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Chapter 1

1 Foreword

1.1 Dairy cow transition period

The period from three weeks before to three weeks after calving is defined as transition period or peripartum period of dairy cattle. It is critically important to health, production, and profitability of dairy cows. This period is the main of dairy cattle cycle production studied during the last years to understand the rapidly changing nutritional requirements and nutrient metabolism from late gestation to early lactation (Drackley et al., 2006). A number of physiologic changes occur in the peripartum period that modifies the endocrine status and metabolism to prepare the cattle for parturition and lactogenesis (Grummer, 1995).

The growth hormone, plasma thyroxine and estrogen of placental origin increases during late gestation, the growth hormone has the higher level at parturition (Grummer, 1995; Kunz et al., 1985). Progesterone concentration during the dry period is elevated for maintenance of pregnancy (Chew et al., 1979). Glucocorticoid and prolactin concentrations increase on the day of calving (Edgerton and Hafs, 1973). Plasma insulin, plasma thyroxine and estrogen concentrations decrease at calving (Grummer, 1995; Kunz et al., 1985). Progesterone decline rapidly two days before calving (Chew et al., 1979). Plasma glucose concentration remain stable or increase slightly during the prepartum period, increase strongly at calving, and then decrease immediately in the postpartum (Kunz et al., 1985; Vazquez-Anon et al., 1994). The increase at calving may result from increased glucagon and glucocorticoid concentrations that promote depletion of hepatic glycogen stores. Although the demand for glucose by mammary tissue for lactose synthesis continues after calving, hepatic glycogen stores begin to replete and are increased by fourteen days postpartum (Vazquez-Anon, 1994) probably due to an increased gluconeogenic capacity to support lactation (Grummer, 1995). Plasma alpha-amino nitrogen concentrations increase during the transition period except for a temporary decrease at calving (Kunz et al., 1985). The majority of metabolic problems associated with the transition period begins by one day postpartum (Grummer, 1995).

During transition period the requirements of energy, protein and minerals for fetal development are very high, and increase during gestation period (Goff and Horst, 1997). At final stage of gestation, fetus development requires about 0.82 Mcal of energy, 117 g of protein, 10.3 g of calcium, 5.4 g of phosphorus, and 0.2 g of magnesium (Bell et al., 1995; House and Bell, 1993). Reynolds et al., (1986) reported that during late pregnancy, the metabolic demands for fetal development are doubled compared that of the mother. However, the fetus metabolic demands are low if compared to the requirements of colostrum formation. The production of just 10 kg of colostrum on the day of calving requires that 11 Mcal of

energy, 140 g of protein, 23 g of calcium, 9 g of phosphorus, and 1 g of magnesium be supplied from the diet or be brought to the mammary gland from body stores (Goff and Horst, 1997). About 85% of whole-body glucose is used for milk synthesis and secretion by mammary gland (Sordillo et al., 2009).

During the peripartum period the dry matter intake, and consequently nutrient intake, are reduced greatly and are unable to cover the bovine needs. Requirements for net energy of lactation and metabolizable protein by healthy cows at four days postpartum exceeded intakes by 26% and 25%, respectively (Drackley, 1999). In these conditions, cows are usually in severe negative energy balance (NEB) and serum or plasma concentrations of several minerals and vitamins are reduced (Bell et al., 1995; Goff, 2006; Ingvartsen and Moyes, 2013). To compensate the inadequate dry matter intake and limit the NEB condition during the transition period, fat from body stores are mobilized and the released as non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) into the blood stream. NEFA and BHB bodies are important sources of energy because the majority of available glucose is redirected to the mammary gland for lactose synthesis (Herdt, 2000). Although mobilization of lipid is a normal physiological adaptation to critical changes occurring in the periparturient period, the excessive mobilization has been linked to an increased risk of diseases (Vernon, 2005; Contreras and Sordillo, 2011). The diet formulation during prepartum should be an important focus to minimize transition stress. The main objective is to meet the nutritional requirements for milk production and reproduction because the physiological state changes rapidly and affect the dry matter intake. Indeed the variability of dry matter intake during the first week post partum is 30-40%, instead after peak of lactation is 6-10% (Drackley, 1999). To maximize the productivity and ensure adequate reproductive performance, rations fed during this time need to be nutrient dense and often expensive due to the higher quantity of concentrates and vitamins integration. Failure in adequately meet this challenge can result in a range of early-postpartum health problems, some potentially fatal and compromised lactation performance (Bell, 1995). In fact, most of the infectious diseases and metabolic disorders as milk fever, ketosis, retained fetal membranes, metritis, and displaced abomasum may occur during the periparturient period and can cause a decreased production (Drackley, 1999).

Moreover the typical immunodepression during the pariparturient period represents the second main factor for increased susceptibilty to mastistis (Mallard et al., 1998). As reported by Wallace et al. (1996) cows with health disorder at calving, produced 7.2 kg less milk per day during the first 20 d postpartum compared to healthy cows. Furthermore, they reported that the retained fetal membranes and subsequent metritis produced 8.2 kg less milk per day,

displaced abomasum and secondary ketosis produced 8.5 kg/d less, than cows with no health disorders. For cows with displaced abomasum and ketosis, the projected 305-d mature equivalent milk yield was significantly lower than that for healthy cows. Hostens et al. (2012) reported that cow with one or more metabolic diseases had lower and delayed peak of lactation mainly on dairy cows at second or more lactation (figures 1,2,3). These authors observed that milk fever, retained placenta, ketosis, and mastitis mainly affected the lactation curve when accompanied by another metabolic disease, whereas metritis and displaced abomasum affected the lactation curve equally with or without another metabolic disease. In addition to the drop in production must also be considered the costs of treatment of the cows and the reproductive success due to negative energy balance and extensive loss of body condition during the periparturient period (LeBlanc et al., 2002; Gilbert et al., 2005). Thus, the important physiological, metabolic and nutritional changes occurring during the transition period making this an interesting time for dairy producers (Drackley, 1999). The success of the transition period effectively determines the profitability of the cow during that lactation. Nutritional and management strategies to facilitate the periparturient transition are based on a thorough understanding of the quality and quantity of nutrients required to support the final stage of pregnancy, calving and milk synthesis during early lactation. The metabolic changes in liver and adipose tissue are other important aspects in peripartum period (Bell, 1995).

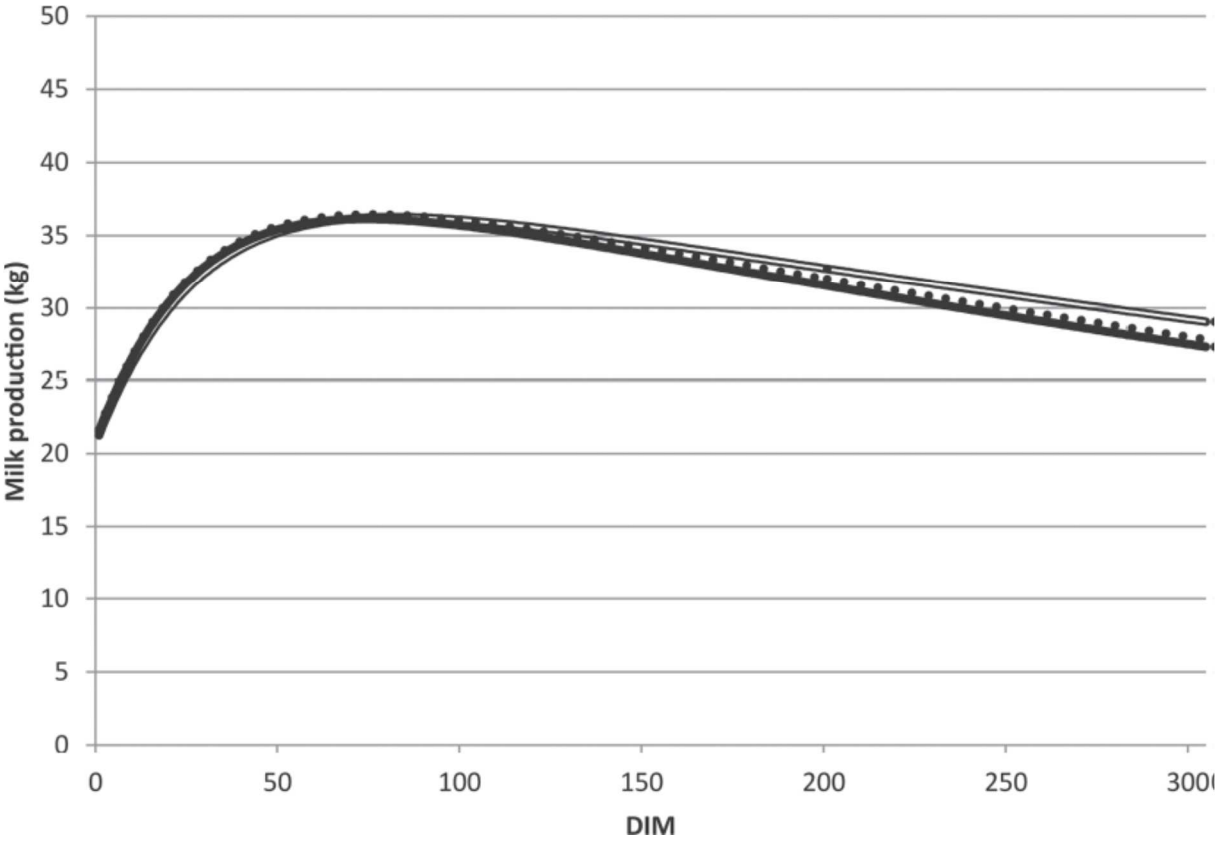


Figure 1. The effect of metabolic problems on lactation curve shape in first-lactation cows: cows that encountered no metabolic problems during the transition period (solid line), cows that encountered 1 metabolic problem during the transition period (dotted line), and cows that encountered >1 metabolic problem during the transition period (double line). (Hostens et al., 2012).

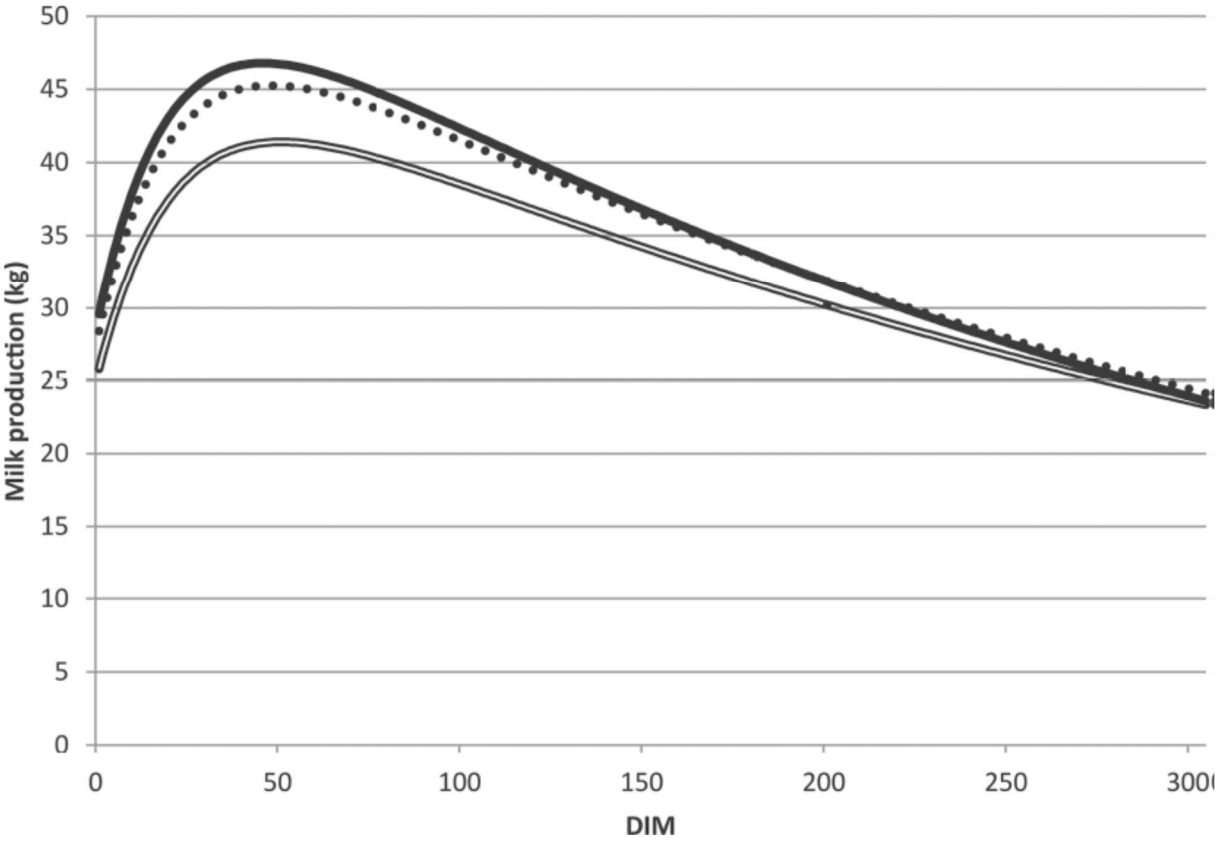


Figure 3. The effect of metabolic problems on lactation curve shape in second-lactation cows: cows that encountered no metabolic problems during the transition period (solid line), cows that encountered 1 metabolic problem during the transition period (dotted line), and cows that encountered >1 metabolic problem during the transition period (double line). (Hostens et al., 2012).

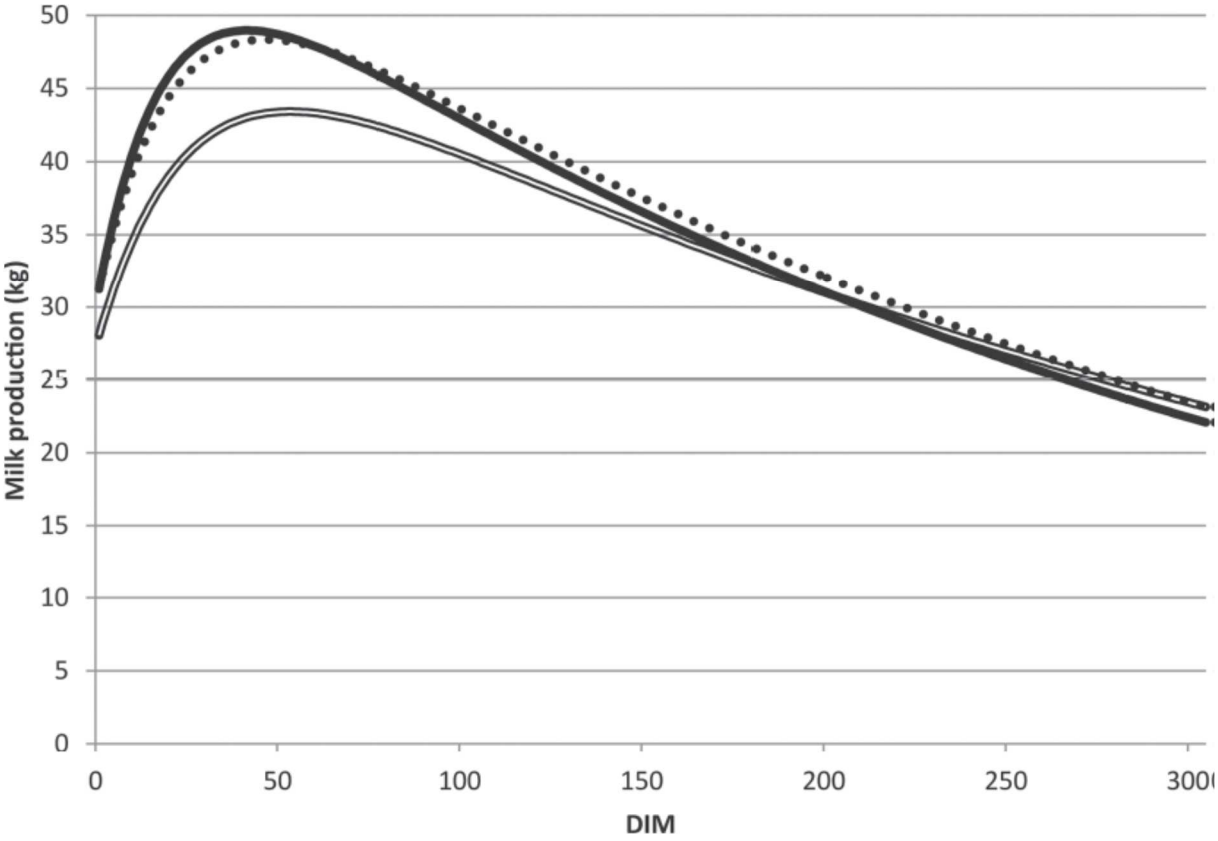


Figure 3. The effect of metabolic problems on lactation curve shape in third- or greater-lactation cows: cows that encountered no metabolic problems during the transition period (solid line), cows that encountered 1 metabolic problem during the transition period (dotted line), and cows that encountered >1 metabolic problem during the transition period (double line). (Hostens et al., 2012).

1.1.1 NEFA and BHB

The blood concentration of serum non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) can be used as indicators to evaluate the negative energy balance during the transition period and consequently the risk of metabolic disease (Adewuyi et al., 2005; LeBlanc, 2010). As reported by Drackley et al. (2001), after calving, there is a decrease in insulin production by the pancreas, which results in decreased glucose utilization by insulin sensitive organs. The combined state of insulin resistance and associated with an increase in adipose tissue sensitivity to catecholamines and an exuberant lipolytic response, allows the mammary gland to have additional glucose for milk production (Herdt, 2000; Holtenius et al., 2003; McArt et al., 2013). For these reasons, alternative fuel sources are needed in the body to maintain normal function during the post-partum period when milk production increased. In response to a decrease in available glucose, there is an increase in lipolysis releases NEFA, which are transported through the blood. The fatty acids released from adipose tissue, can be used directly as a fuel source by various tissues, are used for milk fat synthesis, or are taken up by the liver (Herdt, 2000). Thus, the concentration of NEFA in blood reflects the degree of adipose tissue mobilization. Therefore, greater is the extent of negative energy balance, more NEFA are released from body fat and higher is their concentration in the blood. The liver of cows takes up NEFA from the blood that flows through it at a rate of 15–20% (Drackley and Andersen, 2006). Once taken up by the liver, NEFA can be: 1) completely oxidized to carbon dioxide to provide energy for the liver, 2) partially oxidized to produce ketone bodies (acetone, acetoacetic acid, and BHB), that are released into the blood and serve as fuels for other tissues, or 3) reconverted to triglycerides and included into very low density lipoproteins for transport back to the adipose tissue, or stored as triglyceride (McArt et al., 2013).

Incomplete oxidation of NEFA leads to the formation of ketones, primarily acetoacetate and BHB. The mechanism of ketone body elevation in the blood of ketotic animals is as follows: acetyl-CoA production from fatty acids in the liver exceeds the capacity of the tricarboxylic acid cycle to condensate the acetyl group with oxaloacetate to citric acid. Therefore, acetyl-CoA accumulates, resulting in increased ketone body synthesis. Ketone formation is also favored when blood glucose and insulin concentrations are low, partially because of greater fatty acid mobilization from adipose tissue. Low insulin probably enhances fatty acid oxidation by decreasing hepatocyte malonyl-CoA concentrations and sensitivity of carnitine palmitoyltransferase-1 to malonyl-CoA. Carnitine palmitoyltransferase-1 is responsible for

translocating fatty acids from the cytosol to the mitochondria for oxidation and is inhibited by malonyl-CoA (Li et al., 2006).

NEFA oxidation leads to increased reactive oxygen species (ROS) production and eventually to the development of oxidative stress (Miller et al., 1993) that is an another important factor that may contribute to dysfunctional inflammatory responses in metabolically stressed cows during the periparturient period (Miller et al., 1993; Sordillo and Aitken, 2009). Aerobic cellular metabolism requires oxygen for the efficient production of energy and consequently produces ROS including oxygen ions, free radicals and lipid hydroperoxides. The levels of cellular ROS are maintained within a narrow physiological range to optimize cell performance and prevent cellular damage by a network of antioxidant defense mechanisms. Increased oxygen metabolism during the periparturient period, however, increases the rate of ROS production and the subsequent depletion of important antioxidant defenses (Bell, 1995; Sordillo and Aitken, 2009). As a result, excessive accumulation of ROS can cause cell and tissue injury and lead to a condition referred to as oxidative stress in periparturient dairy cows. Oxidative stress is thought to be a significant underlying factor leading to dysfunctional host immune and inflammatory responses particularly during times of increased metabolic stress.

The threshold limit of pre and post-partum NEFA are 0.3-0.5 mEq/L and 0.7-1.2 mEq/L respectively. Beyond these limits the risk of post-partum health problems increases (LeBlanc et al., 2005; Ospina et al., 2010a,b; Chapinal et al., 2011; Roberts et al., 2012). Chapinal et al. (2011), indicated that pre-partum BHB concentrations 0.8 mmol/L were associated with post-partum problems. Post-partum BHB range concentration that maximize accuracy of disease prediction and production is 0.9-1.6 mmol/L with the majority between 1.2-1.4 mmol/L (LeBlanc et al., 2005; Walsh et al., 2007; Ospina et al., 2010a,b; Chapinal et al., 2011; Seifi et al., 2011; Roberts et al., 2012).

Higher pre-partum NEFA concentration increases the risk of clinical ketosis, metritis and retained fetal membranes and decreases the milk yield in dairy cattle (Ospina et al., 2010a,b; Chapinal et al., 2011; Chapinal et al., 2012). Although few studies reported the association between high post-partum NEFA concentrations and health problems, the literature reported that the risk of metritis, ketosis and culling are higher in dairy cows with elevated post-partum NEFA concentrations (McArt et al., 2013). Chapinal et al. (2012) found that multiparous cows with high concentrations of post-partum NEFA produced less milk than cows with normal NEFA concentrations. Interestingly results were found by Ospina et al. (2010a), where estimated at 120 DIM was decreased in multiparous animals, but increased in

primiparous cattle with elevated post-partum NEFA. Chapinal et al. (2011, 2012) reported that animals with higher pre-partum BHB concentrations show more risk of displaced abomasum with reduced milk production. Roberts et al. (2012) reported that dairy cows with higher pre-partum BHB concentrations had more probability to die within the first 60 DIM than cows with lower BHB. As to post-partum NEFA concentration, also the higher level of BHB increases the risk of health problems (LeBlanc et al., 2005) and reduces the milk yield (Chapinal et al., 2012). Blood NEFA concentrations have been found to be a more accurate measure of NEB than ketone bodies. Studies by Ospina et al. (2010a, 2010b) and Chapinal et al. (2011) reported that blood NEFA concentrations have been found to be the more accurate measure of NEB than BHB, both pre-partum and post-partum, and offer more information on risk of negative health problems, reproductive performance and milk production than blood BHB concentrations.

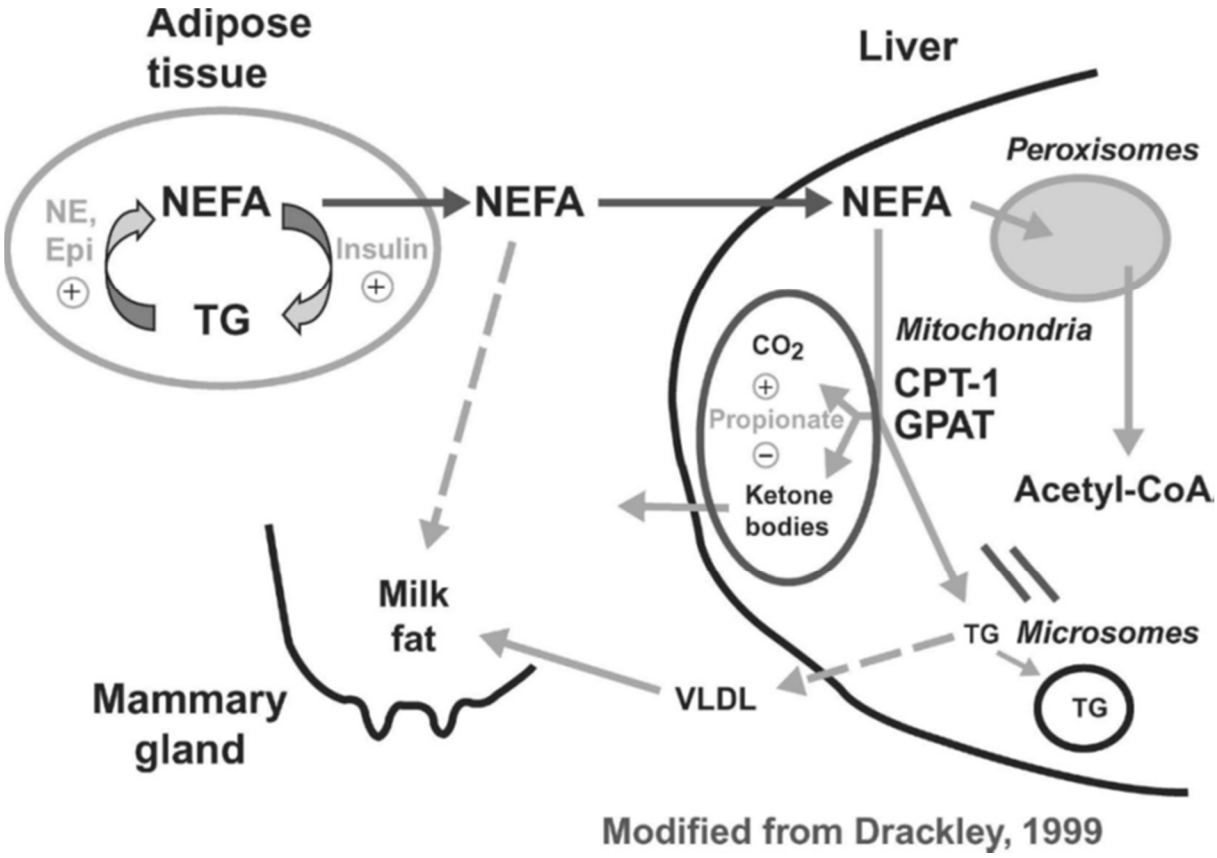


Figure 4. Schematic of metabolic relationships among adipose tissue, liver, and mammary gland during the transition period; NE = norepinephrine, Epi = epinephrine, CPT-1 = carnitine palmitoyltransferase-1,GPAT= glycerol-3-phosphate acyltransferase, TG= triglyceride, CoA = coenzyme A, VLDL = very low density lipoprotein.

1.1.2 Ketosis and Fatty liver

Ketosis is a major disease that occurs during the transition period and is associated with a NEB (Li et al., 2016). This disorder may result from the high productive and reproductive performance of animals under NEB conditions (Radostits et al., 2006). Because the dry matter intake (DMI) is depressed, the glucose demands associated with lactation or/and fetal development increase, the production of propionate during the early postpartum period is not sufficient and insulin concentration is low. Low insulin probably enhances fatty acid oxidation by decreasing hepatocyte malonyl-CoA concentrations that inhibits carnitine acyltransferase, which is the enzyme responsible for transfering fatty acids into the mitochondria for oxidation (Emery et al., 1992). The antiketogenic properties of propionate are likely due to indirect effects as insulin as well as direct effects on hepatic metabolism (Grummer, 1993). Ketosis is characterized by relatively high concentrations of NEFA and ketone bodies (acetoacetate, b-hydroxybutyrate (BHB), acetone) and concurrent low blood glucose concentrations in the blood (Grummer, 1995). If animals are unable to adapt to NEB, due to management factors, disease, or many other factors, the body switches over to fat metabolism forming ketone bodies which are used as fuel for many tissues, sparing glucose for milk production. Ketosis is categorized as clinical and subclinical depending on individual animal ability to tolerate and process ketone bodies. Clinical ketosis is originated from a poor diet with a strong imbalance between energy needs and insufficient nutrient intake. It is recognized as increased blood, urine or milk ketone bodies with visible clinical signs like rapid weight loss, dry feces, dry hair coat and sharp drop in milk yield (Duffield, 2000; Herdt, 2000). Subclinical ketosis is defined as a preclinical stage of ketosis in the absence of the clinical signs. Increased ketone body level reduces milk production, causes delayed reproductive functions return to normal after calving, increased intervals from calving to first and last service, and an increased frequency of ovarian cysts (Andersson, 1988).

