic deterioration (Garon et al., 2006; González-Pereyra et al., 2008; Cavallarin et al., 2011). The pre-harvest contamination of corn crops is well known and has been studied and reviewed by Guo et al. (2008), whereas very few studies have analyzed the post-harvest contamination of corn silage for several reasons, such as the difficulties of recovering AF in the silage matrix (Garon et al., 2006; Cavallarin et al., 2011); the possible increase in concentration of AF due to DM losses during conservation or the feed-out phase; the possible *ex-novo* production by *Aspergillus* during ensiling or the feed-out phase; and the possible degradation or detoxification by lactic acid bacteria (Ahlberg et al., 2015). Furthermore, it is not easy to separate the pre- and post-harvest fungal contamination of whole corn silage, and only a few unclear indications have been reported in literature (Lacey, 1989).

Inoculation with different lactic acid bacteria (LAB) ~~inocula~~ has been used over the years to improve silage fermentation and/or aerobic stability by delaying the development of yeasts and spoilage molds (Muck et al., 2018). Furthermore, it has been documented that LAB can degraded or immobilize aflatoxins during ensiling by binding to their surface (El-Nezami et al., 1998; Oluwafemi et al., 2010; Ma et al., 2017), thereby contribute to improved safety of feed and food. Ogunade et al. (2018) reported that only a few studies have used additives to reduce forage mycotoxin contamination. In one of these studies (Queiroz et al., 2012), the inoculation of corn plants infested with southern rust with a mixture of *Pediococcus pentosaceus* and *Lactobacillus buchneri* at ensiling increased aerobic stability and prevented production of aflatoxins. Some other studies reported that limiting aerobic spoilage by inoculating corn silage with *L. buchneri* can reduce aflatoxin production (Iglesias et al., 2005; Cavallarin et al., 2011). More recently, Ma et al. (2017) reported that, regardless of lactic acid bacterial inoculation, certain silage bacteria can reduce the concentration of AFB_1 that was spiked in corn silage to a safe level within 3 d of ensiling.

Thus, owing to the lack of clear information in literature, the aims of this study were: i) to evaluate whether the presence of *A. flavus* and aflatoxin production in corn silage originates from the field environment or growth of *A. flavus* take place and additional aflatoxins are produced during storage or air exposure after silo opening; ii) to evaluate the effect of different LAB inocula used to improve the aerobic stability of corn silage on reducing *A. flavus* growth and aflatoxin production during fermentation and air exposure; and iii) to characterize the toxigenic potential of *A. flavus* strains isolated from corn silages.

8.3. Materials and methods

8.3.1. Crop and ensiling

The trial was carried out on a commercial farm located in Rocca de' Baldi (CN) in the western Po plain, North West Italy (44°27’18"N, 7°43’19"E, 408 m above sea level). Corn hybrid (P1517W, Pioneer Hi-Bred Italia Srl, Gadesco Pieve Delmona, Cremona, Italy) was sown in April 2015, at an intended planting density of 75,000 seeds/ha. The whole corn crop was harvested at around the 50% milk-line stage and with a DM content of around 34%. Fresh forage was chopped using a precision forage harvester (Claas Jaguar 950, equipped with an 8-row Orbis head, Claas, Harsewinkel, Germany) to a theoretical cutting length of 12 mm. The field was divided in four plots, which were subsequently harvested separately and the crop was chopped in order to obtain four replicates. The fresh herbage of each plot was divided into four 70-kg piles. The piles were either not treated (**C**) or treated with different LAB strains and their combinations. The LAB strains were *L. buchneri* (**LB**) NCIMB 40788 (Lallemand Animal Nutrition, BP 59, Cedex, France) [(theoretical rate of 300,000 cfu/g fresh matter (FM)], *L. hilgardii* (**LH**) CNCM I-4785 (Lallemand Animal Nutrition) (theoretical rate of 300,000 cfu/g FM) and their combination (**LB+LH**) (theoretical rate of 150,000 cfu/g FM of each one). The microbial inoculants were diluted in sterilized water and applied using a hand sprayer, at a rate of 4 ml/kg of forage, by spraying uniformly onto the forage, which was constantly hand mixed. The same amount of water was added to the C treatment. In order to add the targeted amount of LAB, the inocula were plated on MRS agar (Merck, Whitehouse Station, NY), with the addition of natamycin (0.25 g/L) and, on the basis of the measured concentration of LAB, an appropriate amount was used to achieve the desired application rate.

The fresh forage was sampled prior to ensiling after the inoculum had been applied. The untreated and treated forage was then ensiled (11 to 13 kg of wet forage) in 20 L plastic silos equipped with a lid that only enabled the release of gas. The forage was packed by hand, and the final packing densities, on a wet basis, were 627±26 kg FM/m^3. All the laboratory silos were filled within three hours. The silos were weighed, conserved at ambient temperature (20 ± 1°C) and opened after 250 d. At opening, each silo was weighed, and the content was mixed thoroughly and sub-sampled to determine the DM content, the chemical composition, the fermentation profile and the microbial counts. After sampling, the silages were subjected to an aerobic stability test, which involved monitoring the temperature increases due to the microbial activity in the samples exposed to air. About three kilograms from each silo were allowed to aerobically deteriorate at room temperature (20 ± 1°C) in 17 L polystyrene boxes (290 mm diameter and 260 mm height). A single layer of aluminum foil

was placed over each box to prevent drying and dust contamination, but also to allow the air to penetrate. The room and silage temperatures were measured hourly by means of a data logger. Aerobic stability was defined as the number of hours the silage remained stable before its temperature increased by 2°C above room temperature. From silo opening to 14 d of air exposure of silages, peak temperature, hours to reach peak temperature, interval to reach 35°C and time with temperature greater the 35°C were also calculated to better describe the optimum temperature for growth of *A. flavus* in absence of inhibitory conditions. The silage was sampled after 7 d and 14 d of aerobic exposure in order to quantify the chemical, fermentative and microbial changes in the silage during exposure to air, as reported by Tabacco et al. (2011). Other samplings were conducted, on d 21 and d 28, on the silages that did not show any increase in temperature at 14 d of air exposure. The DM losses due to fermentation were calculated as the difference between the weight of the forage placed in each plastic silo 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. The DM losses were calculated after 7 and 14 d of exposure to air using the ash content, as reported by Borreani et al. (2018). Small increases in the ash content of deteriorated silage represent large percentage unit increases in DM loss, as can be seen when the equation for calculating DM losses according to the ash content is used: DM loss (%) = [1 – (ash silage at opening/ash silage after 7 or 14 d of air exposure)] × 100.

8.3.2. Sample preparation and analyses

The pre-ensiled material and the silage were split into five subsamples, at both the opening of the silos and before the exposure to air periods (at 7 and 14 d for all silages, and at 21 and 28 d when needed). One sub-sample was analyzed immediately, for the DM content, by oven drying at 80°C for 24 h. Dry matter was corrected according to Porter and Murray (2001), in order to consider the volatile compound losses that can take place at 80°C. The second subsample was oven-dried at 65°C to a constant weight and was air equilibrated, weighed and ground in a Cyclotec mill (Tecator, Herndon, VA, USA) to pass a 1 mm screen. The dried samples were analyzed for the total nitrogen (TN), according to the Dumas method (method number 992.23, AOAC, 2005), using a Nitrogen analyzer Primacs SN (Skalar, Breda, The Netherlands), for crude protein (CP) (total N x 6.25), for ash by ignition (method number 942.05, AOAC, 2005), and for water soluble carbohydrates (WSC), by the phenol sulphuric acid method, according to DuBois et al. (1956). Neutral detergent fiber (NDF) was analyzed, 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 fiber (ADF) was analyzed and expressed on a DM basis, including residual ash (Robertson and Van Soest, 1981).

A third fresh sub-sample was used to determinate the water activity (a_w), pH, nitrate (NO_3), the ammonia nitrogen (NH_3-N) contents and the buffering capacity. The water activity was measured at 25°C on a fresh sample using an AquaLab Series 3TE (Decagon Devices Inc., Pullman, WA), which adopts the chilled-mirror dew point technique. The fresh forage was extracted for pH, NO_3 and NH_3-N determination, using a Stomacher blender (Seward Ltd, Worthing, UK), for 4 min in distilled water at a 9:1 water-to-sample material (fresh weight) ratio. The total nitrate concentration was determined in the water extract, through semi-quantitative analysis, using Merckoquant test strips (Merck, Darmstadt, Germany; detection limit 100 mg NO_3/kg DM). The ammonia nitrogen content and pH were determined using specific electrodes. The buffering capacity was determined in the water extract, as described by Plaine and McDonald (1966).

A fourth sub-sample was extracted, using a Stomacher blender, for 4 min in H_2SO_4 0.05 mol/L at a 5:1 acid-to-sample material (fresh weight) ratio. An aliquot of 40 ml of silage acid extract was filtered with a 0.20-µm syringe filter and used for quantification of the fermentation products. The lactic and monocarboxylic acids (acetic, propionic and butyric acids) were determined, by means of high performance liquid chromatography (HPLC), in the acid extract (Canale et al., 1984). Ethanol and 1,2-propanediol were determined by means of HPLC, coupled to a refractive index detector, on a Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA).

The fifth subsample was used for the microbial analyses.

8.3.3. Microbial analysis, fungal isolation, macro-morphology and sclerotia production

In order to conduct the microbial counts, 30 g of sample was transferred into a sterile homogenization bag, suspended 1:10 w/v in a peptone salt solution (1 g of bacteriological peptone and 9 g of sodium chloride per liter) and homogenized for 4 min in a laboratory Stomacher blender (Seward Ltd, London, UK). Serial dilutions were prepared, and the mold 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 mold, respectively. The yeast and mold colony forming units (cfu) were enumerated separately, according to their macromorphological features, on plates that yielded 1 to 100 cfu. The LAB were determined on MRS agar 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). Since LAB are facultative anaerobe bacteria, anaerobic incubation was chosen to improve the selectivity of the media against *Bacillus* spp.

A subsample of 5 to 10 colonies per sample, with macromorphological features of *A.* section *Flavi*, was selected for characterization (Table 1). The strains were maintained as a monoconidial culture on 30% glycerol and stored at -80°C. A macro-morphological analysis of each isolate was performed on Yeast Extract Sucrose agar (YES), Malt Extract Autolysate agar (MEA) and Czapek Yeast Autolysate agar (CYA), and observed after 7 days of incubation at 25°C in the dark, according to Samson et al. (2004).

The strains were inoculated on CYA agar and incubated for 14 d at 30°C in the dark to evaluate the sclerotia production (Horn et al., 1996; Horn and Dorner, 1998). The sclerotial size was evaluated by measuring a reticule with a Nikon Eclipse 55i Microscope (Nikon Inc., Garden City, NY, USA) connected to a Samsung color monitor (Samsung Corporation, Seul, Korea), using 10× magnification. The strains were identified as small strains (S) with a sclerotial size <400 µm in diameter, or large strains (L) with sclerotia >400 µm in diameter (Horn, 2003).

8.3.4. Molecular identification

The isolates were grown on Potato Dextrose Broth (27 g PDB, Merck, Germany; 1 L H_2O) and incubated at 28°C in the dark. DNA was extracted from the mycelium using an Omega E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek Inc., Norcross, GA, USA), according to the manufacturer's instructions. Partial amplification of the calmodulin gene was obtained using the cmd5 and cmd6 primer pair (Hong et al., 2006). PCR was carried out in a total volume of 25 µl which contained: 2.5 µl of Buffer 10 X, 0.5 µl of $MgCl_2$, 0.75 µl of dNTPs (10 mM), 1 µl of each primer (10 mM), 0.2 µl of Taq DNA polymerase (Qiagen, Hilden, Germany) and 20 ng of template DNA. A thermal cycling program was performed according to Samson et al. (2014). Amplification was verified by means of electrophoresis on 1% agarose TAE gel, and gel images were acquired using a Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were purified using a QIAquick© PCR purification Kit (Qiagen) and sent to Macrogen, Inc. (Amsterdam, The Netherlands) for sequencing in both directions. The consensus sequences were obtained using the DNA Baser program (Heracle Biosoft S.R.L., Arges, Romania) and compared, using the BLAST program, with those deposited in the RefSeq database of the National Centre for Biotechnology Information (NCBI) for species identification. All the sequences are deposited in the GenBank database with the accession numbers listed in Table 1.

8.3.5. Aflatoxin gene detection and aflatoxin production in vitro

The presence of four genes, three structural genes (*nor-1*, *ver-1* and *omtA*) and one regulatory gene (*aflR*), which were involved in the aflatoxin biosynthesis pathway, and which have been studied extensively and used as a diagnostic tool for the differentiation of aflatoxin producing and non-producing fungi, was verified through a quadruplex PCR assay. Quadruplex PCR was performed using the primers listed in supplementary Table S1. A PCR reaction was carried out in a total volume of 50 µl which contained: 5 µl of Buffer 10 X, 1 µl of $MgCl_2$, 2 µl of dNTPs (10 mM), 1 µl of each primer (10 mM), 0.4 µl of Taq DNA polymerase (Qiagen) and 100 ng of template DNA. A thermal cycling program was performed according to Criseo et al. (2008), with some minor modifications. The PCR products were separated by gel electrophoresis using a TBE buffer with 2% agarose and 5 µl of SYBRSafe® (Invitrogen, Waltham, MA, USA) at 80 V/cm for 4 hours. A Get Pilot 1 kb Plus Ladder (100-10000 bp, Qiagen) was used as a molecular marker and an aflatoxigenic *A. parasiticus* strain AFCAL11 (from the collection of Agroinnova, University of Turin, Italy) was used as positive control. The gel profiles were visualized under UV transilluminator using the Quantity One program (BioRad Labs, Segrate (MI), Italy). The experiment was repeated in triplicate.

The production of aflatoxin was tested for each isolate using a YES Broth medium, according to Visagie et al. (2014): 20 g/L Yeast extract, 150 g/L Sucrose, 0.5 g/L $MgSO_4$ (Merck, Germany) and 1 mL trace elements (1 g $ZnSO_4.7H_2O$ and 0.5 g $CuSO_4.5H_2O$ in 100 mL distilled water). Strains were inoculated with three mycelia plugs from a seven-day-old culture (4 mm diameter) on 50 mL of medium and incubated in the dark at 35°C. After 7 days, the cultures were filtered and extracted to establish the aflatoxin production. The samples were extracted and analyzed according to Prencipe et al. (2018).

8.3.6. Aflatoxin analysis

The aflatoxins were extracted according to the method reported by Cavallarin et al. (2011). Sample extracts were stored at −20°C until HPLC analysis. The HPLC apparatus consisted of a Dionex P680 pump (Dionex, Sunnyvale, CA, USA) equipped with a Rheodyne Model 7725i injection valve (Rheodyne, Rohnert Park, CA, USA), a Dionex RF-2000 fluorimetric detector (λex = 365 nm, λem = 435 nm for AFB1, AFB2, AFG1, AFG2), a Dionex TCC-100 thermostatted column compartment and a Chromeleon®6 data handling system (Dionex). The analytical column was a ProdigyODS 2 (150×4.6 mm, 5 µm particles) (Phenomenex, Torrance, CA, USA), which was preceded by a

SecurityGuard (Phenomenex) guard column.

8.3.7. Statistical analysis

The microbial counts were $\log_{10}$ transformed and were presented on a wet weight basis. The values below the detection limit for yeasts and molds (detection levels: 10 cfu/g of silage) were assigned a value, corresponding to half of the detection limit, in order to calculate the average value.

The data were analyzed for their statistical significance, via analysis of variance, with their significance reported at a 0.05 probability level, using the General Linear Model of the Statistical Package for Social Science (v 24.0, SPSS Inc., Chicago, Illinois, USA). Data were analyzed using the presence or absence of *L. buchneri* (LB+ or LB-) and *L. hilgardii* (LH+ or LH-) as fixed factors, with 4 replications. The used statistical model was as follows: $Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$, where Y_{ijk} = observation, μ = overall mean, α_i = LB effect (i = presence or absence of LB), β_j = LH effect (j = presence or absence of LH), $\alpha\beta_{ij}$ = LB × LH effect, and ε_{ijk} = error. The measured aflatoxins were pooled together for silo opening, 7 d of air exposure and 14 d of air exposure, and were corrected for the DM losses. An unpaired t-test was used to compare the mean values of the measured aflatoxins and DM loss corrected ones.

8.4. Results

8.4.1. Fermentative, chemical and microbial parameters

The chemical and microbial parameters and the AF contamination of the corn forage, prior to ensiling, are summarized in Table 2. The chemical values were typical of corn harvested at the 50% milk-line stage. The DM content was 34% and the NDF, starch and CP were 42.5, 32.3 and 7.8% of DM, respectively. The yeast and mold counts were 6.46 and 5.26 log_{10} cfu/g, respectively. At ensiling, the *A. flavus* count was below the detection limit and the aflatoxins were present with mean values of 0.055, 1.050, 0.095 and 0.044 μg/kg DM for AFB_1, AFB_2, AFG_1 and AFG_2, respectively. Table 3 shows the fermentative parameters after 250 d of conservation at silo opening and after 7 and 14 d of air exposure. All the silages were well fermented with the main acids found at silo opening were lactic and acetic acid, and butyric acid was under the detection limit (0.01 g/kg DM). A slight amount of propionic acid was found in the LB+LH silages. The presence of LB and LH affected the pH, with higher values in the inoculated silages than in the control ones. The LB and LH inocula affected the lactic-to-acetic ratio to a great extent, and this resulted in higher values than 4 in the C silages and values that ranged from 1 and 2 in the treated silages. The LB+LH silages presented about the half of lactic acid and 2.5-fold the acetic acid than the C silage. The 1,2-propanediol was present in all the treated silages and ranged from 7.1 to 12.9 g/kg DM, whereas was not detected in the C silages. After 7 d of air exposure, the lactic acid content decreased and pH increased in all the silages, except for LB+LH treatment. The acetic acid content decreased in C and LH silages, whereas it remained stable in the presence of LB. Ethanol only remained in LB+LH treatment. After 14 days of air exposure, no fermentative products were found in C, LB or LH silages, while 23.4 g/kg DM of lactic acid and 17.9 g/kg DM of acetic acid were found in the LB+LH silages. The aerobic stability, the temperature indices, as observed from silo opening to 14 d of air exposure, DM losses, microbial count and aflatoxin concentrations, after 250 d of conservation at silo opening and after 7 and 14 d of air exposure, are reported in Tables 4 and 5. At opening, C silages showed lower DM losses and LAB count than treated silages. The yeast count was under the detection limit in the treatments containing the LB strain. The aerobic stability was affected by the treatments, with the highest value (365 h) in LB+LH silages. At opening, the mold count was below the detection limit or around 1.00 log_{10} cfu/g in all the silages, and the average *A. flavus* count was under the detection limit in 15 out of 16 silage samples. Only one LH sample showed the presence of *A. flavus*, at a level of 1.24 log_{10} cfu/g. The addition of LB and LH did not affect the AFB_1, AFB_2 or AFG_2 contents, whereas AFG_1 was lower in the treatments containing the LH strains. After 7 d of air exposure,

the yeast count increased in all the silages, with the highest value observed in C silage. The addition of LB and LH affected the mold count, with higher value in C than in the treated silages. Aflatoxin was not influenced by the treatments during air exposure, except for AFG_1 after 7 d in C silage. In 14 d air exposed silages, the *A. flavus* count increased in all the treatments and reached 3.32 log_{10} cfu/g in LB silages. Aflatoxin G_1 was not detected after 14 d of air exposure in any treatment, and the other aflatoxins did not show any significant difference between treatments. After 14 d of air exposure, 2 out of 4 LB+LH samples were still aerobically stable, with the *A. flavus* count below the detection limit. These samples were left to deteriorate and were sampled after 21 and 28 d. The average *A. flavus* count in these samples was 1.33 log_{10} cfu/g and 2.74 log_{10} cfu/g after 21 and 28 d, respectively. During air exposure, when the cumulated hourly difference between silage and ambient temperatures (dT) reached 1000°C h, the yeast had completely depleted the fermentative products, regardless of the treatment and LAB inoculum, causing a rise in silage pH, which was greater than 4 (with most of the values ranging from 5.5 to 7), and in the silage temperatures, which were around 30 to 40°C (Figure 1). A cumulated 1000°C h dT was reached at different times during aerobic exposure, the lowest being for C silages after 176 h and the highest for LB+HB silages after 484 h. When all the inhibiting conditions (pH and the presence of undissociated organic acids) were depleted, the total molds and *A. flavus* quickly started to increase (Figure 2). The DM losses in all the silages increased during exposure to air and reached the highest value in C silage and the lowest in LB+LH silage after 14 d. Since the actual concentration of AF in the silage samples should take into account in the amount of DM losses, Figure 3 reports the AFB_1, AFB_2, AFG_1, and AFG_2 concentrations from ensiling to the end of conservation and after 7 and 14 d of air exposure, for both the potential amount of aflatoxins derived from the field, corrected for DM losses during conservation and air exposure, hypothesizing no new biosynthesis or degradation, and their actual measured contents. The AFB_1 increased during silage conservation and during air exposure, with higher values observed after 14 d of conservation, whereas AFB_2 did not increase significantly during conservation, compared to its increase due to DM losses. On the other hand, the AFG_1 content decreased during conservation and the subsequent exposure to air. Aflatoxin G_2 was not affected by the ensiling process or aerobic deterioration, even though it apparently increased when the DM losses were not taken into account.

The nutritional analyses after 250 d of conservation at silo opening and after 7 and 14 d of air exposure are reported in Table 6. At opening, no differences between treatments were found among the studied nutritional parameters. After 7 day of air exposure, a slight concentration of the starch was observed in C, LB, and LH silages, as well as a reduction in NDF. The crude protein increased after 14 d of exposure to air. After 1000°C h, when mold activity was evident, the starch content, corrected for DM losses, decreased greatly, until reaching values below 10% on DM (Figure 1). After 14 d of exposure to air, a concentration of NDF, ADF, hemicelluloses, CP and ash was observed, and this

was more evident in C, LB and LH silages than in LB+LH silages.

8.4.2. Strain identification, macro-morphology and sclerotia production

A representative subsample of the isolated strains of *Aspergillus* section *Flavi* obtained from silage at opening, after 7 d of air exposure and after 14 d of air exposure, was used for the analysis. A dataset of 532 bp for the partial sequence of the calmodulin gene was obtained for each strain. The majority of isolates (14/20) resulted to belong to the *A. flavus* species, while the other isolates (6/20) were identified as *A. oryzae* var. *effusus*, and they showed 100% identity with the RefSeq deposit in GenBank (Table 1). A second subsample of *Aspergillus* section *Flavi*, obtained from silage after 21 d of exposure to air (4 isolates) and after 28 d of exposure to air (4 isolates), was used for the analysis (data not shown). All 4 isolates from the LB+LH samples at 21 d of exposure to air belonged to the *A. oryzae* var. *effusus* species (9026/1, 9026/2, 9026/3 and 9026/4). After 28 d of exposure to air, 2 out of 4 isolates were identified as *A. flavus* (9031/1 and 9031/2) and 2 as *A. oryzae* var. *effusus* (9031/3 and 9031/4).

The typical morphology of *A.* section *Flavi* was observed, with yellow to green conidia, as well as a mean diameter of 40.7, 37.6 and 35.0 mm for YES, MEA and CYA, respectively. The *A. oryzae* var. *effusus* strains showed poor sporulation, with a white surface and irregular margins, as well as a mean diameter of 40.9, 37.5 and 35.2 mm for YES, MEA and CYA, respectively (Table 1).

Nine out of fourteen *A. flavus* produced sclerotia, while none of the strains of *A. oryzae* var. *effusus* were able to produce them (Table 1). The 8931/2, 9010 and 9016 strains produced both S and L sclerotia, the 8931/7, 9015/1 and 9015/2 strains produced L sclerotia, while the 9002, 9005 and 9006 strains produced S sclerotia. The 8959, 8931/3, 9012 and 9015/3 strains did not produce any sclerotia.

8.4.3. Aflatoxin production in vitro and aflatoxin gene detection

After 14 d of incubation at 35°C in the dark on YES, 6 out 14 *A. flavus* were able to produce AFB_1, whereas not one of the *A. oryzae* var. *effusus* strains seemed to be able to produce them (Table 1). Quadruplex PCR for gene detection produced the expected size amplicons in the positive control, with around 1000 bp, 800 bp, 500 bp and 400 bp for the *aflR*, *omt-A*, *ver-1* and *nor-1*

genes, respectively (Figure 4). All the aflatoxigenic *A. flavus* strains showed a complete molecular pattern with the four analyzed genes. The non-aflatoxigenic *A. flavus* strains showed different banding pattern results. No DNA amplification was found for the *A. flavus* 8959, 9015/1, 9015/2 and 9015/3 strains for any of the genes. As far as the *A. oryzae* var. *effusus* strains are concerned, quadruplex PCR showed three banding patterns: strains 8976, 8931/1 and 8931/4 with 2 bands, corresponding to the *ver-1* and *nor-1* genes, a second group with three bands corresponding to *aflR*, *ver-1* and *nor-1* amplification (8931/5 and 8931/6 strains) and one strain (9004) with the complete pattern.

8.5. Discussion

In light of the potential risk of mycotoxin contamination, it has been considered important to obtain information about the type and distribution of AF in corn silage, which is the main source of forage for lactating dairy cows in Europe and North America. Furthermore, a possible effect of different LAB inocula on the growth of spoilage molds has also been evaluated. The longer the anaerobic phase of silage is, the greater the reduction in yeast and mold counts (Borreani et al., 2014). This implicates that silage is not a favorable environment for mold development during conservation, if anaerobiosis is maintained (Borreani et al., 2018). In the present experiment, the presence of *A. flavus* has been found to be below the detection limit (<1.00 $\log_{10}$ cfu/g) in the fresh forage prior to ensiling, in which the mold count was observed to be higher than 5 $\log_{10}$ cfu/g. At silo opening, after a long ensiling period (250 d), the mold count was around or below 1.00 $\log_{10}$ cfu/g of silage, whereas *A. flavus* was found in 1 out of 16 silage samples (1.24 $\log_{10}$ cfu/g, which represents around 45% of the total molds of that sample). This could suggest that *A. flavus* was already present in the field and that it survived after the anaerobic conservation period, whereas many other mold species did not. This hypothesis is also supported by the presence of AF in the herbage prior to ensiling, which means that *A. flavus* must have developed on the crop during the growing cycle, albeit at a low level, and synthesized AF. Over the last decade, the Po plain environment has been characterized by warmer and drier summers that in the previous decades, and these conditions could have favored the development of *A. flavus* on the corn crops and AF synthesis. This could explain the increased frequency of AFB_1 contamination observed on corn grain and silages in the last few years (Decastelli et al., 2007; Anfossi et al., 2009). The presence of *A. flavus* on corn crops at a low level, at our latitudes, could be explained by the occurrence, of some periods with higher ambient temperatures than 35°C in summer, as this is the optimal growth temperature for the fungus, and these increased temperatures generally cause it to be the predominant species in tropical and subtropical climates (Cheli et al., 2013). In this regard, Gonzales and Pereira (2008) found *A. flavus* as the predominant species, followed by *A. fumigatus* and *A. niger*, in different farm corn silages in the tropical environment of Central Argentina. Keller et al. (2013) found *A. flavus* as the predominant *Aspergillus* species, at both ensiling and at silo opening, in Brazil and El-Shanawany et al. (2005), in a farm survey, found *A. flavus* as the dominant species in corn silage in Egypt. On the other hand, Garon et al. (2006) and Spadaro et al. (2015) did not find *A. flavus* in corn silage in the temperate climates of France or northern Italy, but instead reported the presence of *A. parasiticus* and *A. fumigatus*. However, Richard et al. (2007) detected *A. flavus* in farm corn silage in France after eleven months of conservation. This indicates that the fungus is able to survive during the anaerobic conservation phase of

corn silage, albeit at a low level, and could grow when the environmental conditions become more suitable for its growth, such as during the feed-out phase, or in peripheral areas of the silage, where temperatures increase as a consequence of a deteriorating microflora activity, which depletes the acidic conditions.

In the present experiment, even though *A. flavus* was below the detection limit in the fresh forage prior to ensiling and it was present at a low level at silage opening, aflatoxins were found in both the fresh forage and at opening in all the samples, with a predominance of AFB_2. The presence of AF has been found, at silo opening, in different countries of the world, as reviewed by Alonso et al. (2013), with higher concentrations in warmer climates (Carvalho et al., 2016; Ogunade et al., 2018) than in colder ones (Driehuis et al., 2008; Cavallarin et al., 2011).

In our experiment, the inoculation with heterolactic bacteria, which was aimed at improving the aerobic stability of silage during the feed-out phase, and the long ensiling duration (250 d) influenced the fermentative profile of the silages, with a dominant homolactic fermentation (higher lactic-to-acetic ratio than 4) in the control silages and a heterolactic fermentation (lower lactic-to-acetic ratio than 2) in LB and LH treated silages, as previously reported by Kleinschmit and Kung (2006). The 1,2-propanediol was found in LB silages, as previously reported by Oude Elferink et al. (2001), and in LH silages, in agreement with the results of Assis et al. (2014). The fermentation process, combined with the longer ensiling duration, greatly reduced the yeast and mold count compared to those observed at harvesting. Furthermore, the inocula containing LB determined a reduction in yeast to below the detection limit, as reported by Kleinschmit and Kung (2006). The use of heterolactic inocula determines greater DM losses during fermentation, and greater DM losses could determine a higher concentration of non-degraded components (e.g. ashes). Therefore, in order to obtain a better understanding of the fate of the different aflatoxins in silage, due to the fact that they could be produced both in the field and during ensiling, and at the same time could be degraded or bound by LAB microbial activity (Oluwafemi et al., 2010) or bound by other silage bacteria (Ma et al., 2017), it is important to keep in mind that they could concentrate in silage as a consequence of occurring DM losses. This is evident in Figure 3, where it appears that the AFB_1 increased slightly during the fermentation process and increased significantly at 7 and 14 d of exposure to air. Aflatoxin B_2 and AFG_2 were not affected by the anaerobic fermentation phase or by the subsequent exposure to air, and their higher concentration in deteriorated silages could mainly be attributed to the DM losses. On the other hand, AFG_1 was partially degraded during the ensiling process and almost completely disappeared after 14 d of air exposure. Cavallarin et al. (2011) analyzed the presence of AF in both fresh forage and after ensiling in silage stored under different plastic films and, during feed out, they found that AF were absent in the center of the bunker silos, while they were present with values of up to 6 µg/kg DM in the top layer of the bunker. In contrast to the results of the present

study and those of Cavallarin et al. (2011), Garon et al. (2006) observed, in farm-scale silos, a decrease in the AFB_1 content as the ensiling duration increased. This could be attributable to a detoxification effect during fermentation, as reported by Oluwafemi et al. (2010), who observed that some strains of LAB are able to partially degrade AFB_1 in corn grain via a biological pathway, with a reduction range from 31 to 46%. Ahlberg et al. (2015), reviewing the ability of different LAB species and strains to bind aflatoxins in different food matrices, reported binding effects ranging from 0 to 90%, but this review did not report any research results concerning inoculation trials with *L. buchneri* or *L. hilgardii*. Ma et al. (2017) reported the capacity of binding AFB1 in vitro by some strains of lactic acid bacteria when applied at 10^9 cfu/mL (*L. plantarum*, *L. buchneri*, and *Pediococcus acidilactici*), but they failed to find the same effect on corn silage artificially contaminated with AFB_1, even if they concluded that some silage bacteria could have reduced the AFB_1 to a safe content within 3 d of ensiling, regardless of LAB inoculation. In the present experiment, even if the conservation period was 250 d long, aflatoxin concentrations did not decrease compared to aflatoxin present in the fresh forage.

One of the most relevant issues of corn silage at a world level is its proneness to aerobic deterioration, which negatively influences the silage nutritive value, animal performance, and farm profitability (Borreani et al., 2018). In order to better understand the role of air on the reduction of the silage nutritional value and on the increase in DM losses during aerobic deterioration, silages were left to deteriorate in laboratory trials for up to 14 d. This time period was chosen to represent the average age of silages in the peripheral areas of farm bunker silos at risk to exposure to air when a feed-out rate of 0.7 to 1.4 m/wk is adopted (Borreani and Tabacco, 2008; Weinberg et al., 2009), which results in a potential aerobic exposure of the silage of 20 to 10 d. In our experiment, the inocula improved aerobic stability compared to the control, and this is in agreement with previous studies about the efficiency of *L. buchneri* (Arriola et al., 2011; Comino et al., 2014) and *L. hilgardii* (Assis et al., 2014). In agreement with the findings reported by Tabacco et al. (2011), the deteriorated silages of the present study showed an increase in the yeast and mold counts and a reduction of their nutritive value, with a substantial decrease in starch and an increase in the fiber and ash concentrations.

At the beginning of aerobic deterioration, the mold count values were close to those observed at silo opening, whereas the yeasts increased to higher values than 5 $\log_{10}$ cfu/g; the developing yeast depleted the fermentative products (first the ethanol and then the acetic and lactic acid) and determined an increase in the pH and silage temperature (Figure 1). Irrespective of the treatment, when the hourly cumulated temperature reached 1000°C, all the inhibiting conditions for mold growth (undissociated organic acids) were depleted, and the mean pH was 6.19 ± 0.62. At the same time, the silage temperature increased to over 35°C (with an average value of 38.7 ± 6.3°C). These lowered inhibiting conditions allowed the growth of both the total molds and *A. flavus*, which reached higher

values than 8.00 and 4.00 log_{10} cfu/g, respectively. Those conditions were observed in both the control and LAB treated silages after about 70 hours from the time at which the temperature started to rise above the ambient temperature (data not shown), with LB and LH treated silage showing a longer period of aerobic stability. This means that all the silages (both the control and LAB inoculated ones) are prone to the development of *A. flavus* during their exposure to air, and this development takes place when silage stability has already been compromised (pH and temperature higher than 5 and 35°C, respectively). Cavallarin et al. (2011) provided evidence of aflatoxin accumulation in whole crop corn silage as a result of aerobic deterioration, which had previously been hypothesized by other authors throughout the world, who had found higher contaminations of AF in peripheral areas of commercial silos, which are known to be the most prone to aerobic deterioration (Rosiles, 1978; Richard et al., 2009). The results of this experiment have also shown that the use of LAB inocula, which are able to shift silage fermentation toward a more heterolactic pathway, could delay the onset of aerobic deterioration after exposure to air of the silage, and, as a consequence, could stave off *A. flavus* development and aflatoxin synthesis. Results are in agreement with data of Queiroz et al. (2012) who found that when corn infested with southern rust was inoculated with a mixture of *Pediococcus pentosaceus* and *L. buchneri* at ensiling, its aerobic stability increased and the production of aflatoxins was prevented compared to an untreated control. Results are also in agreement with Cavallarin et al. (2011) who reported that inoculation with *L. buchneri* delayed the onset of aerobic deterioration and the synthesis of aflatoxins. In the present experiment we also observed, as previously reported by Cavallarin et al. (2011) that, when deterioration have took place, the total aflatoxin concentration in *L. buchneri* treated silages increased more than in the control or in *L. plantarum* inoculated silage.

The environment resulting from aerobic deterioration allowed *A. flavus* to grow and AFB_1, which was *ex-novo* produced during ensiling and air exposure, to increase. In order to verify the aflatoxigenic potential of the selected colonies of *A. flavus*, four genes involved in the aflatoxin biosynthesis pathway were analyzed through a quadrupex PCR, and an *in vitro* assay was performed. Nine out of 14 strains of *A. flavus* showed the presence of the complete gene pattern and, of these strains, 6 were able to produce aflatoxins. According to Criseo et al. (2001), some atoxigenic strains could have a quadruplet pattern, and other markers are necessary to discriminate aflatoxigenic strains from atoxigenic ones. Furthermore, the occurrence of S sclerotia was positively related to the production of AF *in vitro*, as previously reported by Cotty (1989). On the other hand, some *A. flavus* strains isolated from the corn silage of this study did not reveal the presence of the complete AF gene pattern and were unable to produce AF *in vitro*. This observation could be a starting point for future investigations on the selection of atoxigenic *A. flavus* to occupy the same niche as the naturally occurring epiphytic toxigenic *A. flavus*, as recently proposed as a strategy to reduce AF contamination in the field (Mauro et al., 2018).

During aerobic deterioration, the molds also degraded starch and part of the hemicelluloses, thus contributing to an increase of the less degradable part of NDF. This was previously described by Tabacco et al. (2011), who reported that when molds started to grow, the nutritional value of the silages decreased, with a strong reduction in the starch content, which represents the main energy component of corn silage, an increase in NDF and a reduction in its degradability.

8.6. Conclusions

Ensiling corn that was naturally contaminated by aflatoxin did not change its aflatoxin concentration at the end of a long conservation period. A. flavus showed to be able to survive in the anaerobic silage and revive when the inhibiting conditions in terms of low pH and temperature were lost. Both the control and inoculated silages deteriorated during aerobic exposure and showed an increase in the A. flavus count and AFB1 concentration. Inoculation with LB and LB+LH increased the aerobic stability of the silages and delayed the onset of aerobic microbial degradation, and this in turn could indirectly reduce the risk of A. flavus outgrowth and AFB1 production after silage opening.

8.7. Acknowledgments

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information, and does not imply either recommendation or endorsement by the University of Turin, Italy.

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8.8. References

Abbas, H. K., M. A. Weaver, R. M. Zablotowicz, B. W. Horn, and W. T. Shier. 2005. Relationships between aflatoxin production and sclerotia formation among isolates of *Aspergillus* section *Flavi* from the Mississippi Delta. Eur. J. Plant Pathol. 112: 283-287.

Ahlberg, S. H., V. Joutsjoki, and H. J. Korhonen. 2015. Potential of lactic acid bacteria in aflatoxin risk mitigation. Int. J. Food Microbiol. 207: 87-102.

Alonso, V. A., C. M. Pereyra, L. A. M. Keller, A. M. Dalcero, C. A. R. Rosa, S. M. Chiacchiera, and L. R. Cavaglieri. 2013. Fungi and mycotoxin in silage: an

overview. J. Appl. Microbiol. 115: 637-643.
Anfossi, L., C. Baggiani, C. Giovannoli, G. D'Arco, C. Passini, and G. Giraudi. 2012. Occurrence of aflatoxin M1 in Italian cheese: results of a survey conducted in 2010 and correlation with manufacturing, production season, milking animals, and maturation of cheese. Food Control 25: 125-130.
AOAC International, 2005. Official Methods of Analysis. 18th ed. Gaithersburg, MD: AOAC International.
Arriola, K. G., S. C. Kim, and A. T. Adesogan. 2011. Effect of applying inoculants with heterolactic or homolactic and heterolactic bacteria on the fermentation and quality of corn silage. J. Dairy Sci. 94: 1511-1516.
Assis, F. G. D., C. L. D. Avila, J. C. Pinto, and R. F. Schwan. 2014. New inoculants on maize silage fermentation. Rev. Bras. Zoot. 43: 395-403.
Borreani G., S. Piano, and E. Tabacco. 2014. Aerobic stability of maize silage stored under plastic films with different oxygen permeability. J. Sci. Food Agric. 94: 2684-2690.
Borreani, G., and E. Tabacco. 2008. Low permeability to oxygen of a new barrier film prevents butyric acid bacteria spore formation in farm corn silage. J. Dairy Sci. 91:4272–4281.
Borreani, G., and E. Tabacco. 2010. The relationship of silage temperature with the microbiological status of the face of corn silage bunkers. J. Dairy Sci. 93: 2620-2629.
Borreani, G., Tabacco, E., Schmidt, R. J., Holmes, B. J., and R. E. Muck. 2018. *Silage review:* Factors affecting dry matter and quality losses in silages. J. Dairy Sci. 101**:** 3952–3979.
Canale, A., M. E. Valente, and A. Ciotti. 1984. Determination of volatile carboxylic acids (C1-C5) and lactic acid in aqueous acid extracts of silage by high performance liquid chromatography. J. Sci. Food Agric. 35: 1178-1182.
Carvalho B. F., C. L. S. Avila, P. M. Krempser, L. R. Batista, M. N. Pereira, and R. F. Schwan. 2016. Occurrence of mycotoxins and yeasts and moulds identification in corn silages in tropical climate. J. Appl. Microbiol. 120:1181-1192.
Cavallarin, L., E. Tabacco, S. Antoniazzi, and G. Borreani. 2011. Aflatoxin accumulation in whole crop maize silage as a result of aerobic exposure. J. Sci. Food Agric. 91: 2419-2425.
Cheli, F., A. Campagnoli, and V. Dell'Orto. 2013. Fungal populations and mycotoxins in silages: from occurrence to analysis. Anim. Feed Sci. Technol. 183: 1-16.
Comino, L., E. Tabacco, F. Righi, A. Revello-Chion, A. Quarantelli, and G. Borreani. 2014. 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. Anim. Feed Sci. Technol. 198: 94-106.
Cotty, P. J. 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathology 79: 808-814.
Criseo G., A. Bagnara, and G. Bisignano. 2001. Differentiation of aflatoxin-producing and non-producing strains of *Aspergillus flavus* group. Lett. App. Microbiol. 33: 291-295.
Criseo, G., C. Racco, and O. Romeo. 2008. High genetic variability in non-aflatoxigenic *A. flavus* strains by using Quadruplex PCR-based assay. Int. J. Food Microbiol. 125: 341-343.
Decastelli, L., J. Lai, M. Gramaglia, A. Monaco, C. Nachtmann, F. Oldano, M. Ruffier, A. Sezian, and C. Bandirola. 2007. Aflatoxins occurrence in milk and feed in

Northern Italy during 2004–2005. Food Control 18: 1263-1266.
Driehuis, F., M. C., Spanjer, J. M., Scholten, and M. C. Te Giffel. 2008. Occurrence of mycotoxins in maize, grass and wheat silage for dairy cattle in the Netherlands. Food Addit. Contam. Part B Surveill. 1: 41–50.
Driehuis, F. 2013. Silage and the safety and quality of dairy foods: a review. Agric. Food Sci. 22: 16-34.
Driehuis, F. M., C. Spanjer, J. M. Scholten, and M. C. te Giffel. 2008. Occurrence of mycotoxins in feedstuffs of dairy cows and estimation of total dietary intakes. J. Dairy Sci. 91: 4261-4271.
DuBois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350-356.
El-Shanawany, A. A., M. Eman Mostafa, and A. Barakat. 2005. Fungal populations and mycotoxins in silage in Assiut and Sohag governorates in Egypt, with a special reference to characteristic Aspergilli toxins. Mycopathologia 159: 281-289.
El-Nezami, H., P. Kankaanpää, S. Salminen, and J. Ahokas. 1998. Physico-chemical alterations enhance the ability of dairy strains of lactic acid bacteria to remove aflatoxin from contaminated media. J. Food Prot. 61: 466-68.
Garon, D, E. Richard, L. Sage, V. Bouchart, D. Pottier, and P. Lebailly. 2006. Mycoflora and multimycotoxin detection in corn silages: experimental study. J. Agric. Food Chem. 54: 3479-3484.
González-Pereyra, M. L., V. A. Alonso, R. Sager, M. B. Morlaco, C. E. Magnoli, A. L. Astoreca, C. A. daRosa, S. M. Chiacchiera, A. M. Dalcero, and L. R. Cavaglieri. 2008. Fungi and select mycotoxins from pre- and post- fermented corn silage. J. Appl. Microbiol. 104: 1034-1041.
Guo, B., Z. Y. Chen, R. D. Lee, and B. T. Scully. 2008. Drought stress and preharvest aflatoxin contamination in agricultural commodity: genetics, genomics and proteomics. J. Integr. Plant Biol. 50: 1281-1291.
Hong, S. B, H. S. Cho, H. D. Shin, J. C. Frisvad, and R. A. Samson. 2006. Novel *Neosartorya* species isolated from soil in Korea. Int. J. System. Evolut. Microbiol. 56: 477-486.
Horn, B. W. 2003. Ecology and population biology of aflatoxigenic fungi in soil. J. Toxicology-Toxins Rev. 22: 355-383.
Horn, B. W., and J. W. Dorner. 1998. Soil populations of *Aspergillus* species from section *Flavi* along a transect through peanut-growing regions of the United States. Mycologia 90: 767-776.
Horn, B. W., R. L. Greene, V. S. Sobolev, J. W. Dorner, J. H. Powell, and R. C. Layton. 1996. Association of morphology and mycotoxin production with vegetative compatibility groups in *A. flavus, A. parasiticus,* and *A. tamarii*. Mycologia 88: 574-587.
Iglesias, C., A. Bach, M. Devant, C. Adelantado, and M. A. Calvo. 2005. The effect of *Lactobacillus buchneri* inoculation on corn silages conservation. Pages 611–613 in Proc. XI Jornadas sobre Producción Animal. Gobierno de Aragón, Servicio de Investigación Agroalimentaria, Zaragoza, Spain.
IRAC, 2012. Agents Classified by the IRAC Monograph. Vol. 1 and 104. International Agency for Research on Cancer. Accessed Mar, 05, 2018. http://monographs.iarc.fr/ENG/Classification/index.php.
Keller, L. A. M., M. L. González Pereyra, K. M. Keller, V. A. Alonso, A. A. Oliveira, T. X. Almeida, T. S. Barbosa, L. M. T. Nunes, L. R. Cavaglieri, and C. A. R., Rosa. 2013. Fungal and mycotoxins contamination in corn silage: monitoring

risk before and after fermentation. J. Stored. Prod. Res. 52: 42-47.
Kleinschmit, D. H., and L. Kung Jr. 2006. A meta-analysis of the effects of *Lactobacillus buchneri* on the fermentation and aerobic stability of corn and grass and small-grain silages. J. Dairy Sci. 89: 4005-4013.
Lacey, J. 1989. Pre- and post-harvest ecology of fungi causing spoilage of food and other stored products. J. Appl. Bacteriol. Symp. Suppl. 67: 11-25.
Ma, Z. X., F. X. Amaro, J. J. Romero, O. G. Pereira, K. C. Jeong, and A. T. Adesogan. 2017. The capacity of silage inoculant bacteria to bind aflatoxin B_1 in vitro and in artificially contaminated corn silage. J. Dairy Sci. 100: 7198-7210.
Mauro, A., P. Battilani, K.A. Callicott, P. Giorni, A. Pietri, and P. J. Cotty. 2013. Structure of an *Aspergillus flavus* population from maize kernels in northern Italy. Int. J. Food Microbiol. 162: 1-7.
Mauro, A., E. Garcia-Cela, A. Pietri, P.J. Cotty, and P. Battilani. 2018. Biological control products for aflatoxin prevention in Italy: commercial field evaluation of atoxigenic *Aspergillus flavus* active ingredients. Toxins 10: 30.
Mitchell, N. J., E. Bowers, C. Hurburgh, and F. Wu. 2016. Potential economic losses to the US corn industry from aflatoxin contamination. Food Addit. Contam. 33: 540-550.
Muck, R. E., E. M. G. Nadeau, T. A. McAllister, F. E. Contreras-Govea, M. C. Santos, and L. Kung Jr. 2018. *Silage review*: Recent advances and future uses of silage additives. J. Dairy Sci. 101: 3980-4000.
Murphy, P. A., S. Hendrich, C. Landgren, and C. M. Bryant. 2006. Food mycotoxins: an update. Food Sci. 71: 51-65.
Nesci, A., and M. Etcheverry. 2002. *Aspergillus* section *Flavi* populations from field maize in Argentina. Lett. Appl. Microbiol. 34: 343-348.
Ogunade, I. M., Martinez-Tuppia, C., Queiroz, O. C. M., Jiang, Y., Drouin, P., Wu, F., Vyas, D., and A. T. Adesogan. 2018. *Silage review*: Mycotoxins in silage: Occurrence, effects, prevention, and mitigation. J. Dairy Sci. 101:4034-4059.
Oluwafemi, F., M. Kumar, R. Bandyopadhyay, T. Ogunbanwo, and K. B. Ayanwande. 2010. Bio-detoxification of aflatoxin B1 in artificially contaminated maize grains using lactic acid bacteria. Toxin Rev. 29: 115-122.
Oude Elferink, S. J. W. H., J. Krooneman, J. C. Gottschal, S. F. Spoelstra, F. Faber, and F. Driehuis. 2001. Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. Appl. Environ. Microbiol. 67: 125-132.
Pahlow, G., R. E. Muck, F. Driehuis, S. J. W. H. Oude Elferink, and S. F. Spoelstra. 2003. Microbiology of ensiling. Pages 31-93 in Silage Science and Technology (Agronomy Series No. 42). Buxton D. R., R. E. Muck, and H. J. Harrison, ed. American Society of Agronomy, Madison, WI.
Playne, M. J., and P. McDonald. 1966. The buffering constituents of herbage and of silage. J. Sci. Food Agric. 17: 264-268.
Porter, M. G., and R. S. Murray. 2001. The volatility of components of grass silage on oven drying and the inter-relationship between dry-matter content estimated by different analytical methods. Grass Forage Sci. 56: 405-411.
Prencipe, S., I. Siciliano, C. Contessa, R. Botta, A. Garibaldi, M. L. Gullino, and D. Spadaro. 2018. Characterization of *Aspergillus* section *Flavi* isolated from fresh chestnuts and the chestnut flour process. Food Microbiol. 69: 159-169.
Queiroz, O. C. M., S. C. Kim, and A. T. Adesogan. 2012. Effect of treatment with a mixture of bacteria and fibrolytic enzymes on the quality and safety of corn silage infested with different levels of rust. J. Dairy Sci. 95: 5285-5291.
Richard, E., N. Heutte, L. Sage, D. Pottier, V. Bouchart, P. Lebailly, and D. Garon.

2007. Toxigenic fungi and mycotoxins in mature corn silage. Food Chem. Toxicol. 45: 2420-2425.

Richard, E., N. Heutte, V. Bouchart, and D. Garon. 2009. Evaluation of fungal contamination and mycotoxin production in maize silage. Anim. Feed Sci. Technol. 148: 309-320.

Robertson, J. B., and P. J. Van Soest. 1981. The detergent system of analysis and its application to human foods. Pages 123-158 in The Analysis of Dietary Fiber in Food. W. P. T. James and O. Theander, ed. Marcel Dekker, New York, NY.

Rosiles, R. M. 1978. Estudio de las Aflatoxinas en ensilado de maiz. (A study of aflatoxins in ensiled corn.) Veterinaria (Mexico). 9: 163-167.

Samson, R. A., C. M. Visagie, and J. Houbraken. 2014. Phylogeny, identification and nomenclature of the genus *Aspergillus*. Stud. Mycol. 78: 141-173.

Samson, R. A., E. S. Hoekstra, and J. C. Frisvad. 2004. Introduction to food and airborne fungi. seventh ed. Utrecht: Centraalbureau voor Schimmelcultures (CBS).

Spadaro, D., M. P. Bustos-Lopez, M. L. Gullino, S. Piano, E. Tabacco, and G. Borreani. 2015. Evolution of fungal population in corn silage conserved under polyethylene or biodegradable films. J. Appl. Microbiol. 119: 510-520.

Spoelstra, S. F., M. G. Courtin, and J. A. C. van Beers. 1988. Acetic acid bacteria can initiate aerobic deterioration of whole crop maize silage. J. Agric. Sci. 111: 127-132.

Tabacco, E., F. Righi, A. Quarantelli, and G. Borreani. 2011. Dry matter and nutritional losses during aerobic deterioration of corn and sorghum silages as influenced by different lactic acid bacteria inocula. J. Dairy Sci. 94: 1409-1419.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods of dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74: 3583-3597.

Varga, J., J. C. Frisvad, and R. A. Samson. 2011. Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. Stud. Mycol. 69: 57–80.

Veldman, A., J. A. C. Meijst, G. J. Borggreve, and J. J. Heeres-van der Tol. 1992. Carry-over of aflatoxin from cows'food to milk. Anim. Prod. 55: 163-168.

Visagie, C. M.; Y. Hirooka, J. B. Tanney, E. Whitfield, K. Mwangen, M. Meijer, A. S. Amend, K. A. Seifert, and R. A. Samson. 2014. *Aspergillus*, *Penicillium* and *Talaromyces* isolated from house dust samples collected around the world. Stud. Mycol. 78, 63-139.

Weinberg, Z. G., Y. Chen, and R. Solomon. 2009. The quality of commercial wheat silages in Israel. J. Dairy Sci. 92:638–644.

World Health Organization, 2002. Report on meetings of expert committees and study groups. WHO Technical Report Series, No. 906.

8.9. Tables

Table 1. Sampling time, accession numbers of the calmodulin sequences, growth on YES, CYA and MEA media, sclerotia production and AF genes pattern of the strains of *Aspergillus* section *Flavi* used in this study.

Strain name	Accession number	Species	Sampling time	YES[1]	MEA[1]	CYA[1]	Sclerotia type[2]	AF genes pattern[3]				AFB_1 production in vitro[4]
								aflR	*omtA*	*ver-1*	*nor-1*	µg/kg DM
8931/2	KY886401	*A. flavus*	Silo opening	4.17	3.57	3.27	S and L	+	+	+	+	++
8931/3	KY886402	*A. flavus*	Silo opening	3.9	3.8	3.38	-	+	+	+	+	-
8931/7	KY886381	*A. flavus*	Silo opening	4.13	3.97	3.53	L	+	+	+	+	-
8959	KY886377	*A. flavus*	7 d air exposure	4.2	3.77	3.6	-	-	-	-	-	-
9002	KY886382	*A. flavus*	14 d air exposure	4.13	3.53	3.63	S	+	+	+	+	+
9005	KY886383	*A. flavus*	14 d air exposure	4.03	3.77	3.33	S	+	+	+	+	++
9006	KY886384	*A. flavus*	14 d air exposure	4.1	3.6	3.44	S	+	+	+	+	++
9010	KY886385	*A. flavus*	14 d air exposure	4.33	3.7	3.61	S and L	+	+	+	+	-
9011	KY886386	*A. flavus*	14 d air exposure	4.17	3.87	3.52	-	+	+	+	+	wk
9012	KY886387	*A. flavus*	14 d air exposure	4.03	3.83	3.56	-	+	-	+	+	-
9015/1	KY886378	*A. flavus*	14 d air exposure	4	3.93	3.54	L	-	-	-	-	-
9015/2	KY886379	*A. flavus*	14 d air exposure	3.67	3.87	3.56	L	-	-	-	-	-
9015/3	KY886380	*A. flavus*	14 d air exposure	3.93	3.73	3.6	-	-	-	-	-	-
9016	KY886388	*A. flavus*	14 d air exposure	4.17	3.7	3.41	S and L	+	+	+	+	+
8931/1	KY886389	*A. oryzae* var. *effusus*	Silo opening	4.2	3.77	3.3	-	-	-	+	+	-
8931/4	KY886390	*A. oryzae* var. *effusus*	Silo opening	3.93	3.83	3.6	-	-	-	+	+	-
8931/5	KY886391	*A. oryzae* var. *effusus*	Silo opening	4.13	3.83	3.67	-	+	-	+	+	-
8931/6	KY886392	*A. oryzae* var. *effusus*	Silo opening	4.13	3.67	3.47	-	+	-	+	+	-
8976	KY886393	*A. oryzae* var. *effusus*	7 d air exposure	4.13	3.63	3.62	-	-	-	+	+	-
9004	KY886394	*A. oryzae* var. *effusus*	14 d air exposure	4	3.77	3.47	-	+	+	+	+	-

[1] Colony diameters (mean cm) of strains grown on YES, CYA and MEA at 25°C for 7 days in the dark. [2] Sclerotia production on CYA after 14 days at 30°C in the dark. S: small sclerotia (<400 µm diameter); L: large sclerotia (>400 µm diameter); -: no sclerotia production. [3] +: amplification in quadruplex PCR; -: no amplification in quadruplex PCR. [4] WK <100 µg/kg DM; + = 101-1000 µg/kg DM; ++ > 1001 µg/kg DM.