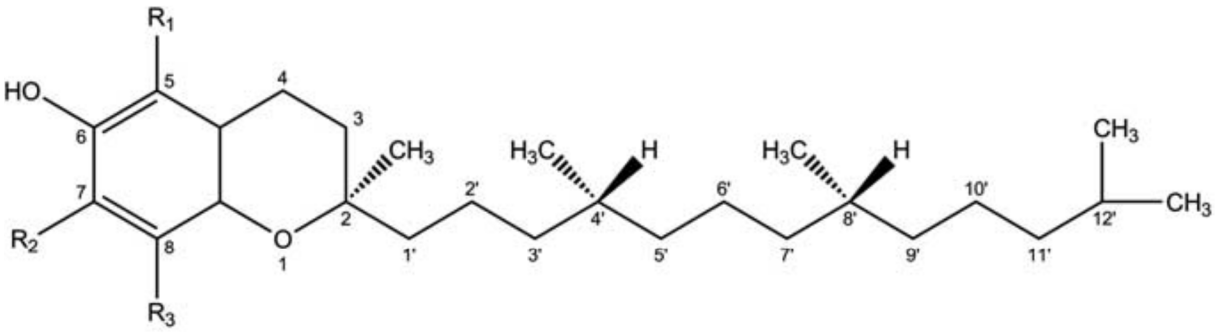
Fatty liver is a disease that develops when the hepatic uptake of lipids exceeds the oxidation and secretion of lipids by the liver and thereby causes accumulation of triglycerides (TG) in the liver, which is associated with decreased metabolic function of the liver. The incidence of fatty liver is strongly associated with the incidence of other metabolic disorders, especially ketosis and displaced abomasum because these metabolic disorders have in common that the cows either are or will be in a severe negative energy balance. Fatty liver can be categorized into normal liver and mild (1-5% liver TG), moderate (5-10% liver TG), or severe fatty liver (>10% liver TG). Insufficient or unbalanced dietary intake, obesity, and elevated estrogen

concentrations are involved in the etiology of fatty liver, which is associated with dystocia, diseases, infections, and inflammations (Bobe et al., 2004).

1.2 Vitamin E

Vitamin E play an important role as a component of cellular antioxidant systems. In nature it is present under eight different forms, four tocopherols (*α*-, *β*-, *γ*-, *δ*) (figure 5) and four tocotrienols (*α*-, *β*-, *γ*-, *δ*), of which *α*-tocopherol is the most bioactive. *α* -tocopherol contains three centers of asymmetry, therefore eight possible stereoisomers exist. Four of the eight possible stereoisomers possess the 2R configuration (RRR, RRS, RSS and RSR) and the other four stereoisomers possess the 2S configuration (SSS, SRR, SSR and SRS) (figure 6). In the natural form of *α*-tocopherol, all three centers of asymmetry possess the R configuration (Politis, 2012). The RRR *α*-tocopherol is the only isomeric form of vitamin E produced by plants and is therefore the only one naturally present in feedstuffs (Vagni et al., 2011). The synthetic form of *α*-tocopherol contains all the eight possible stereoisomers in equal proportions and it is known as the all-rac form (Politis, 2012). The acetate ester of all-rac α-tocopherol is the most common form of supplemental vitamin E fed to dairy cows. Plasma vitamin E levels fall significantly at calving in the dairy cow and normally the concentration levels are under the limit considered adequate for health. It has been suggested that this is one of the mechanisms for the decrease in peripartum immune system efficiency. The liver plays an important role in the release of α-tocopherol into the circulation and consequently its transfer to peripheral tissues. This function requires the mediation of the hepatic, cytosolic 30-kDa α-tocopherol transfer protein (TTP). TTP allows the incorporation of vitamin E into VLDL that are released from the liver for subsequent extrahepatic distribution (Baldi, 2005). The antioxidant functions of *α*-tocopherol in the cell is that of conclude the chain of events of oxidative processes by donation of its phenolic hydrogen to chain propagating lipid peroxyl radicals, resulting in the enhanced formation of the less reactive *α*-tocopheroxyl radical (Zhang and Omaye, 2001). Several studies investigated the effect of vitamin E supplementation during the periparturient period on immune function. The data reported suggesting that vitamin E supplementation mitigates the decline in immune function that occurs during the periparturient period (Hogan et al., 1992; Politis et al., 1995). Plasma concentrations of α-tocopherol have been related to prevalence of retained fetal membranes and mastitis and neutrophil function (Weiss et al., 1994; LeBlanc et al., 2004). Politis et al. (2001) performed an interesting study on which they observed a positive association of vitamin E supplementation with gene expression of urikinase-plaminogen activator present on the cell membrane of neutrophils and that is fundamental to reach the point of inflammation. Baldi et al. (2004) reported a positive effect of *α*-tocopherol in a bovine mammary epithelial

cell line, susceptible to ochratoxin A cytotoxicity. The study also found that *α*-tocopherol significantly inhibited ochratoxin A induced ROS production in other cell lines and that inhibition was concentration dependent. The excess of ROS production is considered one factor causing apoptosis. Inhibition of ROS production by vitamin E should therefore delay this process. The functions of vitamin E is closely related to the supplementation of adequate dietary levels of other nutrients involved in the cell antioxidant system. For example the selenium is important to maintain proper levels of the components of the tissue defense mechanisms against free-radical damage because is a component of the enzyme glutathione peroxidase (GSH). (Vagni et al., 2011). Although the main functions of vitamin E are as an antioxidant, it has other properties. The *α*-tocopherol can influences various signalling cascades at the cellular level by inhibiting protein kinase C (PKC). PKC has an important role in the cell signal transmission induced by growth factors and hormones following receptor interactions (Baldi, 2005). The NRC (2001) reported that the vitamin E intake is adequate to maintain the correct health and immune function in cows when *α*-tocopherol plasma concentrations are above 3-3.5 μg/ml.



Vitamin E form	R_1	R_2	R_3
α-Tocopherol	CH_3	CH_3	CH_3
β-Tocopherol	CH_3	H	CH_3
γ-Tocopherol	H	CH_3	CH_3
δ-Tocopherol	H	H	CH_3

Figure 5: Diagrammatic representation of structural characteristics of the major vitamin E forms (Politis, 2012).

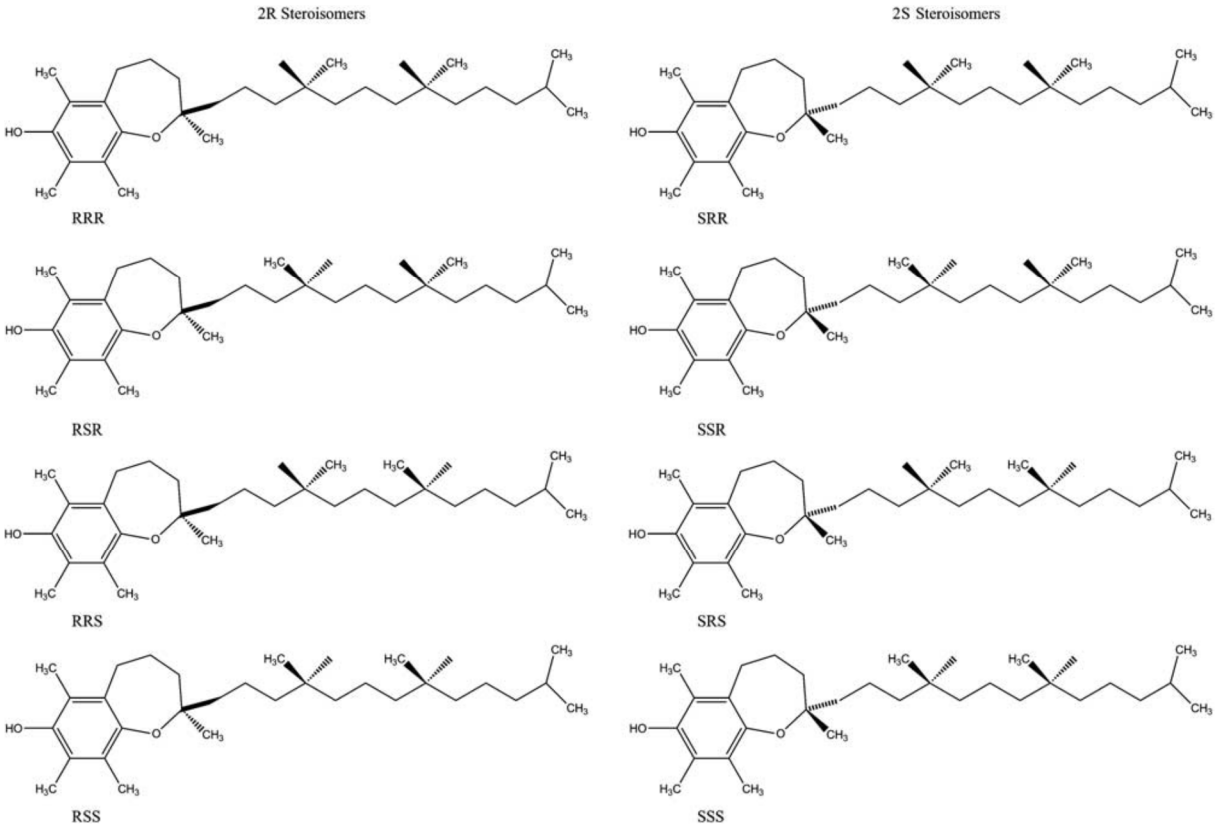


Figure 6: Diagrammatic representation of various stereoisomers of a-tocopherol (Politis, 2012).

1.3 Silage

The silage represent the main raw material administered to dairy cattle. Ensiled grass and corn, often are the major part of the feed ration of cattle. Forage provides the animal with dietary fibre, which is essential for the normal functioning of the rumen. Due to the huge amount of this forage usually used for dairy cow nutrition, the quality of this product, in terms of amount of nutrients provided and safety, is important to support both production and health status of cow. The high quality and safety of silage is obtained primarily avowing undesirable fermentations along the storage period. When the silage is not carefully preserved, severe alterations may occur mostly due to contamination of fungi, bacteria and molds with consequent health problems for the dairy cows and humans. The preservative effect against the growth of harmful microorganisms is due to a first aerobic phase with acetic acid production and consequent anaerobiosis and acidification with lactic acid production. The ensiling process begins with the enzymatic activity of intact plant cells and is called residual respiration. These cells consume the oxygen entrapped in the silage and use carbohydrates such as glucose and fructose (Shao et al., 2005). Early consumption of carbohydrates is detrimental for the subsequent anaerobic lactic acid fermentation, which is the major effect of silage preservation. The acidification of ensiled mass promotes the development of acid-tolerant lactic acid bacteria (LAB) species such as *L. brevis*, *L. plantarum* and *L. buchneri*, which convert water-soluble carbohydrates mainly into lactic acid (Holzer et al., 2003). This is called the fermentation phase, which starts when the ensiled mass has become anaerobic and can last for several days or weeks, during which different groups aerobic or anaerobic microorganisms naturally found in plants compete for available nutrients (Dunière et al., 2013). The aerobic microflora develops until oxygen has been entirely consumed or the silage pH is fairly acid to stop their metabolism. The initial microbial activity is mostly due to epiphytic aerobic flora such as Enterobacteria, yeasts and molds (Dunière et al., 2013). The characteristics of the ensiled crops, the environmental conditions and practices adopted during silage making have a strong impact on the nutritional value and fermentation stability of the silage (McDonald et al., 1991). During acidification and conservation, the oxygen into the silage can cause dry matter and nutritional losses as well as increase the risk of proliferation of potentially pathogenic or otherwise undesirable microorganisms as yeast, clostridia and bacilli that are acid tolerant or survive as spores (Driehuis and Oude Elferink, 2000). The silage microflora plays an important role in the conservation process. It can be divided in two groups. Different types of lactic acid bacteria (LAB) are the desirable micro-organisms and

are involved into the aerobic and anaerobic phases. The undesirable micro-organisms are the organisms involved in anaerobic spoilage (e.g. clostridia and enterobacteria) and aerobic spoilage (e.g. yeasts and moulds). These micro-organisms can decrease the nutritional value of the silage, such as the dry matter reduction or the protein degradation. Furthermore, they can have a detrimental effect on animal health and/or milk quality (Driehuis and Oude Elferink, 2000). Another key factor that ensure the well-preserved nutrient on silage is the aerobic stability. A realistic practical target for silage aerobic stability is 7 days exposure to air without significant temperature rising or visible mould contaminations, including the time in the feeding alley. To reach this target four key objectives should be achieved: 1) Low contamination of the crop with epiphytic yeasts and moulds before the harvesting; 2) achieve the correct density of the silage mass in the trench; 3) effective sealing of the silo, with an oxygen barrier film; 4) remove the silage from the exposed feedout face, to avoid the air oxidation (Wilkinson and Davies, 2012).

1.3.1 Lactobacillus inoculation

To obtain the correct fermentations, bacteria inoculation are used to improve and speed up the process of acidification of the silage mass, creating the ideal environmental conditions for the preservation of forage for long periods. In order to improve the ensiling process, various chemical and biological additives have been developed. The biological additives are natural products not detrimental or dangerous to the environment and human, very easy to use and non-corrosive to machinery. The lactic acid bacteria (LAB) are the main silage inoculants used to control microbial events during silage fermentation (Weinberg and Muck, 1996). The effects of bacteria inoculantion on the preservation and nutritive value of silage had showed positive outcomes such as higher lactate:acetate ratios, lower ammonia N, decreased dry matter losses, increased digestibility, improved aerobic stability and enhanced growth performance have been reported (Henderson, 1993; McAllister et al., 1995, Zahiroddini et al., 2004). The bacteria used for silage inoculation consist of selected strains of LAB, such as *Lactobacillus plantarum*, *Pediococcus species* and *Enterococcus faecium*, which are of different origin (Weinberg and Muck, 1996; Přikryl, 2006).

LAB belong to the epiphytic microflora of plant material. The crop dry matter and water-soluble sugars concentrations, and the characteristics of the LAB, such as maximal growth rate, acid tolerance, osmotolerance, and substrate utilization, are fundamental aspects that can affect the competitiveness of the LAB during silage fermentation (Driehuis and Oude Elferink, 2000). LAB commonly associated with silage belong to the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Enterococcus* (McDonald et al., 1991). The LAB used for silage inoculation grow at temperatures between 20 and 40°C, with best conditions around 30°C. The main characteristic of the LAB is their acid tolerance. They are able to decrease the silage pH to about 4 to 5, depending on the species and the type of forage crop. All LAB are facultative aerobes, but some have a preference for anaerobic conditions. They are classified in three groups based on their sugar metabolism: 1) the obligate homofermentative species, produce more than 85% lactic acid from hexoses such as glucose, but are unable to ferment pentoses such as xylose; 2) the facultative heterofermentative species also produce mainly lactic acid from hexoses, but in addition, they are able to ferment some pentoses to lactic acid and acetic acid and/or ethanol. 3) The obligate heterofermentative species utilize both hexoses and pentoses, but unlike homofermenters they ferment hexoses to equimolar amounts of lactic acid, acetic acid or ethanol, and CO_2 (Driehuis and Oude Elferink, 2000).

1.3.2 Silage contaminations

1.3.2.1 Mycotoxins

Mycotoxins are a large, diverse group of toxic metabolites of fungi. Currently, more than 300 mycotoxins have been identified. They can be found commonly in the crops used as silage, such as corn, wheat and grasses and are of particular concern because of their adverse effects on animal health and productivity. The mycotoxins classification is made between that are formed before and after ensiling. The mycotoxins formed before ensiling are associated with moulds that infect a crop during its growth in the field or with endophytic moulds that lives as symbionts. Usually this contamination is due to adverse environment conditions or not correct field management. In this group, there are tricothecenes, zearalenone, fumonisin, aflatoxins and ergot alkaloid. Mycotoxins formed after ensiling are associated with moulds that develop in silage during storage or feeding out, usually as result of poor silage density or not effective sealing. This group include *Penicillium roqueforti* and *Penicillium paneum* and diverse group of mycotoxins formed by *Aspegillus fumigatus* (Driehuis, 2013).

Almost all the mould species are obligate aerobic microrganisms, they do not develop in well-preserved anaerobic silage. Indeed growth of moulds and development of mycotoxins in silage are associated with the duration and extent of air infiltration that are dependent on the porosity and density of the stored silage and the feeding-out after opening. Moulds not only cause a reduction of feed value and palatability of the silage, but also can have a negative effect on animal health. This relates to the fact that many mould species are capable of producing toxins (Driehuis and Oude Elferink, 2000). Different health problems can affect the animals as digestive upsets, fertility reduction, abortion, reduced immune function, liver and kidney damage, depending on the type and amount of mycotoxin present in the silage (Scudamore, 1998).

The most important mycotoxins contamination in the silage is probably the aflatoxins. Aflatoxins are produced by *Aspergillus flavus* and, to a lesser extent, *Aspergillus parasiticus*. It can infect crops in the field under favorable conditions, especially in subtropical and warm temperate climates. *Aspergillus flavus* and *Aspergillus parasiticus* are associated with aflatoxin production in a number of crops, including maize, sunflower, peanut and several tree nuts. Maize plant can become infected from the environment, usually soil or insects. A high level of insect damage increases the risk of infection. If conditions are favorable, the mould

colonizes the cobs and penetrates into kernels. Aflatoxin development in the kernels occurs within narrow ranges of moisture content and temperature.

Aflatoxins are highly toxic and carcinogenic to man and animals. Aflatoxin B1 is the most prevalent and most toxic form. It is transformed into aflatoxins M1 in the liver of cattle and carry-over into milk (Driehuis, 2013). The European Food Safety Authority (2004) reported that from 1 to 6 percent of aflatoxin B1 ingested by dairy cattle is excreted in milk as aflatoxin M1. This difference is due to milk production of dairy cows. Aflatoxin M1 is less mutagenic and genotoxic than aflatoxin B1, but the cytotoxicity is similar.

The risk of mycotoxin contamination in the feed for animal health and safety of animal food products depends on the carry-over from feed into the milk and meat, due to the mycotoxin metabolism in the animal. After intake, mycotoxins, follow the typical pharmacokinetic cascade of uptake from the gastro-intestinal tract to the blood, internal distribution, metabolism, storage, remobilization and excretion (Driehuis, 2013).

Table 1: Major Mycotoxigenic moulds and mycototoxins in silage crops and silages.

Mycotoxin group	Major toxin(s)	Mould species	Crop(s)	Field or ensilage derived
Aflatoxins	aflatoxin B1(M1), B2, G1,G2	Aspergillus flavus, A. parasiticus	Maize silage	Field
Trichotecens	Type A: T2, diacetoxyscirpenol	Fusarium langsethiae, F. poae, F. sporotrichioides	Maize silage, cereals	Field
	Type B: DON, nivalenol	F. graminearum, F. culmorum	Maize silage, cereals, grass	Field
Fumonisis	Fumonisin B1, B2	F. verticillioides, F. proliferatum	Maize silage	Field
Resorcylic acid lactones	Zearalenone	F. graminearum, F. culmorum	Maize silage, cereals, grass	Field
Ochratoxins	Ochratoxin A	A. ochraceus, Pennicilium verrucosum	Cereals	Field
Ergot alkaloids	Clavines, lysergic acid amide,ergotamine	Claviceps purpurea	Cereals	Field
	Lolitrem B, ergovaline	Neotyphodium lolii, N. coenophialum	Grass	Field
P. roqueforti toxins	Roquefortine C, mycophenolic acid	P. roqueforti, P. paneum	All silages	Ensilage
A. fumigatus toxins	Gliotoxin, fumigaclavines	A. fumigatus	All silages	Ensilage
M ruber toxins	Monacolin K, citrinin	Monascus ruber	All silages	Ensilage

Modified from Driehuis, 2013.

1.3.2.2 Clostridia

The Clostridium bacteria are found in different anaerobic ecosystems. Many species of these endospore-forming obligately anaerobic bacteria ferment carbohydrates, as well as proteins. Clostridium species typically associated with silage are *C. tyrobutyricum, C. butyricum, C. sporogenes, and C. bifermentans* (Driehuis and Oude Elferink, 2000). These anaerobic bacteria are responsible for the secondary fermentation of glucose and lactic acid to butyric acid by saccharolytic species such as *C. butyricum* and for proteolysis by species such as *C. perfringens* (Wilkinson, 1999). Protein and amino acids degradation by clostridia contamination have a negative impact on the feeding value. Spores of clostridia from silage can survive the passage through the digestive tract of the dairy cow. The spores can be transferred into the milk, mainly through faecal contamination of the udder, reducing its quality (Driehuis and Oude Elferink, 2000). Some Clostridium species are extremely pathogenic to animals and humans as *C. botulinum,* that is well known to produce a botulinum toxin that frequently causes death in animals and humans. The botulinum agent is product in poorly made silage and in the gastrointestinal tracts of cattle. This can lead to contamination of the farm environment and raw milk, and further transmission through the dairy chain (Lindström et al., 2010). *C. botulinum* is not acid tolerant thus quick and correct decline of the silage pH decreases the chance of clostridia contamination. The animals are at risk when feeding on anaerobically stored silage at pH > 5.5, or on acidified silage accidentally containing animal carcasses (e.g. dead rodents), which can support growth and toxin production (Johnson et al., 2010). A typical clostridia contaminated silage is characterized by a high pH and high contents of butyric acid, ammonia, and amines. (Driehuis and Oude Elferink, 2000).

1.3.2.3 Yeast

Yeasts are facultative anaerobic eukaryotic micro-organisms and are considered the most important group as they are involved in aerobic spoilage either during the aerobic phase at the beginning of ensiling or during the feed-out, when the silage is exposed to air. (McDonald et al., 1991; Driehuis and Oude Elferink, 2000). Species typically associated with silage are acid-tolerant species belonging to the genera *Candida*, *Hansenula*, *Saccharomyces*, and *Torulopsis* (Middelhoven and Baalen, 1988). Under anaerobic conditions, spoilage yeasts are able to oxidize sugars and lactic acid, resulting in the production of CO_2, H_2O, ethanol and heat. Ethanol production in silage not only decreases the amount of sugar available for acid production, but it also increases dry matter loss during ensilage (Driehuis and van Wikselaar, 2000) and may have a negative effect on the taste of milk (Randby et al., 1999). Yeasts are acid-tolerant and during the unloading phase, silage oxygenation restarts their organic acid metabolism pathways (succinic, citric and lactic acids) inducing a pH increase and allowing the growth of less acid-tolerant microrganisms. A gradual decrease in counts usually takes place during the subsequent storage period (Middelhoven and Baalen, 1988). The presence of yeasts during storage is mainly correlated with the grade of anaerobiosis, the silage pH, and the concentrations of organic acids.

1.3.3 Thermal camera and electronic nose

The evaluation of microbiological and chemical quality of the silage during the feed-out phase required many samples, expensive labor and equipment, qualified personnel, and need long time to laboratory analyses. For these reasons, a simple method is necessary to evaluate the silage quality in short time and with low cost. The consequences of aerobic deterioration of silages include changes in the composition of volatile compounds as organic acids. For these reasons, silages can be evaluated for organic acid content that result from fermentation reactions to assess fermentation quality based on the content of undesired degradation products. Furthermore the growth of spoilage microorganisms in silages due to the aerobic deterioration produces heat, which is a typical indicator of aerobic degradation (Kung et al., 2000). Borreani and Tabacco (2010) evaluated and correlated the temperature silage with chemical composition and microbial count. Samples from peripheral areas showed greater temperatures, pH values, and yeast counts than samples from the central area. Those authors concluded that temperature is linked to microbial activity and can be an important indicator of the early stages of aerobic degradation. Thermal imaging is a non-invasive process analytical technique. It is a two-dimensional, non-contact diagnostic technique for measuring surface temperature of materials, which can be usefully employed in non-destructive quality evaluation. Thermal imaging utilizes the radiation emitted to produce a pseudo image of the thermal distribution of a body's surface. In thermography, a large number of point temperatures are measured over an area and processed to form a thermal map or thermogram of the target surface. The basic principle of thermal imaging is based on the fact that all materials emit infrared radiation. Emissivity, defined as the ratio of energy emitted from an object to that of a black body at the same temperature, can vary from 0 (perfect white body) to 1 (perfect black body). In thermal imaging cameras the infrared energy emitted from a measured object is converted into an electrical signal via IR detectors (e.g. focal plane arrays, microbolometers) in the camera and displayed as a colour or monochrome thermal image. The surface temperature of the body is estimated based on the infrared energy emitted (Gowen et al., 2010). Infrared thermography is a useful tool for detecting variations in temperature over large areas, such as the working face of silos but at moment a there are limited and contradictory information on its application. Schmidt et al. (2015) performed a research where reported that thermal imaging was not appropriate indicator to evaluate the mycotoxins incidence or concentration. Instead Addah et al. (2012) reported that thermal imaging can offers prospects as a practical method for assessing the aerobic stability of silages on farm.

The odor of silage is highly indicative of its intrinsic quality. A rapid technique to assess the silage quality is electronic nose evaluation. Electronic nose represent a group of devices for many chemical and microbiological applications, and they have been widely and successfully used for different commercial industries, including the agricultural, environmental, food, and various scientific research field (Wilson and Baietto, 2009). The electronic nose is an instrument consists of electronic and chemical multisensory array with partial specificity and an appropriate pattern recognition system that is capable of recognising simple or complex odours. This method utilizes the aromatic and volatile characteristic compounds that can be utilized effectively as a marker of the volatile molecules rising from the analysed samples. The presence of volatile compounds induces a variation in the metal oxide semiconductor (MOS) sensor conductivity with respect to the value in standard conditions (zerogas). This variation is due to the reaction between the oxygen species adsorbed on the sensor surface and the gas mix which comes in contact. (Campagnoli et al., 2011; Campagnoli and Dell'Orto, 2013; Masoero et al., 2007). Some authors reported the potential application of use of electronic nose to assess the quality of cereals contaminated with mycotoxins (Cheli et al., 2009; Campagnoli et al., 2009; Campagnoli et al., 2011; Eifer et al., 2011). Masoero et al. 2007 reported a study where compared the use of electronic nose and near infrared techniques to evaluate the silage quality. The results obtained suggest a interesting possibilities of providing quick easy and low-cost information of the main quality parameters in farm silage.

1.4 Feed processing and quality

Animal feed manufacturing involves the use of a variety of raw materials to produce compound feeds. Different animal species require different physical properties for their respective feeds. This means that different quality standards are used. Feed processing includes the treatment (physical, chemical, thermal) of a feed prior to consumption by animals. In general, the variability in processing effects is associated with the choice of equipment, with processing conditions as well as with the processing system, e.g. the combination and sequence of processing equipment. Therefore, processing may involve a simple process such as grinding in the form of mash or can be much more complicated, such as pelleting, crumbling or extruder/expander (Thomas and van der Poel, 1996).

Mash is the easiest feed form. It is a mix of different grinded raw material obtained with two different way, hammer mill and roller mill, and further mixed.

Hammer mills consist of a rotor assembly made from two or more rotor plates fixed to main shaft and enclosed in some form of grinding chamber. The actual working parts are the hammers and screens or grinding plates that encircle the rotor. The rotor can be supported from one end only or on both ends by the shaft and bearings. The hammers are simply flat metal bars with a hole at one or both ends and usually some form of hard facing treatment on the working ends.

Roller mill consist of pairs of rolls mounted horizontally in a frame. Roller mills often are referred to the type of service they perform. A roller mill can be used to crack, crimp flake or grind grain. The roller mill can have two or three pair of rollers. Double pair roller mill can be utilized in several ways. Traditional applications use a pair of coarsely grooved rolls in the top to crack corn, whereas finer grooved rolls on the bottom could crimp small grain or double roll corn to make a finer finished product. When machines use no differential drive, the two rolls turn at same speed, and the processing between the rolls is purely compression. The minimum, mean particle size of corn that can be achieved is 1200µm to 1600 µm. This type of roller mill often is referred to as cracker/crimper. When the roller mill is equipped with differential, the rolls turn at different speed and the grinding action is more shearing and cutting to produce fine, uniform, finished product. The double pair roller mill can be equipped with differential speeds on one pair of rolls only. Triple pair roller mill can be used in some feed mills for more specialized applications. A triple pair machine with differential speeds on all three pairs of rolls can produce a very fine, finished products, down to 350µm. It can

produce a particle size from 600µ to 800µ with 25-30% higher capacity than double pair machine (Feed Manufacturing and Technology, 2005).

After grinding the mash feed is mixed and can be pelleted or extruded.

The pelleting process within the feed manufacturing facility provides a means of molding feed mash into larger particles. Pelleting is accomplished through a mechanical process in combination with moisture, heat and pressure. Feed mash from the bin flows into the feeder and conditioner, where steam and liquids are added. The conditioned mash then flows into the pelleting chamber, where the pellet is formed and sent to the cooler. As the hot pellet passes through the cooler, it is cooled by air movement from a fan. Fines entrained in the cooling air are separated in a collector and returned to the pellet mill for reprocessing. Cool pellets are discharged from the cooler and pass around or through the crumbler, depending on the product being manufactured. The product then passes through a screening mechanism, where separation occurs. The acceptable product goes to the finishes feed bins, while the fines and overs are returned to the pellet mill to be reprocessed (Feed Manufacturing and Technology, 2005). The aim of a pellet mill is to combine heat, moisture and pressure to make a high percentage of cylindrical and solid structures, the pellets, from mash feed. Pellets should be sufficiently durable to resist compression, impact and abrasion forces during conveyance and storage in feed mill and transportation to the farms (Cavalcanti and Behnke, 2005; Mina-Boac et al., 2006). Pellet quality is estimated by assessing pellet durability index (PDI) (McKinney, 2013). Many factors can affect pellet quality in a feed mill. Particle size plays an important role in pellet press operation. Particles larger than 1,000 µm or 1,500 µm may cause fracture of pellets (Franke and Rey, 2006). Increasing levels of added fat in feed also reduce pellet quality (Thomas and van der Poel, 1996). Fat addition reduces the friction between die wall and feed ingredients, and decreases the compression made upon the feed particles inside the die holes (Fahrenholz, 2012).

Extrusion processing has been defined as the process by which moistened, expansible, starchy, and/or proteinaceus materials are plasticized in a tube by a combination of moisture, pressure, heat and mechanical shear. The dry and liquid portions of the feed are introduced separately into a preconditioning device, where they are continuously mixed, heated and moisturized by the injection of hot water (70°C) and/or steam. Preconditioned formulations are discharged directly into the extruder assembly, which consist of the barrel segments and screw configuration. Extruders are classified generally as a single or twin-screw design. This unit can be divided in three zones: feeding, kneading and final cooking. The feeding zone if the extruder is that area where the low-density discrete particles of raw material are

transported into extruder barrel inlet. The kneading zone continues the compression started in the feeding zone, and the flow channels of the extruder screw have higher degree of fill. The final cooking zone is that area where melting and texturizing occur. Temperature and pressure increase most rapidly in this region where shear rates are highest because of the extruder screw configuration and maximum compression of the extrudate (Feed Manufacturing and Technology, 2005).

The physical quality of feed is an important aspect of animal production. It is well known that fine grinding improves nutrient digestibility by enhancing the accessibility of digestive enzymes (Wondra et al., 1995). Thus, many studies revealed increased average daily weight gain (ADG) and/or feed efficiency as a result of enhanced nutrient digestibility (Döll et al., 2007; Healy et al., 1994). Briggs et al. (1999) reported that a poor-quality pellet resulted in a high percentage of fines, which were not consumed well by birds. Moreover, there is evidence that increased extent of technical feed treatment might also adversely affect animal health. Some studies showed that fine grinding induces stomach lesions (Healy et al., 1994). Further, treatments such as pelleting of grain resulted in a secondary decrease of particle size, which appeared to be strongly associated to keratinisation and ulceration in the stomach (Grosse Liesner et al., 2009; Millet et al., 2012).

Different methods are used to evaluate the feed physical quality. The mash is evaluate to check the particle size of grounded feed with sieving procedure define by American Society of Agricoltural Engineer (ASAE), the pellets and extruded are evaluate to check the durability and hardness, to measure the relative ability of feed to resist to the stresses that occur during storage and transportation, up to the administration to animals.

1.5 Ration distribution

Dairy producers are increasingly mindful of factors affecting efficiency of milk production and ultimately their profit margin. Total mixed ration (TMR) was introduced as a means of providing a consistent supply of nutrients to rumen microbes to optimize rumen function and improve the efficiency of nutrient utilization (Coppock et al., 1981). A total mixed ration (TMR) is composed of forages, commodities/byproducts (such as whole cottonseed), grains, protein supplement(s), minerals, and vitamins that have been mixed together to make a balanced ration in which the weight of each ingredient is known. This mixture is then offered to cows as their sole source of feed (Amaral-Phillips et al., 2002). Provision of a consistent ration, with respect to both physical and chemical composition, is an essential part of maximizing cow performance and getting the best value out of the ration. The optimal condition is to provide in each bite of feed the same proportion of forages and concentrates required in order to guarantee the same nutrition. Inconsistencies in nutrient intake have many causes. Sorting of a TMR can result in cows consuming a ration with a different nutrient composition than that delivered (Leonardi and Armentano, 2003), which has been shown to affect production at the herd level (Sova et al., 2013). Observation of the feeding behavior and analysis of the orts revealed that cows typically sort against long particles (Leonardi and Armentano, 2003; DeVries et al., 2007), which could reduce the intake of physically effective NDF and increase the intake of the finer nonstructural carbohydrate fraction. This situation could affect the normal function of the rumen and increase the risk of displaced abomasum, result in fluctuations in feed intake, alter the immune function, increase the incidence of foot lesions, depress milk fat, and reduce rumination activity (Leonardi and Armentano, 2003; Maulfair and Heinrichs, 2013).

Even if cows consume a TMR with high consistency (i.e., no sorting), the ration delivered may not reflect the ration formulated by the nutritionist, nor be consistent in composition from day to day. In practice, there are basically three types of TMR. The first type is feed ration theoretical – calculated by optimization program and quantified sometimes up to two decimal places. The second type is a TMR that leaves the mixer feeder wagon and is discharged into fodder table in barn to animals (depending on the accuracy of loading and homogeneity – mixing of individual components). The third TMR type is the dose that cows actually intake (Šístkova et al., 2015).

The goal of any feeding system or method is to provide the opportunity for cows to consume the amount of feed specified in a formulated diet. Nutrients can be effectively supplied by

feeding either a total mixed ration (TMR) or individual ingredients. A TMR allows for the mixing of all feed ingredients together based on a prescribed amount of each ingredient (NRC, 2001). A uniform TMR has many potential advantages, such as improving the animals health and reproductive, decreasing the feed cost, improving the meat production of beef and milk production of cows and decrease labor requirements. (Buckmaster et al., 2014). The production of TMR requires the use of loading, mixing and feeding machines, feed mixer wagons, that should, within a short time, finely comminute ration components with different physical-mechanical properties, mix them until homogeneous and uniformly distribute structure is obtained (Kowalik et al., 2015). The TMR mixer wagon can have different equipment: horizontal or vertical screw or screws, reel and tumbling action within a drum and/or with chain and paddles.

The horizontal screw mixer come with one, two, three, or four horizontal screws for mixing the feed. With multiple-screw mixers, the mixing action occurs when one or two of the screws counter-rotates, moving the feed opposite to an adjacent screw (or screws). Knife sections attached to the screw flights can cut or tear long-stemmed alfalfa hay into pieces of 7 to 10 cm, which can then be incorporated into the ration. Many horizontal screw mixers do not handle grass hay or alfalfa hay well because these forages tend to wrap around the screws.

The vertical mixer consists of a tub with a single, center screw or a tapered screw. The center screw is powered by a transmission and planetary gearbox. These mixers can make TMR rations in which all the forage is dry hay. (Water or wet byproducts are added to the mix to prevent separation.) Knife sections attached to the flights on the center screw and movable shear or restrictor plates on the tub wall help reduce the particle size of dry hay. Caution should be used so that excessive mixing does not occur. It could decrease the particle size of forages and lead to health problems. Vertical screw mixers can generally handle large round bales of grass or alfalfa hay, but they may cost more than other types of TMR mixers.

The Reel mixer often combines a set of screws and a reel similar to a combine reel in a hopper. The rotary mixing system lifts feed past the wedging point on the lower side of the screw. The lifting action of the rotor is intended to minimize wedging of alfalfa hay and other long-stemmed forages under the lower auger, potentially preventing the particle size of the forages being mixed from being reduced.

The Tumble or chain and paddle mixer use spirals and pans on the interior of the drum to lift and tumble the ration. Loading and unloading occur at different ports on the mixer. Chain and paddle mixers consist of a tub with a chain and paddles on a conveyor that are used to tumble the ration from one end to the other. Some configurations of both types of mixers use a central

screw that circulates the feed, moving it to the front of the mixer, where it is either remixed in the tumbling action or delivered to the unloading port. Dry hay must be chopped to a length of 2.5 to 7 cm before it can be mixed. These mixers wear less and have lower power requirements than screw or reel mixers of the same capacity, are generally stationary, and, for the most part, have been replaced with auger or reel-type mixers by most equipment manufacturers (Amaral-Phillips et al., 2002).

To get the best performance out of cows fed TMR, it is important monitor rations frequently. This could be done using physically or chemically observable tracers or simply particle size distribution. Kononoff et al. (2003) reported the function and results of Penn State Particle Separator (PSPS), which is actually the best instruments to evaluate the particle size distribution of TMR. Factors such as fill order (sequence of putting feedstuffs into the mixer), mixing time, mixing protocol, moisture levels of feedstuffs, and scale maintenance and calibration can all dramatically affect mix uniformity (Buckmaster, 2009). Some authors reported that the mixing time and mixer type significantly affect particle size distribution (Heinrichs et al., 1999; Jordan, 2001; Buckmaster et al., 2014). Quality control issues regarding TMR delivery include: uniformity among/within batches, particle size distribution, minimizing labor requirements, low utilization of energy and long equipment life (Buckmaster, 2009).

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Chapter 2

2 Objectives

Dairy cattle production is an important component of the food industry. Nutrition is a key factor for performance and health of dairy cows. Several factors must be taken into account to provide the proper ration to maintain a healthy state and to obtain the best productive and reproductive performance. The stage of the production cycle is certainly one of the factors to keep into account. Among these, the transition period is the most important period and involved a great number of researchers around the world in the last 20 years. This attention is due to the particularly critical conditions of dairy cows that can affect the entire production cycle, with considerable economic losses for the farmer.

The quality of the feed provided to the animals with the ration is undoubtedly very important. The silage represents the most raw material used for the dairy cattle nutrition, and it is therefore essential that it has an excellent quality. To improve the silage quality, bacteria inoculations are used to accelerate and improve the lactic fermentation for an optimal preservation of the silage. Furthermore, to ensure a good quality of the ration, the forages used for dairy cattle nutrition should be regularly checked. To date the chemical analysis still are the best assessment tool to evaluate the silage quality, but unfortunately, they are very expensive and need much time to provide the necessary information. Therefore, the use of tools that provide a rapid assessment and low cost analysis of forages can be very useful to improve the total mixed ration quality, and consequently reduce the risk of disease, and reach a good milk production.

Besides the forages, also the concentrates play a fundamental role in animal nutrition. The concentrates are an important component to provide the nutrients as complete ration for pigs and poultry or complementary in dairy cows. Their physical form and quality play an important role in animal nutrition to achieve the best results in animal production.

As previously said the quality of the forages and feeds used for dairy cows nutrition are very important, but also the correct administration of the ration is fundamental to provide all the necessary nutrients and their proper balance. The ration administered to the cows may be different from that formulated by the nutritionist due to an incorrect use of the equipment used to prepare the ration, such as mixer wagon, which can affect both health and milk production.

The objective of this thesis was to improve the knowledge and study new technology to provide solutions on the factors that influence animal nutrition previously described. The aims of the experimental trials performed were to:

- Improve the knowledge on relationship between vitamin E and the indicator of negative energy balance during periparturient period NEFA and BHB. Several study

were performed on transition period but few information’s are available about this relationship.

- Evaluate the use of uncommon bacteria inoculation to improve the acidification process of grass silage and the use of two technologies as thermal camera and electronic nose to provide a rapid and cheap analysis of corn silage quality and.
- Evaluate the physical quality of some mashed, pelleted and extruded feeds used in animal nutrition.
- Evaluate the effect of mixing times, cutting times and wagon load on homogeneity of the ration administered to dairy cows, and the differences with the theoretical ration formulated.

Chapter 3

3 Associations between blood fatty acids, β-hydroxybutyrate, and α-tocopherol in the periparturient period in dairy cows.

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3.1 Abstract

The objective of the present study was to examine the relationships between blood concentrations of fatty acids, β-hydroxybutyrate (BHB), and α-tocopherol during the periparturient period in dairy cows. Blood samples were collected from 131 cows belonging to 4 different commercial dairy farms in southeastern Europe (Greece and Italy). We determined blood concentrations of fatty acids, BHB, and α-tocopherol at dry-off, at calving, and 30 d postpartum. Results indicated that fatty acid concentrations were low at dry-off, reached maximum value at calving, and then declined at 30 d postpartum. In fact, fatty acid concentrations at 30 d postpartum were 50% lower than at calving. In contrast, BHB concentrations were low at dry-off, increased by 27% at calving, and continued to increase by another 20% at 30 d postpartum. Overall, we found a weak correlation between fatty acids and BHB throughout the periparturient period. Concentrations of α-tocopherol were lowest at calving, and we detected no differences in α-tocopherol concentrations at dry-off or 30 d postpartum. Negative correlations between fatty acids and α-tocopherol were highly significant at 30 d postpartum and approached the level of significance at dry-off. However, both correlations became nonsignificant following the adjustment of α-tocopherol with cholesterol, indicating that the correlations were a reflection of changes in lipid transport. We found significant negative correlations (strong at dry-off and weak at 30 d postpartum) between BHB and α-tocopherol after adjustment with cholesterol. The physiological basis for the negative correlations between BHB and α-tocopherol, especially that at dryoff, is not known and should not be taken to imply a cause-effect relationship. However, it opens the door to investigating the effects of vitamin E on liver function in dairy cows.

Key words: α-tocopherol, fatty acids, β-hydroxybutyrate

3.2 Introduction

The multitude of disorders that dairy cows face during transition from the dry period to lactation is striking. At this time, milk production is high, but a lag in feed intake creates a negative energy balance. It is well documented that when the negative energy balance in the periparturient period becomes severe, the risk for several postpartum diseases increases. These include retained placenta, milk fever, metritis, mastitis, clinical ketosis, and displaced abomasum (Duffield et al., 2009; LeBlanc, 2010; Suthar et al., 2013). Fatty acids and BHB are both used as markers of negative energy balance during the periparturient period (Konigsson et al., 2008; Ospina et al., 2010a,b; Chapinal et al., 2011; McArt et al., 2012, 2013), but in most studies, the 2 markers are presented together. More recently, McCarthy et al. (2015) showed that the concentrations of fatty acids and BHB are not well correlated during the transition period. More specifically, fatty acid concentrations increased gradually, starting before parturition and up to 9 d after parturition, and then gradually declined. In contrast, BHB concentrations began to increase in the late prepartum period, continued to increase during the first week postpartum, and remained elevated through 21 d postpartum. All correlations between fatty acids and BHB in the transition period were weak or extremely weak. McCarthy et al. (2015) concluded that changes in fatty acids and BHB concentrations should be interpreted with caution, and that changes in the concentration of one metabolite should not be taken to suggest corresponding changes in the concentration of the other. The role of inflammation in metabolic disorders associated with transition from the dry period to lactation is not known with certainty. Bradford et al. (2015) have suggested that all cows experience some degree of systemic inflammation during the first 2 weeks postpartum, but the magnitude and the duration of the inflammatory state vary widely among cows. Bertoni et al. (2015) suggested that early postpartum inflammation can impair cow performance by lowering milk production, DMI, fertility, and energy efficiency. They proposed that providing appropriate nutrients such as antioxidants, n-3 (omega-3) polyunsaturated fatty acids, and vitamin D might be a way of reducing inflammation and avoiding associated conditions, such as tissue damage and digestive or metabolic syndrome–related disorders. Vitamin E is one of the main antioxidant vitamins. Politis (2012) has demonstrated that vitamin E status has a direct influence on reducing the frequency of mastitis and retained placenta. Lower serum α-tocopherol (vitamin E) concentrations are a potential early indicator for the development of left displaced abomasum in multiparous cows (Qu et al., 2013). No study has ever suggested a direct relationship between vitamin E and ketosis. However, in rodent models, antioxidants

can improve the metabolic function of the liver, as well as immunity (Sakaguchi and Furusawa, 2006; Mao et al., 2010). Furthermore, vitamin E improves metabolic function in humans with nonalcoholic fatty liver disease (Rinella, 2015; Sato et al., 2015). Bouwstra et al. (2008) reported that vitamin E supplementation in heifers during the periparturient period reduced oxidative damage in the liver. Because information is limited concerning the relationship between indicators of negative energy balance and α-tocopherol, the objectives of the present study were (1) to determine the relationship between fatty acids and BHB during the periparturient period in dairy cows in farms located in southeastern Europe, where cows are likely to encounter less oxidative stress than cows in North America; and (2) to examine the relationship between blood concentrations of fatty acids, BHB, and α-tocopherol during the periparturient period.

3.3 Materials and methods

A total of 131 Holstein cows from 4 commercial farms participated in an observational field study. Two of the farms were in Italy and the other 2 were in Greece. Of the total, 59 cows belonged to the Italian farms (30 and 29 from each farm) and 72 belonged to the Greek farms (36 from each farm). Diets (DM basis) on the 2 Greek farms during the dry period were 42% corn silage, 40% straw hay, 9.6% soybean meal, and 8.4% molasses. After calving, the diet was 48.5% corn silage, 16.5% soybean meal, 14% alfalfa hay, 12% corn, 3.7% molasses, 1.7% rumen-protected fat, and 3.5% vitamin and mineral premix. Diets on the Italian farms during the dry period were 43.3% corn silage, 35% straw hay, 12.8% soybean meal, 5.9% corn meal, and 3% vitamin and mineral premix. After calving, the diet was 23.8% corn silage, 22.8% corn, 17.6% soybean meal, 13.5% meadow silage, 7.6% meadow hay, 5.8% alfalfa hay, 2% molasses, 1.9% extruded flaxseed, 1.5% rumen-protected fat, and 3.5% vitamin and mineral premix. We collected blood samples from all cows at dryoff, at calving, and at 30 d postpartum. Serum was obtained following centrifugation of the blood samples, and it was frozen at −80°C until analysis. We assayed α-tocopherol levels using reversed-phase HPLC (C18 column, reversed-phase) with UV absorbance detection at 292 nm, as described by Baldi et al. (2000). The extraction phase involved precipitation of plasma proteins with absolute ethanol and liquid-liquid extraction performed with hexane in the presence of butylated hydroxytoluene as a preservative. After centrifugation, the supernatant was evaporated to dryness under a stream of nitrogen, and the residue was dissolved in organic solvents; 50 μL were injected directly onto the HPLC column. Samples were maintained in ice and kept in the dark during the procedure. We prepared standard solutions from a stock solution (100 mg/10 mL) of pure dl-α-tocopherol dissolved in methanol/ butylated hydroxytoluene. Separation was performed on the SupelcoSil LC-18 (25 cm × 4.6 mm, 5 μm) column. The mobile phase consisted of methanol:water 97:3 (vol:vol %) pumped at a flow rate of 1 mL/min at room temperature. We used enzymatic colorimetric methods to determine plasma concentrations of fatty acids and BHB (Stella et al., 2007; Konigsson et al., 2008). Blood fatty acid concentrations were determined using the acyl-CoA synthetase–acyl-CoA oxidase method (Wako Chemicals, Richmond, VA). Plasma concentrations of BHB were determined based on the oxidation of d- 3-hydroxybutyrate to acetoacetate by the enzyme 3-hydroxybutyrate dehydrogenase (Cayman Chemical, Ann Arbor, MI). Total cholesterol was measured using commercial enzymatic colorimetric kits (Instrumentation Laboratory s.p.a, Milan, Italy; Biosis, Athens, Greece) based on the method described by Allain et al. (1974).

We calculated the ratio of α-tocopherol (μmol/L) to cholesterol (μmol/L) multiplied by 1,000 (to provide more meaningful, easily understood numbers) to adjust for changes in lipid transport and stage of lactation (Herdt and Smith, 1996; Qu et al., 2013). Individual milk samples were collected weekly for 4 weeks postpartum. Samples were analyzed for protein and fat by infrared method using a MilkoScan 133B (Foss Electric, Hillerød, Denmark) calibrated against the Kjeldahl method for protein and the Mojonnier method for fat. SCC were determined using a Fossomatic cell counter (Foss Electric).

3.4 Statistical analysis

We performed statistical analysis with ANOVA using a linear mixed model, considering 2 independent fixed factors: farm and time of sampling. For the fixed factor "time of sampling," 3 measures were repeated for each cow. Cow was considered a random factor nested within farm. We tested several covariance structures: compound symmetry was used for fatty acids and BHB, and first-order autoregressive for α-tocopherol, resulting in the smallest Akaike information criterion. The model used was

$$Y_{ijk} = \mu + F_i + T_j + F_i \times T_j + C_{k(i)} + e_{ijk},$$

where Y_{ijk} is the individual value for each dependent variable (fatty acids, BHB, α-tocopherol, logSCC); μ is the overall mean; F_i is the fixed effect of farm (1 and 2 = Italian farms and 3 and 4 = Greek farms); T_j is the fixed effect of 3 repeated measures factor "time of sampling" for each cow (1 = dry-off, 2 = calving, 3 = +30 d of lactation); $C_{k(i)}$ is the random animal effect, nested within farm; and e_{ijk} is the random error assumed to be normally and independently distributed with zero expectation and common variance σ^2. Values in the tables are least squares means (with SE). We used the Bonferroni test for *P*-values when performing multiple comparisons and assigned significance at an α level of 0.05 unless otherwise noted. All analyses were carried out by PROC MIXED in SAS, version 9.0 (SAS Institute, 2004). To examine relationships between data, we estimated the bivariate correlations of Spearman's rho at each sampling time using the PROC CORR statement in SAS.

3.5 Results and Discussion

Milk yield and milk composition from all 4 farms are presented in Table 1. Milk yield was approximately 20% higher in the Italian farms than in the Greek farms. As expected, we observed higher levels of fat and protein in the Greek farms. Somatic cell counts were very low in all farms. None of the animals developed symptoms of clinical or subclinical mastitis. Table 2 shows changes in the concentrations of fatty acids, BHB, and α-tocopherol during the periparturient period in dairy cows. Fatty acids were low at the beginning of the dry period. Fatty acid concentrations at calving were 3.3-fold higher than corresponding values at dry-off. The amount of fatty acids declined by 50% at 30 d postpartum compared with corresponding values at calving. In contrast, BHB concentrations were low at dry-off, increased by 27% ($P < 0.05$) at calving, and continued to increase by another 20% at 30 d postpartum. Concentrations of α-tocopherol at calving were 50% lower than the corresponding values at dry-off; concentrations increased in the postpartum period and essentially reached dry-off levels by 30 d postpartum. Changes in cholesterol and the ratio of α-tocopherol to cholesterol followed similar trends to those of α-tocopherol alone. The lowest values for both parameters were detected at calving. Cholesterol concentrations and the ratio of α-tocopherol to cholesterol were higher at dry-off and 30 d postpartum than at calving. Table 3 shows the Spearman's rho correlation coefficients between the concentrations of blood fatty acids, BHB, and α-tocopherol before and after adjustment with cholesterol at dry-off, calving, and at 30 d postpartum. Correlations between fatty acids and BHB at all 3 sampling points were extremely weak ($P = 0.186–0.732$). We found a negative correlation between fatty acids and blood α-tocopherol at 30 d postpartum (rho value = −0.3; $P < 0.001$) and a negative correlation that approached significance at dry-off (rho value = −0.17; $P = 0.06$). However, both negative correlations became nonsignificant following adjustment of α-tocopherol with cholesterol (ratio of α-tocopherol to cholesterol). In contrast, we found a correlation between BHB and α-tocopherol ($P < 0.001$) before or after adjustment with cholesterol at dry-off. We also found a negative weak correlation between BHB and α-tocopherol after adjustment with cholesterol (rho value = −0.19; $P < 0.05$) at 30 d postpartum. The first finding emerging from the present study is that fatty acid and BHB concentrations are not correlated during the periparturient period. This conclusion is supported by 2 observations. First, BHB and fatty acids followed similar trends in the prepartum period (dry-off vs. calving), even though changes in fatty acids were more pronounced. The 2 parameters followed exactly opposite trends in the postpartum period (calving vs. 30 d postpartum): fatty acid values declined, and

BHB values continued to increase at 30 d postpartum. Second, we detected a weak correlation ($P > 0.05$) between fatty acids and BHB (Table 3). Thus, changes in the concentrations of one metabolite should not be taken to automatically suggest corresponding changes in the concentration of the other. Our data have many similarities with those of McCarthy et al. (2015), which had the advantage of more observations and much more frequent sampling. However, all their experiments were performed in North America, where cows are likely to experience higher levels of oxidative stress than cows in European countries (Allison and Laven, 2000), particularly countries in the Mediterranean region. Even though we have confirmed earlier findings with the present study, the fact that our data were obtained from a different environment and production system makes them novel. The second finding that was significant negative correlations between fatty acids and α-tocopherol at dry-off and 30 d postpartum exist. However, the fact that these correlations disappeared following adjustment of α-tocopherol with cholesterol indicates that they were simply a reflection of changes in lipid transport. Furthermore, we found strong negative correlations at dry-off (rho values = −0.352 and −0.370; $P < 0.001$) between BHB and α-tocopherol before and after adjustment with cholesterol. We also found a weak but significant correlation ($P < 0.05$) between BHB and the ratio of α-tocopherol to cholesterol at 30 d postpartum. It is interesting to observe that α-tocopherol was negatively correlated with BHB at dry-off but that those correlations were nonsignificant or extremely weak at calving and 30 d postpartum. It seems that the association between α-tocopherol and BHB is more prominent at dry-off and less prominent at calving or postpartum. This might be related to the fact that postpartum hyperketonemia was essentially nonexistent in all 4 participating farms. The physiological basis for the negative correlations between α-tocopherol and BHB is not known and should not be taken to imply a cause-effect relationship. Previous research has revealed a direct link between α-tocopherol and incidence of mastitis and retained placenta, and the physiological basis of the link between vitamin E and mastitis is the relationship between α-tocopherol and proper immune function (Politis, 2012). Our results have certain similarities with those of Qu et al. (2014), who reported that elevated BHB concentrations before calving coincided with low α-tocopherol concentrations. Furthermore, they reported that this inverse relationship held only in the prepartum period and served as a potential risk indicator for retained placenta. To date, the prevalent view is that vitamin E status and ketosis are unrelated. However, the negative correlations between BHB and α-tocopherol certainly open the door to more careful investigations of the effects of vitamin E on liver function in dairy cows. In addition to the well-documented link between vitamin E status and immune function, there may be a link between vitamin E status and

metabolic function at the level of the liver in dairy cows, similar to that in rodents and humans. In vivo observations suggest a possible role for oxidative stress and liver function in dairy cows. Mudron et al. (1999) showed that cows with fatty liver have lower antioxidant status and higher hepatic lipid peroxide concentrations than healthy cows. Bouwstra et al. (2008) have reported that vitamin E has a role in recovery from parturition-related oxidative stress in periparturient heifers. Their suggestion that vitamin E reduces oxidative damage in the liver provides indirect support for the concept we propose. The possibility that α-tocopherol affects liver function is one side of the story; it is also possible that liver function affects α-tocopherol secretion and transport in the liver. The latter concept is supported by the work of Mudron et al. (1997), who reported that cows with fatty liver had lower plasma α-tocopherol concentrations but normal or higher α-tocopherol concentrations in the liver. Thus, in cows with fatty liver, α-tocopherol transport out of the liver may be impaired. Indirect support for the concept that fatty acids and α-tocopherol are negatively correlated comes from the work of Pinotti et al. (2003) in an experiment involving choline supplementation in dairy cows. They found that transition cows, receiving rumen-protected choline, had lower plasma fatty acid concentrations and higher α-tocopherol around calving than controls, and they suggested that this finding could be related to more efficient liver function.