Table 2. Chemical and microbiological characteristics of the herbage prior to ensiling

Parameters[1]	Value		SD
DM, %	34	±	1.94
pH	5.8	±	0.32
Buffering capacity, mEq kg^{-1}DM	56	±	9.31
Water activity (a_w)	1	±	0
Nitrate, mg/kg	<100		-
NDF, % of DM	42.5	±	1.94
ADF, % of DM	22.1	±	1.43
CP, % of DM	7.84	±	0.2
Ash, % of DM	5.54	±	0.18
Soluble carbohydrates, % of DM	7.37	±	0.89
Starch, % of DM	32.3	±	1.95
LAB, $\log_{10}$ cfu/g	7.71	±	0.56
Yeasts, $\log_{10}$ cfu/g	6.46	±	0.29
Molds, $\log_{10}$ cfu/g	5.26	±	0.2
A. flavus, $\log_{10}$ cfu/g	<1.00		-
Aflatoxin B_1, µg/kg DM	0.06	±	0.08
Aflatoxin B_2, µg/kg DM	1.05	±	1.5
Aflatoxin G_1, µg/kg DM	0.1	±	0.15
Aflatoxin G_2, µg/kg DM	0.04	±	0.06

[1] Values represent the average of 16 replications and the standard deviation. ADF = acid detergent fiber; CP = crude protein; DM = dry matter; NDF = neutral detergent fiber; NH_3-N = ammonia nitrogen; LAB = Lactic acid bacteria

Table 3. Fermentative parameters of corn silage at opening (after 250 d) and after 7 and 14 d of air exposure.

		DM[1] (%)	DM corrected (%)	pH	NH_3-N (g/kg DM)	Lactic acid (g/kg DM)	Acetic acid (g/kg DM)	Lactic-to-acetic ratio	Propionic acid (g/kg DM)	1,2-Propanediol (g/kg DM)	Ethanol (g/kg DM)
Opening											
C	(LB- LH-)	36.4	37.3	3.57	1.05	60.7	13.4	4.5	<0.01	<0.01	8.9
LB	(LB+ LH-)	32.8	34.2	3.78	0.94	47.9	29.5	1.6	<0.01	9.9	13.5
LH	(LB- LH+)	34.1	35.4	3.69	1.84	52.5	26	2	<0.01	7.1	11.3
LB+LH	(LB+ LH+)	34.4	36	3.88	2.01	35.4	33.4	1.1	1.3	12.9	14.1
SEM		1.094	1.088	0.018	0.082	NS	1.208	0.128	-	0.6	0.989
LB[2]		NS	NS	***	NS	***	***	***	-	***	***
LH		NS	NS	***	***	**	***	***	-	***	NS
*LB*LH*		NS	NS	NS	NS	NS	**	***	-	***	NS
7 d											
C	(LB- LH-)	36.1	36.2	5.84	-	10.7	<0.01	-	<0.01	<0.01	<0.01
LB	(LB+ LH-)	32.1	32.9	4.21	-	34.9	29.5	-	<0.01	7.7	<0.01
LH	(LB- LH+)	34.2	34.6	4.25	-	29	10.3	-	<0.01	5.6	<0.01
LB+LH	(LB+ LH+)	34.1	35.5	3.91	-	35	32.4	-	1.2	12.6	11.3
SEM		1.145	1.158	0.276	-	3.72	2.499	-	-	0.351	-
LB		NS	NS	***	-	***	***	-	-	***	-
LH		NS	NS	**	-	*	*	-	-	***	-
*LB*LH*		NS	NS	*	-	*	NS	-	-	NS	-
14 d											
C	(LB- LH-)	29.8	29.8	5.93	-	<0.01	0.2	-	<0.01	<0.01	<0.01
LB	(LB+ LH-)	28	28.1	6.39	-	<0.01	0.8	-	<0.01	<0.01	<0.01
LH	(LB- LH+)	29.9	29.9	6.53	-	<0.01	0.4	-	<0.01	<0.01	<0.01
LB+LH	(LB+ LH+)	34.9	35.7	4.81	-	23.4	17.9	-	1.1	5.6	4.9
SEM		1.136	1.369	0.373	-	-	4.209	-	-	-	-
LB		NS	0.17	NS	-	-	*	-	-	-	-
LH		*	0.02	NS	-	-	NS	-	-	-	-
*LB*LH*		*	0.02	**	-	-	NS	-	-	-	-

[1] C = control; DM = dry matter; LB = *L. buchneri*; LH = *L. hilgardii*; NH_3-N = ammonia nitrogen; SEM = standard error of the mean. [2] LB = effect of LB; LH = effect of LH; LB+LH = interaction of LB and LH. **P* <0.05; ***P* <0.01; ****P* <0.001; NS = not significant.

Table 4. Hours of aerobic stability and temperature indices of corn silage during air exposure.

		Aerobic stability 2°C (h)	Peak temperature[2] (°C)	Interval to peak temperature[2] (h)	Interval to 35°C[2] (h)	Interval with temp. >35°C [2] (h)
Opening						
C	(LB- LH-)	102	51	213	117	180
LB	(LB+ LH-)	138	46	260	163	143
LH	(LB- LH+)	124	48	251	139	182
LB+LH	(LB+ LH+)	365	35	332	294	37
SEM		50.1	3.33	7.8	12.8	18.2
LB[3]		*	*	***	***	***
LH		*	*	***	***	*
*LB*LH*		NS	NS	*	**	*

[1] C = control; LB = *L. buchneri*; LH = *L. hilgardii*; SEM = standard error of the mean.

[2] as observed from silo opening to 14 d of air exposure.

[3] LB = effect of LB; LH = effect of LH; LB+LH = interaction of LB and LH. **P* <0.05; ***P* <0.01; ****P* <0.001; NS = not significant.

Table 5. Microbial count, DM losses, *Aspergillus flavus* count and aflatoxin B_1, B_2, G1, and G_2 of corn silage at opening (after 250 d) and after 7 and 14 d of air exposure.

		LAB[1]	Yeast	Mold	DM losses	*Aspergillus flavus*	Samples with *A. flavus*	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2
		log_{10} cfu/g	log_{10} cfu/g	log_{10} cfu/g	% of DM	log_{10} cfu/g		µg/kg DM	µg/kg DM	µg/kg DM	µg/kg DM
Opening											
C	(LB- LH-)	6.18	2.17	<1.00	2.3	<1.00	0/4	0.19	0.492	0.215	<0.01
LB	(LB+ LH-)	8.43	<1.00	<1.00	3	<1.00	0/4	0.106	2.038	0.057	0.037
LH	(LB- LH+)	7.44	1.41	0.9	3	0.69	1/4	0.314	2.436	0.013	0.039
LB+LH	(LB+ LH+)	8.58	<1.00	1.05	3.6	<1.00	0/4	0.137	1.878	<0.01	0.039
SEM		0.098	0.289	0.251	0.058	-	-	0.086	1.152	0.049	0.02
LB[2]		***	***	NS	***	-	-	NS	NS	NS	NS
LH		***	NS	NS	***	-	-	NS	NS	*	NS
*LB*LH*		***	NS	NS	NS	-	-	NS	NS	NS	NS
7 d											
C	(LB- LH-)	-	8.03	4.98	7	1.36	1/4	0.148	0.871	0.094	<0.01
LB	(LB+ LH-)	-	3.81	1.34	5.8	<1.00	0/4	0.705	0.551	<0.01	0.053
LH	(LB- LH+)	-	6.56	1.14	6	<1.00	0/4	0.348	1.512	<0.01	0.132
LB+LH	(LB+ LH+)	-	3.24	1.46	6	<1.00	0/4	0.292	1.639	<0.01	0.129
SEM		-	0.777	0.658	2.164	-	-	0.192	1.248	-	0.068
LB		-	***	*	NS	-	-	NS	NS	-	NS
LH		-	NS	*	NS	-	-	NS	NS	-	NS
*LB*LH*		-	NS	**	NS	-	-	NS	NS	-	NS
14 d											
C	(LB- LH-)	-	6.14	8.49	39.6	2.23	2/4	0.972	1.656	<0.01	<0.01
LB	(LB+ LH-)	-	3.74	8.1	28.6	3.32	3/4	3.588	1.415	<0.01	0.203
LH	(LB- LH+)	-	5.24	8.04	30.2	2.61	2/4	0.86	1.851	<0.01	0.092
LB+LH	(LB+ LH+)	-	2.38	4.56	18.9	1.25	1/4	0.586	2.273	<0.01	0.071
SEM		-	0.86	0.792	3.633	0.999	-	1.242	1.417	-	0.094
LB		-	**	*	**	NS	-	NS	NS	-	NS
LH		-	NS	*	*	NS	-	NS	NS	-	NS
*LB*LH*		-	NS	NS	NS	NS	-	NS	NS	-	NS

[1] C = control; DM = dry matter; LAB = lactic acid bacteria; LB = *L. buchneri*; LH = *L. hilgardii*; SEM = standard error of the mean.

[2] LB = effect of LB; LH = effect of LH; LB+LH = interaction of LB and LH. **P* <0.05; ***P* <0.01; ****P* <0.001; NS = not significant.

Table 6. Nutritional analyses of corn silage at opening (after 250 d) and after 7 and 14 d of air exposure.

		Starch	NDF[1]	ADF	Hemicelluloses	CP	Ash
		% of DM	% of DM	% of DM	% of DM	% of DM	% of DM
Opening							
C	(LB- LH-)	34.6	37	19.4	17.6	8.5	4.66
LB	(LB+ LH-)	33.3	39.4	21.1	18.3	8.5	5.16
LH	(LB- LH+)	33.1	39.2	20.9	18.3	8.8	5.14
LB+LH	(LB+ LH+)	35	38.7	20.5	18.2	8.5	5.02
SEM		0.94	1.275	0.81	0.19	0.495	0.158
LB[2]		NS	NS	NS	*	NS	NS
LH		NS	NS	NS	NS	NS	NS
*LB*LH*		NS	NS	NS	NS	NS	NS
7 d							
C	(LB- LH-)	39.3	35	18.8	16.3	7.6	4.88
LB	(LB+ LH-)	36.5	37.3	20	17.3	8.1	5.31
LH	(LB- LH+)	36.4	38.1	20	18.1	8.4	5.29
LB+LH	(LB+ LH+)	35.7	38	20.1	17.9	8.6	5.14
SEM		0.628	0.799	0.525	0.146	0.314	0.169
LB		*	NS	NS	***	NS	NS
LH		*	*	NS	***	***	NS
*LB*LH*		NS	NS	NS	**	NS	NS
14 d							
C	(LB- LH-)	18.1	56.4	35.3	21.1	17.7	7.27
LB	(LB+ LH-)	26.8	51.5	31	20.5	12.8	6.91
LH	(LB- LH+)	26	50.1	30.5	19.7	13.4	6.96
LB+LH	(LB+ LH+)	33.5	42.1	23.4	18.7	9.6	5.9
SEM		2.859	2.734	1.937	0.523	0.869	0.424
LB		*	*	**	***	NS	NS
LH		*	**	**	*	NS	NS
*LB*LH*		NS	NS	NS	NS	NS	NS

[1] ADF = acid detergent fiber; C = control; CP = crude protein; DM = dry matter; LB = *L. buchneri*; LH = *L. hilgardii*; NDF = neutral detergent fiber; SEM = standard error of the mean.

[2] LB = effect of LB; LH = effect of LH; LB+LH = interaction of LB and LH. **P* <0.05; ***P* <0.01; ****P* <0.001; NS = not significant.

8.10. Figures

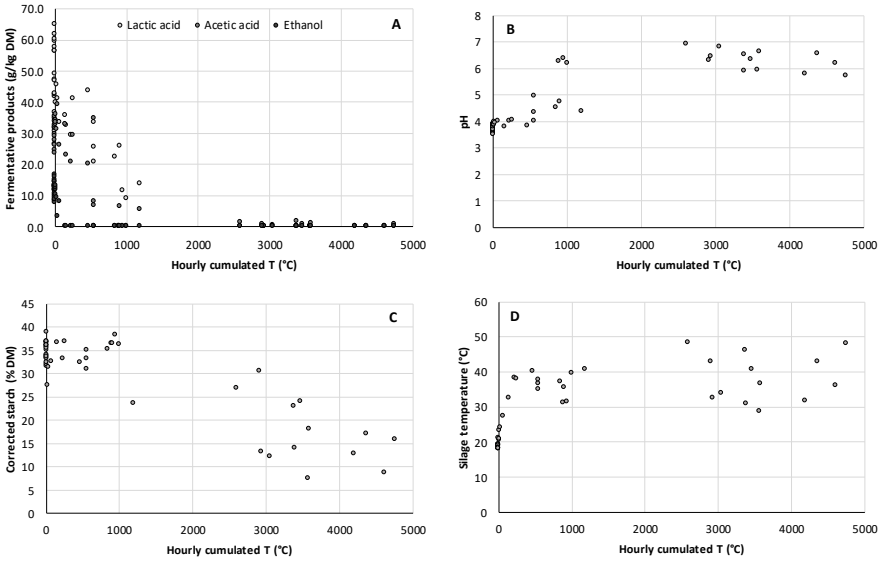

Figure 1. Scatter plot of fermentative products in relation to hourly accumulated temperature rise (°C·h) above the ambient temperature over air exposure, (A); of pH in relation to the hourly accumulated temperature rise (°C·h) above the ambient temperature over air exposure, (B); dry matter losses corrected starch in relation to the hourly accumulated temperature rise (°C·h) above the ambient temperature over air exposure, (C); and silage temperature in relation to the hourly accumulated temperature rise (°C·h) above the ambient temperature over air exposure, (D).

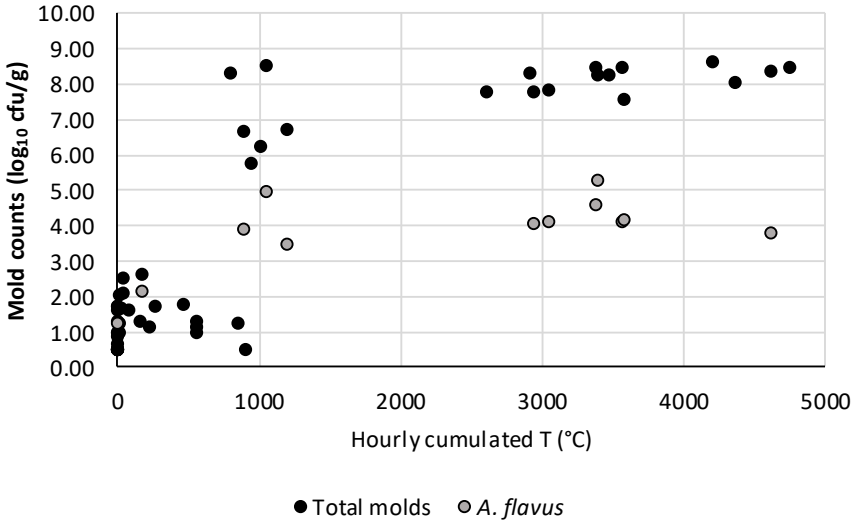

Figure 2. Scatter plot between the total mold and *Aspergillus flavus* counts and the hourly accumulated temperature rise (°C·h) above the ambient temperature over air exposure.

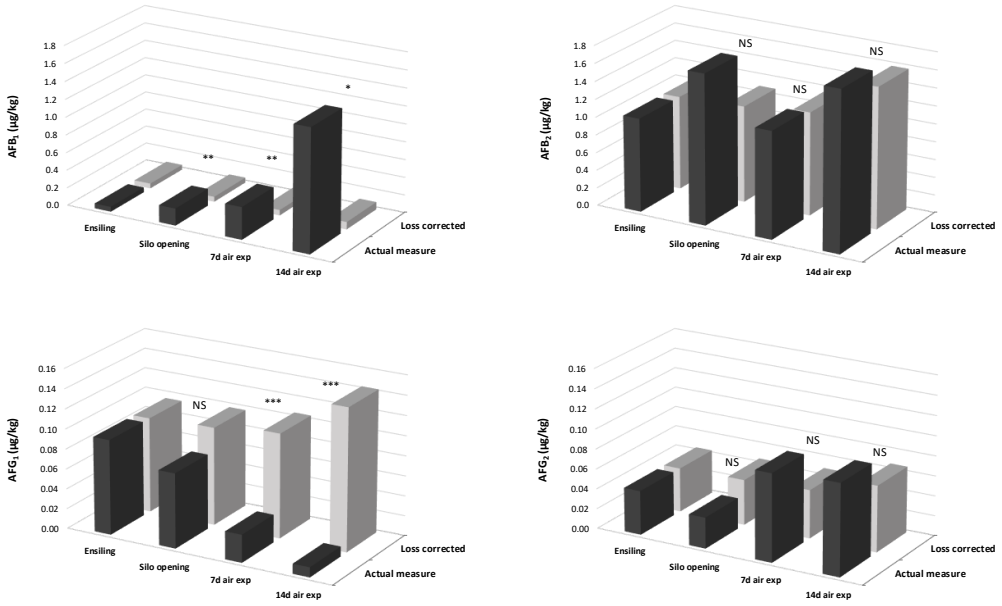

Figure 3. Concentration of AFB_1, AFB_2, AFG_1, and AFG_2 from ensiling to end of conservation and after 7 and 14 d of air exposure data from different treatments are pooled together. In grey the potential amount of aflatoxins derived from the field corrected for DM losses hypothesizing no new biosynthesis or degradation; in black the actual measured content. *P <0.05; **P <0.01; ***P <0.001; NS = not significant.

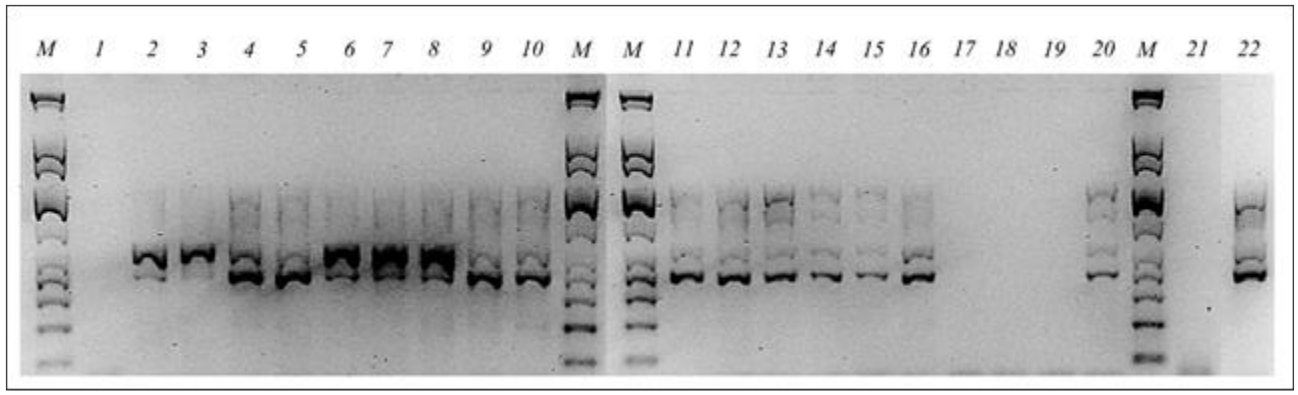

Figure 4. Agarose gel electrophoresis of quadruplex PCR products for strains used in this study. Lane M: molecular marker 1kb Plus Ladder (Quiagen); Lane 1: *A. flavus* 8959; Lane 2: *A. oryzae* var. *effusus* 8976; Lane 3: *A. oryzae* var. *effusus* 8931/1; Lane 4: *A. flavus* 8931/2; Lane 5: *A. flavus* 8931/3; Lane 6: *A. oryzae* var. *effusus* 8931/4; Lane 7: *A. oryzae* var. *effusus* 8931/5; Lane 8: *A. oryzae* var. *effusus* 8931/6; Lane 9: *A. flavus* 8931/7; Lane 10: *A. flavus* 9002; Lane 11: *A. oryzae* var. *effusus* 9004; Lane 12: *A. flavus* 9005; Lane 13: *A. flavus* 9006; Lane 14: *A. flavus* 9010; Lane 15: *A. flavus* 9011; Lane 16: *A. flavus* 9012; Lane 17: *A. flavus* 9015/1; Lane 18: *A. flavus* 9015/2; Lane 19: *A. flavus* 9015/3; Lane 20: *A. flavus* 9016; Lane 21: Negative control; Lane 22: Positive control.

8.11. Appendix

Supplementary Table S1. List of primers used for quadruplex PCR amplification.

Primer name	Target gene	Sequences (5'-3')	Amplicon size (bp)
aflR-1	*aflR*	TATCTCCCCCCGGGCATCTCCCGG	1032
aflR-2		CCGTCAGACAGCCACTGGACACGG	
nor-1	*nor-1*	ACCGCTACGCCGGCACTCTCGGCAC	400
nor-2		GTTGGCCGCCAGCTTCGACACTCCG	
omt-1	*omt-A*	GTGGACGGACCTAGTCCGACATCAC	797
omt-2		GTCGGCGCCACGCACTGGGTTGGGG	
ver-1	*ver-1*	GCCGCAGGCCGCGGAGAAAGTGGT	537
ver-2		GGGGATATACTCCCGCGACACAGCC	

9. Paper IV: *Aspergillus fumigatus* population dynamics and sensitivity to demethylation inhibitor fungicides in whole-crop corn, high moisture corn and wet grain corn silages

Research Article

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Aspergillus fumigatus population dynamics and sensitivity to demethylation inhibitor fungicides in whole-crop corn, high moisture corn and wet grain corn silages

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9.1. Abstract

BACKGROUND: *Aspergillus fumigatus*, the causal agent of aspergillosis in humans, is commonly present as saprophyte in various organic substrates, such as spoiled silages. Aspergillosis is generally combated with demethylation inhibitor (DMI) fungicides, but the recent appearance of resistant medical and environmental strains made current treatment strategies less reliable. The goal of this study was to determine the evolution of *A. fumigatus* populations during the ensiling process of whole-crop corn, high moisture corn and wet grain corn, and to monitor the sensitivity of isolates from treated and untreated fields to one medical and one agricultural DMI fungicide.

RESULTS: *A. fumigatus* was isolated from fresh forage at harvest at rather low concentrations (10^2 cfu/g). The low frequency lingered during the silage process (at 60 and 160 days), whereas it significantly increased during air exposure (at 7 and 14 days of air exposure). Field treatment of corn with a mixture of prothioconazole and tebuconazole did not affect the sensitivity of *A. fumigatus* isolates. One isolate out of 29 coming from the untreated plot was resistant to voriconazole. A unique amino acid substitution (E427K) was detected in the *cyp51A* gene of 10 out of 12 sequenced isolates, but it was not associated to DMI resistance.

CONCLUSION: *A. fumigatus* significantly increased during aerobic deterioration of ensilaged corn after silo opening, compared to the low presence in fresh corn and during ensiling. Field treatment of corn with DMI fungicides did not affect the sensitivity of *A. fumigatus* isolates collected from fresh and ensiled corn.

Keywords: *Aspergillus fumigatus*; corn; ensiling; environmental samples; fungicide treatment; quantification; sensitivity to DMIs

9.2. Introduction

Aspergillus fumigatus (Fresen., syn. *Neosartorya fumigata*, O'Gorman, Fuller and Dyer), common soil inhabiting fungus and saprophyte of decomposing organic matter, is an important human pathogen provoking serious to life-threatening diseases in immunocompromised patients, such as invasive pulmonary aspergillosis.[1,2] It propagates by releasing into the atmosphere a large amount of asexual spores (conidia) which stay viable for a prolonged period of time.[3] A teleomorph of *A. fumigatus* was recently found and named as *N. fumigata*.[4] The conidia are resistant to high temperatures, with the ability to germinate from 20 °C to 50 °C.[5] The fungus can survive in extreme environments at high concentrations of CO_2 and N_2, limited nutrients and oxidative stress.[6] Besides, it represents an important threat for bred animals, especially cattle, due to its capacity to colonize feed and fibre entering the ensiling process.[5,7]

Ensiling is one of the most effective techniques to conserve forages, cereals and other feeds in dairy and beef farm. The conservation of feed is due to an acidification by lactic acid bacteria (LAB) that convert water soluble carbohydrates into lactic and other acids under anaerobic conditions. The acidification and the anaerobic conditions inhibit the growth of aerobic microorganisms, such as yeast and moulds. During the feed-out, silages are exposed to the air and, after an initial stable phase, the silages begin to deteriorate as a result of the activity of aerobic microorganisms, first yeast and later moulds.[8,9] High concentration of *A. fumigatus* capable of producing thermogenic mycotoxins have been found repeatedly in aerobic deteriorated silages and forages for dairy cows.[10-13]

Demethylation inhibitor (DMI) fungicides, chemically known as “azoles”, are the main antifungals used for prophylactic and therapeutic treatments of diseases caused by *A. fumigatus* in humans and animals.[14] DMIs are also commonly used in agriculture as fungicides against a wide range of fungal pathogens of plants.[15] DMIs bind the cytochrome P450 mediated lanosterol 14-α demethylase, encoded by two paralogues (A and B) of the *cyp51* gene, thus preventing the biosynthesis of ergosterol, the principal fungal cell membrane sterol.[16,17]

Soon after the introduction of medical DMIs for therapy and prophylaxis of aspergilloses, resistant isolates were recorded.[18] Resistance was attributed to few mutations in the *cyp51A* gene and started to expand in DMI treated patients in several countries. The main mutations involved in DMI resistance occurred in *cyp51A* gene causing amino acid changes at positions 98, 121 and 289 (L98H, Y121F and T289A), together with modifications of the promoter region by the presence of a tandem repeat (TR_{34} or TR_{46}). DMI-resistant strains recently isolated from DMI naïve patients indicated that resistance might

originate not only from clinical treatments, but may also have environmental sources.[19,20] DMI resistant isolates carrying *cyp51A* mutations and TR promoter modifications were also reported to occur in different environmental matrices, such as soil and compost enforcing the suggestion that DMI resistance in patients may originate also from the application of similar DMIs in agriculture which were used to control plant diseases but have hit also *A. fumigatus* as collateral effect thereafter producing resistant conidia which were inhalated by the patient.[21]

Since DMI sensitivity evaluation of *A. fumigatus* from agricultural and environmental habitats, e.g. compost[22,23] is still limited, we carried out this study in corn silage, one of the preferred habitats of *A. fumigatus* on dairy farms. Silage is considered one of the major sources of conidia release of *A. fumigatus* into air[24] and emphasis has been given to different corn silage types (whole-crop corn, high moisture corn and wet grain silages) from corn harvest to conservation and aerobic deterioration of silage during feed-out phase.

9.3. Materials and methods

9.3.1. Field experiment and corn harvest

The experiment was carried out at the experimental farm of the University of Turin in the western Po plain, northern Italy (44°53' N, 7°41' E, altitude 232 m a.s.l.) on corn (*Zea mays* L.). The field was cultivated with maize under fungicide spray programme on the previous year. Corn (P1517W, Pioneer Hi-Bred Italia Srl, Cremona, Italy) was sown in April 2016, at expected planting density of 75,000 seeds/ha. At the beginning of flowering (61 BBCH scale), half of the plots were treated with a commercial fungicide (Prosaro®, Bayer Crop Science: 12.7 g prothioconazole and 12.7 g tebuconazole per 100 g) applied at the dose of 1.0 l/ha (twice the commercial dose). Corn was harvested at around 2/3 milk line stage as whole-crop corn and at the black line stage for high moisture corn and wet grain silages. Fresh forage for whole-crop corn and high moisture corn were harvested, using a precision forage harvester (Claas Jaguar 950, Claas, Harsewinkel, Germany) and then ensiled in 20 l plastic silos. Wet grain was harvested with a grain harvester (Wintersteiger Quantum plot combine, Wintersteiger AG, Ried, Austria).

9.3.2. Sample preparation and analyses

Corn plants were harvested and conserved as: a) whole-crop corn silage **(WCC)** = ensiling of the whole chopped plant; b) high moisture corn silage **(HMC)** = ensiling of the chopped ear (cob and grain); c) wet grain silage **(WG)** = ensiling of the whole wet grain.