Table 1. Daily milk yield and milk composition from the 4 participating farms[1].

Trait	Farm A	Farm B	Farm C	Farm D
Milk yield (L/d)	45.65 ± 0.87	46.47 ± 0.88	38.20 ± 1.12	39.40 ± 1.14
Fat (%)	3.59 ± 0.07	3.14 ± 0.09	4.15 ± 0.02	4.16 ± 0.02
Protein (%)	2.91 ± 0.03	2.99 ± 0.04	3.45 ± 0.03	3.43 ± 0.03
log SCC / mL	1.60 ± 0.07	1.49 ± 0.08	1.96 ± 0.04	1.86 ± 0.06

[1]Farms A and B are the Italian farms and farms C and D are the Greek farms. All values are means ± SEM

Table 2. Changes in levels of blood fatty acids, BHB, α-tocopherol (α-T), total cholesterol (TC), and the ratio of α-T to total cholesterol during the periparturient period in dairy cows[1]

Time of sampling	Fatty acids (mmol/L)	BHB (mmol/L)	α-T (μmol/L)	TC (mmol/L)	α-T (μmol/L):TC (μmol/L) (x10^3)
Dry-off	0.155^{a} ± 0.017	0.394^{a} ± 0.024	8.900^{a} ± 0.206	3.898^{a} ± 0.099	2.422^{a} ± 0.061
Calving	0.511^{b} ± 0.017	0.512^{b} ± 0.024	4.372^{b} ± 0.206	2.471^{b} ± 0.099	1.863^{b} ± 0.062
30 d postpartum	0.255^{c} ± 0.017	0.620^{c} ± 0.024	9.062^{a} ± 0.212	3.988^{a} ± 0.099	2.361^{a} ± 0.063

[a-c]Means within the same column followed by different letters differ at $P < 0.05$.

[1]Dairy cows from 4 herds, 2 of them in Italy and 2 in Greece; all values are LSM ± SEM

Table 3. Spearman's rho correlations between the levels of blood fatty acids, BHB, α-tocopherol (α-T), and the ratio of α-T to total cholesterol (TC) during the periparturient period in dairy cows[1]

Time	Item		Fatty acids	BHB	α-T	α-T:TC
Dry-off	Fatty acids	Rho	1	0.114	−0.169	−0.002
		P-value	_	NS	NS	NS
	BHB	Rho		1	−0.370	−0.352
		P-value		_	***	***
	α-T	Rho			1	0.348
		P-value			_	***
	α-T:TC	Rho				1
		P-value				_
Calving	Fatty acids	Rho	1	0.116	−0.084	0.053
		P-value	_	NS	NS	NS
	BHB	Rho		1	−0.010	0.165
		P-value		_	NS	NS
	α-T	Rho			1	0.207
		P-value			_	*
	α-T:TC	Rho				1
		P-value				_
30 d postpartum	Fatty acids	Rho	1	−0.030	−0.300	0.028
		P-value	_	NS	***	NS
	BHB	Rho		1	−0.104	−0.188
		P-value		_	NS	*
	α-T	Rho			1	0.388
		P-value			_	***
	α-T:TC	Rho				1
		P-value				_

[1]Dairy cows from 4 herds, 2 of them in Italy and 2 in Greece.

*Correlation is significant at $P < 0.05$ or ***$P < 0.001$ (2-tailed).

3.6 Conclusions

The present study documents a weak nonsignificant correlation between fatty acids and BHB during the periparturient period in dairy cows. Negative correlations between fatty acids and α-tocopherol were mainly a reflection of changes in lipid transport. The most interesting finding of our correlation analysis was the relatively strong correlation between BHB and α-tocopherol at dry-off. Future studies will examine whether, in addition to the well-documented link between vitamin E and the immune system in dairy cows, we may have forgotten to examine the effects of vitamin E on the metabolic function of the liver, similar to that in rodents and humans.

3.7 References

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Chapter 4

4 Appling new analytical technologies for the efficacy of a Lactobacilli-based compound added to forages at ensiling

4.1 Abstract

The aims of the study was to understand the effects of lactic acid bacteria (LAB) inoculation on fermentation products and quality of alfalfa and ryegrass silage. Wilted ryegrass and alfalfa silage were divided in control (CTR), and treated (T) groups and stored in triplicate mini silos. The treated group was inoculated with a commercial product containing *Lactobacillus Rhamnosus* and *Lactobacillus Farciminis*. The silos were opened after 2, 5, 30, 60, and 90 days and silages were collected. For each sample chemical analysis and electronic nose analysis were performed. At each sampling time, the temperature of all mini silos was detected by thermal camera. The alfalfa silage pH value was lower in T silage with higher content of lactic acid ($P \leq 0.01$). Unexpected higher values of NH_4 were observed in alfalfa T silage ($P \leq 0.01$). No significant differences of LAB concentration were observed between CTR and T alfalfa silages. Strong negative correlation between pH and lactic acid content (97%; $P \leq 0.01$) and positive correlation between pH and LAB concentration (93%; $P \leq 0.01$) were observed in alfalfa silage. In ryegrass silage the pH value and the LAB concentration were lower in T compared to CTR silages ($P \leq 0.01$). No difference of lactic acid content was observed between CTR and T. The NH_4 content was higher in CTR silage ($P \leq 0.01$). The LAB concentration was positive correlated with pH value 94% ($P \leq 0.01$). Negative correlation was observed between pH and NH_4 content (33%; $P \leq 0.05$). No temperature differences were observed between CTR and T silage. Further analysis will be needed to understand the ability of the electronic nose to evaluate the silage.

Key words: bacteria inoculation, electronic nose, silage, thermal camera

4.2 Introduction

Ensiling technique is based on converting water-soluble carbohydrates into organic acids such as lactic acid, acetic acid and preventing high ammonia nitrogen levels in anaerobic conditions by lactic acid bacteria (LAB). A number of factors can influence silage quality and nutrient content such as crops, ensiling technologies, machinery and additives used for manipulating fermentation processes (Davies et al., 2005; Jatkauskas et al., 2015). If the silage is not properly sealed, unfavorable fermentations may occurs, leading to a deterioration of the mass and consequently to a poor quality (Whitlock et al., 2000). Among other factors, the aerobic stability of a silage mass is of great importance because if not guaranteed, losses of DM and nutrients together with potential development of molds, having the potential to produce mycotoxins which can occur with consequent health hazards for animals and humans (Driehuis and Oude Elferink, 2000; Schmidt and Kung, 2010). In a well preserved silage, epiphytic microflora may be overwhelmed by the inoculant LAB, which results in a fast decrease in pH, high lactic to acetic acid ratio, low ethanol and ammonia nitrogen content and improvement in dry matter recovery (Weinberg and Muck, 1996; Li and Nishino, 2011a).

When a silage is not properly sealed, the addition of enzymes that are able to increase the amount of substrate available for fermentation has been suggested: ideally, the addition of enzymes can rapidly hydrolyze polysaccharides enough to produce glucose, which would be converted to lactate by the LAB (Sheperd at al., 1995).

To enhance lactic acid fermentation in the silo, many species of LAB have been selected as silage additives. The LAB used for silage treatment can be divided in homo-fermentative and hetero-fermentative. The homo-fermentative bacteria such as *L. plantarum, E. faecium* and *P. acidilactici*, have the function to ensure a rapid and efficient fermentation of water-soluble carbohydrates into lactic acid, a rapid decrease in pH, and improved silage conservation with minimal fermentation losses (Arriola et al., 2011; Weinberg et al., 1993; Weinberg and Muck 1996). The hetero-fermentative bacteria such as *L. Buchneri and L. Brevis,* on the other hand improves the aerobic stability of silages, converting lactic acid to acetic acid under anaerobic conditions (Oude Elferink et al., 2001; Taylor et al., 2002).

L. farciminis CNCM I-3699 and *L. rhamnosus* CNCM I-3698 are a probiotic with a scientific and commercial interest. These two LAB have been approved by EFSA as zootechnical feed additive for post-weaning piglets (Commission regulation, 2008). To date, there are no reports on the ability of combined use of *L. farciminis* and *L. rhamnosus* to improve the fermentations of alfalfa and ryegrass silage.

The chemical analysis of raw materials for animal nutrition is definitely the best and most accurate method to control their safety and quality. Provide a good silage quality is very important for dairy cattle nutrition. Unfortunately, chemical analysis are very expensive and take a long time to run and then provide the necessary information. Instruments that can perform quick and low cost analysis would be very useful for silage quality evaluation.

Some authors reported the correlation between the aerobic deterioration and temperature measurement of the silo (Ruppel et al., 1995; Borreani and Tabacco, 2010). The thermal camera allows detecting the temperature changes in front of the silo in short time and this enables performing the silage samples for the subsequent analysis quickly.

Electronic nose may represent a promising analytical tool to be used for an early detection of wrong fermentation in silage. Electronic nose consist of non-specific chemical detectors, which interact with different volatile molecules and provide an electronic signal that can be utilized effectively as a fingerprint of the volatile molecules associated with the product (Cheli at al., 2009). The aim of the present study was to evaluate the effects of inoculation of these two LAB on alfalfa and ryegrass silage quality and assess the use of electronic nose and the thermal camera for silage analysis.

4.3 Materials and methods

Two experiments were conducted to evaluate the efficacy of a product based on *Lactobacilli* and added to alfalfa and ryegrass silages. The trials were run in micro fermenters under natural conditions. Forages at ensiling time were taken from appropriate commercial farms located in Northern Italy.

The whole alfalfa and ryegrass plants were chopped with a conventional forage harvester to a theoretical length of 1.5 cm for alfalfa and 1.3 for ryegrass and sealed to triplicate mini-silos to each sampling time. Fresh forages were taken from a wagon–loader immediately after chopped and divided in two piles for both silages. Each pile was assigned to one of the following groups: no added product (control, CTR), *L. farciminis and L. rhamnosus* added silage (Treatment, T). The product concentration was $\geq 1.10^8$ CFU/g and was added respectively 0.5 kg/Ton on ryegrass and 0.8 kg/Ton on alfalfa. Samples of fresh forage were collected from each initial pile for chemical analysis. The treated silages were mixed with *Lactobacilli*. The silages were manually compacted with 40 atm pressure on micro fermenters, moved to University experimental facilities (Polo Veterinario of Lodi), and taken under ambient conditions during the trial. Six sampling times (0, 2, 5, 30, 60, and 90 days post-ensiling) were considered. The samples were collected by a corer (Master forage probe) and rapidly vacuum-sealed and frozen at -20°C. On all collected samples the following parameters were analysed to evaluate the fermentation characteristics: DM, pH, and organic acids (Lactic acid, Acetic acid, Butyric acid, Propionic acid, Isobutyric acid and Valeric acid), NH_4, NH_3-N, Total Nitrogen, soluble Nitrogen, crude protein, Ethanol, lactic acid bacteria (LAB) content. For each sample a FLIEG score was assigned. Moreover, on fresh forages samples and at 90 days of ensiling DM, CP, EE, CF, NDF, ADF, ADL and Ash content were determined to evaluate the nutritional characteristics.

All the analyses were performed with ACCREDIA certified internal method laboratory.

At each sampling time, the mass temperature was detected with thermal camera (AVIO mod. ThermoGEAR G120EX). The images were analyzed with the dedicated program InfReC Analyzer NEC AVIO, which allows obtaining the maximum, the medium and lowest temperature value for the areas of interest by drawing directly on the thermal images. For each analysis in each sampling day, the environmental temperature of the sampling day was included and an emissivity of 0.97 was considered to correct the data. The environmental temperature is very important because as reported by Schmidt et al. (2015), the environmental conditions may affect the temperature detection. During the thermal scan, humidity and

internal temperature of the silage were measured through a probe (Fortester by Isoelectric) on each sampling point.

Each collected sample during the trial was also analysed with an electronic nose to evaluate the off-flavor by EN PEN2 (10 MOS). Electronic nose consist of non-specific chemical detectors, which interact with different volatile molecules and provide an electronic signal that can be utilized effectively as a fingerprint of the volatile molecules associated with the product. Sensor details are reported in table 1. The air flux method was used in this trial. The fluxed aroma was obtained using an output needle inserted into a 20 ml vial covered with crimp seal, containing 1 g of silage, it was then thawed for 24 hours. A second needle allowed aspiration of the charcoal-filtered air at a 400 ml/min flux rate. The run of each sample lasted 120 seconds, and was followed by a 240 seconds flush time to clean the instruments. After each sample run was performed a white run for 120 seconds with empty vial to clean the needle, followed by another flush time to clean the instruments. Each measurement, carried out in triplicate, was controlled and recorded on a text file by WinMunster v.1.6 software.

Table 1: PEN2 sensors details and applications.

Sensor name	Description	Reference material
Aromatic1	Aromatic compound	Toluene, 10 ppm
Broadrange	Broad range sensitivity react on nitrogen oxides and ozone very sensitive with negative signal	NO2, 10 ppm
Aromatic2	Ammonia, used as sensor for aromatic compounds	Benzene, 10 ppm
Hydrogen	Mainly hydrogen, selectively (breath gases)	H2, 100 ppb
Arom-aliph	Alkanes, aromatic compounds, less polar compounds	Propane, 1 ppm
Broad-methane	Sensitive to methane (environment) ca. 10 ppm. Broad range, similar to n°8	CH4, 100 ppm
Sulphur-organic	Reacts on sulphur compounds H2S, 1 ppm Otherwise sensitive to many terpenes and Sulphur organic compounds, which are important for smell, limonene, pyrazine	H2S, 1 ppm
Broad-alcohol	Detects alcohols, partially aromatic compounds, broad range	CO, 100 ppm
Sulphur-chlor	Aromatics compounds, sulphur organic compounds	H2S, 1 ppm
Methane-aliph	Reacts on high concentrations > 100 ppm, sometime very selective (methane)	CH4, 10 ppm

Modified from Cheli et al. 2009.

4.4 Statistical analysis

The data obtained with chemical analysis were analysed with a GLM procedure of SAS v. 9.4 (SAS Institute Inc., Cary, NC) for a completely randomized design in 3 x 6 factorial arrangement (inoculation and days of fermentation) to assess the silage quality during the trials and the differences of silage composition. Pearson correlation coefficients (SAS v9.4, The SAS Institute, Cary, NC) were tested to evaluate silage chemical composition and silage temperature. Differences were considered significant for $P \leq 0.05$. The obtained results from electronic nose were analyzed with PCA procedure (JMP 12 pro, The SAS Institute, Cary, NC, 2015). This technique transforms the entire set of *n*-correlated variables to *n*-uncorrelated linear functions of the original measurements. The first principal component is the linear combination of all variables showing the maximum variation among the samples. The second, third and further components are similarly linear combinations representing the next-largest variations, irrespective of those represented by the previous combination. Orthogonal transformations such as PCA have been utilized to mitigate possible negative effects of a feature-selection process, such as the correlation among variables of datasets (Jain and Zongker, 1997; Campagnoli et al., 2011).

4.5 Results and Discussion

Ryegrass silage

In ryegrass silage, both CTR and T silage samples showed decreased DM content during the trial (table 2). The CTR group had higher DM content than T for almost the whole trial ($P \leq 0.01$) with exception of second sampling time. The lactobacilli inoculum in ryegrass significantly lowered pH values ($P \leq 0.01$), the CTR silage had higher pH value than T silage especially in the last two sampling time (table 4). The decreased pH treated silage was not due to higher content of lactic acid, how reported in literature (Holzer et al., 2003; Jatkauskas et al., 2015; Rodríguez Amado et al., 2012) although the correlation analysis showed significant negative correlation of pH value with lactic acid (71% $P \leq 0.01$) and acetic acid (40% $P \leq 0.01$). The lower pH value in the treated silage could be due to the higher content of ammonia and ammonia nitrogen in CTR group ($P \leq 0.01$). The levels of ammonia and ammonia nitrogen are constantly lower in T than CTR (tables 7 and 9), with significant differences at 30, 60 and 90 days of ensiling. This is an important aspect because the ammonia, ammonia nitrogen and ethanol content have to be low for well-preserved silage (Weinberg and Muck, 1996; Li and Nishino, 2011a).

The lactic acid and acetic acid content were not different between CTR and T (tables 5-6) with the exception for higher acetic acid content in CTR ($P \leq 0.01$) at 30 days of ensiling. The ethanol content resulted not significant different between treatment (table 11). The LAB concentration (table 12) was higher CTR than T ($P \leq 0.01$) with significant differences in the last two sampling times (60 and 90 days). This result could be explained by the observed significant decrease of pH values in T that might have created not favorable environmental conditions for indigenous bacteria communities (Li and Nishino, 2011a; McDonald et al., 1991). The data analysis showed strong and significant positive correlation of LAB concentration with pH value (94%; $P \leq 0.01$), and negative correlation with lactic acid (67%; $P \leq 0.01$) and acetic acid (38%; $P \leq 0.05$). The soluble nitrogen (table 10) resulted constantly higher in treated silage ($P \leq 0.01$). No effects of LAB inoculation on crude protein content (table 3) total nitrogen content (table 8), and FLIEG score (table 13) were observed.

The analysis of the data showed a significant influence of the sampling time for all the parameters considered with the exception of ethanol and FLIEG score.

The chemical analysis performed at 90 days of ensiling to evaluate the silage nutritional characteristics (table 26) showed that T ryegrass silage had higher content of EE ($P \leq 0.01$),

NDF (P≤0.01), ADL (P≤0.01), ash (P≤0.01), calcium (P≤0.01) and phosphorus (P≤0.05) but lower content of DM (P≤0.01).

Ryegrass is widely distributed throughout temperate and tropical or subtropical regions and is one major forage crops used either as fresh green-chop or as hay or silage (Shao et al., 2005). However, there are lack information's on fermentation quality of ryegrass silage.

Alfalfa silage

Alfalfa is a forage crop with high nutritive value and is one of the major components of diets for high producing dairy cows. This forage crop can be difficult to ensile due to the low content of soluble carbohydrates and the high contents of organic acids, salts, proteins, and minerals, resulting in a high buffering capacity which may limits the ensiling process (McDonald et al., 1991; Sheperd at al., 1995).

In our trial the LAB inoculum on alfalfa silage had effect on CP, pH value, lactic acid, ammonia and ammonia nitrogen. The T silage showed higher CP content than CTR (P≤0.05) (table 15). The CTR silage had higher pH value with lower lactic acid content than T (tables 16 and 17) with significant differences (P≤0.01) from 30 days of ensiling until the end of the experimental study. The correlation analysis showed higher and stronger negative correlation of pH value with lactic acid (97% P≤0.01) and acetic acid (89% P≤0.01). These results are in accordance with Wang et al. (2006), although they used different lactobacilli inoculation. Unexpected results were observed on ammonia and ammonia nitrogen content (tables 19-21) that were constantly higher in T silage during the whole trial period (P≤0.01). Kung et al. (2003) reported similar results of ammonia content in silage treated with *L. buchneri*. Also the total N was higher in T silage (table 20) although only the last sampling time had significant difference (P≤0.01). Although high contents of ammonia and ammonia nitrogen are not favorable for silage, in the present trial did not affect the acidification and preservation of the mass, probably due to the higher content of lactic acid in LAB inoculated silage. No effect of LAB inoculation were observed on dry matter, acetic acid, soluble nitrogen, ethanol, LAB concentration and FLIEG score in alfalfa silage. The LAB concentration showed positive correlation with pH value (93% P≤0.01) and negative correlation with lactic acid (89% P≤0.01) and acetic acid (92% P≤0.01). The data analysis showed a significant influence of the sampling time for almost all the parameters considered with the exception of DM, soluble N and FLIEG score.

The chemical analysis performed at 90 days of ensiling to evaluate the silage nutritional characteristics (table 27) showed that the CTR alfalfa silage had higher content of CF P≤0.05,

NDF (P≤0.05), ADF (P≤0.01) and ADL (P≤0.01), but had lower content of DM (P≤0.05), CP (P≤0.05), EE (P≤0.01), calcium (P≤0.05) and UFL (P≤0.01).

The content of propionic acid, butyric acid, isobutyric acid and valeric acid, were also analyzed to evaluate the fermentation quality both in ryegrass and alfalfa silage, but in all analysis performed they were not detected.

Thermal camera and electronic nose

The thermal camera scan and probe have not detected differences of temperature between T and CTR in both silages. Although no differences were observed, the data analysis showed some correlations of temperature with fermentation parameters. In ryegrass silage the temperature was negative correlated with pH value (82% P<0.01) and positive correlated with lactic acid (84% P≤0.01), acetic acid (58% P≤0.01), NH_4 (66% P≤0.01), total nitrogen (72% P≤0.01), ammonia nitrogen (32% P≤0.05) and soluble nitrogen (34% P≤0.01). In alfalfa silage the temperature was negative correlated with pH (45% P≤0.01) and total nitrogen (32% P≤0.05), and positive correlated with lactic acid (53% P≤0.01), NH_4 (41% P≤0.05) and ammonia nitrogen (42% P≤0.01).

The PCA analysis applied to electronic nose data showed that the variance of the ten sensors was explained by two factors. The two factors were able to explain 88.9% of total data variance for ryegrass silage and 87.2% for alfalfa silage (figures 1and 3).

The data analysis with GLM procedure showed two different results. In ryegrass silage was observed significant difference between T and CTR samples in 7 of 10 sensors. Aromatic1, Aromatic 2 and Arom-aliph were higher in CTR samples with P≤0.01, instead Broadrange, Broad-methane, Broad-alcohol and Sulphclor were higher in T samples (P≤0.01). No differences were observed in alfalfa silage. This result is probably due to the greater acidification and pH value differences between T and CTR samples of ryegrass silage.

Table 2: Ryegrass silage dry matter content during the experimental study.

DM %	CTR	T	P-value		
			t	period	t*p
treatment	33.15	30.66	≤0.01	≤0.01	≤0.01
period:					
2 d	35.26^{A}	32.04^{B}			
5 d	33.99	34.08			
30 d	33.23^{a}	31.78^{b}			
60 d	32.99^{A}	28.02^{B}			
90 d	30.29^{A}	27.38^{B}			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 3: Ryegrass crude protein content during the experimental study.

CP (%DM)	CTR	T	P-value		
			t	period	t*p
treatment	7.79	7.90	NS	≤0.01	NS
period:					
2 d	6.95	7.00			
5 d	7.13	6.92			
30 d	8.38	8.24			
60 d	8.45^{a}	8.96^{b}			
90 d	8.02	8.37			

The points with different letter at same sampling time differ.
A,B P≤0.01
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 4: Ryegrass silage pH value during the experimental study.

pH	CTR	T	P-value		
			t	period	t*p
treatment	4.28	3.96	≤0.01	≤0.01	≤0.01
period:					
2 d	4.62^{A}	4.46^{B}			
5 d	4.49	4.44			
30 d	4.26	4.26			
60 d	4.19^{A}	3.55^{B}			
90 d	3.84^{A}	3.11^{B}			

The points with different letter at same sampling time differ.
A,B P≤0.01
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 5: Ryegrass silage lactic acid content during the experimental study.

Lactic acid (%DM)	CTR	T	P-value		
			t	period	t*p
treatment	3.23	3.20	NS	≤0.01	NS
period:					
2 d	2.44	2.29			
5 d	2.35	2.40			
30 d	3.69	3.55			
60 d	3.73	3.92			
90 d	3.90	3.81			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 6: Ryegrass silage acetic acid content during the experimental study.

Acetic acid (%DM)	CTR	T	P-value		
			t	period	t*p
treatment	0.87	0.78	NS	≤0.01	NS
period:					
2 d	0.62	0.51			
5 d	0.52	0.42			
30 d	1.20^{A}	0.88^{B}			
60 d	1.12	1.13			
90 d	0.88	0.98			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 7: Ryegrass silage NH_4 content during the experimental study.

NH_4 (mg/Kg DM)	CTR	T	P-value		
			t	period	t*p
treatment	685.58	633.72	≤0.01	≤0.01	NS
period:					
2 d	575.33	556.50			
5 d	567.40	541.87			
30 d	680.13^{a}	601.88^{b}			
60 d	797.50	762.20			
90 d	807.33^{A}	706.20^{B}			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 8: Ryegrass silage total N content during the experimental study.

Total N (%)	CTR	T	P-value		
			t	period	t*p
treatment	1.25	1.26	NS	<0.01	NS
period:					
2 d	1.11	1.12			
5 d	1.14	1.08			
30 d	1.34	1.32			
60 d	1.35	1.43			
90 d	1.28	1.34			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 9: Ryegrass silage NNH3 content during the experimental study.

NNH_3 (% total N)	CTR	T	P-value		
			t	period	t*p
treatment	4.51	4.12	≤0.01	≤0.01	NS
period:					
2 d	4.25	4.09			
5 d	4.09	4.03			
30 d	4.19^{a}	3.76^{b}			
60 d	4.85^{a}	4.38^{b}			
90 d	5.17^{A}	4.34^{B}			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 10: Ryegrass silage soluble N content during the experimental study.

Soluble N (% DM)	CTR	T	P-value		
			t	period	t*p
treatment	0.59	0.63	≤0.01	≤0.01	NS
period:					
2 d	0.57[a]	0.62[b]			
5 d	0.59	0.63			
30 d	0.58	0.60			
60 d	0.60	0.62			
90 d	0.62	0.67			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 11: Ryegrass silage ethanol content during the experimental study.

Ethanol (mg/100g as fed)	CTR	T	P-value		
			t	period	t*p
treatment	0.17	0.12	NS	NS	NS
period:					
2 d	0.18	0.10			
5 d	0.20	0.10			
30 d	0.19	0.12			
60 d	0.14	0.17			
90 d	0.12	0.13			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 12: Ryegrass silage LAB content during the experimental study.

LAB log^{10} (cfu/g)	**CTR**	**T**	**P-value**		
			t	**period**	**t*p**
treatment	8.81	8.33	≤0.01	≤0.01	≤0.01
period:					
2 d	9.11	9.10			
5 d	9.18	9.04			
30 d	8.63	8.62			
60 d	8.50^{A}	7.67^{B}			
90 d	8.61^{A}	7.19^{B}			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 13: Ryegrass silage FLIEG score during the experimental study.