Fresh forages were sampled and ensiled (about 10 to 12, 13 to 15, 16 to 18 kg of wet forage for WCC, HMC and WG, respectively) into 20 l plastic silos equipped with a lid that only enabled gas release. The forages were hand-packed and final packing densities, on a wet basis, were 490 ± 33 kg fresh matter (FM)/m^3, 698 ± 23 kg FM/m^3, 852 ± 22 kg FM/m^3 for WCC, HMC and WG, respectively. All silos were filled within three hours. The silos were weighed, conserved at ambient temperature and opened after 60 and 160 days. At opening, the content of each silo was mixed thoroughly and sub-sampled to determine the DM content, the fermentative and chemical characteristics and the microbial counts. After sampling, the silages were subjected to an aerobic stability test and the silages were sampled after 7 d and 14 d of aerobic exposure in order to quantify the microbial changes in the silages during air exposure.

Pre-ensiled material and silages were split into four subsamples. One sub-

sample was immediately analysed for DM content by oven drying at 80 °C for 24 h. Dry matter was corrected according to Porter and Murray[25], in order to consider the losses of volatile compounds that can take place at 80 °C. The second fresh sub-sample was used to determine the water activity (a_w), pH and nitrate (NO_3^-) concentration. The water activity was measured at 25 °C on a fresh sample using an AquaLab Series 3TE (Decagon Devices Inc., Pullman, WA), which adopts the chilled-mirror dew point technique. The fresh forage was extracted for pH, and NO_3^- determination, using a Stomacher blender (Seward Ltd, Worthing, UK), for 4 min in distilled water at a 9:1 water-to-sample material (fresh weight) ratio. The total nitrate concentration was determined in the water extract, through semi-quantitative analysis, using Merckoquant test strips (Merck, Darmstadt, Germany; detection limit 100 mg NO_3/kg). The pH was determined using specific electrodes. The third subsample was used for the microbial analyses. For the microbial counts, 30 g of sample was transferred into a sterile homogenization bag, suspended 1:10 w/v in a peptone salt solution (1 g of bacteriological peptone and 9 g of sodium chloride per litre) and homogenized for 4 min in a laboratory Stomacher blender (Seward Ltd, London, UK). Serial dilutions were prepared and the yeast and mould 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 yeasts and moulds, respectively. Yeast and mould colonies were divided in morphotypes, based on the macro and micro morphological features observed. Subsequently, yeast and moulds were counted separately on plates that yielded 1 to 100 colony forming units (cfu). The LAB 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. Since LAB are facultative anaerobic, the choice of anaerobic incubation was made to improve the selectivity of the media against *Bacillus* spp. For each type of matrix, sampling was carried out in 6 replicates, 3 replicates for treated plant material (T) with DMIs, and 3 replicates for untreated plants (NT) (Table 1). The fourth sub-sample was used to identification, quantification and selection of *Aspergillus fumigatus*. Sub-amples from which *A. fumigatus* was isolated are indicated in Table 2.

9.3.3. Morphological and molecular identification, and quantification of Aspergillus fumigatus isolates

Morphological identification was performed by isolation of *Aspergillus fumigatus* according to the protocol by Franceschini *et al.*[26] Samples (30 g) were collected from each of the three corn mixtures (DMI-treated and

untreated) at pre-silage and silage conservation periods. The samples were resuspended (1:10 w/v) in a peptone salt solution (1 g of bacteriological peptone and 9 g of sodium chloride/l) and placed into a sterile bag for a 4 min homogenization by laboratory Stomacher blender (Seward Ltd, London, UK). One hundred µl of suspension and its serial dilutions (with a factor 10) were grown in triplicate onto potato dextrose agar (PDA, Merck, Darmstadt, Germany) amended with streptomycin (50 mg/l; Merck). Plates were sealed with parafilm and incubated at 50 °C for 4-5 days to select *A. fumigatus* from other fungal species.

Identification of Aspergillus-like colonies was based on the selection of the grey-green and powdery colonies and observation of their macro- and micro-morphological characteristics. Surviving colonies of *A. fumigatus* were quantified by considering the dilution factor and the average of three replicates.

Three monoconidial cultures were prepared per each isolate and they were stored at –80 °C in 30% glycerol. Fifty isolates of *A. fumigatus* initially isolated from the different matrices were chosen for further study: molecular characterization of the β-tubulin (*tub2*), and *cyp51A* genes, molecular identification of the mating type and DMI sensitivity assays (Table 3). Fungal DNA was extracted from 100 mg fungal mycelium using EZNA® Fungal DNA extraction kit (Omega Bio-Tek, Darmstadt, Germany) according to the manufacturer protocol. The *tub2* gene portion was amplified following the PCR protocol described by Glass and Donaldson[27], and subsequent sequencing of the tub2 amplicons.

9.3.4. Mating types identification

A. fumigatus mating types were determined by multiplex PCR as decribed by Paoletti *et al.*[28] using specific primers *MAT-1* (AFM1, 5′-CCTTGACGCGATGGGGTGG-3′) and *MAT-2* (AFM2, 5′-CGCTCCTCATCAGAACAACTCG-3′), along with common primer AFM3 (5′-CGGAAATCTGATGTCGCCACG-3′). The PCR-25 µl reaction included 10 ng of fungal DNA, 1× PCR buffer, 1.4 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each primer (AFM1 and AFM2), 0.8 µM primer AFM3, and 1 U Taq DNA polymerase (Qiagen, Hilden, Germany). PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR products (5 µl) were stained with RedGel (Biotium, Hayward, CA, USA) in a 1.2% agarose gel and following the electrophoresis visualized under UV light. The mating type of isolates was determined by the amplicon size: 834 bp (MAT-1), and 438 bp (MAT-2).

9.3.5. DMI sensitivity of A. fumigatus by in vitro assays

Sensitivity of *A. fumigatus* against imazalil (agricultural/veterinary DMI), and voriconazole (medical DMI) was evaluated by using the EUCAST protocol[29] with some modifications applying the FRAC standard protocol regarding plant pathogens (http://www.frac.info/monitoring-methods). Three reference environmental isolates were also included in the study: a wild-type (WT) and resistant isolate (TR_{34}+L98H) from NL, and resistant isolate (TR_{46}+Y121F+T289A) from UK (kindly provided by B. Fraaije, Rothamsted Research, UK).

Imazalil (Pestanal® analytical standard; Sigma-Aldrich, Milan, Italy) and voriconazole (Vetranal™ analytical standard; Sigma-Aldrich) were applied in five concentrations (50, 10, 2, 0.4 and 0.08 mg/l) and in two replicates. The PMI 1640/L-glutamine medium (Sigma-Aldrich) supplemented with 2% glucose and 3-(N-morpholino) propanesulfonic acid (MOPS) at a 0.165 mol/l concentration (pH 7.0), was loaded together with each fungicide in flat-bottom Nunc™ 96-well microplate (100 µl/well; Thermo Fisher Scientific, Wilmington, USA). Then, 100 µl of the fungal spore suspension at 2 to 5×10^5 conidia/ml was loaded per well. The substrates without fungicide and without *A. fumigatus* were used as controls. The microplates were kept at 37 °C for 48 hours and then the mycelial growth was estimated visually[29] by a turbidity-grade scale (0-5), where 0 indicates optically clear well and 5 refers to no turbidity change with respect to the turbidity of the fungicide-free control.

Percent growth inhibition (GI) was determined by formula as % GI = (G_c – G_f / G_c) x 100 where G_c refers to the control growth and G_f refers to the growth percentage at each concentration of fungicide. A log/logit dose response was chosen for EC_{50} calculation (concentrations inducing 50 % of growth inhibition) by GraphPadPrism® software (7.02 v.; La Jolla, CA, USA). A fungicide concentration (log) vs. normalized response-variable (percentage of the growth inhibition) was calculated as: Y = Bottom + (Top-Bottom) / {1+10[(LogEC_{50}-X) × HillSlope]} where Y indicates the response (GI) and X the fungicide concentration. Top and Bottom refers to the plateaus of the Y axis units. Hillslope indicates the steepness of the curve.[30] According to the sensitivity of *A. fumigatus* to imazalil and voriconazole, an isolate was considered as less sensitive if its EC_{50} was at least five times the mean EC_{50} of sensitive (S) isolates (0.26 and 0.31 µg/ml for imazalil and voriconazole, respectively), and resistant (R) if the EC_{50} showed 50 times the mean EC_{50} of S) isolates. The sensitivity distribution of all 50 isolates were then estimated for both fungicides and compared to the reference isolates.

9.3.6. Molecular characterization of the cyp51A gene sequences

PCR amplification was carried out for the *cyp51A* coding sequence of 12 *A. fumigatus* isolates. The primers P450-A1 (5'-ATGGTGCCGATGCTATGG-3') and P450-A2 (5'-CTGTCTCACTTGGATGTG-3')[31] were used for the PCR applying the conditions described by Snelders *et al.*[32] Initial denaturation at 95 °C for 5 min, was followed by 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 2 min for 40 cycles, and by a final extension at 72 °C for 7 min.

9.3.7. Sequence analyses

The PCR products of the *tub2* and *cyp51A* genes were sequenced at BMR Genomics (Padua, Italy) and deposited in GenBank with the accession numbers reported in Table 3 for *tub2* and for *cyp51A*. The sequences were compared with those at NCBI GenBank database. Nucleotide and amino-acid alignments were performed with Vector NTI Advance 11 software (InforMax, North Bethesda, Maryland, USA) by the Clustal W algorithm.[33] Phylogenetic analyses were carried out by MEGA 7 software[34] by creating the neighbour-joining (NJ) trees at 1000 bootstrap replications.

9.4. Results

9.4.1. Silage characteristics

The chemical characteristics and microbial counts on corn plants and in silages at harvest, silo opening and after air exposure are summarized in Table 1. At harvest, the DM content was on average around 46%, 65% and 75% for WCC, HMC and WG, respectively. The WG showed slightly higher pH and lower LAB, yeast and mould counts compared to WCC and HMC. After 60 d of conservation the pH reached values below 4 in WCC and HMC, in which the LAB counts increased whereas the yeast and mould counts decreased. The pH of WG at opening did not show significant differences compared to harvest although an increase of LAB was detected. After 160 d of conservation, the silages showed characteristics similar to those detected at 60 d except for a further reduction of the mould counts (on average from 3.16×10^2 cfu/g to below 0.32×10^2 cfu/g). After 7 days of air exposure, both for 60 and 160 d of conservation, yeast counts increased in all the matrices. A further development of aerobic microorganisms was detected after 14 d of air exposure, with mould counts higher than 1.00×10^7 cfu/g in WCC and WG. After 7 days of air exposure, HMC had a pH close to that at silo opening; mould counts were below the detection limit, whereas in WCC and WG, these parameters increased after 14 d of air exposure.

9.4.2. Quantification of A. fumigatus in silages

A. fumigatus frequency was expressed as cfu/g dry weight of silage (Fig. 1). *A. fumigatus* was isolated from the corn samples at harvest in rather low concentrations: 2.60×10^3 cfu/g in WCC and 0.20×10^3 cfu/g in WG, while in HMC it was below the detection limit (<10 cfu/g). During silage conservation, *A. fumigatus* counts was stable after 60 and 160 days of conservation, whereas it significantly increased during air exposure (at 7 and 14 days of air exposure after silo opening). In the 60 days of conservation samples, the fungal presence was quite high in WCC and HMC (3.50×10^7 and 1.37×10^7 cfu/g, respectively) after 14 days of air exposure. The highest concentration of *A. fumigatus* was measured after 160 days of conservation, on the samples after 14 days of air exposure in WCC (2.63×10^8 cfu/g), followed by wet grain (1.28×10^7 cfu/g).

9.4.3. Molecular identification of A. fumigatus isolates and mating type

Fifty *Aspergillus*-like colonies were chosen for molecular identification based on morphological characterization and survival at 50 °C. One hundred percent of isolates collected from corn samples at harvest and after ensiling were confirmed as *A. fumigatus* by BLASTn analysis of the β-tubulin sequences (Table 3), and selected for further studies.

The isolates were further analysed by mating-type specific PCR resulting in 26% MAT1-1 and 74% MAT1-2 idiomorph (Table 3). No isolate showed simultaneously amplification of both mating-types.

9.4.4. Sensitivity of A. fumigatus isolates to imazalil and voriconazole

Fifty *A. fumigatus* isolates collected at harvest, silage opening and subsequent air exposure were evaluated for sensitivity to imazalil and voriconazole (Table 3). The intrinsic antifungal activity of the two DMI fungicides was similar: for imazalil the mean EC_{50} was 0.31 mg/l, ranging from <0.01 to 1.47 mg/l, and for voriconazole the mean EC_{50} was 0.26 mg/l, ranging from <0.01 to 1.48 mg/l.

The sensitivity distribution of *A. fumigatus* isolates was rather continuous for both fungicides without a clear separation between sensitive (EC50 <1.50 mgl) and less sensitive isolates (EC50 >1.51 mg/l), only one resistant isolate against voriconazole (EC50 >10 mg/l) was detected (Fig. 2). No significant differences in sensitivity to either imazalil or voriconazole were found between the isolates coming from the treated and those coming from the untreated plots (Fig. 2). The resistant reference isolate (TR_{34}+L98) was clearly separated from all silage isolates for imazalil, but it grouped together with one silage isolate (HMC.N.60S.1C) for voriconazole (Fig. 2 and Table 3). Ten (for imazalil) and three (for voriconazole) isolates were distributed in an intermediate group between sensitive and resistant isolates, together with another reference isolate (TR_{46} +Y121F +T289A) representing the group of intermediate resistant isolates. The resistant reference isolate TR_{34}+L98 was 34 fold less sensitive against voriconazole and 138 fold less sensitive against imazalil, with respect to the value of the WT reference isolate (Table 3).

9.4.5. The cyp51A molecular characterization

Out of 12 isolates, the cyp51A amino acid substitution (E427K) was found in 10 corn-silage *A. fumigatus* isolates (either DMI sensitive or less sensitive isolates, originating from either treated or untreated plots), including the silage isolate HMC.N.60S.1C that showed resistance to voriconazole. No other cyp51A mutations including those coding for DMI resistance (L98H, Y121F, and T289A) present in the resistant reference isolates[21,35] were found in the studied *A. fumigatus* isolates from corn silage.

The E427K polymorphism was found in the isolates of one subcluster of the main cluster, while the second subcluster included two other silage isolates (WCC.N.H.3B and HMC.T.60S.2B) with the reference resistant and sensitive isolates (Fig. 3).

9.5. Discussion and conclusions

Emphasis in this study has been given to corn harvest as whole-crop corn, high moisture corn and wet grain which was conserved as silage for different time periods since deteriorated silages could be considered a major source of release of *A. fumigatus* spores into air in the farm environment.[13,36,37] *A. fumigatus* is a saprophytic fungus that thrives on organic debris. It is ubiquitous worldwide and is frequently present in silage,[7,37,38] mainly under aerobic conditions (Dolci et al., 2011), where the heat derived from degradation of the organic matter favours the development of thermophilic microorganisms. The occurrence of *A. fumigatus* in whole-crop corn silage has already been documented in different reports,[13,22,36,37] but the growth evolution of the fungus through different conservation periods in high moisture corn and wet grain silages is still poorly investigated.

In this study, *A. fumigatus* was detected both at crop harvest and after ensiling. In the fresh forage at harvest, it was found at rather low concentrations in wet grains (0.11×10^3 cfu/g) and whole-crop corn (2.60×10^3 cfu/g). It was not detected in the high moisture corn. This was probably due to insufficiently sensitive detection levels of the used technique (10 cfu/g). There is still limited information on the presence of *A. fumigatus* on corn plants and grains in the field, where it was detected only by more sensitive methods like molecular techniques, however without fungal quantification.[39]

Thermophilic competence of *A. fumigatus* is evident through surviving at temperatures that approach the upper limit for eukaryotes, thanks to unique mechanisms of stress resistance, useful to bypass high-temperature processes and starting a re-colonization of the substrate in absence of competition with other microbial species.[13] Thus, in the present study, the highest number of contaminated samples by *A. fumigatus* was found after 60 days of silage conservation, indicating that the fungus was able to survive 60 days under anaerobic conditions. Whereas, after longer conservation period (160 days of anaerobic condition), the number of samples containing *A. fumigatus* was low as previously reported by Ferrero *et al.*[40]. The occurrence of *A. fumigatus* in corn silages at opening was previously described by Dolci *et al.*[10] and Spadaro *et al.*[13] after 110 and 146 days of conservation. The highest counts of *A. fumigatus* were detected during aerobic deterioration of silages when the inhibiting conditions to the growth of the fungus (absence of oxygen and low pH) were depleted by the previous activity of yeast allowing the fungus to develop reaching frequencies over 1.00×10^8 cfu/g. These data are in accordance with a high amount of *A. fumigatus* found in corn silage by Santoro *et al.*[22]. During the experiments, a fungicide treatment was performed in the corn field at the beginning of flowering with a mixture of prothioconazole and tebuconazole, both are DMIs. The DMI mixture was applied at twice the

commercial dose. The aim was to evaluate if a high-dose fungicide treatment would affect the occurrence of *A. fumigatus* and the sensitivity of the isolates coming from treated plots. Neither a lower frequency of *A. fumigatus*, nor a significantly different sensitivity profile of the isolates to the two fungicides, imazalil and voriconazole, were detected in the treated plots. The only isolate resistant to voriconazole was found in silage sample originating from a plot which was not treated with DMI. The low number of treatments of field crops like corn with DMIs per cropping season does not seem to be a factor favouring the selection of fungal pathogens or *A. fumigatus* resistant to DMIs. In other crops, such as grapevine or apple, up to four DMI sprays per cropping season are made (FRAC, www.frac.info), potentially representing a higher risk ofresistance selection.

The presence of *A. fumigatus* teleomorph stage seems rare in nature.[4] The genes related to the mating type expression were found in the *A. fumigatus* genome. Mating type 1 seems to be attributed to a higher invasiveness of *A. fumigatus* in human populations, while the mating type 2 is found more frequently in environmental habitats.[22,41] In this study, the isolates did not show a simultaneous expression of both mating types, most of them belonged to mating type 2. The isolate resistant to voriconazole belonged to mat 2. This could be related to a higher aggressiveness of silage isolates as was also described previously for compost isolates (both environmental origin), where mat 2 was predominant and a high sensitivity to DMI fungicides was reported in northern Italy.[23]

Out of 50 silage isolates evaluated, nine and three were less sensitive to imazalil and voriconazole, respectively, while only one isolate showed resistance to voriconazole. However, none of these isolates harbored the mutations known to code for DMI resistance in *A. fumigatus* as reported from other European countries and India for DMI resistant environmental isolates (TR_{34}+L98H, TR_{46}+Y121F and T289A).[21,42-45] In this survey, ten silage *A. fumigatus* isolates contained the *cyp51A* E427K amino acid mutation. The polymorphism E427K has been already found in our previous reports in *A. fumigatus* deriving from composts of kitchen and garden wastes, and orange compost[23,26] and brown compost[22] from Italy and Spain. This mutation, detected now also in several corn silage isolates, is not related to DMIs resistance, it rather represents a specific genotype existing at certain geographic sites.The silage isolate 9378C resistant to voriconazole probably possesses some additional mechanisms associated with DMI resistance such as overexpression of *cyp 51* gene.[46]

While field treatment with DMIs did not influence the sensitivity of *A. fumigatus* isolates from fresh and ensiled corn, more investigation should be done on the presence and sensitivity of *A. fumigatus* in corn silage and samples from other crops including vegatables, due to the risk of contamination of food samples.

9.6. Acknowledgments

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9.7. References

1. Heinekamp T, Schmidt H, Lapp K, Pähtz V, Shopova I, Köster-Eiserfunke N *et al.*, Interference of *Aspergillus fumigatus* with the immune response. *Semin Immunopathol* **37**:141–152 (2015).
2. Fiore M, Cascella M, Bimonte S, Maraolo AE, Gentile I, Schiavone V, et al., Liver fungal infections: an overview of the etiology and epidemiology in patients affected or not affected by oncohematologic malignancies. *Infect Drug Resist* **11**:177-186 (2018).
3. Debeaupuis JP, Sarfati J, Chazalet V and Latgé JP, Genetic diversity among clinical and environmental isolates of *Aspergillus fumigatus*. *Infect Immun* **65**:3080-3085 (1997).
4. O'Gorman CM, Fuller H and Dyer PS, Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Nature* **457**:471–474 (2009).
5. Millner PD, Marsh PBR, Snowden RB and Parr JF, Occurrence of *Aspergillus fumigatus* during composting of Sewage Sludge. *Appl Env Microbiol* **34**:765-772 (1997).
6. Alonso V, Cavaglieri L, Ramos AJ, Torres A and Marin S, Modelling the effect of pH and water activity in the growth of *Aspergillus fumigatus* isolated from corn silage. *J Appl Microbiol* **122**:1048-1056 (2017).
7. Dos Santos V, Dorner J and Carreira F, Isolation and toxigenicity of *Aspergillus fumigatus* from moldy silage. *Mycopathologia* **156**:133–138 (2003).
8. Borreani G, Tabacco E, Schmidt RJ, Holmes BJ and Muck RE, Silage review: Factors affecting dry matter and quality losses in silages. *J Dairy Sci* **101**:3952–3979 (2018).
9. Muck RE, Nadeau EMG, McAllister TA, Contreras-Govea FE, Santos MC and Kung Jr L, Silage review: Recent advances and future uses of silage

additives. *J Dairy Sci* **101**:3980-4000 (2018).
10. Dolci P, Tabacco E, Cocolin L and Borreani G, Microbial dynamics during aerobic exposure of corn silage stored under oxygen barrier or polyethylene films. *Appl Environ Microbiol* **77**:7499–7507 (2011).
11. González Pereyra ML, Chiacchiera SM, Rosa CAR, Sager R, Dalcero AM, Cavaglieri LR, Comparative analysis of mycobiota and mycotoxins contaminating corn trench silos and silo bags. *J Sci Food Agric* **91**:1474–1481 (2011).
12. Pena G. A., Pereyra M., Armando M. R., Chiacchiera S. M., Magnoli C. E., Orlando J. L., *et al.*, *Aspergillus fumigatus* toxicity and glio-toxin levels in feedstuff for domestic animals and pets in Argentina. *Lett Appl Microbiol*, **50**:77–81 (2010).
13. Spadaro D, Bustos-Lopez MP, Gullino ML, Piano S, Tabacco E and Borreani G, Evolution of fungal populations in corn silage conserved under polyethylene or biodegradable films. *J Appl Microbiol* **119**:510–520 (2015).
14. Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, Marr KA *et al.*, Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. *Clin Infect Dis* **46**:327–360 (2008).
15. Brancato A, Brocca D, De Lentdecker C, Erdos Z, Ferreira L, Greco L, *et al.*, Reasoned opinion on the review of the existing maximum residue levels for imazalil according to Article 12 of Regulation (EC) No 396/2005. *EFSA J* **15** (9):4977 (2017).
16. Ghannoum MA and Rice LB, Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin Microbiol Rev* **12**:501–517 (1999).
17. Mellado E, Garcia-Effron G, Buitrago MJ, Alcazar-Fuoli L, Cuenca-Estrella M and Rodriguez-Tudela JL, Targeted gene disruption of the 14-α sterol demethylase (*cyp51A*) in *Aspergillus fumigatus* and its role in azole drug susceptibility. *Antimicrob Agents Chemother* **49:**2536–2538 (2005).
18. Denning DW, Venkateswarlu K, Oakley KL, Anderson MJ, Manning NJ, Stevens DA *et al.*, Itraconazole resistance in *Aspergillus fumigatus. Antimicrob Agents Chemother* **41**:1364–1368 (1997).
19. Stensvold CR, Jørgensen LN and Arendrup MC, Azole-Resistant Invasive Aspergillosis: Relationship to Agriculture. *Curr Fungal Infect Rep* **6:**178-191 (2012).
20. Snelders E, Camps SM, Karawajczyk A, Rijs AJ, Zoll J, Verweij PE, *et al.*, Genotype-phenotype complexity of the TR46/Y121F/T289A cyp51A azole resistance mechanism in *Aspergillus fumigatus*. *Fungal Genet Biol*. **82**:129-35 (2015).
21. Snelders E, Huis In 't Veld RA, Rijs AJ, Kema GH, Melchers WJ and Verweij PE, Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. *Appl Environ Microbiol* **75**:4053–4057 (2009).
22. Santoro K, Matić S, Gisi U, Spadaro D, Pugliese M and Gullino ML, Abundance, genetic diversity and sensitivity to demethylation inhibitor fungicides of *Aspergillus fumigatus* isolates from organic substrates with special emphasis on compost. *Pest Manag Sci* **73**:2481-2494 (2017).
23. Pugliese M, Matić S, Prethi S, Gisi U and Gullino ML., Molecular characterization and sensitivity to demethylation inhibitor fungicides of *Aspergillus fumigatus* from orange-based compost. *PLoS One* **13**:e0200569. (2018).

24. Gisi U, Assessment of selection and resistance risk for demethylation inhibitor fungicides in *Aspergillus fumigatus* in agriculture and medicine: a critical review. Pest Manag Sci **70**:352–364 (2014).
25. Porter MG and Murray RS, The volatility of components of grass silage on oven drying and the inter-relationship between dry-matter content estimated by different analytical methods. *Grass Forage Sci* **56**:405-411 (2001).
26. Franceschini S, Chitarra W, Pugliese M, Gisi U, Garibaldi A and Gullino ML, Quantification of *Aspergillus fumigatus* and enteric bacteria in European compost and biochar. *Compost Sci Util* **24**:20–29 (2016).
27. Glass NL and Donaldson GC, Development of Primer Sets Designed for Use with the PCR To Amplify Conserved Genes from Filamentous Ascomycetes. *Appl Environ Microbiol* **61**:1323–1330 (1995).
28. Paoletti M, Rydholm C, Schwier EU, Anderson MJ, Szakacs G, Lutzoni F, *et al.*, Evidence for sexuality in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Curr Biol* **15**:1242-1248 (2005).
29. Lass-Flörl C, Cuenca-Estrella M, Denning DW and Rodriguez-Tudela JL, Antifungal susceptibility testing in *Aspergillus* spp. according to EUCAST methodology. *Med Mycol* **44**:S319–S325 (2006).
30. Motulsky H, GraphPad Prism Regression Guide. San Diego, CA, USA, GraphPad Software Inc. Available at: http://www.graphpad.com (verified May 5, 2019) (2007).
31. Diaz-Guerra TM, Mellado E, Cuenca-Estrella M and Rodriguez-Tudela JL, A point mutation in the 14α-sterol demethylase gene *cyp51A* contributes to itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob Agents Chemother* **47:**1120–1124 (2003).
32. Snelders E, Karawajczyk A, Schaftenaar G, Verweij PE and Melchers WJ, Azole resistance profile of amino acid changes in *Aspergillus fumigatus* CYP51A based on protein homology modeling. *Antimicrob Agents Chemother* **54**:2425–2430 (2010).
33. Thompson JD, Higgins DG and Gibson TJ, CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**:4673–4680 (1994).
34. Kumar S, Stecher G and Tamura K, MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol* **33**:1870–1874 (2016).
35. Kuipers S, Brüggemann RJ, de Sévaux RG, Heesakkers JP, Melchers WJ, Mouton JW *et al.*, Failure of posaconazole therapy in a renal transplant patient with invasive aspergillosis due to *Aspergillus fumigatus* with attenuated susceptibility to posaconazole. *Antimicrob Agents Chemother* **55**:3564–3566 (2011).
36. Alonso V, Díaz Vergara L, Aminahuel C, Pereyra C, Pena G, Torres A, *et al.*, Physiological behaviour of gliotoxigenic *Aspergillus fumigatus sensu stricto* isolated from maize silage under simulated environmental conditions, *Food Addit Contam A* **32**:236-244 (2005).
37. Dogi C, Alonso V, Fochesato A, Poloni V and Cavaglieri L, Comparison of toxicogenic and immunosuppressive capacity of *Aspergillus fumigatus* strains isolated from clinical and corn silage samples. *J Appl Microbiol* **118**:175-181 (2015).
38. González Pereyra ML, Alonso VA, Sager R, Morlaco MB, Magnoli CE,

Astoreca AL, *et al.*, Fungi and selected mycotoxins from pre- and post-fermented corn silage. J Appl Microbiol **104**:1034–1041 (2008).
39. Zulkifli NA and Zakaria L, Morphological and molecular diversity of *Aspergillus* from corn grain used as livestock feed. *HAYATI Journal of Biosciences* **24**:26e34 (2017).
40. Ferrero F, Prencipe S, Spadaro D, Gullino ML, Cavallarin L, Piano S, *et al.*, Increase in aflatoxins due to *Aspergillus* section *Flavi* multiplication during the aerobic deterioration of corn silage treated with different bacteria inocula. *J Dairy Sci* **102**:1176-1193 (2019).
41. Alvarez-Perez S, Blanco JL, Alba P and Garcia ME, Mating type and invasiveness are significantly associated in *Aspergillus fumigatus*. *Med Mycol* **48**:273–277 (2010).
42. Van der Linden JW, Camps SM, Kampinga GA, Arends JP, Debets-Ossenkopp YJ and Haas PJ, Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. *Clin Infect Dis* **57**:513–520 (2013).
43. Van Ingen J, van der Lee HA, Rijs TA, Zoll J, Leenstra T, MelchersWJ et al., Azole, polyene and echinocandin MIC distributions for wild-type, TR34/L98H and TR46/Y121F/T289A *Aspergillus fumigatus* isolates in the Netherlands. *J Antimicrob Chemother* **70**:178–181 (2015).
44. Mortensen KL, Mellado E, Lass-Flörl C, Rodriguez-Tudela JL, Johansen HK and Arendrup MC, Environmental study of azole-resistant *Aspergillus fumigatus* and other aspergilli in Austria, Denmark, and Spain. *Antimicrob Agents Chemother* **54**:4545–4549 (2010).
45. Chowdhary A, Kathuria S, Xu J, Sharma C, Sundar G, Singh PK *et al.*, Clonal expansion and emergence of environmental multiple-triazole-resistant *Aspergillus fumigatus* strains carrying the TR34/L98H mutations in the cyp51A gene in India. *PLoS One* **7**:e52871 (2012).
46. Verweij PE, Zhang J, Debets AJ, Meis JF, van de Veerdonk FL, Schoustra SE *et al.*, In-host adaptation and acquired triazole resistance in *Aspergillus fumigatus*: a dilemma for clinical management. *Lancet Infect Dis* **16**:e251–e260 (2016).