FLIEG score	**CTR**	**T**	**P-value**		
			t	**period**	**t*p**
treatment	87	88	NS	NS	NS
period:					
2 d	86	86			
5 d	85	87			
30 d	88	88			
60 d	88	88			
90 d	88	88			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 14: Alfalfa silage dry matter content during the experimental study.

DM %	CTR	T	P-value		
			t	period	t*p
treatment	40.67	41.06	NS	NS	NS
period:					
2 d	40.40	41.10			
5 d	41.40	41.20			
30 d	40.40	41.00			
60 d	41.20	40.80			
90 d	40.00	41.20			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 15: Alfalfa silage crude protein (CP) content during the experimental study.

CP (%DM)	CTR	T	P-value		
			t	period	t*p
treatment	18.52	18.83	≤0.05	≤0.01	≤0.05
period:					
2 d	19.06	19.47			
5 d	19.10	19.11			
30 d	18.64	18.21			
60 d	18.48	18.85			
90 d	17.37^{A}	18.54^{B}			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 16: Alfalfa silage pH value during the experimental study.

pH	CTR	T	P-value		
			t	period	t*p
treatment	4.92	4.75	≤0.01	≤0.01	≤0.01
period:					
2 d	5.78	5.73			
5 d	5.48	5.45			
30 d	4.63^{A}	4.34^{B}			
60 d	4.53^{A}	4.28^{B}			
90 d	4.18^{A}	3.95^{B}			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 17: Alfalfa silage lactic acid value during the experimental study.

Lactic acid (%DM)	CTR	T	P-value		
			t	period	t*p
treatment	3.27	3.56	≤0.01	≤0.01	≤0.01
period:					
2 d	2.32	2.27			
5 d	2.25	2.30			
30 d	3.92^{A}	4.35^{B}			
60 d	3.85^{A}	4.39^{B}			
90 d	4.00^{A}	4.49^{B}			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 18: Alfalfa silage acetic acid content during the experimental study.

Acetic acid (%DM)	CTR	T	P-value		
			t	period	t*p
treatment	0.47	0.52	NS	≤0.01	NS
period:					
2 d	0.18	0.23			
5 d	0.22	0.19			
30 d	0.45	0.49			
60 d	0.62	0.71			
90 d	0.89	0.98			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 19: Alfalfa silage NH_4 content during the experimental study.

NH_4 (mg/Kg DM)	CTR	T	P-value		
			t	period	t*p
treatment	717.19	808.43	≤0.01	≤0.01	NS
period:					
2 d	553.93^{a}	594.06^{b}			
5 d	603.63	621.73			
30 d	900.73	932.83			
60 d	899.1^{a}	938.7^{b}			
90 d	878.57^{A}	954.8^{B}			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 20: Alfalfa silage total N content during the experimental study.

Total N (%)	CTR	T	P-value		
			t	period	t*p
treatment	2.96	3.01	≤0.05	≤0.01	≤0.05
period:					
2 d	3.05	3.11			
5 d	3.05	3.06			
30 d	2.98	2.91			
60 d	2.95	3.02			
90 d	2.78^{A}	2.97^{B}			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 21: Alfalfa silage NNH3 content during the experimental study.

NNH_3 (%total N)	CTR	T	P-value		
			t	period	t*p
treatment	2.14	2.22	≤0.01	≤0.01	NS
period:					
2 d	1.50	1.57			
5 d	1.62	1.67			
30 d	2.49^{a}	2.64^{b}			
60 d	2.50	2.57			
90 d	2.61	2.65			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 22: Alfalfa silage soluble N content during the experimental study.

Soluble N (%DM)	CTR	T	P-value		
			t	period	t*p
treatment	0.57	0.55	NS	NS	NS
period:					
2 d	0.55	0.51			
5 d	0.53	0.53			
30 d	0.54	0.56			
60 d	0.58	0.57			
90 d	0.67	0.59			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 23: Alfalfa silage ethanol content during the experimental study.

Ethanol (mg/100g AF)	CTR	T	P-value		
			t	period	t*p
treatment	0.26	0.28	NS	≤0.01	NS
period:					
2 d	0.10	0.10			
5 d	0.10	0.10			
30 d	0.30	0.29			
60 d	0.31	0.30			
90 d	0.49^{A}	0.61^{B}			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 24: Alfalfa silage LAB content during the experimental study.

LAB log^{10} (cfu/g)	**CTR**	**T**	**P-value**		
			t	**period**	**t*p**
treatment	8.53	8.56	NS	≤0.01	≤0.05
period:					
2 d	9.48	9.41			
5 d	9.28	9.44			
30 d	8.34^{A}	8.70^{B}			
60 d	7.97	7.76			
90 d	7.59	7.48			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 25: Alfalfa silage FLIEG score during the experimental study.

FLIEG score	**CTR**	**T**	**P-value**		
			t	**period**	**t*p**
treatment	88	87	NS	NS	NS
period:					
2 d	88	86			
5 d	88	88			
30 d	88	88			
60 d	88	88			
90 d	88	88			

The points with different letter at same sampling time differ.
A,B P≤0.01
a,b P≤0.05
CTR= control, T= treated
t= treatment effect, period= time effect, t*p= treatment for time effect

Table 26: Ryegrass silage composition at 90 days.

Ryegrass silage				
ITEM	CTR	T	SE	P-Value
DM %	30.3	27.4	0.57	≤0.01
CP (%DM)	8.02	8.37	0.06	NS
CF (%DM)	34.54	34.48	0.4	NS
EE (%DM)	1.24	2.32	0.15	≤0.01
NDF (%DM)	55.37	58.08	0.45	≤0.01
ADF (%DM)	39.87	39.33	0.28	NS
ADL (%DM)	3.71	4.47	0.14	≤0.01
Ash (%DM)	6.23	7.88	0.31	≤0.01
Ca (%DM)	0.3	0.46	0.07	≤0.01
P (%DM)	0.21	0.26	0.02	≤0.05
UFL (%DM)	55.6	56.8	0.97	NS
Energy (%DM)	1.83	2.07	0.03	≤0.01

CTR= control, T= treated, SE= standard error
NS= no significant

Table 27: Alfalfa silage composition at 90 days.

Alfalfa silage				
ITEM	CTR	T	SE	P-Value
DM %	40.0	41.2	0.42	≤0.05
CP (%DM)	17.4	18.5	0.21	≤0.01
CF (%DM)	29.8	27.2	0.64	≤0.05
EE (%DM)	1.56	2.47	0.08	≤0.01
NDF (%DM)	46.1	42	1.38	≤0.05
ADF (%DM)	34.0	31.0	0.6	≤0.01
ADL (%DM)	5.13	4.39	0.01	≤0.01
Ash (%DM)	9.88	9.92	0.18	NS
Ca (%DM)	1.45	1.58	0.04	≤0.05
P (%DM)	0.3	0.32	0.02	NS
UFL (%DM)	51.9	56	1.04	≤0.01
Energy (%DM)	1.3	1.36	0.04	NS

CTR= control, T= treated, SE= standard error
NS= no significant

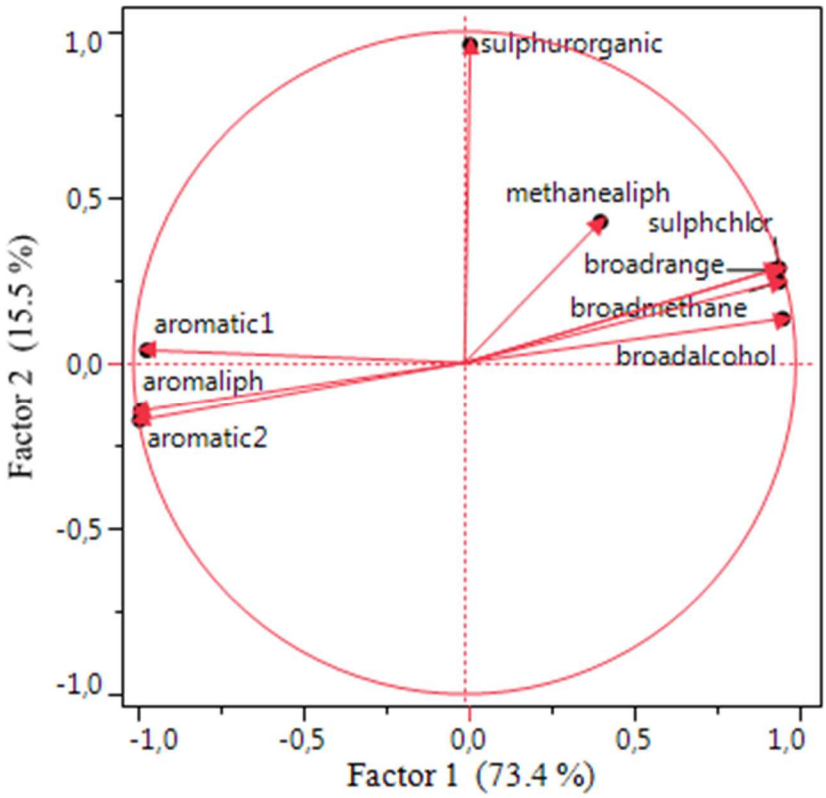

Figure 1: PCA factor plot of ryegrass silage.

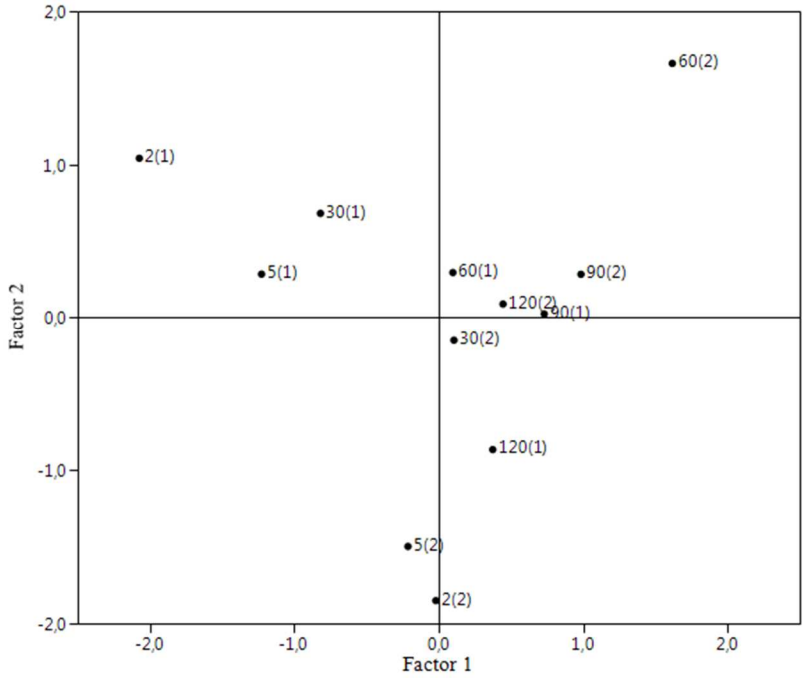

Figure 2: PCA factor score of ryegrass silage.

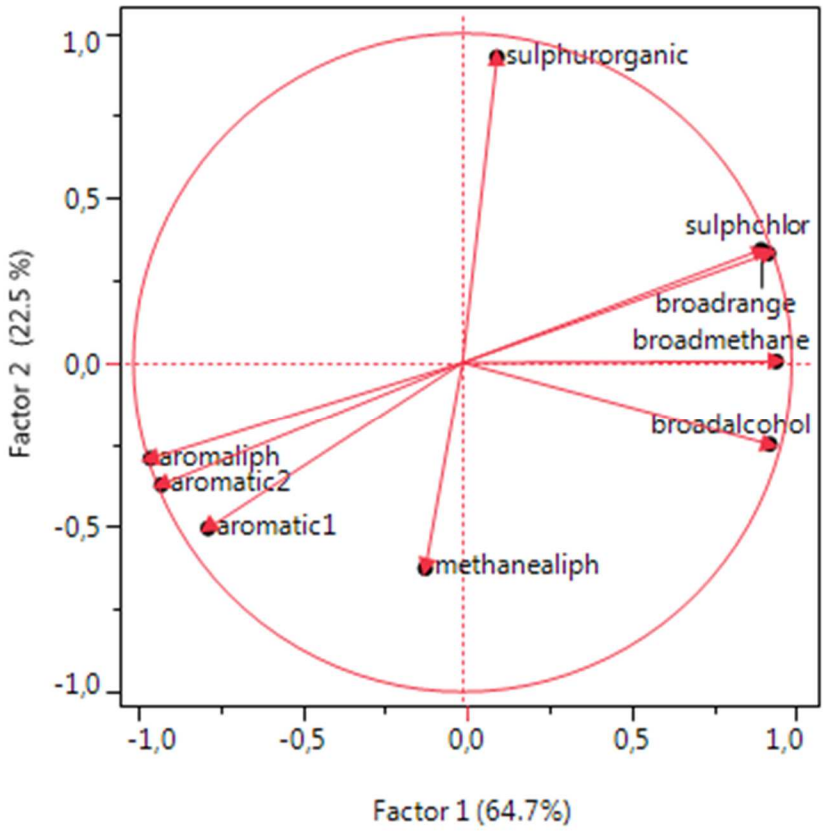

Figure 3: PCA factor plot of alfalfa silage.

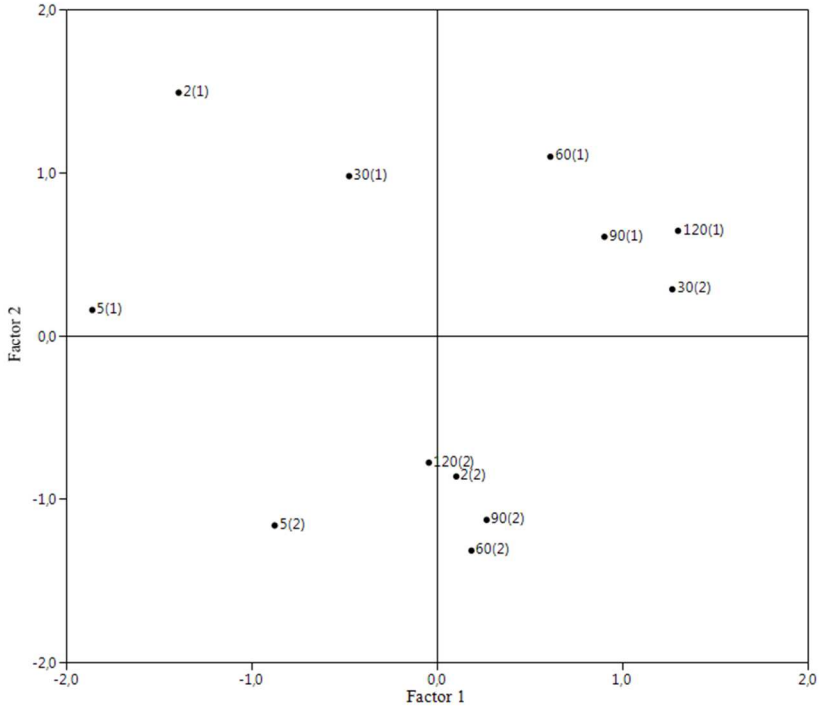

Figure 4: PCA factor score of alfalfa silage.

4.6 Conclusions

This study showed that the microbial inoculants with *L. farciminis and L. rhamnosus* had a positive effect on silages characteristics and sped up the acidification of the mass. The pH value was lower in both types of treated silages and this help to preserve the mass (Schmidt et al., 2009). The inoculation with lactobacilli increased the lactic acid content in the alfalfa treated silage. This result is very significant because this forage crop can be difficult to ensiling for its high contents of organic acids, salts proteins and minerals result in a high buffering capacity (McDonald et al., 1991). The electronic nose has been shown to be used for the analysis of the silage quality although the results obtained with alfalfa silage suggest that further studies will be needed to assess the accuracy and sensitivity of the instrument.

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Chapter 5

5 New technologies for the rapid assessment of silage quality

5.1 Abstract

The objectives of the present study to were evaluate the silage quality and the combined use of thermal camera and electronic nose for rapid assessment of unfavorable fermentations that can occur in a corn silage mass. From April to September 2015, five sampling times were performed to collect silage samples. At each sampling time, the temperature were detected by thermal camera and five representative samples of silage with temperature near to the average of the whole silage front (Average Temperature Samples, ATS) and up to 3 extra samples with higher temperature (High Temperature Samples, HTS) were collected. Chemical analyses over the collected samples were performed to evaluate the content of DM, CP, EE, Starch, Ash, pH, NH_4, molds, yeast and mycotoxins (Aflatoxin B1) and an electronic nose equipped with metal-oxide-semiconductor (MOS) was used to perform the off-flavors analysis. The silage quality was found to be constant and no differences were observed between the ATS throughout the trial. Significant differences were observed between HTS and ATS. The HTS showed higher temperatures detected with thermal camera ($P=0.01$), higher pH values ($P<0.01$) and lower lactic acid content ($P<0.05$) compare to ATS. The results obtained on off-flavor analyses indicate that electronic nose was able to distinguish the strong unfavorable fermentations, and the combined use with thermal camera can provide an indication of silage quality in short time and at a lower cost than classical analytical methods.

Key words: silage, thermal camera, electronic nose

5.2 Introduction

Corn silage is the major source of forage for lactating dairy cows. Most of the silage stored in horizontal silos is exposed to air penetration, especially in the upper parts near the walls, which are difficult to pack and seal properly. The characteristics of the ensiled crops, the environmental conditions and practices adopted during silage making have a strong impact on the resulting nutritional value and fermentation stability (McDonald et al., 1991). If the sealing procedure of the silo is not appropriate, air can penetrates the silage mass and resulting in aerobic deterioration with dry matter and nutritional losses as well as increase the risk of proliferation of potentially pathogenic or otherwise undesirable microorganisms (Driehuis and Oude Elferink, 2000). The preservative effect against the growth of detrimental microorganisms results from a first aerobic phase with lactic acid and acetic acid production and consequent anaerobiosis and acidification of the ensiled mass.

The presence of oxygen in the silage may lead to the growth of molds and fungi that can cause several diseases to the animals such as respiratory problems, unfavorable ruminal fermentations, decreased reproduction indexes, low production, kidney damage, and skin and eye irritation. The dry matter intake of deteriorated material is often restricted, and animals may refuse the feed completely (McDonald et al., 1991).

Moreover some molds that contaminate forages can produce mycotoxins that can be transferred in the milk or meat and can cause huge health problems to humans beside animals (Amigot et al., 2006; Schmidt et al., 2014).

The consequences of aerobic deterioration include potentially marked changes in the composition of volatile compounds. For these reasons, silages can be evaluated for volatile compounds that result from fermentation reactions to assess fermentation quality based on the content of undesired degradation products (Borreani et al., 2007). The electronic nose exhibited advantages for the evaluation of fermentation characteristics like volatile fatty acids and ammonia nitrogen (Masoero et al., 2007). A large number of electronic-nose technologies have been developed including acoustic sensors, such as Quartz crystal microbalance; surface and bulk acoustic wave, Carbon black composite detectors, Catalytic bead, Catalytic field-effect, Calorimetric, Complementary metal oxide semiconductor, Conducting polymer, Electrochemical, Electrical porous silicon sensor, Fluorescence, Infrared, Metal oxides semiconducting, and Optical sensors. Among all of these e-nose types, Metal oxide semiconductor (MOS) sensors are the most widely used class of gas sensors for environmental pollution detection because they are capable of detecting both organic and

inorganic toxins. MOS e-noses also have good reproducibility and limited manufacturing costs (Wilson, 2012). Roβ et al. (2012) conducted an experiment to assess correlation between the DMI and volatile composition of silage and verifying the ability of the electronic nose to evaluate silage quality. Fermentation qualities, hygiene status, stage of deterioration, and preference behavior by goats and DMI of silages with different lengths of aerobic exposure were compared, based on the signal pattern obtained from the electronic nose. Chemical and microbial compositions were correlated with the signals given by the chemosensor system and with the feed assumption. The authors observed a relationship between changes in the sensor pattern and changes in the composition of the silage gas and a direct relationship between the sensor signals and the concentration of the measured gases.

On farm, the silage deterioration is usually manifested by an increase in temperature. Some authors reported the correlation between the aerobic deterioration and temperature measurement of the silo (Ruppel et al., 1995; Borreani and Tabacco, 2010). The thermal camera allows detecting the temperature changes in front of the silo in short time and this enables performing the silage samples for the subsequent analysis quickly. At the moment few and contradictory information in the literature are reported on the use of the camera as a tool for assessing the quality of silages. Novinski et al. (2012) performed a trial to assess the use of thermal camera to identify heating sites in the silo faces, and to correlate the temperatures with mycotoxin content in maize silages. They reported that thermal camera is ineffective for detecting the presence of mycotoxins, suggesting that temperature of silo face is not a good indicator of these compounds in corn silages. Completely different results were published by Addah et al. (2012), they used the thermal imaging to assess the impact of an inoculant with feruloyl esterase activity on the aerobic stability and digestibility of barley silage. The data obtained showed a higher temperature of uninoculated as compared with inoculated silage exposed to air and suggests that this technique has potential for documenting heating in silage ensiled in farm-scale systems.

No studies are available on the combined use of the electronic nose and thermal camera for the evaluation of the silage. The aim of present study was to assess the use of both instrument for rapid analysis of the entire silage front in short time with low cost and evaluate the silage quality during the entire period of use.

5.3 Materials and methods

The present study was applied to a corn silage mass, chopped at 18 mm theoretical length, inoculated with commercial product based of *L. buchneri* and *L. casei*, and ensiled in bunker silos. Fresh silage samples were collected in September 2014 when the silage was placed in the bunker. From April 2015 to September 2015, the thermic measurements with the thermal camera were performed (figure 1 and 2) and corn silage samples were collected monthly. At each sampling time, the height and the width of the silo were measured to determine exactly the sampling points. Five samples were collected at each sampling time in well-defined points (two for each right and left side in the upper and the lower part of the mass, and one in middle to obtained a representative sample of the whole front). Three additional samples were collected in the case the thermal camera (AVIO ThermoGEAR G120EX model, equipped with uncooled microbolometer detector of 320x240 pixels, with a temperature resolution of 0.04 ° C and spatial 1.78 mrad) scan evidenced high differences of temperature in others points of the mass front.

Thermal images were taken in front of the silage surface and were analyzed with the dedicated program InfReC Analyzer NEC AVIO, which allows to obtain the temperature of the areas of interest by drawing directly on the thermal images (figure 3 and 4). For each analysis, the environmental temperature of the sampling day was included and an emissivity of 0.97 was considered to correct the data. The environmental temperature is very important because as reported by Schmidt et al. (2015), the environmental conditions may affect the temperature detection. Furthermore, in our study the sampling times were performed only in sunny days to reduce the environmental effect due to rain, fog, cloudy and wind and at same hour (7.30 AM), immediately after the removal of silage from the front to prepare the daily ration. During the thermal scan, humidity and internal temperature of the silage were measured through a probe (Fortester by Isoelectric) on each sampling point.

The samples were collected by a corer (Master forage probe). For each sample 500g (250g to chemical analyses, 250g to electronic nose analyses), were rapidly vacuum-sealed, and frozen at -20°C to ensure their characteristics remain unaltered until laboratory assays.

The collected silage samples were subsequently analyzed to evaluate the content of DM, CP, EE, Starch, Ash, pH, NH_4, molds, yeast and mycotoxins (Aflatoxin B1) in accordance with the Association of Official Agricultural Chemists (AOAC 2005). The corn silage NDF and ADF were evaluated in according with the procedures described by Van Soest et al. (1991). To evaluate the lactic acid, acetic acid, propionic acid, butyric acid and free water content the

ACCREDIA certified internal method laboratory was used. To evaluate the clostridia content the ISO 15213:2003 method was used. For each sample a FLIEG score was assigned.

Furthermore, the corn silage samples were analyzed to assess the off-flavors by Electronic nose EN PEN2 (10 MOS). Electronic nose consist of non-specific chemical detectors, which interact with different volatile molecules and provide an electronic signal that can be utilized effectively as a fingerprint of the volatile molecules associated with the product. Sensor details are reported in table 1. The air flux method was used in this trial. The fluxed aroma was obtained using an output needle inserted into a 10 ml vial covered with crimp seal, containing 1 g of silage. A second needle allowed aspiration of the charcoal-filtered air at a 400 ml/min flux rate. The sample run lasted 120 seconds, and was followed by a 240 sec flush time to clean the system. After each sample run was performed, a white run for 120 seconds with empty vial to clean the needle, followed by flush time. Each measurement, carried out in triplicate, was controlled and recorded on a text file by WinMunster v.1.6 software.

Table 1: PEN2 sensors details and applications.

Sensor name	Description	Reference material
Aromatic1	Aromatic compound	Toluene, 10 ppm
Broadrange	Broad range sensitivity react on nitrogen oxides and ozone very sensitive with negative signal	NO2, 10 ppm
Aromatic2	Ammonia, used as sensor for aromatic compounds	Benzene, 10 ppm
Hydrogen	Mainly hydrogen, selectively (breath gases)	H2, 100 ppb
Arom-aliph	Alkanes, aromatic compounds, less polar compounds	Propane, 1 ppm
Broad-methane	Sensitive to methane (environment) ca. 10 ppm. Broad range, similar to n°8	CH4, 100 ppm
Sulphur-organic	Reacts on sulphur compounds H2S, 1 ppm Otherwise sensitive to many terpenes and Sulphur organic compounds, which are important for smell, limonene, pyrazine	H2S, 1 ppm
Broad-alcohol	Detects alcohols, partially aromatic compounds, broad range	CO, 100 ppm
Sulphur-chlor	Aromatics compounds, sulphur organic compounds	H2S, 1 ppm
Methane-aliph	Reacts on high concentrations > 100 ppm, sometime very selective (methane)	CH4, 10 ppm

Modified from Cheli et al. 2009.