9.8. Tables

Table 1. Characteristics of whole-crop corn (WCC), high moisture corn (HMC) and wet grain (WG) corn silages at harvest, silo opening and after 7 and 14 days of air exposure

Sampling time	Matrices	Treatment*	DM (%)	a_w	pH	LAB (cfu × 10^5/g)	Yeast (cfu × 10^6/g)	Mould (cfu × 10^6/g)
Harvest	WCC	T	46.0	0.985	5.87	15.85	2.63	1.7
		NT	47.3	0.984	5.89	26.3	5.5	2.24
	HMC	T	64.2	0.976	5.93	19.5	3.8	1.12
		NT	67.7	0.978	5.96	7.08	3.72	1.62
	WG	T	73.3	0.967	6.35	0.05	0.33	0.04
		NT	76.3	0.954	6.33	0.01	0.26	0.03

			Silo opening						7 days of air exposure				14 days of air exposure			
			DM (%)	a_w	pH	LAB (cfu × 10^7/g)	Yeast (cfu × 10^3/g)	Mould (cfu × 10^3/g)	DM (%)	pH	Yeast (cfu × 10^7/g)	Mould (cfu × 10^3/g)	DM (%)	pH	Yeast (cfu × 10^7/g)	Mould (cfu × 10^3/g)
60 d of conservation	WCC	T	45.4	0.982	3.79	0.3	0.79	0.19	48.7	4.97	30.9	0.09	52.7	6.33	44.7	501.2
		NT	47.1	0.98	3.82	0.98	0.28	0.32	47.9	4.1	16.22	7.59	54	6.57	54.95	691.8
	HMC	T	64.1	0.969	3.78	1.05	2	0.68	64.6	3.81	0.63	0.01	68.6	5.35	19.50	77.62
		NT	66.4	0.963	3.82	1.2	0.55	0.35	67.2	3.83	0.04	0	68.6	4.09	2.69	0.21
	WG	T	71.7	0.973	5.93	1.02	955	1.38	73.4	5.98	15.85	275.4	75.3	5.95	8.13	478.6
		NT	74.6	0.971	6.16	0.38	794.3	0.66	76.2	5.84	10.47	208.9	76.6	5.94	13.49	181.9
60 d of conservation	WCC	T	46.3	0.984	3.79	0.2	2.95	0.15	48	4.02	15.5	2.51	50.4	6.26	5.01	3630
		NT	46.6	0.978	3.81	0.03	10.96	0.03	48.1	4.29	25.12	20.89	46.9	6.79	4.27	5011
	HMC	T	62.7	0.963	3.8	0.42	0.05	0.01	64	3.83	0.35	n. d.	63.6	4.73	11.48	7.76
		NT	65.8	0.953	3.86	0.46	0.13	0.01	67	3.86	0.01	n. d.	68.4	3.95	2.04	0.01
	WG	T	71.8	0.964	5.56	0.21	575.44	n. d.	74.8	6.14	13.8	1174.9	76.9	6.01	11.75	537.0
		NT	74.0	0.957	5.46	0.09	691.83	n. d.	76.5	5.8	10.23	0.06	77.7	5.92	14.79	436.5

*T= treated with Prosaro® (Bayer Crop Science: 12.7 g prothioconazole and 12.7 g tebuconazole per 100 g); NT= not treated; WCC = whole-crop corn; HMC = high moisture corn; WG = wet grain; DM = dry matter; aw = activity water; LAB = lactic acid bacteria; cfu = colony forming unit; n.d. = not detected or below 10 cfu/g.

Table 2. List of samples in different corn mixtures (DMI-treated and non-treated) through the different stages: pre-silage and silage conservation (selected samples for molecular characterization and *in vitro* sensitivity assays are highlighted in grey)

Silage type		Harvest	60 d conservation			160 d conservation		
			Silo opening	7 d air exposure	14 d air exposure	Silo opening	7 d air exposure	14 d air exposure
Whole-crop corn	**T***	WCC.TH	WCC.T.60S	WCC.T.607	WCC.T.6014	WCC.T.160S	WCC.T.1607	WCC.T.16014
	NT	WCC.N.H	WCC.N.60S	WCC.N.607	WCC.N.6014	WCC.N.160S	WCC.N.1607	WCC.T.16014
High misture corn	**T**	HMC.T.H	HMC.T.60S	HMC.T.607	HMC.T.6014	HMC.T.160S	HMC.T.1607	HMC.T.16014
	NT	HMC.N.H	HMC.N.60S	HMC.N.607	HMC.N.6014	HMC.N.160S	HMC.N.1607	HMC.N.16014
Wet grain	**T**	WG.T.H	WG.T.60S	WG.T.607	WG.T.6014	WG.T.160S	WG.T.1607	WG.T.16014
	NT	WG.N.H	WG.N.60S	WG.N.607	WG.N.6014	WG.N.160N	WG.N.1607	WG.N.16014

*T= treated twice with Prosaro® (Bayer Crop Science: 12.7 g prothioconazole and 12.7 g tebuconazole per 100 g), NT= not treated

Table 3. Sensitivity to two DMI fungicides (EC_{50}) of *Aspergillus fumigatus* isolates collected from different silage samples

				EC_{50} (mg/L)		EC_{50} (mg/L)		Mating type	
Isolate	**Period**	**Accession no. (β-tubulin)**	**Accession no. (*cyp51A*)***	**Imazalil**	**Reaction****	**Voriconazole**	**Reaction****	**1**	**2**
WCC.T.H.1D		MK879472		0.04	S	1.09	S	+	
WCC.T.H.3B		MK879473		<0.01	S	<0.01	S		+
WCC.N.H.1C	**Harvest**	MK879474		0.27	S	0.02	S	+	
WCC.N.H.3B		MK879475	MK879460 (-)	3.21	LS	1.29	S		+
WG.T.H.1A		MK879476		0.06	S	<0.01	S		+
WG.T.H.2A		MK879477		<0.01	S	<0.01	S	+	
WCC.T.60S.1A		MK879478	MK879466 (+)	0.18	S	0.09	S	+	
WCC.T.60S.2C		MK879479		0.65	S	0.06	S		+
WCC.T.60S.3B		MK879480		1.45	S	1.2	S	+	
WCC.N.60S.1C		MK879481		2.5	LS	0.29	S	+	
WCC.N.60S.3A	**60 d conservation**	MK879482	MK879465 (+)	0.09	S	0.08	S		+
HMC.T.60S.1B		MK879483	MK879461 (-)	0.09	S	0.08	S		+
HMC.T.60S.2B		MK879484	MK879467 (+)	3.92	LS	5.45	LS		+
HMC.T.60S.3C		MK879485	MK879470 (+)	2.34	LS	4.46	LS		+
HMC.N.60S.1C		MK879486	MK879468 (+)	2.42	LS	10.84	R	+	
HMC.N.60S.2A		MK879487		0.2	S	0.08	S	+	
HMC.N.60S.3B		MK879488		0.28	S	0.21	S		+
WG.T.60S.3A		MK879489	MK879471 (+)	0.26	S	0.09	S		+
WG.N.60S.3A		MK879490		0.32	S	0.15	S	+	
WCC.T.607.1A		MK879491		0.21	S	0.04	S	+	
WCC.T.607.2A		MK879492		0.05	S	0.02	S		+
WCC.T.607.3A		MK879493		<0.01	S	0.04	S		+
WCC.N.607.1A	**60 d conservation (+ 7 d air exposure)**	MK879494		0.01	S	<0.01	S	+	
WCC.N.607.3A		MK879495		<0.01	S	0.04	S		+
HMC.T.607.1A		MK879496		<0.01	S	0.02	S		+
HMC.T.607.2B		MK879497	MK879463 (+)	0.32	S	0.1	S		+
HMC.T.607.2A		MK879498	MK879469 (+)	2	LS	1.16	S	+	
WG.T.607.1B		MK879499		0.04	S	0.08	S	+	
WG.T.607.2B		MK879500		0.03	S	0.09	S		+
WG.T.607.3C		MK879501		0.05	S	0.1	S	+	
WG.N.607.1C		MK879502		0.03	S	0.03	S		+
WCC.T.6014.1A	**60 d**	MK879503		1.19	S	1.12	S		+
WCC.N.6014.1A	**conservation**	MK879504		1.51	LS	1.47	S		+
WCC.N.6014.2D	**(+ 14 d air**	MK879505		2.86	LS	0.43	S		+
HMC.T.6014.1B	**exposure)**	MK879506		0.03	S	0.04	S		+
HMC.T.6014.3A		MK879507		0.05	S	0.05	S		+
WCC.T.160S.1A		MK879508		0.28	S	0.11	S		+
WCC.T.160S.2A		MK879509		0.01	S	0.04	S		+
WCC.N.160S.2B	**160 d conservation**	MK879510		0.06	S	<0.01	S		+
WCC.N.160S.3A		MK879511		0.99	S	3.67	LS		+
HMC.T.160S.1A		MK879512		0.05	S	<0.01	S		+
HMC.T.160S.2A		MK879513		0.06	S	<0.01	S		+
HMC.N.160S.2A		MK879514		0.05	S	0.05	S	+	
HMC.N.160S.3A		MK879515		1.48	S	1.25	S	+	
WCC.T.1607.2D	**160 d conservation (+ 7 d air exposure)**	MK879516		0.14	S	0.08	S	+	
WCC.T.16014.3A	**160 d**	MK879517		0.07	S	<0.01	S		+
WCC.N.16014.1A	**conservation**	MK879518	MK879462 (+)	3.05	LS	1.14	S		+
WCC.N.16014.3C	**(+ 14 d**	MK879519		1.41	S	0.76	S		+
HMC.N.16014.2B	**air**	MK879520		1.81	LS	1.2	S	+	
WG.T.16014.1B	**exposure)**	MK879521	MK879464 (+)	0.05	S	<0.01	S	+	
	Mean EC_{50}*			**0.26**		**0.31**			
	WT			**0.09**	S	**0.33**	S	nt	
	TR_{34} +L98H			**12.49**	R	**11.33**	R	nt	
	TR_{46} +Y121F +T289A			**1.71**	LS	**4.42**	LS	nt	

* The presence of the E427K mutation is indicated by a +, while the absence by a -.

**Less sensitive isolates (EC_{50} between 1.51 and 10.0) and resistant isolates (EC_{50} higher than 10.0) were not included in calculation of mean EC_{50}.

9.9. Figures

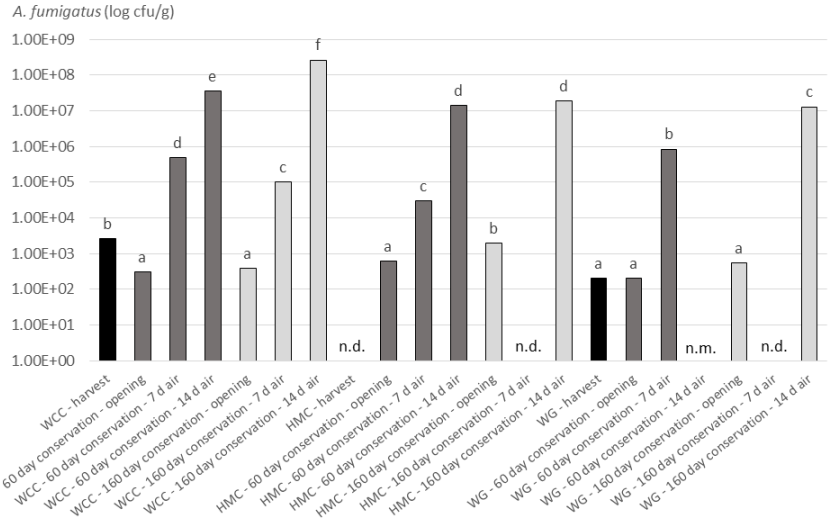

Figure 1. Abundance (cfu/g dry weight) of *Aspergillus fumigatus* in different types of silage and its period of conservation, at silo opening, and at 7 days and 14 days of air exposure (n.d. = not detected or below 10 cfu/g; n.m. = not measured).

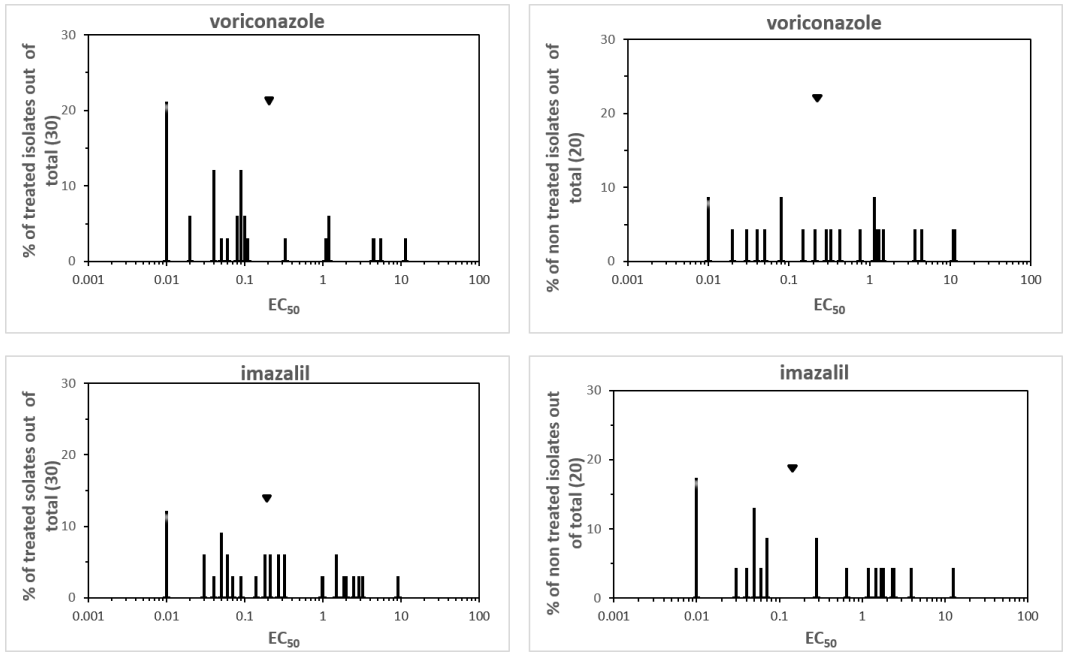

Figure 2. Sensitivity distribution of *A. fumigatus* silage isolates and reference isolates to voriconazole and imazalil. Mean EC_{50} is shown by inverted triangle.

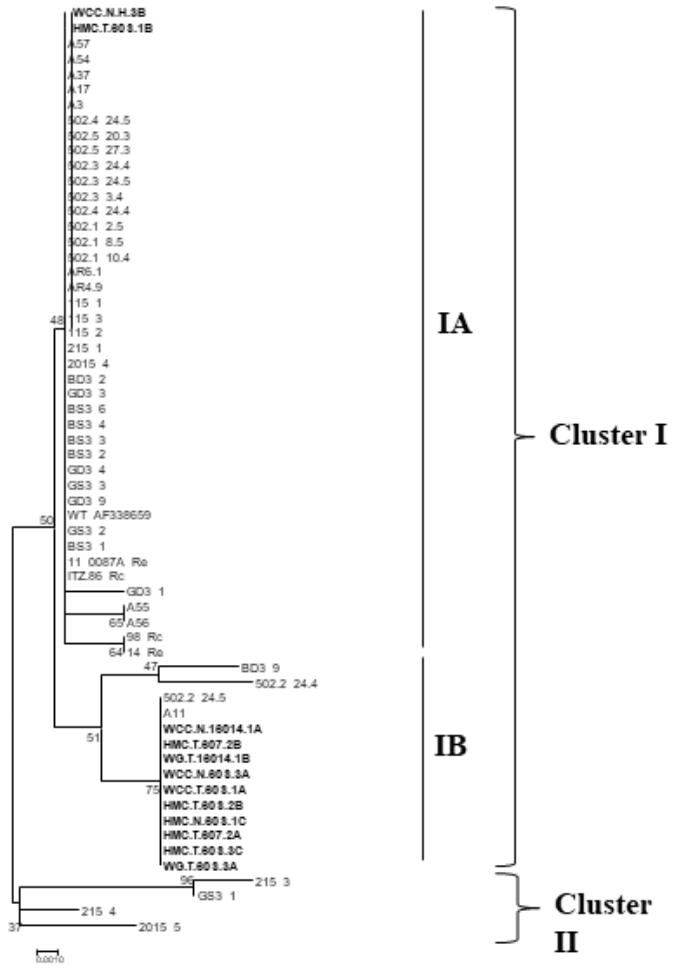

Figure 3. Phylogenetic relatedness of 12 *Aspergillus fumigatus* isolates from corn silage and reference environmental isolates based on the *cyp51A* gene sequence inferred by Neighbour-joining analysis. Bootstrap analysis is supported with 1000 replications.

10. Paper V: Dairy farm management practices and the risk of contamination of tank milk from *Clostridium* spp. and *Paenibacillus* spp. spores in silage, total mixed ration, dairy cow feces and raw milk

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Dairy farm management practices and the risk of contamination of tank milk from *Clostridium* spp. and *Paenibacillus* spp. spores in silage, total mixed ration, dairy cow feces, and raw milk

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10.1. Abstract

The occurrence of *Paenibacillus* and *Clostridium* spores in silage is of great concern for dairy producers, because their spores can contaminate milk and cause the damage of processed milk and semi-hard cheeses. Spoiled silage is considered to be the main contamination source of the total mixed ration (TMR), the feces of dairy cows and consequently bulk tank milk, via the contamination of cow teats by dirt during milking. The presence of an anaerobic and facultative anaerobic sporeformer population in different matrices (soil, corn silage, other feeds, TMR, feces and milk) and its transmission pathway has been studied on 49 dairy farms, by coupling plate count data with 16S-DNA identification. The different matrices have shown a high variability in the anaerobic and facultative anaerobic spore count, with the highest values being found in the aerobically deteriorated areas of corn silages. *Clostridium tyrobutyricum, Paenibacillus macerans* and *P. thermophylus* were detected in all the matrices. The TMR spore count was influenced by the amount of spoiled corn silage in the TMR and by the care taken when cleaning the spoiled silage before feed-out. Most of the farms that prevent the presence of visible moldy silage in the silo and carefully clean to remove molded spots were able to maintain their TMR spore counts below 4.0 log spores/g. When a level of 4.5 log spores/g of TMR was exceeded, the feces presented a greater contamination than 3.0 log spores/g. Moreover, the higher the number of spores in the feces was, the higher the number of spores in the milk. Most of the farms that presented a greater feces contamination than 5.0 log spores/g, had a higher milk spore contamination than 1,000 spores/L. A careful animal cleaning and good milking practices have been found to be essential to maintain low levels of contamination in bulk tank milk, but it has emerged that only by coupling these practices with a correct silage management and cleaning during TMR preparation, can the contamination of milk by spores be kept at a low level. It has been found that aerobically deteriorated silage has a great capacity to contaminate TMR and consequently to increase the risk of milk spore contamination, even when routine milking practices are adopted correctly.

Keywords: silage aerobic deterioration, management practice, *Paenibacillus* spp., *Clostridium* spp.

10.2. Introduction

The occurrence of *Paenibacillus* and *Clostridium* spores in silage is of great concern for dairy producers, because their spores can survive milk and cheese processing, thus, after germination, some strains are capable of spoiling processed milk and dairy products (te Giffel et al. 2002; Masiello et al., 2017). The ubiquitous nature of these sporeformers and the shift caused by the pasteurization processes may result in sporeformers being the dominant viable microflora, even when the total microbial count in raw bulk milk is at least 100 to 1,000-fold higher than the spore count (Wells-Bennik at al., 2016). Butyric acid fermentation in cheese (late-blowing), caused by the outgrowth of the clostridial spores present in raw milk (Klijn et al., 1995; Le Bourhis et al., 2005), which commonly originate from silage (Vissers et al., 2007c; Julien et al., 2008), can create a considerable loss of product, especially in the production of semi-hard cheeses (Stadhouders et al., 1993; Colombari et al., 2001). Several studies have reported that the initial contamination of milk with spore-forming bacteria occurs on dairy farms (Vissers et al., 2007c; Julien et al., 2008; Masiello et al., 2017) and is mainly due to the presence of spores in soil, silage and other feeds. Spores are concentrated in the feces of dairy cows and transferred to the raw milk via the contamination of cow teats with dirt during milking (Visser et al., 2007b). It has been shown that the high risk of sporeformer contamination of bulk tank milk could be attributable to aerobically deteriorated silages, which can contribute to increasing the spore contamination of TMR and consequently of cow feces (Jonsson, 1991; Vissers et al., 2007c; Driehuis et al., 2016). When the aerobic deterioration of silages takes place, oxygen penetration indirectly stimulates the growth of anaerobic microorganisms (Jonsson, 1989; 1991). Aerobic microorganisms, mainly yeasts, consume oxygen close to the surface and cause an increase in the silage temperature. Moreover, the O_2/anO_2 zone moves toward the surface of silage exposed to air and anaerobiosis is restored in the deeper parts (Jonsson, 1989). *Clostridium tyrobutyricum* and other anaerobic or facultative anaerobic sporeformers (mainly clostridia and *Paenibacillus* spp.) can grow and multiply in this ecosystem in micro-niches with less inhibitory activity (Jonsson, 1989; Borreani et al., 2013). The increase in the anaerobic spore content of silage, due to air penetration, has been observed for whole-plant corn silage (Vissers et al., 2007a; Borreani and Tabacco, 2008), wilted alfalfa (Colombari et al., 2001), whole-plant grain sorghum (Tabacco et al., 2009) and grass silage (Jonsson, 1991; Vissers et al., 2007a). Furthermore, the multiplication of *Paenibacillus* spp. has also been reported in aerobically deteriorated corn (Borreani et al., 2013) and grass silages (Driehuis et al., 2016). This leads to a different distribution of spores at the silage feed-out face when aerobic deterioration takes place (Tabacco and Borreani, 2002; Vissers 2007a). The improper incorporation of any deteriorated part from the top layers of the silo in the feed

mixer could increase contamination of the ration with filamentous fungi and anaerobic spores (Borreani and Tabacco, 2014; Borreani et al., 2018). Borreani and Tabacco (2014) found that the anaerobic spore count in corn silage increased linearly with an increase in the mold count, thus showing that the inclusion of parts of the silage that are visually spoiled (higher mold count than 5.0 log cfu/g) increases the risk of worsening the spore contamination of the TMR fed to lactating dairy cows.

When dairy cows are fed with contaminated feeds or TMR, the sporeformers are concentrated in the gastrointestinal tract, due to digestive processes, and they then pass to the feces, whereby the risk of milk contamination becomes greater, especially when low hygiene practices are adopted during milking (Stadhousers and Jørgensen, 1990; Vissers et al., 2007b).

Among the clostridia that are considered of concern for milk and cheese processing, *Clostridium tyrobutyricum* is the most frequently detected in late-blown cheeses, and it is considered the principal causative agent of butyric acid fermentation in cheese (Klijn et al., 1995). However, spores of other *Clostridium* species, such as *C. butyricum*, *C. beijerinckii* and *C. sporogenes*, which occur in raw milk, have been associated with butyric acid fermentation and are believed to be responsible for causing late-blowing defects (Le Bourhis et al., 2005; Driehuis et al., 2016). Spores of other clostridia, and *C. bifermentans, C. perfringens* and *C. tertium* in particular, have also been isolated from natural and processed cheeses and raw milk, and their role as enhancers of the late-blowing defect has been suggested (Le Bourhis et al., 2007). In the last few decades, studies have shown that many organisms that were formerly classified in the *Bacillus* genus actually represent several genera of the Bacilli class (Durak et al., 2006). Members of the *Paenibacillus* genus can survive pasteurization in spore form and are able to grow under refrigeration, thus resulting in product spoilage and limiting the shelf life of high-temperature short-time pasteurized fluid milk (Ivy et al., 2012). Among the *Paenibacillus* spp., the most frequently reported are *P. odorifer*, *P. graminis*, *P. amylolyticus*, *P. c.f. peoriae and P. polymixa* in milk (Ivy et al., 2012; Driehuis et al., 2016), and *P. polymyxa*, *P. pabuli* and *P. macerans* in silages (Borreani et al., 2013; Driehuis, 2013; Driehuis et al., 2016).

The objectives of this study were: i) to verify on farm the role of the aerobic deterioration of corn silage on the proliferation of *Clostridium* and *Paenibacillus* spores; ii) to identify, through 16S-DNA sequencing, the dominant species of anaerobic and facultative anaerobic sporeformers along the milk chain from the farm soil to the bulk tank milk; iii) and to evaluate whether careful management practices, aimed at reducing the amount of aerobic deteriorated corn silage that reaches TMR could mitigate the carry-over of anaerobic and facultative anaerobic spores in milk.

10.3. Materials and methods

10.3.1. Farm selection and survey design

A survey was carried out over a two year period (2013-2014) in the western Po plain (Italy) on forty-nine dairy farms (Italian Friesian breed) that produced milk destined for Grana Padano PDO cheese. The farms were selected in agreement with the technicians from the "Consorzio di Tutela Grana Padano" in order to be representative of the Grana Padano production area (around 150,000 t of cheese produced in 2017 in 127 dairy plants in the Lombardia, Veneto, Piemonte and Emilia-Romagna regions of Italy). The farms were selected on the basis of the farmers' willingness to participate in the survey, and a previous classification of the anaerobic spore levels in the bulk tank milk from a group of 156 farms that supply milk to two cheese factories in Lombardy (Italy), and in which MPN spores are tested monthly. All the farmers were fully informed about the design of the experiment, the nature of the data being collected and their future use. A detailed questionnaire (with questions on feed production and management practices) was presented to the farmers on each farm and the information collected on each farm is summarized in Table 1.

10.3.2. Sample preparation and analyses

The number of sub-samples and resulting pooled samples collected on the farms are reported in Table 1.

The corn silages used in the lactating cow rations at the time of the visits were sampled on each farm. Samples were collected from three areas in each silo: the core (**C**), that is at least 1 m from the surface (n = 49), and two peripheral areas, close to the sealant film (**A1)**, that is the outermost 0.15 m of visibly spoiled silage, which is usually discarded by farmers before feeding (n = 47, since two farms did not present any visible molded area), and below A1 (**BA1**, 0.15 to 0.30 m immediately below the A1 layer, which is always fed to dairy cows; n = 49). The samples were collected using a sterile corer (45 mm diameter; 250 mm depth) in C area (mixing 5 randomly collected samples) and by means of five hand grabs (around 100 g) in the A1 and BA1 sections. The temperature was measured at each sampling point of the silo to a depth of 200 mm (Borreani and Tabacco, 2010) using a probe thermometer. The working face area that was visibly molded/spoiled was also determined and measured to calculate the percentage of the working face that was aerobically deteriorated. Each silo was then inspected to determine whether the aerobically deteriorated silage that was discarded before silage was included in the TMR, and the farms were classified into 3 categories (farms on which deteriorated silage was not,

partially or well discarded). The resulting TMR fed to the lactating cows was collected immediately after unloading (5 to 6 sub-samples of about 200 g, pooled in one farm sample; n = 49). Other feeds were also sampled, if their incidence in the TMR was higher than 20% on a dry matter (DM) basis (n = 46, of which 19 were commercial mixes, 14 were corn grain meals, 1 was a soybean meal and 12 were grass or alfalfa hays).

Five to 10 random soil sub-samples (0-300 mm) were collected on each farm and pooled into one composite soil sample, which was representative of where corn silage was grown (n = 49). Feces from a random 10% of lactating cows were collected and pooled into one farm sample (n = 49). A pooled drinking and cleaning water sample was collected from the farm taps (n = 49). One milk sample (1 liter) from the farm bulk tank was also collected after 15 min of continuous mechanical mixing on each farm (n = 49).

Each corn silage was analyzed for its chemical, microbial and fermentative characteristics, whereas the other feeds, TMR, feces, soil, bulk tank milk and water were only analyzed for anaerobic and facultative anaerobic sporeformers.

Each silage sample was mixed thoroughly and divided into three sub-samples. The first sub-sample was analyzed to determine the DM concentration, which was achieved by oven drying the sample at 65°C until constant weight was reached.

Thirty grams of the second wet silage sub-sample was transferred to a sterile homogenization bag, suspended 1:10 w/v in a peptone physiological salt solution (1 g of neutralized bacteriological peptone and 9 g of sodium chloride per liter) and homogenized for 4 min in a laboratory Stomacher blender (Seward Ltd, London, UK) for the microbial counts. The mold and yeast counts were determined by preparing serial dilutions and using the pour plate technique with 40.0 g/L of yeast extract glucose chloramphenicol agar (YGC agar, DIFCO, West Molesey, Surrey, UK). Petri dishes were incubated at 25°C for 3 and 5 d for yeast and mold, respectively, and then the mold and yeast colony-forming units (cfu) were enumerated separately on plates that yielded 1-100 cfu per Petri dish. The mold and yeast cfu were enumerated separately, according to their macro-morphological features. The anaerobic and facultative anaerobic spores were counted after pasteurization of the serial dilutions at 80°C for 10 min to inactivate vegetative cells and to trigger the germination of spores, and this was followed by the streak plate technique, which was conducted with 50.0 g/L of reinforced clostridial medium agar (RCM; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 0.005% of neutral red (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and 200 ppm of D-cycloserine (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) incubated anaerobically for 7 d at 35°C (Jonsson, 1990). Colonies showing evidence of gold-yellow fluorescence under UV light were considered for identification.

Thirty grams of the third wet silage sub-sample was transferred to a homogenization bag, suspended 1:10 w/v in deionized water and homogenized

for 4 min in a laboratory Stomacher blender, for quantification of the pH value and nitrate content. The pH value was determined through the use of specific electrodes. The nitrate content was determined in the water extract, through semi-quantitative analysis, using Merckoquant test strips (Merck KGaA, Darmstadt, Germany) (Borreani and Tabacco, 2008).

About 50 g of the same sub-sample was homogenized and extracted for 4 min in a Stomacher blender in H_2SO_4 0.05 M at an acid-to-sample material (fresh weight) ratio of 5:1. The solution was filtered with a 0.20-μm syringe filter and used for quantification of the lactic and monocarboxylic acids (acetic, propionic, and butyric acids) with an HPLC (Agilent Technologies, Santa Clara, CA) (Canale et al., 1984). Ethanol and 1,2-propanediol were determined by means of HPLC, coupled to a refractive index detector, on an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA). The analyses were performed isocratically under the following conditions: mobile phase 0.0025M H_2SO_4, flow rate 0.5 ml/min, column temperature 37°C and injection volume 100 μl. Duplicate analyses were performed for all the determined parameters.

Anaerobic and facultative anaerobic spores of the farm soils, other feeds, TMR, feces, water and tank milk were measured and identified, as reported for the silage samples. The detection limit of the anaerobic and facultative anaerobic spores in the silage, TMR, soil, and feces was 50 spores/g and it was 62 spore/L in the milk. In order to improve the detection limit of milk collected on farms with low levels of spores, 40 mL of milk was centrifuged (3,600 × *g* for 15 min), the supernatant was removed and the resultant pellet was re-suspended in 1 mL of a peptone physiological salt solution, in Eppendorf vials (Eppendorf AG, Hamburg, Germany), and re-centrifuged (17,980 × *g* for 3 min). The pellet was then re-suspended in 0.5 mL of a peptone physiological salt solution, which was plated onto two RCM streak plates (1 colony in 0.2 mL (2 plates), and this resulted in a detection limit of 62 spores/L).

The milk fat, protein, lactose, urea, and casein contents were measured on a subsample by means of Fouriertransform infrared spectroscopy (MilkoScan FT6000; Foss Electric A/S, Hillerød, Denmark). The SCC and total bacteria counts (TBC) were automatically determined in the same subsamples by the mean of Fossomatic FC (Foss Electric A/S) and BactoScan FC (Foss Electric A/S), respectively.

10.3.3. Bacterial isolation and identification

Up to 6 yellow colonies were selected from each sample by visually identifying and picking out the distinct morphologies. Up to 10 colonies were selected in the milk with more than 1,000 spore/L. Each colony was purified by streaking on RCM agar and subsequently incubated for 7 d at 37°C under anaerobiosis. Phenotypic characterization was performed by conducting routine

laboratory tests, and the isolates were analyzed by observing the colony morphology, Gram staining, the ability to grow anaerobically on RCM, and the presence of spores. Only Gram-positive, obligate anaerobic or facultative anaerobic endospore forming isolates were then studied in detail. Pure cultures were grown in BHI broth (Merck KGaA, Darmstadt, Germany) for 7 d at 37°C under anaerobiosis before freezing in 20% glycerol at –80°C. The cultures were grown in BHI for 7 days in anaerobic chambers (Anaerocult Kit- Biomerieux-Milan, IT) and the DNA was then extracted following the extraction kit procedures (Ultra Clean (R) Microbial Isolation Kit Mo.Bio laboratories Inc.-Cabru, Milan, IT). Genomic DNA was amplified using 16S universal primers (527 bp), according to the manufacturer's instructions (Micro seq 500 16S rDNA Bacterial Sequencing Kit). The used primers, that is, primer sequence 5'-*TGGAGAGTTTGATCCTGGCTCAG*-3' and reverse primer sequence 5'-*TACCGCGGCTGCTGGCAC*-3', have already been published by Hall et al (2003). PCR reactions were performed using a TGradient thermal cycler (VWR, Milan, Italy). Each 25 µL of PCR reaction contained 2.5 µl of DNA template (50 ng), 200 mM of deoxynucleotide triphosphate, 5 µL of 10X buffer (Taq DNA Polymerase, Qiagen, Chatsworth, CA, USA), 0.7 mM of primer and 1.0 U of Taq DNA Polymerase (Qiagen). The PCR program was: 95°C, 3 min; 34 cycles: 94°C, 15 s; 55°C, 45 s; 72°C, 55 s; and a final extension at 72°C for 7 min. A 10 µL aliquot of the PCR products from each reaction was electrophoresed in 1.5% agarose gel and then stained with SYBR SAFE (Invitrogen, Eugene, OR, USA). Gel images were acquired using a Gel Doc 1000 System (Bio-Rad Laboratories, Hercules, CA, USA). The amplifications generated by 16S rDNA PCR were purified and then sent to an external laboratory (IGA Technology Services, Udine, Italy) for sequencing. The obtained chromatograms were visually inspected for the absence of background noises, and only sequences with clear and unambiguous peaks were used for the subsequent analysis steps. Any areas of the sequence characterized by unresolved peaks were removed from the analysis. The generated sequences were compared with those present in the GeneBank database, using BLASTn sequence similarity searching (http://www.ncbi.nlm.nih.gov/16S Ribosomal RNA Sequences (Bacteria and Archaea)). The highest identity was selected as the identified species or genus when sequence similarity was ≥ 98% (with no gaps). A match was considered appropriate when the query sequence only matched the selected level of one bacterial species. If more species matched at the same similarity level, identification was not considered to have been achieved at the species level, but only at the genus level.

10.3.4. Statistical analysis

The microbial counts were $\log_{10}$ transformed and were presented on a wet weight basis. The values below the detection limit (that is, 50 spores/g in the soil, silage, TMR and feces, and 62 spores/L in the milk) were assigned a value

corresponding to half of the detection limit in order to calculate the average value.

The data were analyzed for their statistical significance, via analysis of variance, with their significance reported at a 0.05 probability level, using the General Linear Model of the Statistical Package for Social Science (v 25.0, SPSS Inc., Chicago, Illinois, USA) with the following statistical model: $Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$, where Y_{ij} = observation, μ = overall mean, α_i = the corn silage spore content (Low, Medium, High) or management practices (well, partially or not discarded molded silage), and ε_{ij} = error. When the calculated values of F were significant, the Tukey post-hoc test ($P < 0.05$) was used to interpret any significant differences among the mean values. Pearson correlation coefficients of the milk spores, the % of molded silo face, the TMR spores, feces spores and animal cleaning score, and their level of significance were determined for each management practice (well, partially or not discarded molded silage).

10.4. Results

10.4.1. Farm characteristics

The number of lactating cows per farm ranged from 16 to 301 (mean value of 105 cows) and the main cow breed was Holstein. All the farms milked twice daily, as reported in the production disciplinary regulations for Grana Padano PDO cheese. Cows were housed in freestalls (77%) or tiestalls (23%). The average milk production across farms was 8,400 kg per cow per year and ranged from 4,150 to 10,970 kg. The utilized agricultural area of the surveyed farms ranged from 8 ha to 208 ha (mean value of 48 ha). The milk intensity reported as the fat protein corrected milk (FPCM) produced per hectare of utilized agricultural area was on average 24 t/ha, with values ranging from 4 to 61 t FPCM/ha. All the farms included corn silage in their milking cow TMR. The average inclusion was 33% on a DM basis, with values ranging from 9 to 49% of the DM intake. Some farms (n = 14) had other silages (e.g. sorghum silages, Italian ryegrass silage), but they utilized them exclusively for replacement heifer feeding.

10.4.2. Anaerobic and facultative anaerobic sporeformers isolated in different matrices

The anaerobic and facultative anaerobic sporeformer counts (log spores/g) in the soil, corn silage, other feeds, TMR and feces of the 49 surveyed dairy farms are reported in Figure 1. The spore content was below the detection limit in all the drinking and cleaning water samples. The soil spore content had a median value of around 2.9 log spores/g, with 50% of the samples ranging from 2.7 to 4.1 log spores/g. The core area of the corn silages had a median spore count of 2.6 log spores/g, with 75% of the samples below 3.4 log spores/g. The peripheral samples, that is, BA1 and A1, had median values of 4.8 and 6.0 log spores/g, respectively, with only 25% of the samples being below 3.8 and 5.3 log spores/g. Other feeds included in the ration of lactating cows presented average spore counts of 1.98 ± 0.40 and 2.06 ± 0.48 log spores/g for dry forages (hays and straw) and concentrates (corn grain, soybean meal and commercial mixes), respectively. TMR had a median value of 4.3 log spores/g, with 50% of the samples ranging from 3.9 to 5.0 log spores/g. Feces showed a similar box plot to that of TMR, but with slightly lower values, and with a median value of 3.8 log spores/g.

Overall, the dominant species of anaerobic and facultative anaerobic

sporeformers identified in the different matrices belonged mainly to the *Clostridium* and *Paenibacillus* genera. All the identified species and their number are reported in Table 2, where they are clustered according to the different studied matrices (soil, corn silage, other feeds, TMR, feces and bulk tank milk). Overall, 26 species of *Clostridium* and 12 species of *Paenibacillus* were found among the studied matrices. Furthermore, *Bacillus* spp., *Brevibacillus* spp. and *Lysinibacillus* spp. were also identified as facultative anaerobic bacteria. *Clostridium tyrobutyricum*, *P. macerans* and *P. thermophylus* were observed in all of the matrices. Five species of clostridia (*C. tyrobutyricum*, *C. sporogenes*, *C. aerotolerans*, *C. celerecrescens*, *C. xylanolyticum*) and 2 species of *Paenibacillus* (*P. macerans*, *P. thermophylus*) were found in at least four matrices. Twelve species of clostridia and 4 of *Paenibacillus* were found in the corn silage.

10.4.3. Spore population in farm soils

Figure 2 reports the distribution of the dominant species found in the soil, corn silage, other feeds, TMR and feces. The anaerobic and facultative anaerobic spore-forming population of the soils was made up of 49% of clostridia species and 30% of *Paenibacillus* species. Of the dominant species, *C. tyrobutyricum*, *C. aerotolerans*, *C. celerecrescens*, *P. macerans* and *P. thermophylus* presented higher frequencies than 5%.

10.4.4. Spore population in corn silages

The corn silage samples were split, in relation to their anaerobic and facultative anaerobic spore-former contents, into three classes: low spore contamination, that is, below 3.0 log spores/g; medium spore contamination, between 3.0 and 5.0 log spores/g and high spore contamination, that is, above 5.0 log spores/g. The mean fermentative and microbial characteristics of the three silage classes are reported in Table 3. The low contaminated silages are mainly constituted by core silages (89.5%), and to a lesser extent by BA1 silages (10%); no silage collected from the A1 zone was present in this class. Most of the silages in the highly contaminated silage class were collected in the peripheral areas of the silo (34.5% and 63.8% from BA1 and A1, respectively), whereas only one sample was from the silage core. The fermentative characteristics of the low contaminated silages were typical of well fermented lactic acid silages, with a low incidence of butyric acid (only 2 samples had a higher concentration than 1 g/kg DM), whereas the medium and high spore contamination silage classes presented higher numbers of samples containing butyric acid, with 3 samples (one from A1 and two from BA1) having a higher

butyric acid content than 10 g/kg DM. Most of the medium and highly contaminated silages presented the typical fermentative profiles of aerobic deteriorated silages, with mean temperatures above 30°C, higher values of pH, yeast and mold counts than the low contaminated silages, and low concentrations of fermentative products inhibiting the spoilage microorganisms (lactic and acetic acids and ethanol). Nitrates were present in 32% of the low contaminated silages, whereas a lower frequency (9.6% of the samples) was observed in the medium and highly contaminated samples.

The dominant clostridia found in the corn silages were: *C. tyrobutyricum*, *C. aerotolerans*, *C. aminovalericum*, *C. celerecrescens*, *C. jejuense*, *C. sporogenes* and *C. xylanolyticum*, whereas the *Paenibacillus* species were *P. macerans*, *P. thermophylus* and *P. cookii*. Six species (*C. tyrobutyricum*, *C. sporogenes*, *C. celerecrescens*, *C. xylanolyticum*, *P. macerans* and *P. thermophylus*) were present in all the zones of the silages (data not shown). The low contaminated silages were characterized by five dominant clostridia species, with *C. xylanolyticum* representing 38% of the total population. *Clostridium tyrobutyricum*, *C. sporogenes*, *C. aerotolerans* and *C. celerecrescens* were observed, but at lower frequencies. *Paenibacillus macerans* was the main species of this genus, and represented 25% of the total anaerobic and facultative anaerobic sporeformers of the low contaminated silages. The medium contaminated silages presented greater frequencies of *C. celerecrescens* and *P. macerans* than the lower contaminated silages. *C. tyrobutyricum* and *C. sporogenes* increased in frequency in the highly contaminated silages, whereas the frequency of *C. xylanolyticum* reduced from 40% in the medium contaminated silages to 3%.

10.4.5. Spore populations in other feeds, TMR and feces

The spore content of the other feeds ranged from 1.40 to 3.06 log spores/g. The dominant population was represented by the *Paenibacillus* genus (67%) and by the *Clostridium* genus (33%). The most frequently detected dominant species were *P. macerans* (42%), *P. thermophylus* (18%) *and C. sporogenes* (11%).

The TMR spore counts ranged from 2.11 to 5.89 log spores/g. Moreover, 5 farms presented a TMR of below 3.0 log spores/g, 34 farms a TMR of 3.0 to 5.0 log spore/g and 10 farms a greater TMR than 5.0 log spores/g.

The distribution of the dominant species for the TMR contamination level was divided into three classes (as suggested in previous works by Stadhousers and Jørgensen (1990) and Stadhousers and Spoelstra (1990)): a low spore contamination below 3.0 log spores/g; a medium spore contamination between 3.0 and 5.0 log spores/g; and a high spore contamination above 5.0 log spores/g (Figure 2). Clostridia represented 63%, 54%, and 52% and *Paenibacillus* spp.

19%, 17% and 19% of the total anaerobic and facultative anaerobic sporeformers for the three contamination classes, respectively. The dominant species of the low contaminated TMR were *C. tyrobutyricum*, *C. beijerinckii* and *P. macerans*. A greater variability of the dominant species was observed in the medium and high contamination classes. *Clostridium xylanolyticum*, *C. tyrobutyricum* and *P. thermophylus* had frequencies that were greater than or equal to 10% in the medium contamination class, and *C. aerotolerans*, *C. beijerinckii*, *C. celerecrescens*, *C. butyricum*, *C. sporogenes* and *P. macerans* were present at lower frequencies. *Clostridium xylanolyticum*, *C. celerecrescens* and *P. thermophylus* had greater frequencies than 10% in the high contamination class, and *C. aerotolerans*, *C. beijerinckii*, *C. butyricum* and *C. tyrobutyricum* were present at lower frequencies.

The spore contamination of the feces ranged from 2.15 to 5.32 log spores/g. The anaerobic and facultative anaerobic spore-forming population of the feces was represented by 39% of the clostridia species and 33% of the *Paenibacillus* species. *Clostridium xylanolyticum*, *C. aerotolerans*, *P. macerans* and *P. thermophylus* presented higher frequencies than 5% in the dominant species (Figure 2). *Clostridium aerotolerans*, *C. celerecrescens*, *C. beijerinckii*, *C. butyricum* and *C. tyrobutyricum* were present at lower frequencies.

10.4.6. Spore population in bulk tank milk

The milk samples ranged from <1.79 to 3.82 log spores/L, with a median value of 1.79 log spores/L. The milk samples were split, in relation to the spore contamination level, into three classes: low spore contamination, that is, below 200 spores/L; medium spore contamination, between 200 and 1,000 spores/L; and high spore contamination, above 1,000 spores/L. These classes represented two critical contamination levels which could influence the hard cheese making process: without any additive <200 spores/L (Bottazzi and Battistotti, 1978; Bergère and Sivelä, 1990) or with additives (nitrates or lysozyme) >1,000 spores/L (Walstra et al., 2005). The farms were distributed into classes as follows: 35 farms <200 spores/L (of which 20 farms were below 62 spores/L), 8 farms between 200 and 1,000 spores/L and 6 farms >1,000 spores/L.

Among all the spore-forming species in the milk, clostridia represented 36, 58 and 0% of the three classes, respectively. The dominant anaerobic and facultative anaerobic sporeformers of the bulk tank milk are reported in Figure 3. The most frequently identified species in the low and medium contaminated milk was *C. tyrobutyricum*, whereas no clostridia were found as the dominant species in the highly contaminated milk. *Clostridium butyricum*, *C. beijerinckii* and *C. sporogenes* were present at lower frequencies. The *Paenibacillus* species represented 60, 38 and 100% in the milk of the three contamination classes, respectively. *Paenibacillus macerans* and *P. thermophylus* were the most frequently identified species.

10.4.7. Spore contamination pathway from silage to tank milk

The scatter plot of the TMR spore count and the visibly molded silo face, as affected by the different management practices adopted to discard deteriorated silage, is reported in Figure 4. The higher the presence of moldy areas in the silage was, the higher the anaerobic and facultative anaerobic spore count in the TMR. Four out of 15 farms that managed aerobic deterioration in corn silage well were found to be able to keep the TMR spore count below 3.0 log spores/g, whereas the other farms that managed the aerobic deterioration of silage well (reduced aerobic spoiled silage and good cleaning/discarding of the spoiled part, if present) were always below 5.0 log spores/g. Farms that did not carry out an effective aerobic deterioration management or cleaning of the silo before feed out always presented higher TMR spore counts than 3.0 log spores/g, with 18 of 22 farms having higher spore counts than 4.0 log spore/g, and the highest contamination level reaching 5.89 log spores/g.

The scatter plot of the anaerobic and facultative anaerobic spore count in the TMR and of the spores in the feces is reported in Figure 5. The higher the spore count in TMR is, the higher the spore count in the feces. Most of the farms that did not manage corn silage in an appropriate way presented higher levels of spores in both the TMR and feces, but a lower TMR spore count did not always implicate a low spore count in the feces.

A scatter plot of the anaerobic and facultative anaerobic spore count in the feces and in the milk is reported in Figure 6. The higher the spore count in the feces is, the higher the spore count in the milk. When feces contamination was greater than 5.0 log spores/g, most of the farms presented greater milk spore counts than 3.0 log spores/L (6 out of 7 farms). Farms with greater bulk tank milk spore counts than 3.0 log spores/L had generally not managed their silages well.

The practical management practices applied on farm that contribute to the safety of the entire milk chain, from the feed production in the field to the milking routine, are summarized in Table 4. Farms were divided into three groups according to their silage management practices (well, partially or not discarded molded silage), and the proportion of farms applying each of the selected management practices in each group was calculated accordingly. The farms that discarded molded silages well before feeding had a lower spore count in the TMR, feces and milk, compared to those that partially or did not discard molded silages. Farms that did not discard silages before feed out usually adopted fewer silage and milking management practices (accurate silo covering, number of film to cover the silo top, the use of film on the silo walls, pre-dipping treatments, treatments with additive) than those that discarded molded silages partially or totally.

The Pearson correlation coefficients of the spore contamination of the milk and visibly molded silo face, the spore contamination of the TMR and feces and

the hygienic score on farms that used well, partially or not discarded corn spoiled silage before TMR preparation are reported in Table 5. The spore count in the milk of farms that did not discard molded silages before TMR preparation was positively correlated to the percentage of molded silo face and the feces spore count. A relation between the percentage of molded silo face, spores in the TMR and feces was also determined. No significant effect was found for the analyzed parameters on farms that discarded the molded silages completely before TMR preparation.

10.5. Discussion

Recent evidence from several milk production chains for PDO cheeses, milk powder and for long conservation pasteurized milk throughout the world has pointed out the need to reduce the sporeformer contamination of bulk tank milk to a minimum because of the difficulties in reducing their contamination level later on in the dairy plant, in part due to the fact that milk heat treatments exacerbate the activation of spore germination (Doyle et al., 2015; Masiello et al., 2017).

This study has analyzed the contamination pathways of anaerobic and facultative anaerobic sporeformers, from silage to bulk tank milk, on 49 dairy farms in northern Italy that produce milk destined for Grana Padano PDO cheese, and has coupled the plate count method with 16S-DNA sequencing of the dominant isolated anaerobic and facultative anaerobic sporeformer species.

In the present experiment, the low contaminated silages presented a very similar anaerobic and facultative anaerobic spore population to those of the considered soil samples. Most of the species observed in the soil (7 vs. 11) were found in both the well conserved silage (with low pH, high lactic acid content and an absence of butyric acid) and in the deteriorated silage. However, most of the samples from the peripheral areas of the corn silage, which were found to be aerobically spoiled, had a higher number of anaerobic and facultative anaerobic spores that was several magnitudes higher (up to 7.76 log spores/g) than the core samples. The dominant *Clostridium* and *Paenibacillus* species in the spoiled samples were: *C. aerotolerans*, *C. celerecrescens*, *C. jejuense*, *C. sporogenes*, *C. tyrobutyricum*, *C. xylanolyticum*, *P. macerans* and *P. thermophylus*. This suggests that these species have the capacity to outgrow in silage during aerobic deterioration, as previously suggested for *C. tyrobutyricum* in grass silage (Jonsson, 1991). Other authors (Vissers et al., 2007a; Borreani and Tabacco, 2008) suggested, on the basis of tests conducted with the most probable number (MPN) method, that the aerobic deterioration of corn silage led to an increase in butyric acid bacteria (BAB), which in turn produced gas under anaerobiosis on lactate acetate agar. Borreani et al. (2013), in trials on the deterioration of corn silage, then found the outgrowth of *Paenibacillus macerans*, which was identified, by 16S-DNA sequencing, as the dominant anaerobic and facultative anaerobic sporeformer isolated on an RCM medium. Driehuis et al. (2016) found *C. tyrobutyricum*, *C. beijerinckii* and *Paenibacillus spp*. (identified by means of a PCR assay on *C. tyrobutyricum*, *C. beijerinckii* and *Paenibacillus spp*.) as the dominant species, in both low and highly contaminated corn silage (less or more than 4 log MPN/g). This result was achieved using MPN tubes in which gas formation was observed in a Van Beynum and Pette medium. Zucali et al. (2015), in a survey on 23 dairy farms in northern Italy, reported the presence of *C. tyrobutyricum*, *C. beijerinckii*, *C.*

butyricum and *C. sporogenes* in corn. This result was achieved using a multiplex PCR of colonies purified from MPN gas positive tubes (lactate-acetate agar plus reconstituted skimmed milk). In the present study, *C. beijerinckii* was not found among the dominant anaerobic and facultative anaerobic sporeformers in any of the corn silage samples, even though it was present in some of the TMR fed to dairy cows. The contrasting results between the present study and the studies of Zucali et al. (2015) and Driehuis et al. (2016) about the presence of *C. beijerinckii* among the dominant anaerobic sporeformers in corn silage could be due to the MPN media, containing lactate acetate, that was used in those studies, which could have favored a selective multiplication of saccharolytic clostridia.

Most of the dominant sporeformer species in the low contaminated TMR (below 3.0 log spores/g) were found to belong to *C. tyrobutyricum*, *C. beijerinckii* and *P. macerans*. The higher contaminated TMR (>3.0 log spores/g) instead showed less incidence of *C. tyrobutyricum* and *P. macerans* and an equal presence of *C. beijerinckii*. Furthermore, *C. aerotolerans*, *C. butyricum*, *C. celerecrescens*, *C. xylanolyticum* and *P. thermophylus* were among the dominant isolated species. The frequency of most of these clostridia species (except *C. aerotolerans*) increased in the highly contaminated TMR (>5.0 log spores/g), and it seems that *P. thermophylus* partially substituted *P. macerans*.

No *Clostridium* species were found in milk with a higher spore count than 1,000 spores/L. All the identified anaerobic and facultative anaerobic species belonged to *Paenibacillus* spp., with *P. macerans* and *P. thermophylus* representing 62% of the sporeformer population. Bermúdez et al. (2016) found, on five commercial dairy plants in Uruguay, that *C. tyrobutyricum* was the most frequently isolated species in milk (ranging from 50 to 58%), *C. sporogenes* was the second (17 to 21%), while *C. beijerinckii* and *C. butyricum* were present at lower levels, and they also observed several other species of clostridia, that is, *C. bifermentans*, *C. acetobutylicum*, *C. xylanolyticum* and *C. aminovalericum*. Masiello et al. (2017), in a longitudinal assessment of dairy farms in New York State, found that *Paenibacillus* spp. was widely present in bulk tank milk. *Paenibacillus* spp. (which is a facultative anaerobe) is one of the main sporeformer genera that limits the shelf life of pasteurized fluid milk under refrigeration (Ivy et al., 2012).