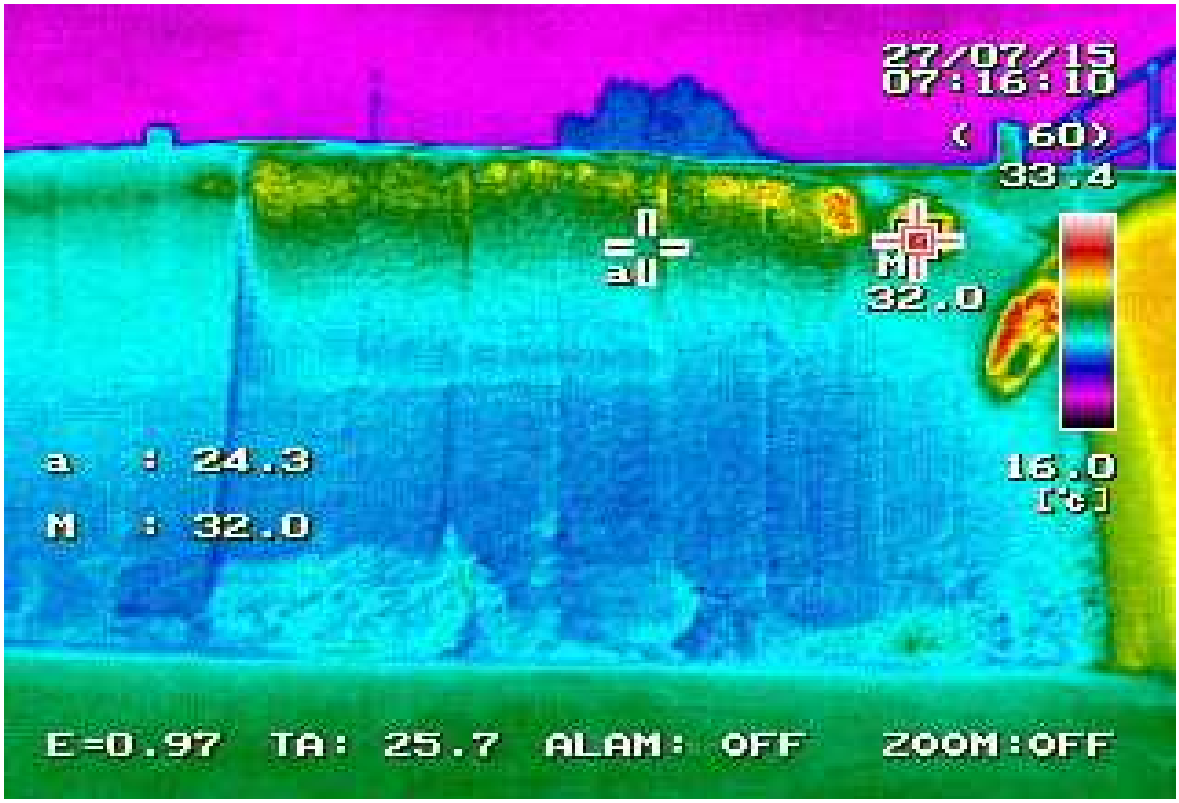

Figure 1 and 2: Example of thermal camera temperature detected on the front of the bunker.

a: Average temperature detected

M: Highest temperature detected

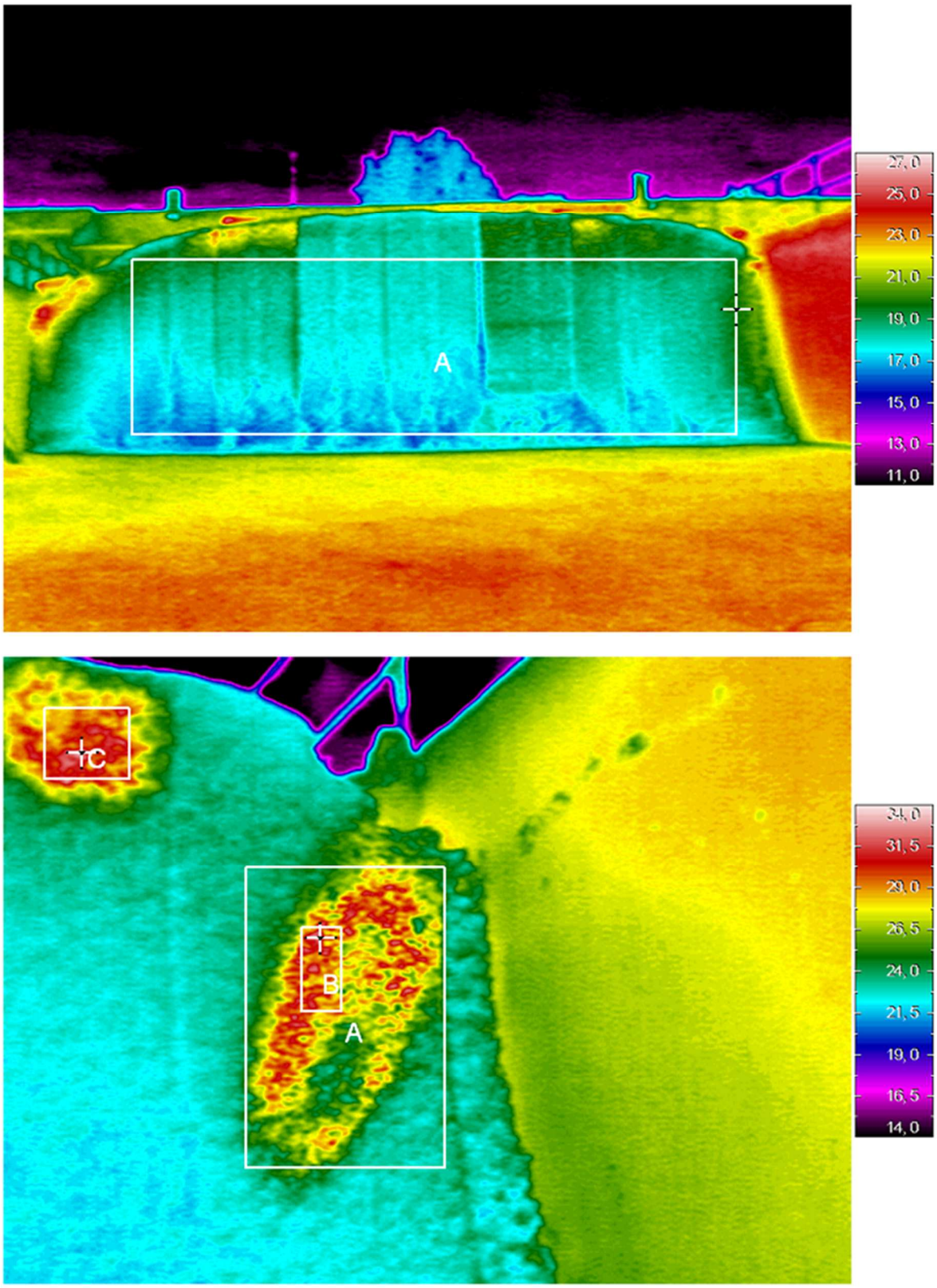

Figure 3 and 4: Example of thermal camera images analyzed with InfReC Analyzer NEC AVIO program.

A-B-C: are the specific zone selected to evaluate the temperature.

5.4 Statistical analysis

The data obtained with chemical analysis were analyzed with GLM procedure of SAS (SAS, v. 9.4, SAS Institute Inc., Cary, NC, 2012) to assess the silage quality during the trials and the differences of silage composition between the samples with normal and higher temperature at all sampling time. Pearson correlation coefficients (SAS v9.4, SAS Institute Inc., Cary, NC, 2012) was applied to evaluate the correlation between corn silage chemical composition, and between them and silage temperature. The obtained results by electronic nose were analyzed with a Principal Component Analysis (PCA) procedure by JMP 12 pro (SAS Institute Inc., Cary, NC, 2015). This technique transforms the entire set of *n*-correlated variables to *n*-uncorrelated linear functions of the original measurements. The first principal component is the linear combination of all variables showing the maximum variation among the samples. The second, third and further components are similarly linear combinations representing the next-largest variations, irrespective of those represented by the previous combination. In the literature, orthogonal transformations such as PCA have been utilized to mitigate possible negative effects of a feature-selection process, such as the correlation among variables of datasets (Jain and Zongker, 1997; Campagnoli et al., 2011).

5.5 Results and Discussion

The detected temperature with thermal camera and the chemical composition of corn silage at different sampling times are reported in the table 2. Obtained results on silage dry matter and starch content are in accordance with the literature, while only in isolated High Temperature Samples (HTS), lower values of crude protein, ash and ether extract than recommended were observed (Gallo et al., 2012). Higher values, with a not well-defined trend were observed for NDF and ADF content (Gallo et al., 2012), probably due to the late harvesting in the field. The level of ammonia nitrogen was over the recommended limits only in the Average Temperature Samples (ATS) at the second sampling time, but in the first one and three months after ensilaging, the values were optimal (Borreani et al., 2007). The chemical analysis showed that some HTS had high pH values and low lactic acid content during the trial, while all ATS samples had optimal values (Kung and Shaver, 2001), although only the difference between ATS and HTS at second sampling time was significant (table 2). The HTS collected at the 2nd sampling time (May), shows a very low content of lactic acid (1.85 %DM) and consequently a high pH value (5.15). Moreover in this sample the presence of butyric acid (0.9 %DM), propionic acid (1.15 %DM), molds (140,000 cfu/g) and yeasts (260,000 cfu/g) has been detected, differently from all the other performed samples. The collected samples were also analyzed to detect the presence of Clostridia and free water content but in both cases, no changes or high presence was observed. The FLIEG score assigned was excellent for almost all samples, with the exception of three samples where the score was below 80 for two of them and in one case it was not possible to assign a score.

Data analysis performed with GLM procedure showed not significant difference of DM content, NDF, ADF, starch, crude protein, fat and ash content in the silage between sampling times during the whole experimental study, which indicates a good quality for the entire period of use of the trench. Significant differences were observed between the average values of ATS and HTS for pH value (HTS 3.99 - ATS 3.41; $P \leq 0.01$), lactic acid (HTS 3.56 – ATS 4.16; $P \leq 0.05$), and temperature (HTS 27.8 – ATS 20.6; $P \leq 0.01$). The ATS had higher content of aflatoxins B1 compare to HTS ($P=0.08$), but the values during the trial were always under the limit of attention reported (Reg. EU No 574/2011). Although unfavorable fermentations were found by chemical assays in some HTS, no significant differences on silage composition were observed between ATS and HTS. The correlation analysis showed negative correlation of lactic acid with pH value (87%; $P \leq 0.01$) and acetic acid (55%; $P \leq 0.05$), and positive correlation with FLIEG score (77%; $P \leq 0.01$). As reported in literature the inoculation with *L.*

buchneri decreased the lactic acid content and increased the acetic acid content improving aerobic stability (Dunière et al., 2013, Li and Nishino, 2011). The FLIEG score was negative correlated with temperature detected with thermal camera (60%; $P \leq 0.05$), relative humidity (56%; $P \leq 0.05$), pH value (66%; $P \leq 0.01$) and acetic acid content (79%; $P \leq 0.01$). The temperature detected with thermal camera was negatively correlated with aflatoxin B1 (70.5%; $P \leq 0.01$), but as previously reported the aflatoxins B1, concentration was always under the limit of attention. Positive correlation were observed between the temperatures detected with thermal probe and thermal camera (83.1%; $P<0.001$), that confirm the data obtained with the thermal camera detection. The temperature detected with thermal probe was also negative correlated with CP (54.5%; $P<0.05$).

Obtained results with electronic nose analysis and processed with PCA procedure are reported in figures 5 and 6. Principal component analysis applied to electronic nose data showed that the variance of the ten sensors was explained by two factors. The two factors were able to explain 83.5% of total data variance. Factor 1 explained 65.6% and factor 2 explained 17.9%. Figure 6 reported the samples score obtained with PCA analysis. The sample distribution on the graphic is not uniform because, as already showed for the chemical analysis, not all the extra samples had unfavorable fermentation. The samples with the most unfavorable fermentation are isolated on the left side of the graphic (2e1 and 3e1). Furthermore, the GLM procedure showed significant differences between normal and extra samples in 7 of 10 sensors. Aromatic1, Aromatic 2 and Arom-aliph were higher in extra samples ($P<0.01$), instead Broadrange, Broad-methane, Sulphur-organic and Broad-alcohol were higher in normal samples ($P \leq 0.01$).

The results obtained in our trial are in according with the literature that reported the correlation between the aerobic deterioration and temperature measurement of the silo (Ruppel et al., 1995; Borreani and Tabacco, 2010). Although the use of thermal camera is still debated (Addah et al. 2012; Novinsky et al., 2012), our results show the ability of this technique to detect heating in silage at farm level as reported by Addah et al. (2012).

As reported by Roβ et al. (2012) the results obtained with electronic nose showed a relationship between changes in the sensor pattern and changes in the composition of the silage gas suggesting the possible use of this technique to detect the silage quality as reported in literature by Masoero et al. (2007) and Cheli at al. (2009).

Due to lack information's available further studies aimed to study the accuracy of both instruments are necessary.

Table 2: Corn silage composition at different sampling time

ITEM	sampling time 0	sampling time 1		sampling time 2		sampling time 3			sampling time 4				sampling time 5		
	fresh	ATS	HTS 1	ATS	HTS 1	ATS	HTS 1	HTS 2	ATS	HTS 1	HTS 2	HTS 3	ATS	HTS 1	HTS 2
DM%	36.79	33.27	33.67	34.77	37.13	36.54	27.91	34.43	37.92	32.15	29.64	33.99	37.9	33.11	34.98
NH_4 (mg/Kg DM)	215.2	945.7	920	1040.8	564.3	906.3	666.3	802.8	798.6	693.2	743.8	970.4	804.3	988.7	885.3
Lactic acid (%DM)	1.45	4.33	4.5	4.26^{A}	1.85^{B}	4.03	3.39	3.95	4.15	3.6	4.02	4.15	4.04	3.85	3.93
Acetic acid (%DM)	0.2	1.69	1.63	1.74	2.47	1.64	2.37	1.71	1.28	2.1	1.31	1.22	1.13	2.79	0.97
Butirric acid (%DM)	NA	NA	NA	NA	0.9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Propionic acid (%DM)	NA	NA	NA	NA	1.15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Total N (%)	0.98	1.19	1.06	0.76	0.96	1.19	0.94	0.92	1.02	1	0.64	1.08	1.08	1.03	1.08
NNH_3 (total N %)	1.81	6.56	7.16	11.25	4.86	6.29	5.83	7.2	6.43	5.73	9.5	7.4	6.14	7.9	6.75
pH	4.14	3.34	3.36	3.39^{A}	5.15^{B}	3.26	3.64	3.38	3.53	4.18	3.93	3.68	3.54	4.11	3.95
starch (%DM)	36.62	34.97	27.4	23.33	30.5	34.72	37.21	29.11	34.54	34.33	46.62	25.94	29.83	39.21	34.96
yeast (cfu/g)	<10	<10	<10	<10	260000	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
mold (cfu/g)	<10	<10	<10	<10	140000	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
Aflatoxins B1 (µg/Kg DM)	2	11	13	15	8.9	7	6.6	5.8	5.6	3.7	3.1	7.5	9.4	2	4.1
ash(%DM)	3.78	4.01	3.92	3.53	3.83	3.47	2.35	3.36	3.54	3.86	1.82	3.82	3.48	3.93	3.67
CP(%DM)	6.49	7.85	7.68	6.98	7.65	6.68	6.59	7.47	5.67	6.43	3.97	7.08	6.82	6.53	6.42
EE(%DM)	2.31	3.1	2.92	2.64	2.84	2.74	3.2	2.16	2.39	2.68	2.82	2.75	2.45	2.07	2.86
NDF(%DM)	36.1	40.6	46.8	46.1	42.0	37.9	42.5	47.2	40.9	37.3	44.1	39.3	37.3	36.7	35.4
ADF(%DM)	20.9	24.2	21.0	23.6	23.2	20.7	25.7	22.6	24.2	23.0	22.8	22.3	19.4	22.8	22.0
FLIEG score	88	86	88	88	NA	86	68	86	86	74	82	86	84	64	84
Thermal camera (°C)	NA	15	24	18	24	22.5	31.5	23.7	23.1	30	27.9	29.8	24.5	32.6	35.7
Thermal probe (°C)	NA	18	17.7	23	24.5	23	31.5	23.7	24.5	36.4	36.5	36.9	26.5	34	37

The points with different letter differ.

A,B $P \leq 0.01$
a,b $P \leq 0.05$

Table 3: Differences between ATS and HTS.

ITEM	LSMEANS	
	ATS	HTS
DM%	36.10	33.60
CP(%DM)	6.80	6.93
EE(%DM)	2.66	2.73
pH	3.41^{A}	3.99^{B}
Starch	31.48	32.75
NDF(%DM)	40.55	41.98
ADF(%DM)	22.41	22.70
ash(%DM)	3.61	3.51
Lactic acid (%DM)	4.20^{a}	3.60^{b}
Acetic acid (%DM)	1.50	1.91
NH_4 (mg/Kg DM)	899.14	791.66
Total N (%)	1.05	0.98
NNH_3 (total N %)	7.33	6.68
Aflatoxins B1 (µg/Kg DM)	9.60	7.20
Thermal camera (°C)	20.6^{A}	27.8^{B}

The points with different letter differ.
A,B $P \leq 0.01$
a,b $P \leq 0.05$

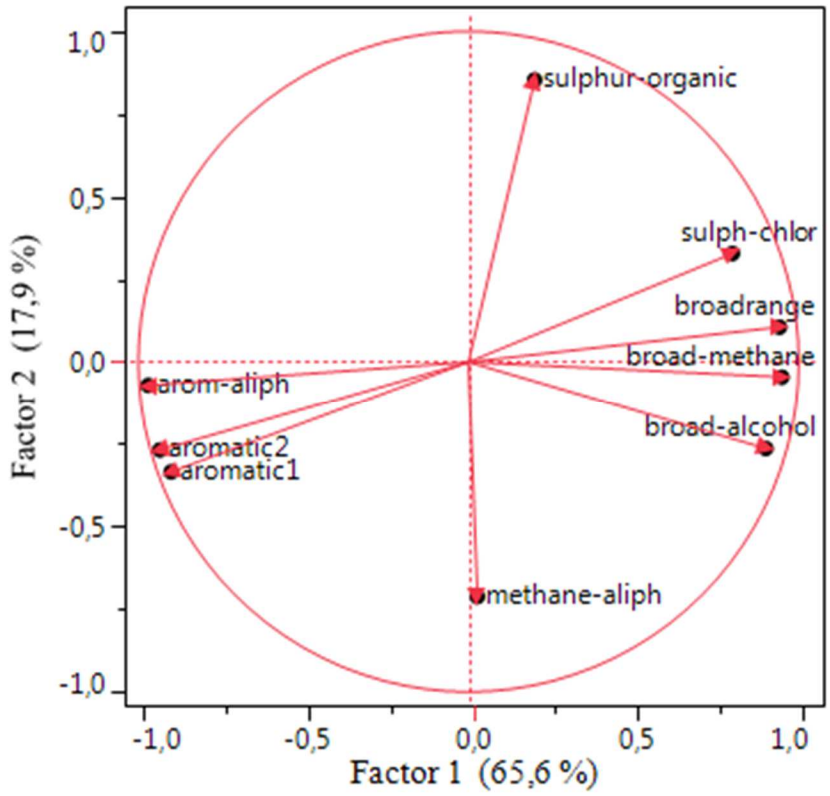

Figure 5: PCA factor plot produced by sensor responses.

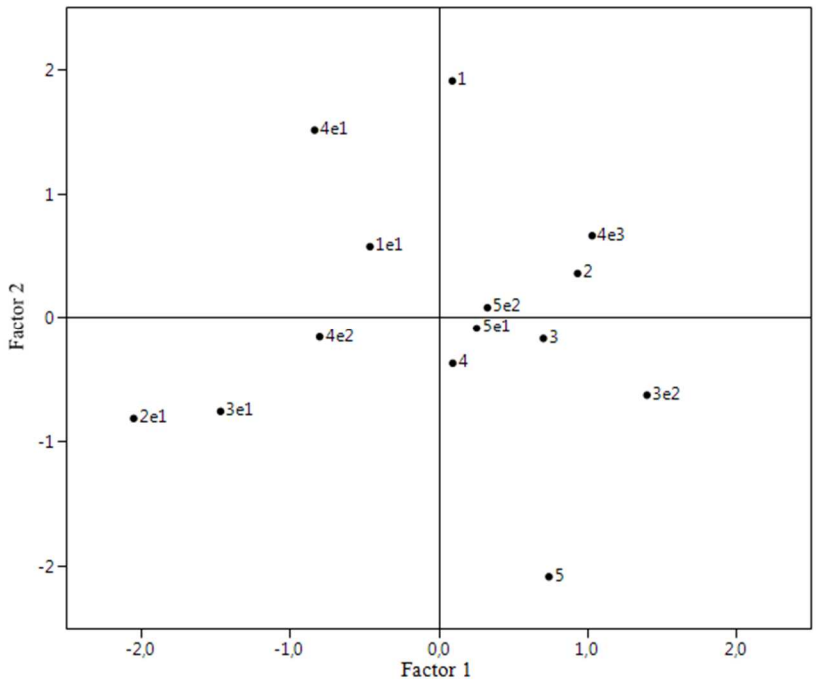

Figure 6: PCA score plot produced by sensor responses.

1,2,3,4,5: ATS samples obtained from five samples collected at predetermined points (two on each side and one in the center) representing the front of the silage.
1,2,3,4,5 with e1, e2, e3: HTS collected at the points where the thermal camera detected a temperature higher than the front of the silage.

5.6 Conclusions

The present study shows how detected differences in temperature for silage samples using a thermal camera are not even related to unfavorable fermentations in the silage mass or to poor silage quality. This leads to the necessity to further analyze the silage for quality. In this view, although the electronic nose is recognized not to have the same accuracy of the chemical official methods of analysis, it has shown to provide useful indications especially in case of strong unfavorable fermentations that can affect the silage quality.

The combined use of thermal camera and electronic nose allow to check the silage quality status in short time and with low costs with easily transportable instruments directly at the farm that can represent a first screening on the silage mass, eventually associated with subsequent classical chemical methods of analysis when huge variation or unfavorable fermentations are detected.

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Chapter 6

6 Physical characteristics of feed collected from Italy and Serbia

Research accepted and in press:

Čolović R., Ottoboni M., Caprarulo V., **Pilotto A.**, Banjac V., Vukmirovic D., Pinotti L. "Physical characteristics of feed collected from Italy and Serbia". Agro Food Industry Hi-Tech.

6.1 Abstract

The aim of this study was to investigate the physical quality of a selected lot of twenty animal feed samples collected in Italy and Serbia. Granulation of cattle and pig feed was finer in Italian than in Serbian samples. Flowability of samples from both countries in mash form were rated from fair to good (angle of repose >30°) while granulated samples (pellets and extrudates) had improved flowability (angle of repose < 20°). Extruded products had higher hardness (>10 kg) than pelleted products (<8 kg). Durability of most of the extruded and pelleted products was higher than 95%. Generally, it was observed that most of the physical characteristics of the samples responded to recommendations, which showed that the similar practices are in use in both countries.

Key words: feed, physical quality

6.2 Introduction

The industrial compound feed sector is a key segment in the agro–food sector in general and in the chain of food products from animal origin in particular. According to den Hartog (2003), an important aspect of feed quality (besides nutritional quality, safety for animals, environment and consumers, and emotional quality) is technical quality, i.e. physical properties of compound feed. Physical properties of single ingredients and the resulting pre-mix and/or compound feeds play a significant role in their resulting storage and flow behaviour, and are therefore essential to design appropriate, efficient, and economic bulk solids handling and storage equipment and structures (Ganesan et al. 2008), which are able to guarantee a good final quality, and which will respond to animals' preferences.

Important physical characteristic of feedstuffs within compound feed is particles size distribution. Goodband et al. (2002) reported that particles size reduction increases the surface area per unit volume allowing greater access to digestive. Moreover particles size reduction may affect handling and mixing of the ingredients (Koch, 2002). However, optimal particle size largely differs in respect of the target species.

Pelleting is a manufacturing process that is commonly used to densify, improve handling characteristics, nutritive and economical value of granular materials (Theerarattananoon et al., 2011). Additionally, pelleting improves microbial stability of the product (Čabarkapa et al., 2010). Pellet quality mostly depends on raw materials that comprise compound mixture, but also on parameters of pellet press and up- and downstream equipment (Čolović et al., 2011).

Extrusion process is mostly used for pet food and fish feed production. In the extruder barrel the material is exposed to thermal and mechanical treatment, plasticizing and shaping the material. Unlike pelleting process, extrusion process can be used for setting required density of the product, with high digestibility and improved physical characteristics (Rokey, 2007).

Considering that the manufacture of compound animal feeds involves nowadays a wide variety of ingredients (Pinotti et al., 2014), which should respond to animals' nutritional demands, but also have significant effect on physical quality of feed (Thomas et al., 1998), and that wide variety of process technologies can be utilized in compound feed production, it is clear that physical quality analysis of single ingredients as well of compound feed assume great importance in ensuring high quality product.

Starting from these assumptions, the aim of this study was to investigate the physical quality/properties of a selected lot of samples of animal feed collected in the frame of an Italian and Serbian bilateral project.

6.3 Materials and methods

Specific survey was conducted during the year 2014 for the purposes of the "Feedneeds" bilateral project. One of the subtasks of the survey was to obtain information about most representative product from Italian and Serbian animal feed companies. For this purpose, 10 samples from Italy and 10 samples from Serbia were collected. The samples belonged to the following categories: cattle feed (dairy cows), poultry feed (broilers and laying hens), pig feed, pet food (dog food), and fish feed (carp feed). Before the physical analysis determination, samples were categorized according to physical form of the samples (related to the production process) into the three groups: mash, pellet, and extrudate.

For testing of flowability of feed samples, angle of repose method was used (Carr, 1965). The angle of repose, α° was calculated using the following equation:

$$\alpha^0 = \left(\frac{180}{\pi}\right) * arc\ tan\frac{h}{r}$$

Where α° is the angle of repose (°), h is the height of the cone formed by material (cm), and r is the radius of the base of the cone (cm). According to the angle of repose, samples were classified into several categories related to their flowability (Table 1).

Table 1. Flowability ratings according to the angle of repose

Flow rating	**Angle of repose (degree)**
Excellent	25-30 (or less)
Good	31-35
Fair	36-40
Passable	41-45
Poor	46-55
Very poor	56-65
Extremely poor	66-90

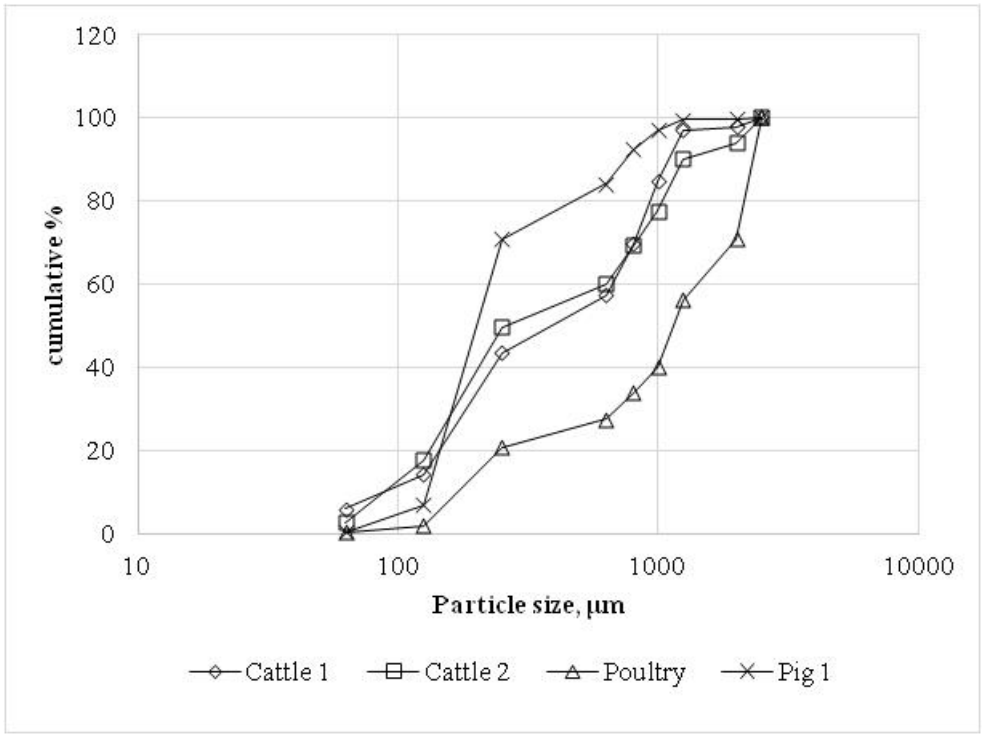
The mash samples were subjected to the sieving analysis, which was performed according to ISO 2591-1 standard, using laboratory sieves ranging from 63 to 2500 µm (Endecotts Ltd., United Kingdom). Geometric mean diameter (dgw) was determined for evaluation of particle size of different samples (ASAE standards 319.3. 2006). Pellet hardness was determined with Kahl Pellet Hardness Tester (Amandus Kahl GmbH & Co. KG, Germany) and fifteen replications were performed per each sample.