Clostridium spp. species that were most frequently identified in the different matrices considered in the present research belonged to two clusters (I and XIVa), according to the classification proposed by Collins et al. (1994). Cluster I is a family of *Clostridiaceae*, which included the following identified species: *C. tyrobutyricum*, *C. beijerinckii*, *C. butyricum* (in the group of non-liquefying gelatin), *C. sporogenes* and *C. acetobutylicum* (liquefying gelatin). The XIVa family cluster of *Ruminococcaceae*, which included the following species: *C. aerotolerans*, *C. aminovalericum*, *C. celerecrescens* and *C. xylanolyticum* (Collins et al., 1994) and *C. jejuense* (Jeong et al., 2004). *Clostridium*

aerotolerans, *C. celerecrescens* and *C. xylanolyticum* are able to hydrolyze and then hydrogenate hydroxycinnamic acids (ferulic and p-coumaric acids), which are esterified to arabinoxylan in plant cell walls and are thus involved in the anaerobic biotransformation of the fiber components linked to lignin (Chamkha et al., 2001). Furthermore, *C. xylanolyticum* is also able to utilize a wide range of carbohydrates and lactate as an energy source. In the present study, these *Clostridium* species were commonly identified in soil, deteriorated corn silages and feces samples. It can be speculated that these species are involved in the advanced degradation of organic matter in deteriorated silages together with filamentous fungi. The pH is not too acidic (around 6.0) in these silages, the temperature is around 37°C and there is a limited availability of oxidizable free soluble nutrients, such as organic acids and sugars. Therefore, the main sources of substrate for microbial growth are linked to the structural carbohydrates of fiber, which are less available, due to cell wall lignification (McSweeney et al., 1999). Phenolic acids are ester-linked to arabinoxylan and lignin, or cross-linked to lignin and polysaccharide through ether and ester bonds, respectively. It is thought that the ester linkages between phenolic acids and polysaccharide limit the anaerobic degradation of fiber through the microorganisms in the gut of ruminants, and these groups of clostridia share a role in the degradation of fiber in the gastrointestinal tract of cows (Dowd et al., 2008).

The role of corn silage management in increasing the risk of bulk tank milk contamination from anaerobic and facultative anaerobic spores has been analyzed in detail on 49 commercial dairy farms. It has been stated, in recent farm surveys, that corn silage is the main source of the anaerobic and facultative anaerobic spore contamination of TMR, in particular when aerobically spoiled areas are included during feed out (Tabacco and Borreani, 2002; Vissers et al., 2007c; Driehuis et al., 2016). The present research has shown a high variability in the anaerobic and facultative anaerobic spore counts in the different studied matrices, with greater values being observed in samples from the peripheral areas of corn silage, especially when aerobic deterioration had taken place. Most of the forage conserved in the core mass of the silo (75% of the farms) contained lower numbers of spores than 3.0 log spores/g. This unavoidable contamination has mainly been attributed to soil contamination during corn growth and harvesting (Julien et al., 2008). The passage of spores from manured soil to silage has been proved in several studies on grass silage, and many of these studies related clostridia spore contamination to the slurry application timing, rates and methods (e.g. Östling and Lindgren, 1991; Davies et al., 1996). Östling and Lindgren (1991) enumerated the clostridia in manure and in manured crops used for silage and found clostridia counts of around 4.2 log spores/g in manure, a number that reduced by 83% in grass silage crops at harvesting. Lango and Heinonen-Tanski (1995), in a work on cattle slurry on Finnish dairy farms, reported that the occurrence of *Clostridium tyrobutyricum* spores in an anaerobic and facultative anaerobic sporeformer population was unexpectedly low (around 5%).

In the present study, the TMR spore count was found to be influenced by the

amount of deteriorated corn silage that was included in the TMR during feed-out and preparation. Figure 4 clearly suggests this hypothesis: the spore count of TMR increased as the incidence of spoiled corn silage (visible moldy silage) present in the silo increased, and it was also connected to the care taken in cleaning the spoiled silage before feed-out. Most of the farms (9 out of 13 farms) that coupled a good silage management, to prevent the presence of spoiled silage in their silos (below 5% of the silage front face), with a careful discarding of all the molded spots, were able to keep their TMR spore counts below 4.0 log spores/g.

The influence of TMR on the anaerobic and facultative anaerobic spore content in the feces of dairy cows indicates that the feces presented a greater contamination than 3.0 log spores/g when the TMR spore count was greater than 4.5 log spores/g. Nineteen farms out of the 21 that had feces contaminations with more than 4.0 log spores/g did not clean the silo face properly before feed-out, and the majority of them (18 out of 19) presented a higher TMR spore count than 4.0 log spores/g. A higher concentration in feces than in TMR is generally explained by the digestion of feed components during passage through the intestinal tract (Driehuis, 2013). Driehuis et al. (2016) reported a ratio between spores in feces and in TMR of around 3. In the present study, four farms showed a higher ratio than 4 between the spores in the feces and the spores in the TMR . This large increase in spores in the feces (Feces/TMR spore ratio from 14 to 59) could be explained, other than by sampling problems that could have led to an overestimation of the feces spore count on these farms, but also by a multiplication of clostridia in the digestive tracts, as previously suggested by Contrepois et al. (1971). It has recently been reported that clostridia generally account for approximately 20% of the total microbial population in the intestinal tract of cattle (Dowd et al., 2008; Callaway et al., 2010) and can become problematic in the case of dietary stress, injury, changes in management, parasitism or other unusual circumstances (McGuirk, 2015). Therefore, new roles of clostridia in the intestinal tract may be speculated and would suggest their beneficial function in improving the digestion of complex organic matter, such as cellulose (Dowd et al., 2008).

The passage of spores into milk occurs from the exterior of the cow's teats, through feces contamination, and they are transferred to milk during the milking process (Vissers at al., 2007c). Therefore, the critical points are feces spore contamination and the accuracy of the cleaning routines during milking (Vissers at al., 2007c; Zucali et al., 2015).

The data of the present study have confirmed the role of feces in spore milk contamination and the role of management practices to reduce the risk of carry-over of spores from spoiled corn silages to milk. In particular, a positive correlation was found between milk spore count in farms that not discarded molded silages before TMR preparation and feces spore count. On the other hand, no correlation among the analyzed parameter were found in farms that well discarded molded silages confirming that the spores in milk came from

spoiled silages included in the TMR. There is clear evidence that the majority of farms that discarded spoiled silages well before feed out also adopted management practices which were considered to be effective in reducing silage spoilage and the carry-over of anaerobic and facultative anaerobic spores from feeds to milk, with more than 70% of the farms having milk spore contamination values under the detection limit. On the other hand, more than 50% of the farms that partially discarded or did not discard spoiled silage failed to reach this goal (Table 4). The silage management practices (such as corn silage conserved in a bunker with concrete walls, at least 2 plastic films used to cover the silo top, plastic film on the silo walls, a feed-out rate of at least 0.8 m/week) helped to reduce the incidence of aerobic deterioration (Borreani et al., 2018) and, consequently, the reduction of the discarded spoiled silage led to lower spore contamination of the TMR, faeces and milk. It is also evident that farms that carried out cleaning management procedures before silage feeding also adopted pre- and post-milking procedures to reduce the risk of microbial contamination of milk during milking.

The present data have confirmed that the anaerobic and facultative anaerobic spore count of milk is influenced to a great extent by the inclusion of aerobically deteriorated corn silage in TMR. This leads to the conclusion that it is not the presence of silage itself in the TMR that increases the risk of milk contamination, but the undervalued capability of aerobic deteriorated silage to contaminate the TMR. When spoiled silage is included in the TMR, it contributes to a great extent to increasing the spore content of TMR and consequently to increasing the risk of a higher feces spore count. This makes any further control of spore contamination along the milk production chain very difficult, even when the milking and cleaning practices are done properly. Processing milk to reduce spore contamination in dairy plants is expensive, time consuming and labor intensive, and even though it is impossible to eliminate the risk of spore contamination of milk on farms, the results of this work show that it is possible to reduce this risk through the implementation of good farm management practices and specific processing steps from silage to milking.

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10.7. References

Bergère, J. L., and S. Sivelä. 1990. Detection and enumeration of clostridial spores related to cheese quality – Classical and new methods. Bulletin IDF, n. 251, International Dairy Federation, Bruxelles, Belgium, pp. 18-23.

Bermúdez, J., M. J. González , J. A. Olivera, J. A. Burgueño, P. Juliano, E. M. Fox, and S. M. Reginensi. 2016. Seasonal occurrence and molecular diversity of clostridia species spores along cheesemaking streams of 5 commercial dairy plants. J. Dairy Sci. 99: 3358-3366. https://doi:10.3168/jds.2015-10079.

Borreani, G., P. Dolci, E. Tabacco, and L. Cocolin. 2013. Aerobic deterioration stimulates outgrowth of spore-forming *Paenibacillus* in corn silage stored under oxygen-barrier or polyethylene films. J. Dairy Sci. 96: 5206-5216. https://doi:10.3168/jds.2013-6649.

Borreani, G., and E. Tabacco. 2008. Low permeability to oxygen of a new barrier film prevents butyric acid bacteria spore formation in farm corn silage. J. Dairy Sci. 91: 4772-4281. https://doi:10.3168/jds.2008-1151.

Borreani, G., and E. Tabacco. 2010. The relationship of silage temperature with the microbiological status of the face of corn silage bunkers. J. Dairy Sci. 93:2620–2629. https://doi:10.3168/jds.2009-2919.

Borreani G., and E. Tabacco. 2014. Improving corn silage quality in the top layer of farm bunker silos through the use of a next-generation barrier film with high impermeability to oxygen. J. Dairy Sci. 97: 2415-2426. https://doi:10.3168/jds.2013-7632.

Borreani, G., E. Tabacco, R. J. Schmidt, B. J. Holmes, and R. E. Muck. 2018. Silage review: Factors affecting dry matter and quality losses in silages. J. Dairy Sci. 101: 3952-3979. https://doi.org/10.3168/jds.2017-13837.

Bottazzi, V., and B. Battistotti. 1978. Indicazioni per il controllo del gonfiore tardivo del formaggio Grana. [Indication to control late blowing of Grana cheese]. Scienza Lattiero Casearia, 25, 313-325.

Callaway, T. R., S. E. Dowd, T. S. Edrington, R. C. Anderson, N. Krueger, N. Bauer, P. J. Kononoff, and D. J. Nisbet. 2010. Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing. J. Anim.

Sci. 88: 3977-3983. https://doi:10.2527/jas.2010-2900.
Chamkha, M., J.-L. Garcia, and M. Laba. 2001. Metabolism of cinnamic acids by some Clostridiales and emendation of the descriptions of *Clostridium aerotolerans*, *Clostridium celerecrescens* and *Clostridium xylanolyticum*. Int. J. Syst. Evol. Microbiol., 51: 2105-2111. https://doi:10.1099/00207713-51-6-2105
Collins, M. D., P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. A. E. Farrow. 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol. 44: 812-826.
Colombari, G., G. Borreani, and G. M. Crovetto. 2001. Effect of ensiling alfalfa at low and high dry matter on production of milk used to make Grana cheese. J. Dairy Sci. 84: 2494-2502. https://doi: 10.3168/jds.S0022-0302(01)74700-5.
Contrepois, M., P. Gouet, and D. Sauvant. 1971. Comportement des spores de bactéries anaérobies fermentant le lactate dans le tractus digestif du ruminant. II. Évolution du nombre de spores de *C. tyrobutyricum* introduites au niveau du rumen, au cours du transit digestif chez le ruminant. Ann. Biol. Anim. Bioch. Biophys. 11: 139-154.
Davies, D. R., R. J. Merry, and E. L. Bakewell. 1996. The effect of timing of slurry application on the microflora of grass, and changes occurring during silage fermentation. Grass Forage Sci. 51: 42-51.
Doyle, C. J., D. Gleeson, K. Jordan, T. P. Beresford, R. P. Ross, G. F. Fitzgerald, and P. D. Cotter. 2015. Anaerobic sporeformers and their significance with respect to milk and dairy products. Int. J. Food Microbiol. 197: 77-87. https://doi: 10.1016/j.ijfoodmicro.2014.12.022.
Dowd, S. E., T. R. Callaway, R. D. Wolcott, Y. Sun, T. McKeehan, R. G. Hagevoort, and T. S. Edrington. 2008. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). BMC Microbiol. 8:125-132. https://doi:10.1186/1471-2180-8-125.
Driehuis, F. 2013. Silage and the safety and quality of dairy foods: a review. Agric. Food Sci., 22:16-34. https://doi.org/10.23986/afsci.6699.
Driehuis, F., J. Hoolwerf, and J. L. W. Rademaker. 2016. Concurrence of spores of *Clostridium tyrobutyricum*, *Clostridium beijerinckii* and *Paenibacillus polymyxa* in silage, dairy cow faeces and raw milk. Int. Dairy J. 63: 70-77. https://doi.org/10.1016/j.idairyj.2016.08.004.
Durak, Z., H. Fromm, J. Huck, R. Zadoks, and K. J. Boor. 2006. Development of molecular typing methods for *Bacillus* spp. and *Paenibacillus* spp. isolated from fluid milk products. J. Food Sci. 71: M50-M56. https://doi.org/10.1111/j.1365-2621.2006.tb08907.x.
Hall, L., K. A. Doerr, S. L. Wohlfiel, and G. D. Roberts. 2003. Evaluation of the MicroSeq system for identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory. J. Clin. Microbiol. 41: 1447-1453.
Ivy, R. A., M. L. Ranieri, N. H. Martin, H. C. den Bakker, B. M. Xavier, M. Wiedmann, and K. J. Boor. 2012. Identification and characterization of psychrotolerant sporeformers associated with fluid milk production and processing. Appl. Environ. Microbiol., 78: 853-1864. https://doi:10.1128/AEM.06536-11
Jeong, H., H. Yi, Y. Sekiguchi, M. Muramatsu, Y. Kamagata, and J. Chun. 2004. *Clostridium jejuense* sp. nov., isolated from soil. Int. J. Syst. Evol. Microbiol. 54: 1465-1468. https://doi:10.1099/ijs.0.63128-0.
Jonsson, A. 1989. The role of yeasts and clostridia in silage deterioration. PhD

Dissertation, Swedish University of Agricultural Science. Report 42. Uppsala, Sweden.

Jonsson, A. 1990. Enumeration and confirmation of *Clostridium tyrobutyricum* in silages using neutral red, D-cycloserine and lactate dehydrogenase activity. J. Dairy Sci., 73: 719-725.

Jonsson, A. 1991. Growth of *Clostridium tyrobutyricum* during fermentation and aerobic deterioration of grass silage. J. Sci. Food Agric. 54: 557-568.

Julien, M.-C., P. Dion, C. Lafrenière, H. Antoun, and P. Drouin. 2008. Sources of clostridia in raw milk on farms. Appl. Environ. Microbiol. 74: 6348-6357. https://doi:10.1128/AEM.00913-08.

Klijn, N., F. F. J. Nieuwenhof, J. D. Hoolwerf, C. B. V. D. Waals, and A. H. Weerkamp. 1995. Identification of *Clostridium tyrobutyricum* as the causative agent of late blowing in cheese by species specific PCR amplification. Appl. Environ. Microbiol. 61: 2919-2924.

Lango, Z., and H. Heinonentanski. 1995. Occurrence of *Clostridium tyrobutyricum* in cattle slurry and fresh forage grasses. Bioresour. Technol. 53:189-191.

Le Bourhis, A. G., J. Doré, J. P. Carlier, J. F. Chamba, M. R. Popoff, and J. L. Tholozan. 2007. Contribution of *C. beijerinckii* and *C. sporogenes* in association with *C. tyrobutyricum* to the butyric fermentation in Emmental type cheese. Int. J. Food Microbiol. 113: 154-163. https://doi:10.1016/j.ijfoodmicro.2006.06.027.

Le Bourhis, A. G., K. Saunier, J. Doré, J. P. Carlier, J. F. Chamba, M. R. Popoff, and J.-L. Tholozan. 2005. Development and validation of PCR primers to assess the diversity of *Clostridium* spp. in cheese. Appl. Environ Microbiol. 71: 29-38. https://doi:10.1128/AEM.71.1.29-38.2005.

Masiello, S. N., D. Kent, N. H. Martin, Y. H. Schukken, M. Wiedmann, and K. J. Boor. 2017. Longitudinal assessment of dairy farm management practices associated with the presence of psychrotolerant Bacillales spores in bulk tank milk on 10 New York State dairy farms. J. Dairy Sci. 100: 8783-8795. https://doi:10.3168/jds.2017-13139.

McGuirk S. M. 2015. Managing clostridial diseases in cattle. University of Wisconsin, School of Veterinary Medicine, Madison, US. Accessed Feb., 04, 2019: https://www.vetmed.wisc.edu/dms/fapm/fapmtools/7health/manage-clostridial.pdf.

McSweeney, C. S., A. Dulieu, R. I. Webb, T. Del Dot, and L. L. Blackall. 1999. Isolation and characterization of a *Clostridium* sp. with cinnamoyl esterase activity and unusual cell envelope ultrastructure. Arch. Microbiol. 172: 139-149.

Muck, R. E., E. M. G. Nadeau, T. A. McAllister, F. E. Contreras-Govea, M. C. Santos, and L. Kung, Jr. 2018. Silage review: Recent advances and future uses of silage additives. J. Dairy Sci. 101: 3980-4000. https://doi.org/10.3168/jds.2017-13839.

Östling, C. E., and S. E. Lindgren. 1991. Bacteria in manure and on manured and NPK-fertilised silage crops. J. Sci. Food Agric. 55: 579-588.

Stadhouders, J., G. Hup, and F. F. J. Nieuwenhof. 1983. Silage and cheese quality. NIZO mededeling M19A. Netherlands Institute for Dairy Research, Ede, The Netherlands.

Stadhousers, J., and K. Jørgensen. 1990. Prevention of the contamination of raw milk by a hygienic milk production. Bull. Int. Dairy Fed. 251: 31-35.

Stadhousers, J., and S.F. Spoelstra. 1990. Prevention of the contamination of raw milk

by making a good silage. Bull. Int. Dairy Fed. 251:24-31.
Tabacco, E. and G. Borreani. 2002. Extent of aerobic deterioration in farm maize silage as affected by silo management. Pages 178-179 in Proc. 13th International Silage Conference, Auchincruive, Scotland. SAC, Auchincruive, Scotland UK.
Tabacco, E., S. Piano, L. Cavallarin, T. F. Bernardes, and G. Borreani. 2009. Clostridia spore formation during aerobic deterioration of maize and sorghum silages as influenced by *Lactobacillus buchneri* and *Lactobacillus plantarum* inoculants. J. Appl. Microbiol. 107: 1632-1641. https://doi: 10.1111/j.1365-2672.2009.04344.x.
te Giffel, M. C., A. Wagendorp, A. Herrewegh, and F. Driehuis. 2002. Bacterial spores in silage and raw milk. Antonie van Leeuwenhoek 81: 625-630.
Vissers, M. M. M., F. Driehuis, M. C. te Giffel, P. De Jong, and J. M. Lankveld. 2006. Improving farm management by modeling the contamination of farm tank milk with butyric acid bacteria. J. Dairy Sci. 89: 850-858. https://doi:10.3168/jds.S0022-0302(06)72148-8.
Vissers, M. M. M., F. Driehuis, M. C. te Giffel, P. D. Jong, and J. M. G. Lankveld. 2007a. Concentrations of butyric acid bacteria spores in silage and relationships with aerobic deterioration. J. Dairy Sci. 90: 928-936. https://doi:10.3168/jds.S0022-0302(07)71576-X.
Vissers, M. M. M., F. Driehuis, M. C. te Giffel, P. D. Jong, and J. M. G. Lankveld. 2007b. Quantification of the transmission of microorganisms to milk via dirt attached to the exterior of teats. J. Dairy Sci. 90: 3579-3582. https://doi:10.3168/jds.2006-633.
Vissers, M. M. M., F. Driehuis, M. C. te Giffel, P. D. Jong, and J. M. G. Lankveld. 2007c. Minimizing the level of butyric acid bacteria spores in farm tank milk. J. Dairy Sci. 90: 3278-3285. https://doi:10.3168/jds.2006-798.
Walstra, P., J. T. M. Wouters, and T. J. Geurts. 2005. Dairy Science and Technology. 2nd Edition. Taylor and Francis, New York.
Wells-Bennik, M. H. J., F. Driehuis, and S. A. F. T. van Hijum. 2016. Prospects for improved control of dairy-relevant sporeformers using -omics technologies. Curr. Opin. Food Sci. 10: 38-44. https://dx.doi.org/10.1016/j.cofs.2016.08.005.
Zucali, M., L. Bava, S. Colombini, M. Brasca, M. Decimo, S. Morandi, A. Tamburini, and G. M. Crovetto. 2015. Management practices and forage quality affecting the contamination of milk with anaerobic spore-forming bacteria. J. Sci. Food Agric. 95: 1294-1302. https://doi:10.1002/jsfa.6822.

10.8. Tables

Table 1. Summary of the surveyed management practices and samples collected during farm visits to 49 dairy farms in Lombardy (Italy)

Factor	Description	Number of samples
Soil	Information: crop succession in the last 5 years; slurry and manure application rates, method and timing of the application; Samples: 5 to 10 randomly selected 0-30 cm cores from a representative cultivated area.	49 (Soil)
Corn silage	Information: amount of silage consumed daily and feeding rates, filling and packing methods, silo cover, materials used to hold the cover on the top of the silo surface, silo face area, visible spoiled area (area visibly spoiled/face area), temperatures at the silo face according to Borreani and Tabacco (2010); Samples: samples collected from the core (C) of the silo (pool of 5 cores), from the top close to the sealant film (A1, pool of 5 grabs), from the zone immediately above the top (BA1, pool of 5 grabs).	49 (C); 47[1] (A1); 49 (BA1)
Total mixed ration	Information: composition of the rations of dairy cows, number of distributions per day; Samples: 5 to 6 randomly selected grabs, pooled to form 1 sample per farm.	49 (TMR)
Herd and cow hygiene	Information: Number of milking cows, average milk production per cow, average milk production per year, cow hygiene score in the housing area, percent dirty of cow udders (Teat cleanliness scoring system: 5 = clean (no visible dirt); 4 = almost clean (approximately <10% of the area dirty); 3 = slightly dirty (10 to 20% of the area dirty); 2 = dirty (20 to 50% of the area dirty); and 1 = extremely dirty (>50% of the area dirty); Samples: feces from 10% milking cows randomly selected from the herd and pooled.	49 (Feces)
Milking routine	Information: Gloves worn during milking, average milking time, use, type and product used for pre-milking teat disinfection, if and how udders were dried, use of forestripping, and use, type and product of post-milking dipping; Samples: pooled milk sample from the farm tank, pooled sample of drinking and cleaning water drawn from the tap.	49 (milk); 49 (water)

[1] Two farms did not present any visible molded area

Table 2. Number of isolates of the predominant anaerobic and facultative anaerobic spores from farm soil, corn silage, other feeds, TMR, feces, and tank milk.

Bacterial species	Soil	Corn silage	Other feeds	TMR	Feces	Milk	Total isolates
***Clostridium* spp.**							
Clostridium acetobutylicum		2					2
C. acidisoli						1	1
C. aerotolerans	13	10		10	14		47
C. alginifaecis					7		7
C. aminovalericum		16				2	18
C. amygdalinum		9			1		10
C. beijerinckii				20		1	21
C. bifermentans			2			4	6
C. butyricum				5	9	3	17
C. celerecrescens	16	37	1	12	8		74
C. felsineum		3					3
C. frigidicarnis					8		8
C. guangxiense		1		2			3
C. isatidis	6						6
C. jejuense		16		2			18
C. neuense		4		1			5
C. perfringens	15		1				16
C. saccharolyticum				2			2
C. sardiniense			1				1
C. sartagoforme				1	1		3
C. schirmacherense			1				1
C. sporogenes	8	22	5	3	7		46
C. subterminale			1		7		8
C. sulfidigenes					5		5
C. tyrobutyricum	23	27	1	24	9	20	104
C. xylanolyticum	8	132	1	37	21		199
Other *Clostridium* spp.[1]	9	23	1	22	17	2	74
***Paenibacillus* spp.**							
Paenibacillus amylolyticus	14						14
P. barcinonensis				2			2
P. barengoltzii		3				1	4
P. cookii				4		2	6
P. fonticola					7		7
P. ginsegagri				2			2
P. lactis					13		13
P. lautus				2			2
P. macerans	18	173	19	16	13	42	281
P. pabuli					6		6
P. polymyxa			1	3		2	6
P. thermophylus	15	23	8	32	25	3	106
Other *Paenibacillus spp.*[1]	17	36	2	20	32	26	133
Others							
Bacillus spp.	28	26	2	27			83
Brevibacillus spp.	15			8			23
Lysinibacillus spp.		11		2			13
Other unclassified			4	5	79	12	100
Total *Clostridium* and *Paenibacillus* species	**162**	**537**	**45**	**224**	**210**	**111**	**1289**
Total isolates	205	574	51	266	289	123	1508

[1] The species were identified by means of 16S universal primers when sequence similarity was ≥ 98% in GeneBank database, using BLASTn sequence similarity searching. Other *Clostridium* and *Paenibacillus* species that was not possible to classify at the specie level.

Table 3. Mean values (ranges in parentheses) of chemical and microbiological characteristics when samples (n = 145) were partitioned into three classes on the basis of their spore content (low, medium, high). Samples collected in 49 dairy farms of the Po plain of Italy, two farms did not present visible spoiled silage. Proportion of samples from core, BA1 and A1 are reported for each contamination class.

Items	Corn silage spore content			**SE**	***P*-value**
	Low (<3.0 log spores/g) **(n = 38)**	**Medium** (3.0 to 5.0 log spores/g) **(n = 49)**	**High** (>5.0 log spores/g) **(n = 58)**		
Anaerobe spore, log spores/g	2.16^{c}	4.11^{b}	6.10^{a}	0.143	<0.001
Core samples (%)	89.5	28.6	1.7	-	-
BA1 samples (%)	10.5	51.0	34.5	-	-
A1 samples (%)	0	20.4	63.8	-	-
DM content, %	35.7^{a} (27.9-43.3)	32.6^{a} (17.8-46.2)	26.1^{b} (13.8-44.6)	0.646	<0.001
pH	3.78^{c} (3.50 – 4.15	4.40^{b} (3.45 – 7.56)	6.01^{a} (3.81 – 8.19)	0.122	<0.001
Yeast, log cfu/g	1.53^{c} (<1.00 – 4.76)	3.75^{b} (<1.00 – 7.78)	5.89^{a} (3.15 – 8.02)	0.206	<0.001
Mold, log cfu/g	1.30^{c} (<1.00 – 4.41)	2.84^{b} (<1.00 – 8.07)	6.14^{a} (<1.00 – 8.44)	0.216	<0.001
Nitrates, mg/kg DM	308^{a} (<100 – 2,200)	103^{ab} (<100 – 2,988)	52^{b} (<100 – 980)	36.2	0.014
Lactic acid, g/kg DM	48.2^{a} (10.8 – 94.4)	32.2^{b} (<0.1 – 84.1)	9.5^{c} (<0.1 – 68.7)	2.01	<0.001
Acetic acid, g/kg DM	24.7^{a} (9.8 – 49.3)	23.0^{a} (<0.1 – 102.8)	8.8^{b} (<0.1 – 43.9)	1.30	<0.001
Propionic acid, g/kg DM	3.1^{ab} (<0.1 – 12.2)	5.4^{a} (<0.1 – 42.4)	1.9^{b} (<0.1 – 11.7)	0.416	0.002
Butyric acid, g/kg DM	0.1 (<0.1 – 1.7)	1.0 (<0.1 – 24.7)	0.8 (<0.1 – 16.5)	0.238	0.385
Ethanol, g/kg DM	6.8^{a} (<0.1 – 21.5)	2.5^{b} (<0.1 – 14.4)	0.3^{c} (<0.1 – 5.3)	0.363	<0.001
1,2 propanediol, g/kg DM	7.6^{a} (<0.1 – 25.9)	4.3^{b} (<0.1 – 27.8)	1.1^{c} (<0.1 – 11.6)	0.477	<0.001
Sample temperature, °C	24.4^{c} (17.8 – 48.8)	31.7^{b} (17.59 – 50.0)	37.6^{a} (22.4 – 59.8)	0.806	<0.001
Number of samples with butyric acid between 1 to 10 g/kg DM	2/38	3/49	7/58	-	-
Number of samples with butyric acid above 10 g/kg DM	0/38	2/49	1/58	-	-

In the same row, mean values with the same letter are not significantly different for P <0.05.