The Holmen Pellet Tester (NHP100, TekPro Ltd., Norfolk, UK) has been used for determining durability of both, pelletized and extruded feed. Granulated sample was pre-sieved, before put into the test chamber of the tester, to remove fines and impurities. A sample was circulated in the air stream around a perforated test chamber for 30 s. The remaining pellets were collected, weighed, and the pellet durability index (PDI) was calculated as the ratio of the weight after testing over the weight before the testing, and given as percent.

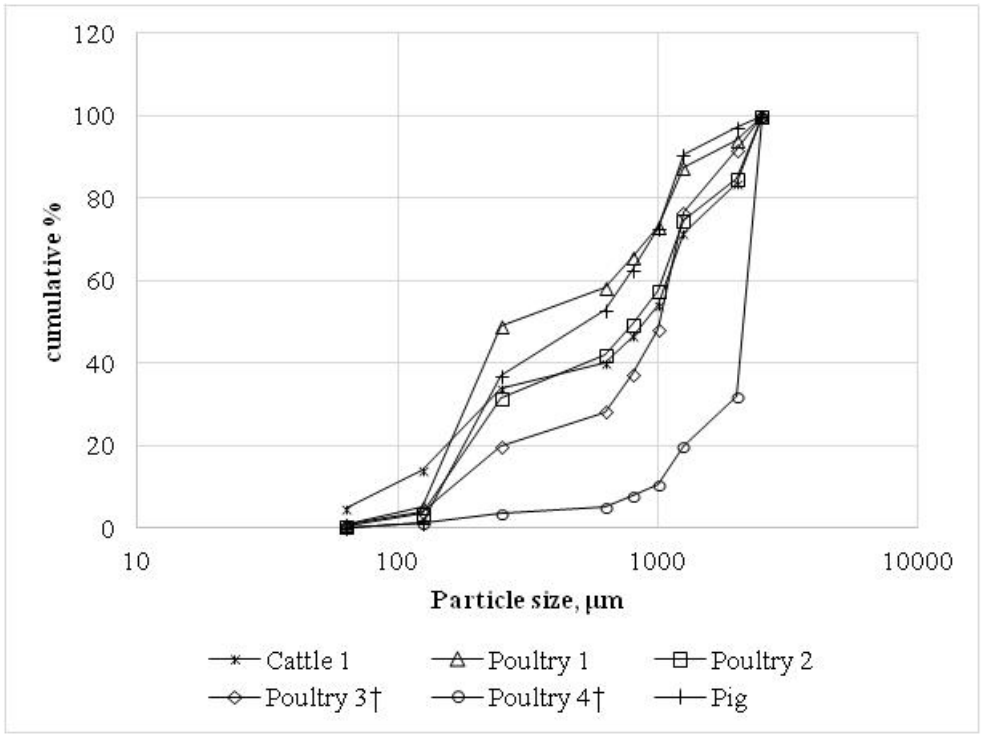
6.4 Results and Discussion

Italian and Serbian animal feed factories provided their most representative product for the purposes of this survey. Selection of representative sample of each factory was done according to the production aims and current market position.

Sieving analysis of Italian samples showed coarser structure of poultry feed comparing cattle and pig feed (Figure 1), which is in line with animals' requirements. Two different Italian cattle feed samples had almost the same particle size distribution, as well as the geometric mean diameter (Figure 2). On the other hand, granulation of cattle and pig feed was finer in Italian than in Serbian samples. When comparing Serbian poultry feed samples, samples Poultry 3 and Poultry 4 were in form of crumbled pellets, and consequently those samples had the coarsest structure and the highest geometric mean diameter, especially sample Poultry 4. This sample had almost 70% of particles larger than 2 mm. This feed was produced specially for laying hens, concerning that for layers medium and coarse particle size is preferable (Amerah et al., 2007; Safaa et al., 2009).



(a)



(b)

Figure 1. Cumulative particle size distribution curves of Italian (a) and Serbian (b) mash samples.

†Crumbled Pellets

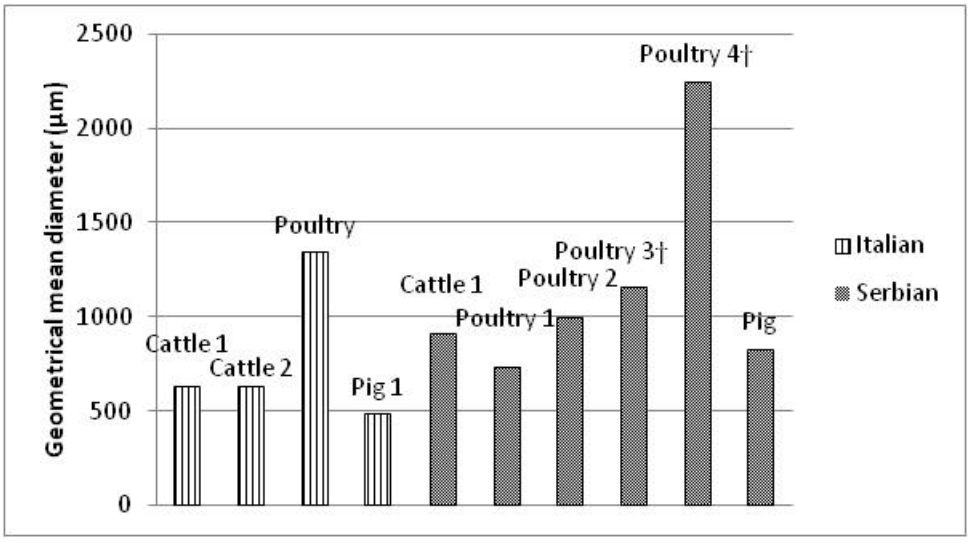
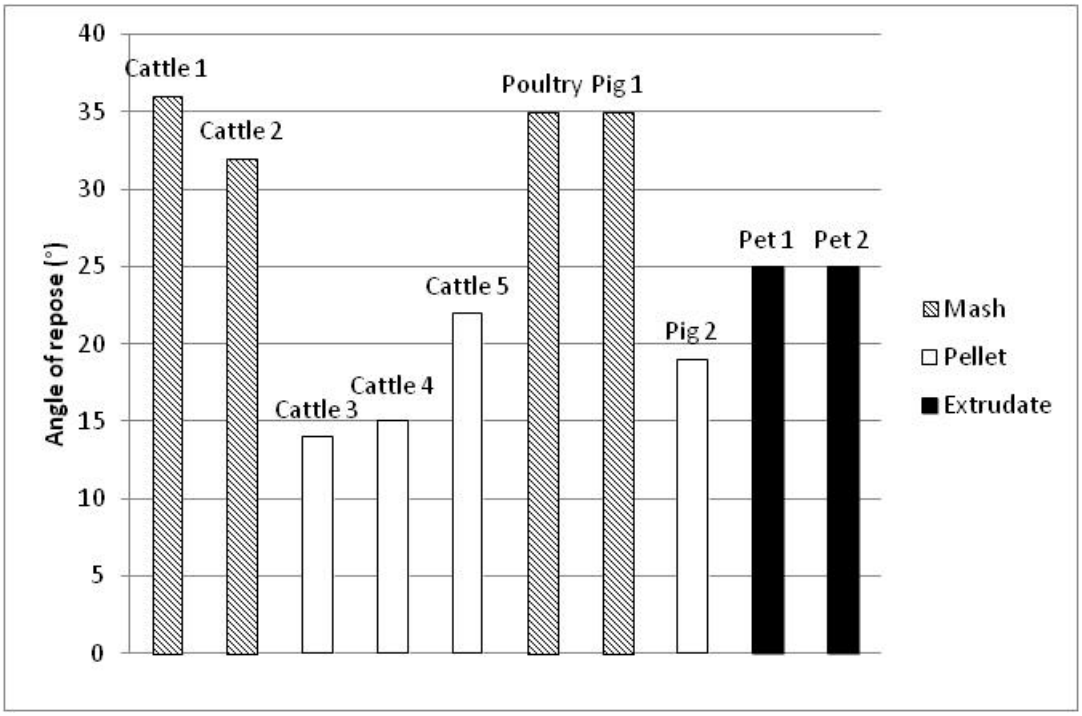


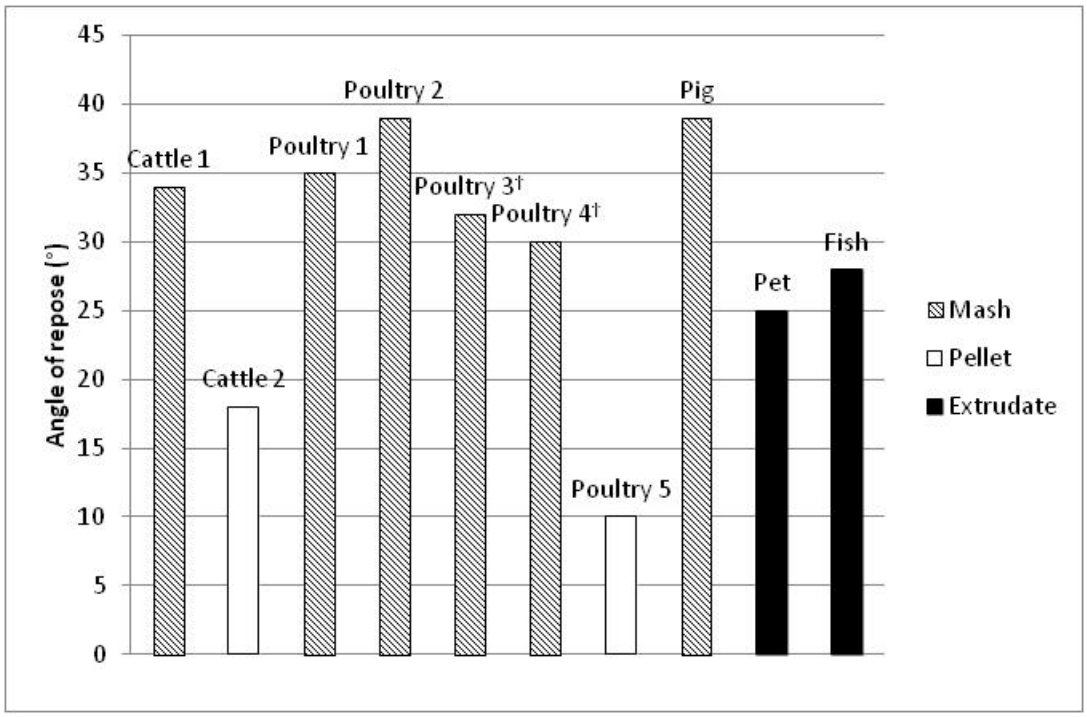
Figure 2. Particle geometrical mean diameters of mash samples.

†Crumbled Pellets

Animal feed is a mixture of up to 40 components (Kirchner, 2013) that differ in terms of their flow properties, which determine the ease of transport in conveying equipment and the flow out process (e.g. from silo cells) (Fürll and Hoffmann, 2013). The flow properties of feed depends of its particle size distribution, particle form, as well as crop-specific properties such as fiber components and the dry matter content (Fürll, 1985). Flowability of all collected samples from both countries in mash form were rated from fair to good (Figure 3), according to obtained values of angle of repose and flowability ratings taken from Table 1. Among Italian samples, sample Cattle 1 had the poorest flowability, rated as fair. The ruminant feeds generally have more fiber, which may have negative influence on flow properties of feed (Fürll, 1985). Compared to the Serbian samples in mash form, Italian samples have slightly lower value of angle of repose within the same category of feed, indicating better flowability. Comparing the Serbian poultry feeds, samples in form of crumbed pellets had lowest values of angle of repose and thus better flowability since these samples had higher percent of coarser particles and higher geometrical mean diameter of particles all which influences better flow properties (Fürll, 1985). Pellets normally have better flow properties than mash (Thomas and van der Poel, 1996), thus all collected samples of pellets from both Italy and Serbia had excellent flowability, as well as extruded samples of pet food and fish feed.



(a)



(b)

Figure 3. Angle of repose of Italian (a) and Serbian (b) samples

†Crumbled Pellets

Pellet hardness is important from the nutritional point of view since it may play a role with preference of animals. The pig feed pellets had lowest value of hardness (Figure 4). This result can be satisfying since, according to a study of Skotch et al. (1983), pigs prefer softer pellets over the harder ones. Samples of cattle feed had higher values of hardness, ranging from 2.83 kg up to 7.83 kg, than samples of pig and poultry feed. The highest values of hardness were observed for samples of extruded pet food. During extrusion process, basic components of raw materials, such as proteins and starch, undergo physical and chemical changes, creating the shaped product which is harder than products obtained within the pelleting process (Rokey et al., 2005).

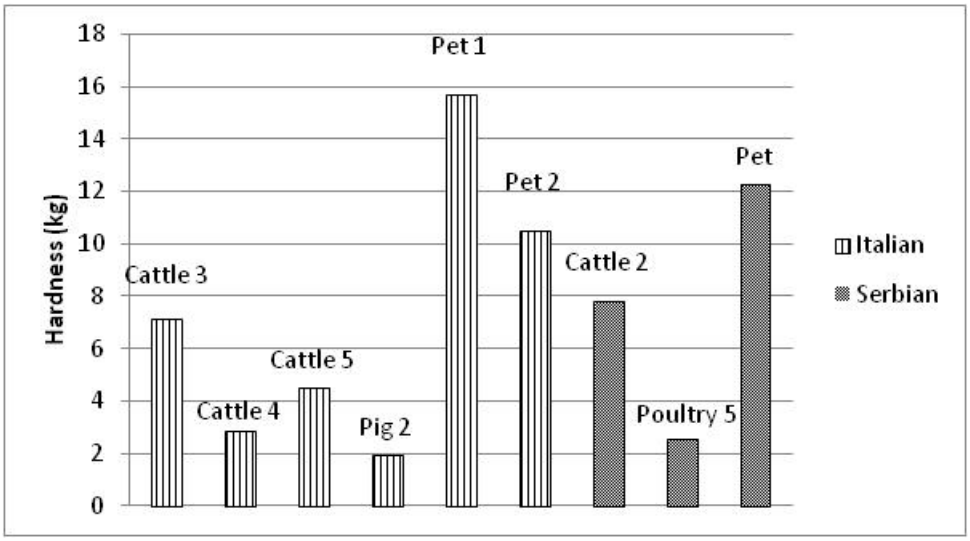


Figure 4. Hardness of pelleted and extruded samples

Results of durability test showed that almost all collected pellets had good durability (Figure 5) and therefore good resistance against the stresses exerted on them during transportation and distribution to the animals. Among Italian sample, the lowest PDI had sample of cattle feed (Cattle 4), slightly below 90% which is limit of tolerance for good pellet durability. This sample also had softest pellets, so it may be that the production parameters or composition of starting mixture were not adequate and resulted in the lowest pellet quality of all collected samples. The sample of poultry feed from Serbia had the lowest durability of all samples, since almost 22% of fines were produced from starting amount of pellets after durability test. This result was unacceptable in terms of pellet quality, indicating that improper setup of pelleting parameters was done in the production process (Thomas and van der Poel, 1996).

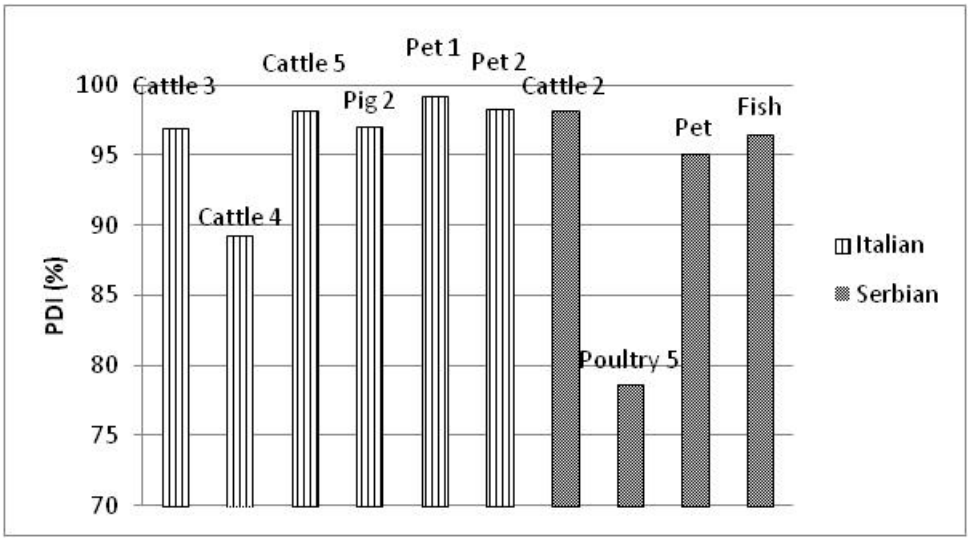


Figure 5. Pellet durability index of pelleted and extruded samples

6.5 Conclusions

Based on the physical analysis of collected animal feed samples, it was observed that most of the physical characteristics of the samples responded to recommendations for specific categories of the animals, no matter if the samples were collected in Italy or Serbia, which showed that the similar practices are in use in both countries. Pelleted and extruded samples had better flowability than mash samples which makes transport and handling of granulated products much easier. Hardness of granulated samples was the lowest for pig feed which is in line with animals' preferences. Most of granulated samples had good durability which is very important from the nutritional and economical aspect.

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Chapter 7

7 Evaluating homogeneity of TMR in dependence on feed mixing times and different loads of a TMR wagon.

Presented at FEED 2016 congress (Geel, Belgium):

Agazzi Alessandro, Tangorra Francesco, **Pilotto Adriano**, Pietro Cesare Martino Tosoni, Luciano Pinotti, Costa Annamaria. “Mixing times and wagon load affects total mixed ration homogeneity in dairy cows”.

7.1 Abstract

The aim of the trial was to determine the influence of cutting and mixing time on homogeneity and particle size distribution of a dairy cow total mixed ration (TMR), considering different loading levels of the mixing wagon.

The TMR particle size distribution, intended as homogeneity of the diet, and the chemical correspondence to the designed diet where investigated applying a Central Composite Design (CCD) in a randomized block design generated by JMP 11 Pro (SAS Institute, Cary, NC, 2015) software considering: three mixing wagon loads (40%, 70% and 100% of the maximum nominal load, 21m^3), b) three cutting times (4, 5 and 6 min) and c) three mixing times (4, 5 and 6 min). The mixing wagon was a two-screws vertical type, and the diet was based on corn silage (57%) and hay (14.7%). Samples of TMR were collected at the beginning, in the middle and at the end of the feeding alley for dry matter (DM) content, ether extract (EE), crude protein (CP), neutral detergent fiber (NDF), and ash content determination. At the same time TMR particle size distribution was determined in triplicate for each sampling point by Penn State Particle Separator. The homogeneity of the diet was evaluated in terms of variation coefficient (CV %) of DM, CP, NDF to evaluate the multiple effect of loading level, cutting and mixing time.

The CCD allowed the 3D response surfaces for CV of DM, CP, and NDF on loading levels and mixing times, since cutting time was not a significant parameter.

Data were also analysed by a Principal Component Analysis PCA, (SAS Institute, Cary, NC, 2015), keeping into account TMR chemical composition of samples collected along the alley and the chemical composition of the designed diet. The higher level of similarity of the real and the designed diet was reached with a 70% nominal loaded mixing wagon, together with 5 minutes of cutting and 5 minutes of mixing.

Key words: TMR, mixing time, filling time, cutting time

7.2 Introduction

Despite the significant time and effort spent formulating total mixed rations (TMR), the ration delivered by the producer and consumed by the cow may not accurately reflect that originally formulated. A correct balanced between the components of the rations is an essential requisite for dairy cows diets to allow suitable rumen conditions. There are several chemical parameters to control the diet composition (i.e., dry matter, crude protein, starch, NDF, ether extract) but these analytical parameters are not ever enough for a complete dietetic evaluation, as the rumination stimuli and the intensity of rumen fermentation are also related to the dietary particle dimension and to the physical effectiveness of the diet. Having the proper particle size distribution of feeds is an important part of ration formulation because the ration uniformity play an important role on dairy cows nutrition. One of the challenge to the dairy cattle nutrition is how to provide feeding of energy-dense diets, which are necessary for supporting milk production, with adequate amounts of dietary physically effective fiber, which is needed to prevent rumen disorders (Zebeli et al., 2011). Finding an optimal balance between physically effective fiber and readily degradable carbohydrates in the diet is difficult but crucial not only for maintaining proper rumen metabolism (Zebeli et al., 2006; Plaizier et al., 2008), but also for maintaining a stable metabolic health status and enhancing the productivity of dairy cattle (Ametaj et al., 2010; Zebeli et al., 2011). Failure of these balancing efforts contributes to the occurrence of metabolic disorder in dairy cows.

The TMR is the most popular feeding method for dairy cows nutrition. The TMR was introduced as a means of providing a consistent supply of nutrients to rumen microbes to optimize rumen function and improve the efficiency of nutrient utilization (Coppock et al., 1981). The TMR is composed of forages, byproducts, grains, protein, minerals and vitamins that have been mixed together to make a balanced ration. In practice, there are basically three types of TMR. The first type is feed ration theoretical calculated by optimization program and quantified sometimes up to two decimal places. The second type is a TMR that leaves the mixer wagon and is discharged into feeding alley in barn to animals (depending on the accuracy of loading and homogeneity – mixing of individual components). The third TMR type is the dose that cows eat. The TMR should provide the desired nutritional requirements for the animal to be fed. The TMR should be uniformly blended so that when an animal takes a mouthful of feed, it receives a homogeneous sample of the combined ingredients and to prevent sorting of feeds by the animal and mask less palatable feeds. Sword and Buckmaster

(2002) showed that harvest or processing method is important because shredded silage, even though it contained long particles, was sorted less than chopped silage. Even if cows consume a TMR with high consistency, which prevents or limits the sorting, the ration delivered may not reflect the ration formulated by the nutritionist, neither be consistent in composition from day to day (Sova et al., 2014). The TMR is obtained with mixer wagons that provide feed, which any given amount contains components added to feed mixed at consistently identical proportions. These machines, depending on the used equipment, may perform many technological functions: loading and weighing of components, cutting and mixing of components, blending and distribution of the prepared TMR. The mixed ability of the feeding equipment to mix a ration can be limited by several factors such as the feed ingredients and feed size, fill order, mixing time, mixing protocol, moisture levels of feedstuffs and scale maintenance and calibration (Buckmaster et al., 2009, Mikus, 2012). Mixing requires motion of the particles. This is done by mechanically moving particles with screws, reels, chains, and drums. The mechanical forces applied to mix the ration can also cause a certain amount of particle size reduction (Heinrichs et al., 2011). This particle size reduction may or may not be a beneficial or desired function of the mixing operation. A uniform TMR has many potential advantages, such as improving the animals' health, decreasing the feed cost, and improving the milk production of cows (Buckmaster et al., 2014). James and Cox (2008) showed strong evidence that feeding programs are not well managed and that greater variation in feed delivered leads to reduced milk production. When considering mix uniformity, there are two distinct types of variation: variation among similar batches and variation within a single batch (Buckmaster et al., 2009).

The management of TMR delivery system is very important and requires careful controls to provide the right amount and balance of TMR to dairy cows. As reported by Heinrichs and Kononoff (2002), the mixing and distribution equipment can reduce particle size of feeds and forages and it must be considered for the correct nutrition of dairy cows.

For these reasons, the aim of the trial was to determine the influence of loading level of the mixing wagon, cutting and mixing time on homogeneity and particle size distribution of a dairy cow total mixed ration (TMR) using chemical and physical analysis of delivered TMR to evaluate the feed and forages distribution.

7.3 Materials and methods

The study was carried out in a dairy farm using a total mixer ration (TMR) wagon Sgariboldi Grizzly 8100 model 8122/2. The wagon capacity was 21 m^3 and was equipped with two-screws vertical type with knives. The milling head dimensions were 1900 x 600 mm (width x diameter) with silage clamp height up to 5 m. The loading system was controlled by an electronic weighing system, in which the different formulas for different groups of cattle were recorded. The weighing system was able to record the actual loads of each food introduced into the formula, and the amount of TMR downloaded to the various production groups. The TMR prepared was discharged along the feeding alley and was controlled by electro magnet for detecting foreign metal. The TMR as fed was 57.3% corn silage, 8.1% corn meal, 3.9% wheat flour middlings, 5.3% meadow hay, 5.3% alfalfa hay, 4.5% ryegrass hay, 3.7% ground corn silage, 1.8% molasses, 1.5% mineral and vitamins and was kept constant throughout the experimental trial. Total mixed ration theoretical composition was 54.3 % dry matter, 12.8% crude protein, 2.6% ether extract, 35.1% neutral detergent fiber and 6.8% ash. The dry matter of the ration was provided for 57.3% from forages and 42.3% from concentrates. The experimental design was based on the adoption of a Central Composite Design (CCD) model, that consisted in the evaluation of the homogeneity and chemical composition of the diet, using different combinations of three main producing factors on the mixing wagon: 1. The filling level of the mixing wagon; 2. The cutting time of roughage; 3. The mixing time of TMR. For each factor, three different levels were tested: the mixing wagon loads (40%, 70% and 100%), cutting time (4, 5, 6 minutes), mixing time of TMR (4, 5, 6 minutes). The wagon load level has been intended as the percentage compared to the maximum nominal load of the machine used (21 m^3). Cutting time was calculated as the time between the loading of long-fiber roughage and stopping the chopping devices, before loading of concentrated products. The mixing time was defined as the period between the addition of the last ingredient of the ration (corn silage) and the stop of mixing system before discharge into the feeding alley. The CCD has allowed to compare and analyse the effectiveness of each possible combinations of the three factors considered in a randomized sequence generated by JMP statistical software 12 Pro (SAS, Cary, NC), as reported in the table 1.

The trial was performed for 16 consecutive days. Every day one different combination of factors was tested as reported in table 1 and three samples of TMR at beginning, middle and at the end of the feeding alley were collected. At each sampling point, 800 g of TMR were collected, rapidly vacuum and frozen at -20°C, to evaluate the ration homogeneity with

chemical analysis. The particle size analysis of TMR was performed in triplicate (700g x 3) for each sampling points and carried out immediately at farm, according with method described by Heinrichs e Kononoff (2002). The chemical analysis were performed to assess the content of dry matter (DM), crude protein (CP) and ether extract (EE), in accordance with the Association of Official Agricultural Chemists (AOAC 2005), and neutral detergent fiber (NDF) according to procedures described by Van Soest et al. 1991. To evaluate the TMR homogeneity the averages of all collected samples for each combination was calculated. Furthermore the results obtained from chemical analysis were computed to evaluate the differences between the rations performed at different filling level, mixing time and cutting time and the theoretical ration formulated using. For the data analysis was used the coefficient of variation (CV) of the averages for DM, CP, EE and NDF to evaluate the multiple effect of loading level, cutting and mixing time. The CV<5% was considered acceptable (Buckmaster et al., 2014; James and Cox, 2008).

Table 1: Trial design: combination of factors tested during the experimental trial, randomly generated by JMP PRO (The SAS, Inst.).

Day	Factor 1 (filling) %	Factor 2 (cutting time) min	Factor 3 (mixing time) min	Factor 1 coding	Factor 2 coding	Factor 3 coding
1	70	5	4	0	0	-1
2	40	6	6	-1	1	1
3	100	4	6	1	-1	1
4	100	4	4	1	-1	-1
5	70	5	5	0	0	0
6	40	4	6	-1	-1	1
7	70	6	5	0	1	0
8	40	5	5	-1	0	0
9	100	6	6	1	1	1
10	100	5	5	1	0	0
11	40	4	4	-1	-1	-1
12	40	6	4	-1	1	-1
13	70	4	5	0	-1	0
14	70	5	6	0	0	1
15	100	6	4	1	1	0
16	70	5	5	0	0	0

7.4 Statistical analysis

Homogeneity of the diet, was investigated applying a Central Composite Design (CCD) in a randomized block design generated by JMP 12 Pro (SAS Institute, Cary, NC, 2015) software considering: three mixing wagon loads (30%, 40 and 70%), three cutting times (4, 5 and 6 min) and three mixing times (4, 5 and 6 min). The central point was formed by the average of the samples carried at day 5 and 16 (table 1). Principal Component Analysis (PCA) was performed to evaluate the differences between the theoretical formulated ration and that administered to cows. GLM procedure of SAS statistical software version 9.4 was applied to evaluate the CV of different TMR obtained during the experimental study from the theoretical ration formulated.