Table 4. Practices adopted for corn silage management, pre- and post-milking cleaning procedures, spore contamination of TMR, feces and milk, and milk quality on farms that WELL, PARTIALLY or NOT discarded molded corn silage before TMR preparation. Between brackets the percentage of the farms applying the procedure within each group.

	NOT **(n = 22)**	PARTIALLY **(n = 12)**	WELL **(n = 15)**	SE	*P*-value
Silage management factors					
Number of farms making corn silage in bunker with concrete walls	18 (81.8)	10 (83.3)	15 (100.0)	-	-
Number of farms using at least 2 plastic films to cover the silo top	10 (45.5)	9 (75.0)	12 (80.0)	-	-
Number of farms using film on the silo walls	6 (27.3)	5 (41.7)	6 (40.0)	-	-
Number of farms using soil/gravel to weigh down the silo cover	6 (27.3)	3 (25.0)	5 (33.3)	-	-
Number of farms having a feed-out rate of at least 0.8 m/week	12 (54.5)	4 (33.3)	13 (86.7)	-	-
Number of farms with silage treated with additive[1]	3 (13.0)	3 (25.0)	3 (20.0)	-	-
Number of farms applying at least 3 silo management practices	12 (54.5)	7 (58.3)	13 (86.7)	-	-
Number of farms applying at least 4 silo management practices	3 (13.6)	4 (33.3)	8 (53.3)	-	-
Number of farms with silage with molded area below 5% of the front face	11 (50.0)	4 (33.3)	11 (73.3)	-	-
Average feed out rate, m/week	0.82	0.86	1.32	0.114	0.067
Incidence of moldy silage on silage feed-out face, %	6.07	6.23	3.27	0.705	0.126
Amount of corn silage in TMR, % on DM	32.5	31.0	35.1	1.10	0.317
TMR spore, log spores/g	4.35^{a}	4.55^{a}	3.71^{b}	0.123	<0.001
Feces spore, log spores/g	4.35^{a}	3.88^{ab}	3.56^{b}	0.101	0.014
Udder hygiene management					
Number of farms with free stall and cubicles	13 (59.1)	9 (75.0)	14 (93.3)	-	-
Number of farms with milking parlour	12 (54.5)	10 (83.3)	14 (93.3)	-	-
Number of farms only wiping teat with a dry paper towel before milking machine attachment	13 (59.1)	4 (33.3)	4 (26.7)	-	-
Number of farms applying a germicidal solution to the teats before milking machine attachment	4 (18.2)	4 (33.3)	8 (53.3)	-	-
Number of farms applying two pre-dipping treatments	4 (18.2)	5 (41.7)	7 (46.7)	-	-
Number of farms applying manual post-milking teat dipping with an iodophor teat dip	14 (66.3)	10 (83.3)	12 (80.0)	-	-
Number of farms with milk spore contamination under detection limit	4 (18.2)	5 (41.7)	11 (73.3)	-	-
Animal cleaning score[2]	2.61^{b}	2.89^{ab}	3.78^{a}	0.144	0.009
Structural and milk quality parameters					
Milking cows per farm (n)	76^{b}	96^{ab}	150^{a}	10.5	0.010
Annual milk yield (kg/cow)	7,946^{b}	8,502^{b}	9,975^{a}	223.7	0.001
Milk intensity (t FPCM/ha)[3]	21.0	21.4	31.0	1.82	0.054
Milk spore count, log spores/L	2.30^{a}	1.90^{ab}	1.62^{b}	0.086	0.006
Milk protein, %	3.37	3.44	3.44	0.020	0.159
Fat content, %	3.79	3.76	3.83	0.043	0.789
Lactose, %	4.97	5.01	4.98	0.008	0.111
Total bacterial count, log/mL	4.18	4.06	4.07	0.037	0.260
SCC, cells/mL	261,000	247,000	236,000	9.86	0.354

[1] Corn silage treated with inocula (*L. buchneri* based inocula or mixed inocula), or other silage additives (propionic acid, propionate, sodium chloride, milk liquid whey);
[2] Teat cleanliness scoring system: 5 = clean (no visible dirt); 4 = almost clean (approximately <10% of the area dirty); 3 = slightly dirty (10 to 20% of the area dirty); 2 = dirty (20 to 50% of the area dirty); and 1 = extremely dirty (> 50% of the area dirty);
[3] FPCM = fat protein corrected milk;
In the same row, mean values with the same letter are not significantly different for P <0.05.

Table 5. Pearson correlation coefficients of milk spore contamination, silo face visibly molded, TMR and feces spore contamination and animal cleaning score on the 49 dairy farms. Data are reported aggregated or split out by farms that well, partially or not discard corn spoiled silage before TMR preparation.

Items	Milk spores	% of molded silo face	TMR spore	Feces spores
All farms *(n = 49)*				
% of molded silo face	0.423**[1]			
TMR spores	0.419**	0.504**		
Feces spores	0.766**	0.420**	0.553**	
Animal cleaning score	-0.503**	-0.075	-0.339*	-0.470**
Spoiled silage WELL discarded *(n = 15)*				
% of molded silo face	-0.003			
TMR spores	0.290	0.210		
Feces spores	-0.293	0.188	0.074	
Animal cleaning score	-0.313	0.202	0.040	0.221
Spoiled silage PARTIALLY discarded *(n = 12)*				
% of molded silo face	-0.089			
TMR spores	0.571	0.477		
Feces spores	0.711**	0.385	0.717**	
Animal cleaning score	-0.649*	-0.171	-0.620*	-0.480
Spoiled silage NOT discarded *(n = 22)*				
% of molded silo face	0.473*			
TMR spores	0.128	0.531*		
Feces spores	0.746**	0.427	0.453*	
Animal cleaning score	-0.299	0.148	-0.084	-0.313

[1] * P <0.05; ** P <0.01.

10.9. Figures

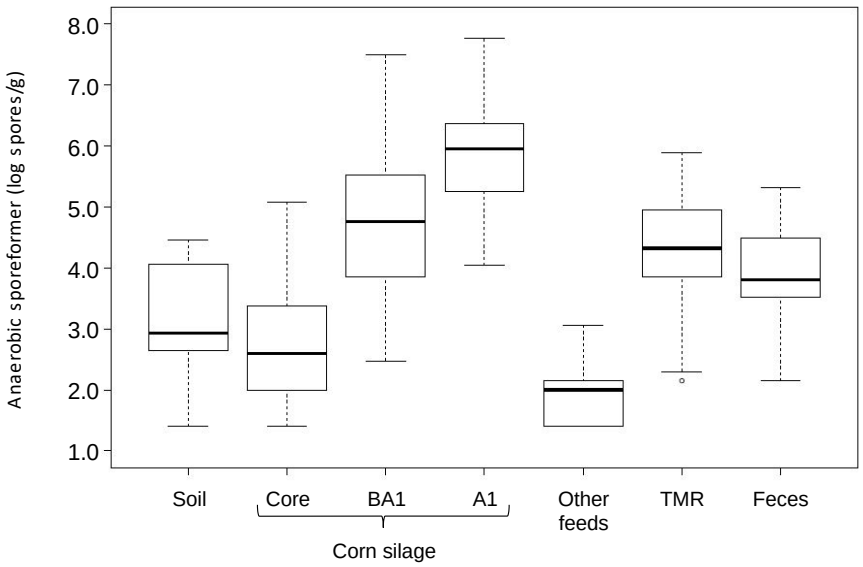

Figure 1. Box plot of the anaerobic and facultative anaerobic spore-forming bacteria of the soil, corn silage, other feeds, TMR and feces of the 49 commercial dairy farms.

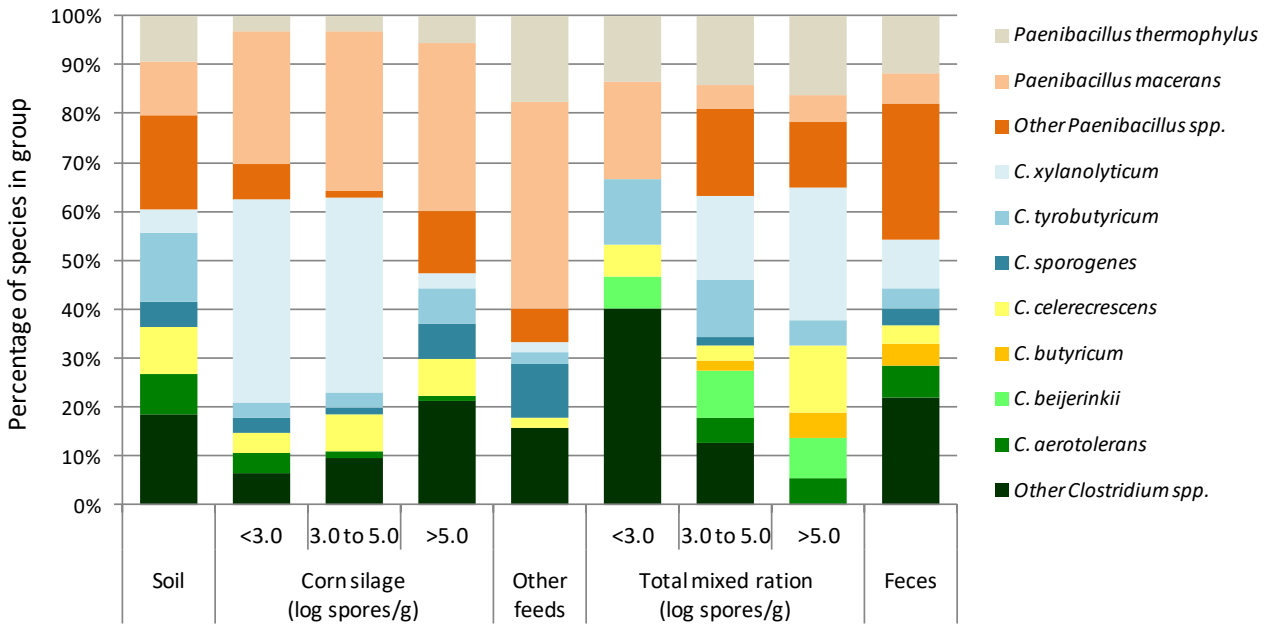

Figure 2. Distribution of the dominant anaerobic and facultative anaerobic spore-forming bacteria isolates in the soil, corn silages (divided into three contamination classes), other feeds, TMR (divided into three contamination classes) and feces of the 49 commercial dairy farms in northern Italy.

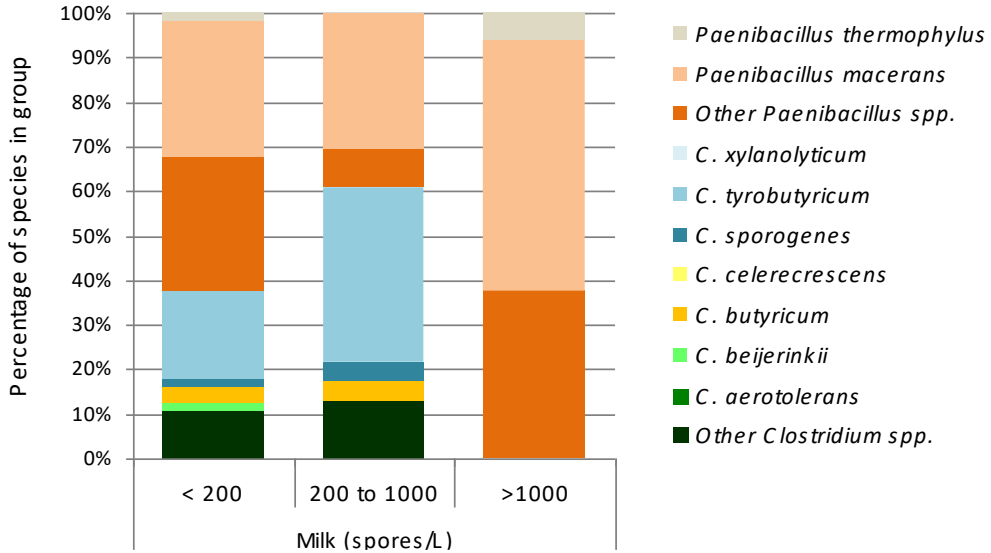

Figure 3. Distribution of the dominant anaerobic and facultative anaerobic spore-forming bacteria in the tank milk (divided into three contamination classes) of the 49 commercial dairy farms in northern Italy.

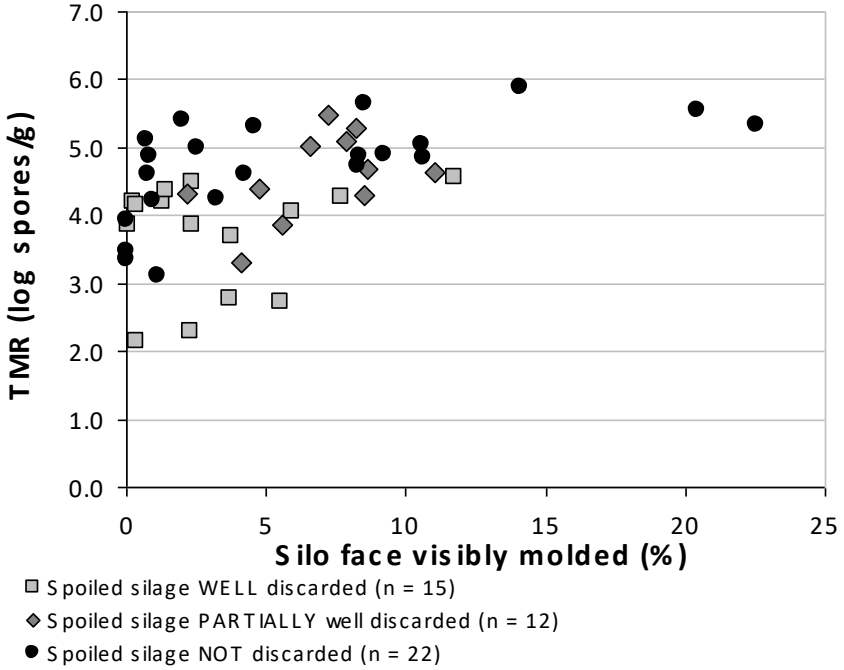

Figure 4. Scatter plot of the anaerobic and facultative anaerobic sporeformers in the TMR and the percentage of visible aerobic deteriorated corn silage fed to the dairy cows on the 49 commercial dairy farms in northern Italy, in relation to the silage management practices before feed-out.

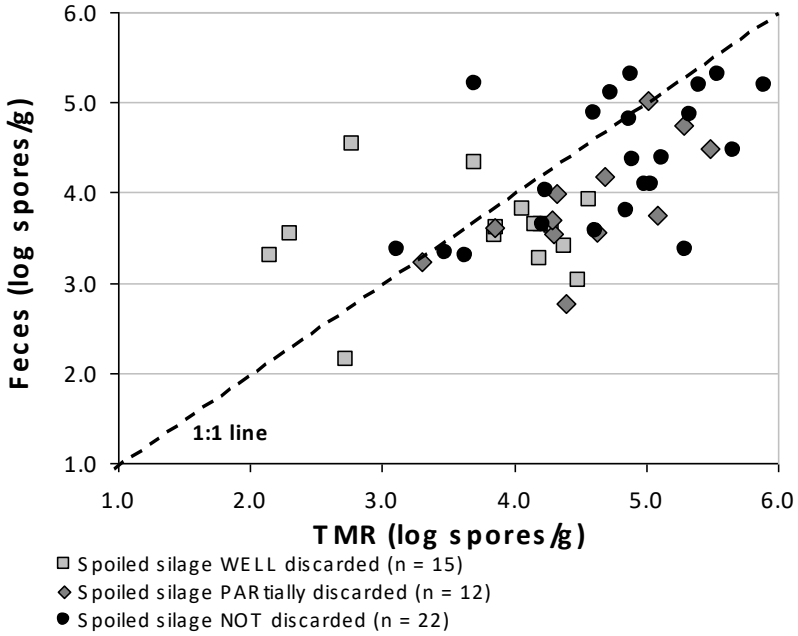

Figure 5. Scatter plot of the anaerobic and facultative anaerobic sporeformers in the TMR and feces of the 49 commercial dairy farms in northern Italy, in relation to the silage management practices before feed out. The dotted line is the 1:1 line.

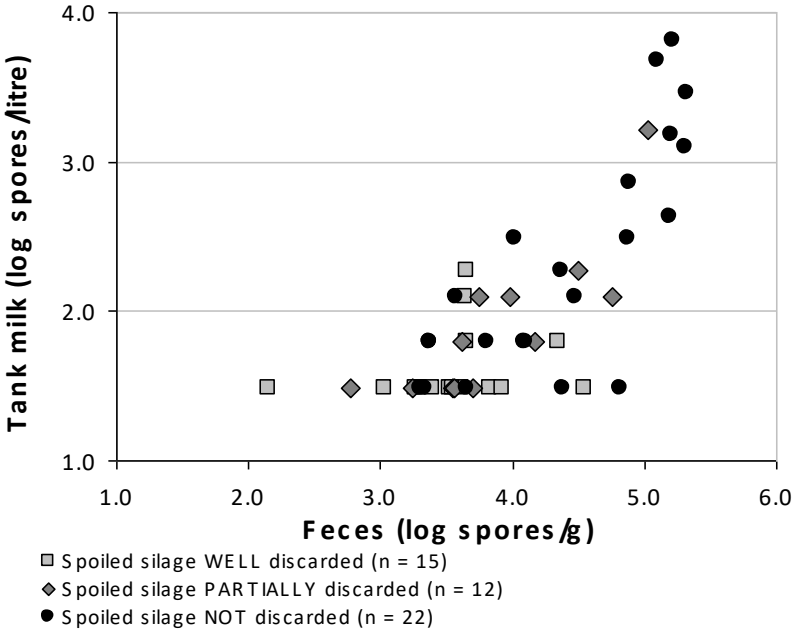

Figure 6. Scatter plot of the anaerobic and facultative anaerobic sporeformers in the feces and milk of the 49 commercial dairy farms in northern Italy, in relation to the silage management practices before feed-out.

Conclusion Part

11. General Conclusion

The purpose of this thesis has been to extend the current understanding of the negative impact of aerobic deterioration on corn silage and to analyze different solutions to help avoid it. Whole-crop corn is one of the most common crops conserved as silage throughout the world because of its optimal characteristics for direct ensiling, high DM yields per hectare and supplies of both fiber and starch in the animal ration. Corn silages rarely undergo butyric acid fermentation. However, the main problem for the correct management of corn silage is that of aerobic deterioration during feed-out. Much of the effort made to produce high yields per hectare of high-quality silages is nullified when aerobic deterioration occurs.

The preservation of the nutritive, fermentative, microbiological and hygienic quality of silages during the feed-out phase of ensiling should begin from the first phases of ensiling. The quality of silage conservation, from fermentation to feed-out, depends on the competition between different groups of microorganisms. The fermentation that a crop undergoes can be explained by considering the interaction that takes place between the microbial population and other factors, such as oxygen, moisture content, buffering capacity and the sugar content of the crop. In each phase of ensiling, many microorganisms can sequentially find optimal conditions for growth, and can be activated or inhibited, with a considerable impact on silage quality. The fermentation profile of silage influences the activity of microorganisms. Aerobic microorganisms, such as yeast, mold and acetic acid bacteria, represent the main spoiling agents of silage during aerobic exposure. Therefore, several procedures could be adopted to modulate the fermentation process in order to inhibit spoiling microorganisms and to avoid aerobic deterioration. In particular, the activity of lactic acid bacteria (LAB) inocula, the addition of chemical additives and the adoption of correct silage management practices can reduce the presence of aerobic deterioration and its negative impact in microbial, fermentative, nutritional and hygienic terms.

Silage additives can be useful to correctly target silage fermentation and to improve aerobic stability as they modify the final fermentative profile. Thus, biological and chemical additives with antifungal proprieties have been widely studied and used over the years (Muck et al., 2018). The type and amount of fermentative silage products have a strong effect on the inhibition of the spoiling microorganism. Acetic acid has been shown to have a strong effect on the reduction of the yeast count. The yeasts are considered as the initial agent of aerobic spoilage, and many studies have confirmed the relationship between acetic acid and aerobic stability (Kleinschmit and Kung, 2006b; Comino et al., 2014). The results of **paper I** confirm the effect of acetic acid on a reduction of the yeast count. However, the results show that aerobic stability is low for large amounts of acetic acid (i.e. greater than 40 g/kg DM). The main reason for this

is that acetic acid bacteria belonging to the *Acetobacter* genus may have started the deterioration process, instead of yeasts, as previously observed by Dolci et al. (2011) and Spoelstra et al. (1988).

Heterofermentative LAB have been developed to modify the fermentative profile in order to improve aerobic stability because of their ability to produce acetic acid. LAB inocula require specific conditions to multiply and to be able to dominate fermentation. In these conditions, they perform a good action at a low cost. Heterofermentative *Lactobacillus buchneri* (LB) is the most commonly adopted inoculum to improve aerobic stability. *L. buchneri* needs a long conservation period (i.e. more than 90 d) to be effective (Driehuis et al., 1999; Kleinschmit and Kung, 2006b). The results of **paper I** show that when LB was inoculated, it did not show any effect on aerobic stability after 15 and 30 days of conservation on either sorghum or on corn. Furthermore, inconsistent results have emerged from the experiments dealt with in **papers I, II** and **III,** concerning the effect of LB on improving aerobic stability after a longer conservation period than 100 d. These differences could be attributable to the DM content of silages. As reported by other authors (Hu et al., 2009; Comino et al., 2014; Xu et al., 2019a), LB shows a limited activity in silages with a higher DM than 38%. Because LB needs a long conservation period to be effective, a new strain of *L. hilgardii* (LH) was developed to be active after a short conservation period. The results of **paper I** show that the addition of LH alone or in combination with LB increases the aerobic stability of a silage after short and long conservation periods. Moreover, as shown in **paper I** and **III,** the new strain has shown the possibility of having a complementary and synergistic effect with LB, even though the results were not consistent between trials.

The variable effectiveness of microbial inoculants from year to year is one of the main issues of their use, since they are dependent on the environmental conditions and on the forage characteristics. Therefore, chemical additives have been developed over the years because their action is independent of the microbial activity. Chemical additives used to improve aerobic stability are composed of acids or their salts, which show antifungal proprieties, and the strongest effect has been shown for propionic acid and its mixtures. One of the problems about the use of chemical additives is that they often require a high application rate to be effective, and this in turn leads to management and economic concerns. Moreover, there is an increasing need to find chemical additives with low levels of toxicity. In this regard, **paper II** analyzes the effect of a low toxic additive, composed of a mixture of monopropionine and monobutyrin, on improving the aerobic stability of corn silage. The results have shown that the new additive is able to reduce the yeast count and improve aerobic stability.

The results of **paper I, II** and **III** suggest that the addition of additives can help to reduce the yeast count and improve the aerobic stability of silages, especially when a natural decrease in the yeast count is not easily obtained (e.g. short conservation period, high DM content of the silages, high environmental

temperature). However, the use of additives should never be regarded as a substitute for good silage-making practices. The adoption of good management practices plays a key role in obtaining a good quality silage. The results of **paper V**, which analyzes the role of the aerobic deterioration of corn silage on the proliferation of anaerobe spores, point out the effect of silage management practices on the reduction of aerobic deterioration. As previously reported by several authors (Muck and Holmes, 2000; Borreani et al., 2018), it has been shown that reaching a high silage density, conserving corn silage in a bunker with concrete walls, covering the silo top with at least 2 plastic films, using the plastic film on the silo walls, weighting down the silage cover, ensuring a feed-out rate of at least 0.8 m/week, and discarding all the spoiled silages allow the presence of aerobic deterioration to be avoided and, consequently, the anaerobic sporeformer count to be lowered.

The presence of spoiled silages on a farm represents a problem that results in a reduction of the fermentative and nutritive value of the silage and an increase in the risks to animal and human health. Borreani et al. (2018) showed that when molds are visible on a silage (mold count greater than 5.0 log cfu/g), the DM losses are greater than 20% of the original ensiled DM. Accordingly, **paper II** and **III** have confirmed a relationship between mold count and DM losses during aerobic deterioration. Furthermore, **paper III** reports that when molds started to grow during aerobic deterioration, the nutritional value of the silages decreased, with an increase in NDF and a great reduction in the starch content, which represents the main energy component of corn silage.

Unlike properly made and managed silage, poorly made or contaminated silages can harbor pathogens that reduce animal performance, cause cattle diseases, reduce the safety of dairy products and constitute a threat to human health. The occurrence of *Paenibacillus* and *Clostridium* spores in silage is of great concern for dairy producers, because their spores can contaminate milk and cause a reduction in the shelf life and can damage processed milk and semi-hard cheeses. The results of **paper V** show that the *Clostridium* and *Paenibacillus* spores in milk are influenced to a great extent by the inclusion of aerobically deteriorated corn silage in TMR. This indicates that although clostridial fermentation is not common in corn silage, the presence of aerobic deterioration allows spores to contaminate the milk.

Spoiled corn silage allows pathogenic and/or mycotoxigenic fungi to grow. **Paper IV** analyzed the deveolpment of *Aspergillus fumigatus*, the causal agent of aspergillosis in humans, during conservation and the aerobic exposure of silages. The results show the role of aerobic deterioration on the proliferation of *A. fumigatus*, with potential issues for farm safety (farm workers and animals). Furthermore, the increase in problems related to the risk of developing genes resistant to some fungicides used at a hospital level (e.g. demethylation inhibitors) makes it necessary to understand the role of high pressures treatments with fungicides in crop fields on the development of the resistance of some strains of *A. fumigatus*. The paper has shown that the presence of aerobic

deterioration determines a favorable environment for the development of *A. fumigatus*. However, treatments with DMI fungicides did not affect the sensitivity of *A. fumigatus* isolates collected from corn before and after ensiling.

Among the mycotoxins, aflatoxins produced by *Aspergillus flavus* represent a risk for animal and human health due to their carry-over into milk (Veldman et al., 1992). Silage is not a favorable environment for the survival and development of *A. flavus* during conservation, if anaerobiosis is maintained. However, the results of **paper III** show that, during aerobic deterioration, when the inhibiting conditions for mold growth were depleted, a high pH coupled with a high temperature allowed *A. flavus* to grow, thereby determining the *ex-novo* production of aflatoxins. Interestingly, the analysis of the gene pattern of isolated colonies of *A. flavus* showed that only 43% of the selected colonies were able to produce aflatoxins in vitro.

The thesis has shown that the several microorganisms present in silage play a key role in the successful outcome of silage conservation. As a result of the importance of the microbial populations associated with the ensiling process, the rapid development of techniques that can be used to quantitatively and qualitatively analyze microbial populations has made it possible to improve the knowledge of silage science. Further advances in new generation RNA sequencing (metatranscriptomics) will allow us to analyze only live populations in silage. Moreover, further advances in new generation sequencing will allow us to identify the genes coding for enzymes involved in silage acid production and in the metabolism, proteolysis or the production of mycotoxins. Furthermore, an in-depth analysis will be useful to identify various antimicrobial compounds at a low level (e.g. bacteriocins) that may influence the course of fermentation (McAllister et al., 2018). More efforts are necessary to analyze fermentation products and this will help to clarify the effect of the volatile organic compounds that come from silages on the environmental sustainability of the process and to understand whether these compounds are caused directly by microorganisms or indirectly by chemical interactions that take place during storage.

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