The CV<5 was considered acceptable.

7.5 Results and Discussion

The table 4 shows diet particle size distribution in the different factorial combination resulting from Penn State Particle Separator analysis.

The values reported for the 3 sieves (upper, middle and lower) and for the bottom collector (bottom) are shown as a percentage relative to the weight of the samples collected.

These data are not in accordance with the Penn State University guidelines (Heinrichs, 2013), that suggest different distribution percentages: upper sieve 2-8%, middle sieve 30-50%, lower sieve 10-20%, bottom collector 30-40%. These discrepancies may be due to the differences existing between American forages and Italian forages: the overall difference seems to be relative at the cutting length of the forages that is lower in USA and this can be reflect on over almost the sieves. Anyway, the obtained results agree with previous finding by Agazzi et al., (2014) where the Penn State Particle Separator was used on typical Italian dairy cow diets.

Beside differences from American guidelines, in the present study no significant differences in the particle size distribution of the rations were showed accounting for the cutting and mixing times (table 2). Although not statistically significant, the wagon filling level affected the particle size distribution of the diet (table 3). The best results were obtained at 70% of the filling level compared with 40% and 100%.

The first data analysis were performed to understand which parameters between filling level, mixing time and cutting time had greatest influence on ration homogeneity. The figures 1, 2 and 3 show the ration homogeneity, using as a benchmark the coefficient of variation of the dry matter (DM %), crude protein (CP %) and neutral detergent fiber (NDF %) at different mixing time and the filling level of the wagon. No significant differences were observed at different cutting time. The filling level of mixing wagon goes from 0% to 100% and the level of mixing time goes from 4 to 6 minutes.

The range of DM (figure 1) was reported as coefficient of variation (CV) from 1 (best) to 7 (worst). Considering only the filling level the best results was obtained with a filling of the wagon that goes from 50 to 100% with the best overall value around 70%. At 40% the TMR was not enough to cover completely the first part of the augers that did not allow to perform the entire mixing cycle. At 100% the two augers were completely covered and a small part of TMR floated without undergoing mixing.

The mixing time had no significant influence on the homogeneity of the ration, probably due to the short time period used during the test (4-6 minutes), although 6 minutes of mixing showed the best results. Considering the contemporary effect of the two parameters the best

value was obtained with a filling at 70% and a mixing time at 6 minutes. The range of CP (figure 2) was reported as coefficient of variation (CV) from 7 (best) to 11.5 (worst). For CP was considered the lowest CV obtained because the results were always over the acceptable limit (CV<5). As observed in figure 1, the best values were obtained with a filling of the wagon between 60% and 70% and a mixing time between 5.5 and 6 minutes. The mixing time had effect with filling from 40% to 80% whereas no significant changes were observed with higher loads. The ration component with higher content of CP was the pelleted feed that has the higher specific gravity of the ration and therefore more subject to precipitation. This characteristic could explain the results. The 100% filling showed the worst homogeneity due to the higher weight of feed loaded that was able to greater precipitation without effect of mixing time. At 40% of filling, the mixing time reduced the variability, probably because the less feed quantity loaded in the wagon were mixed better reducing the precipitation.

The range of NDF (figure 3) was reported as coefficient of variation (CV) from 3 (best) to 11 (worst), how reported in previous figures also the ration homogeneity by neutral detergent fiber was best with the wagon loaded to 70%. The mixing time showed a different effect on the three types of fill. With the tank filled to 100% the homogeneity was improved by increasing the mixing time, because the higher volume need more time to be mixed correctly. At 70%, the best results was obtained with 5 minutes of mixing, while at 40% the homogeneity decreases with the increase of mixing time probably due to the demixing effect.

The figure 4 reported the score plot obtained by principal component analysis performed to evaluate the differences between the theoretical formulated ration and that obtained with the wagon at different filling level, cutting time and mixing time. The data showed that the first two factors were able to explain 71.2% of total data variance. The best results were obtained by filling level at 70% without effect of mixing and cutting time. In fact, the rations prepared with 70% filling level are close to the theoretical ration (number 17).

Subsequent analysis were performed to evaluate the effect of filling level, mixing time and cutting time on TMR obtained compared to theoretical ration (table 4). The data are reported as point of percentage difference from theoretical ration. The mixing time had significant effect on dry matter ($P \leq 0.05$). Significant difference were observed between 40% and 100% filling level. The filling level is the main statistical significant effect on CP ($P \leq 0.05$), EE ($P \leq 0.05$) and NDF ($P \leq 0.05$) content variantion, with a tendency for ash ($P=0.08$). Crude protein and ash content showed significant differences between 40% and 100% filling level ($P \leq 0.05$), instead EE and NDF showed statistical differences between 40% with 70% ($P \leq 0.01$) and with 100% ($P \leq 0.05$). In the figures 5, 6, 7, 8 and 9 were reported the differences

of each parameters considered (DM, CP, EE, NDF and ash) from theoretical ration to each combination of filling level mixing time and cutting time considered.

In the table 5 were reported the sum of the CV of DM, CP, EE, NDF and ash obtained for each combination of filling level, mixing time and sampling time. Filling level at 70% obtained the overall best results with three combination at first three position (CV<5), all of them had five minutes of mixing time, and one no much over (CV=5.12). Only one 100% filling level obtained a good CV (4.08), with four minutes of cutting time and six minutes of mixing time. The worst results were obtained from 40% filling level, with best CV much higher than the acceptable limit (6.15).

Table 2: Penn State Particle Separator data.

sampling	filling	Cutting time	Mixing time	Average upper (%)	Average middle (%)	Average lower (%)	Average bottom (%)
1	70	5	4	18,6	33	29	19,4
2	40	6	6	21,3	31,5	27,4	19,8
3	100	4	6	20,3	32,2	29,9	17,5
4	100	4	4	21,8	32,4	29,5	16,3
$\bar{5}$	70	5	5	19,3	34,3	29,2	17,2
6	40	4	6	17,9	33,2	30,7	18,3
7	70	6	5	16,4	31,6	30	22
8	40	5	5	22,1	32,4	31,3	14,2
9	100	6	6	24,9	26,8	32	16,3
10	100	5	5	22,2	29,6	32	16,1
11	40	4	4	23,7	32,4	30,4	13,4
12	40	6	4	16,7	31,8	32	19,5
13	70	4	5	16,2	30,4	32,2	21,2
14	70	5	6	16,7	35,3	31,3	16,7
15	100	6	4	20,1	31,3	32,1	16,4

Table 3: Penn State Particle Separator data at different filling level.

filling	average upper (%)	average middle (%)	average lower (%)	average bottom (%)
40	20,34	32,26	30,36	17,04
70	17,44	32,92	30,34	19,3
100	21,25	31,35	30,9	16,45

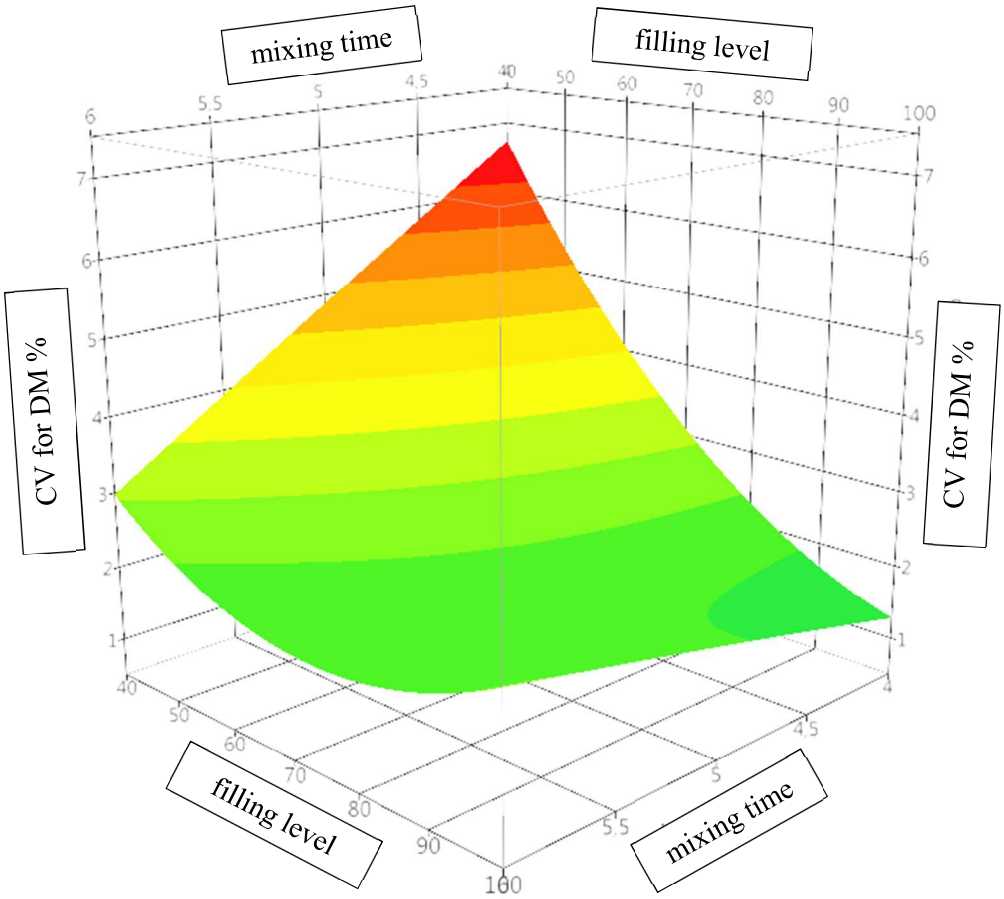

Figure 1: Ration homogeneity by dry matter percentage (DM%) at different mixing time and filling level.

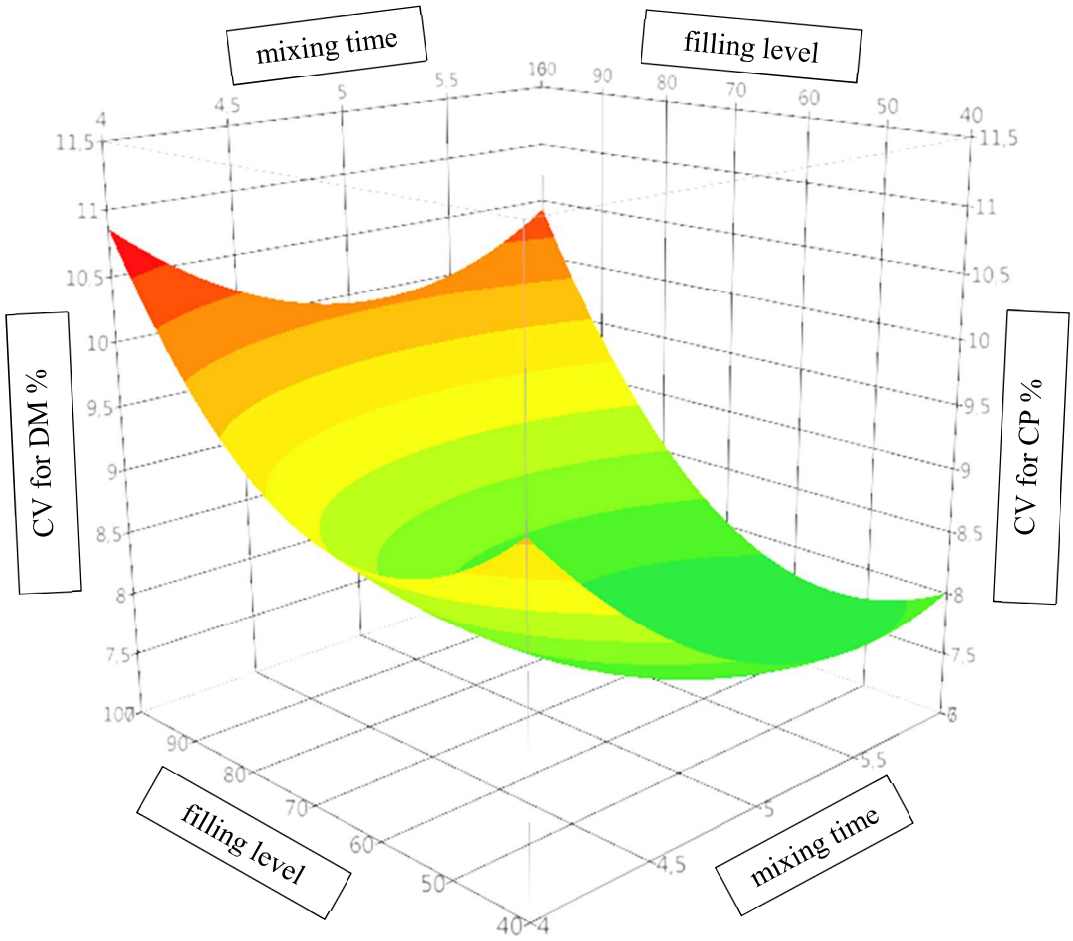

Figure 2: Ration homogeneity by crude protein percentage (CP %) at different mixing time and filling level.

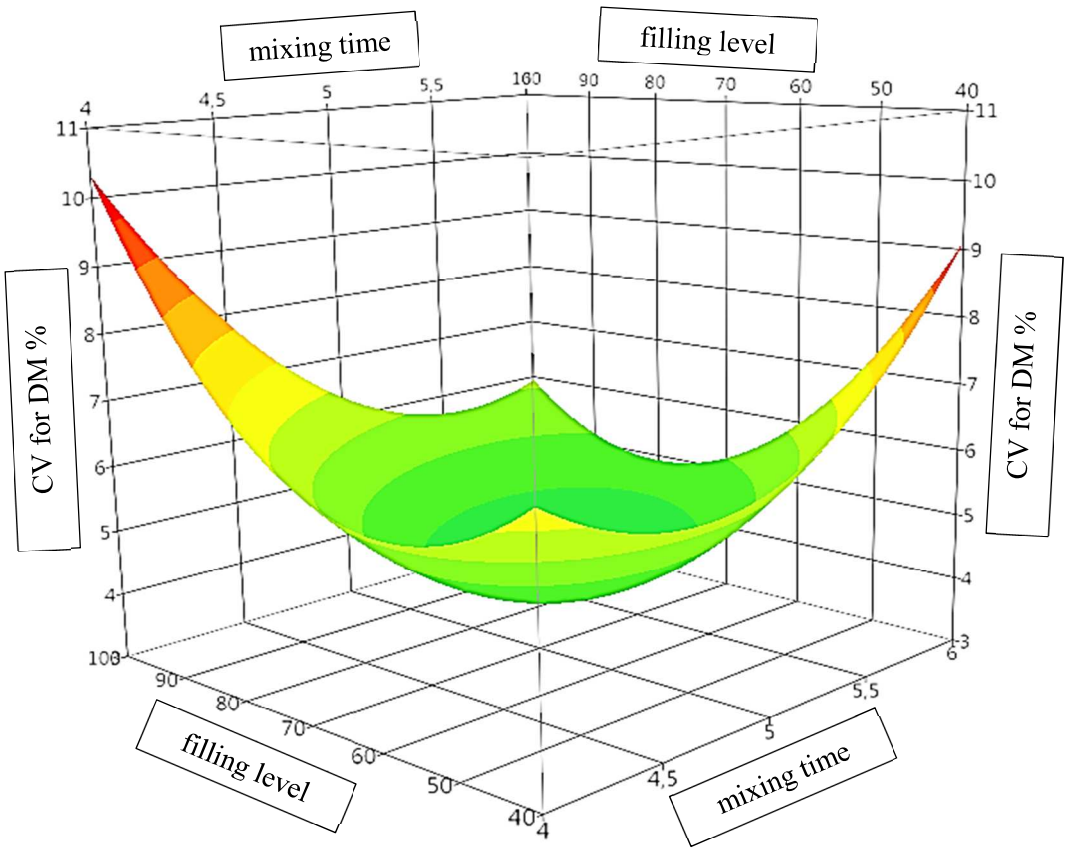

Figure 3: Ration homogeneity by neutral detergent fiber percentage (NDF %) at different mixing time and filling level.

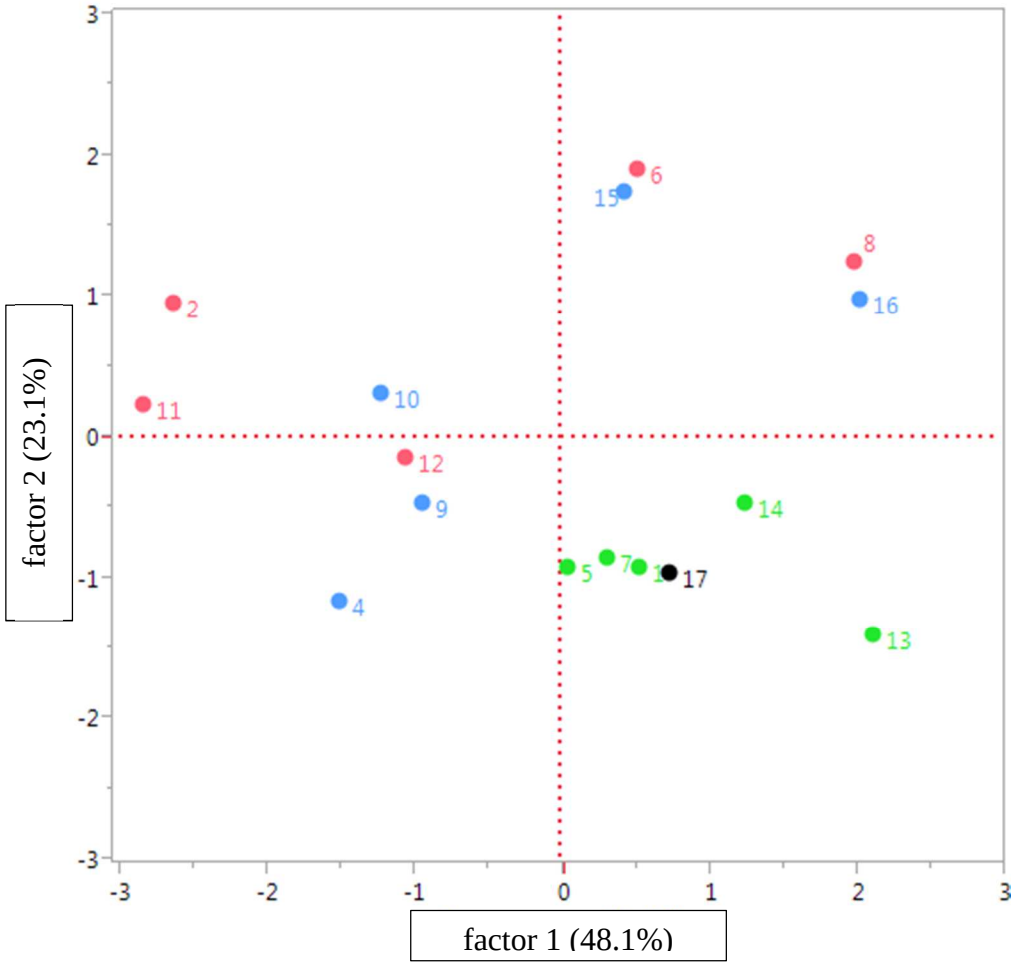

Figure 4: PCA score plot of ration obtained with different filling level, mixing time and cutting time.

● 40% ● 70% ● 100% filling level

● theoretical ration

Table 4: Differences from theoretical ration formulated at different filling level with effect of mixing time and cutting time.

			filling level (%)		
ITEM	Main effect	P	40	70	100
DM (% as fed)	mixing time	<0.05	0.19^{a}	1.68	1.57^{b}
CP (% DM)	filling level	<0.05	-0.54^{a}	0.38	0.70^{b}
EE (% DM)	filling level	<0.05	-0.07^{Aa}	0.33^{b}	0.34^{B}
NDF (% DM)	filling level	<0.01	4.88^{Aa}	-0.59^{B}	0.72^{b}
ash (% DM)	filling level	0.08	0.13^{a}	-0.05	-0.23^{b}

Differences from theoretical ration are reported as percentage.

A,B P≤0.01

a,b P≤0.05

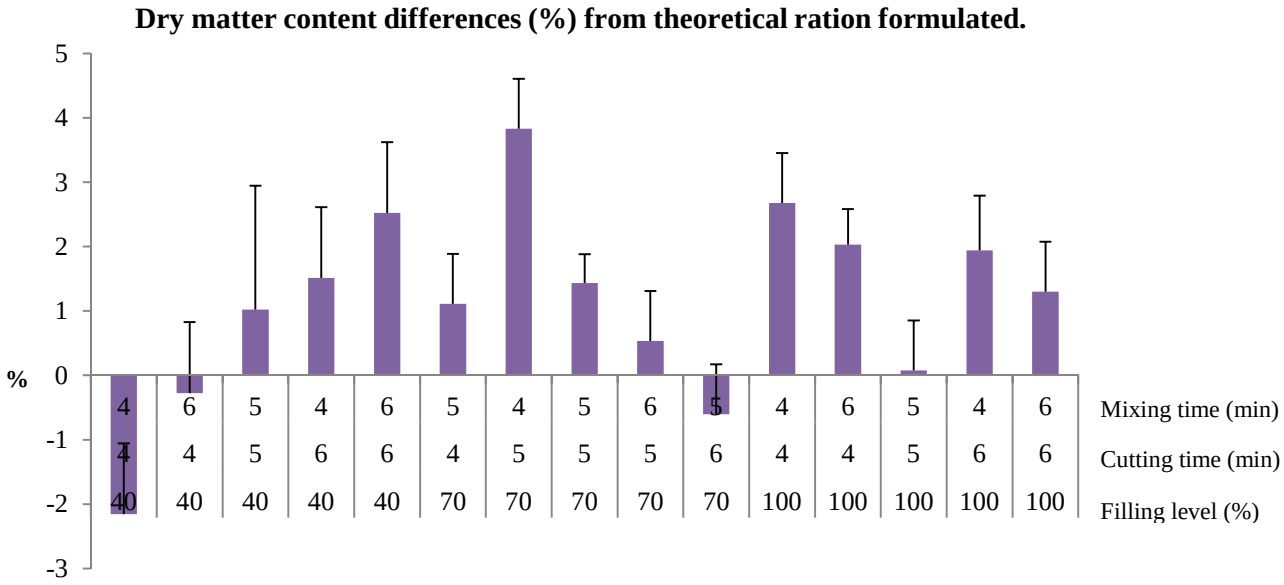

Figure 5: Effect of filling level, mixing time and cutting time on dry matter content, compared to theoretical ration. The differences from theoretical ration are reported in percentage.

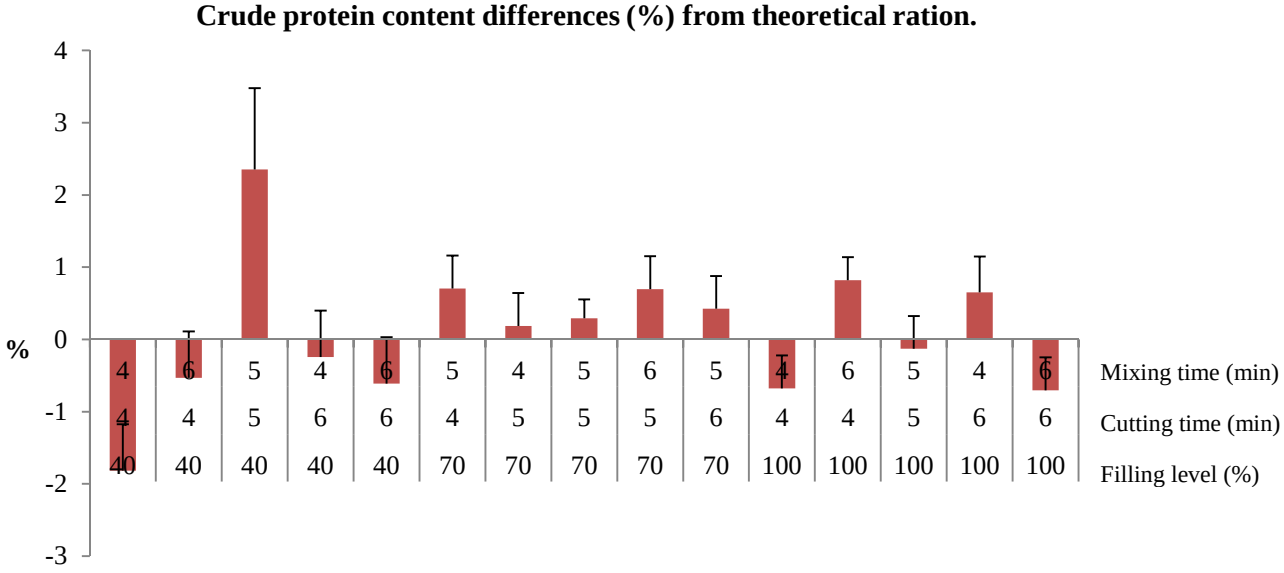

Figure 6: Effect of filling level, mixing time and cutting time on crude protein content, compared to theoretical ration. The differences from theoretical ration are reported in percentage.

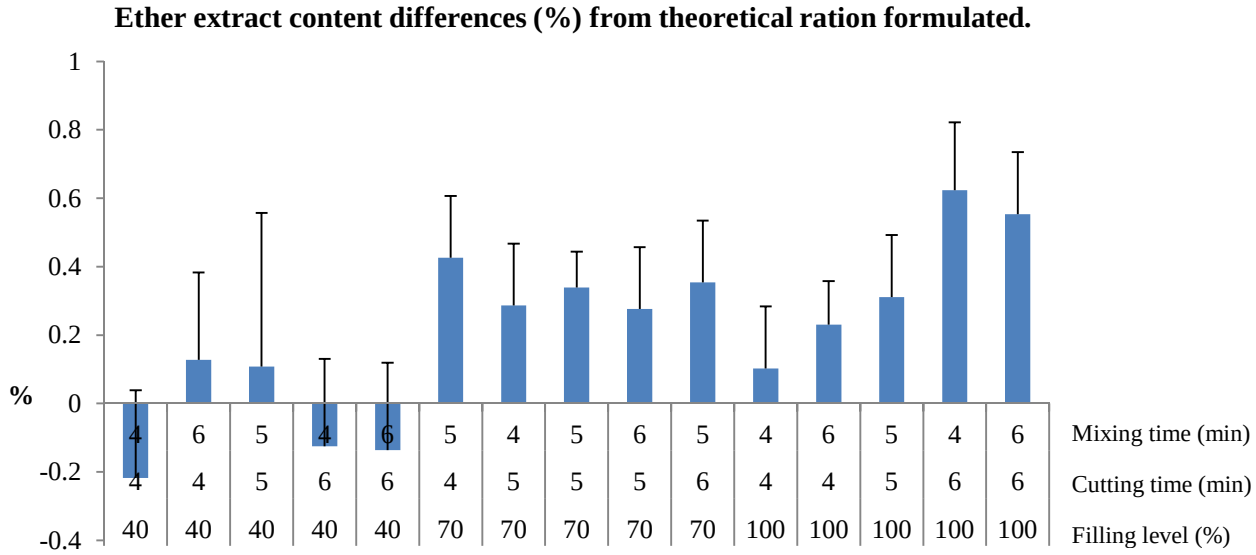

Figure 7: Effect of filling level, mixing time and cutting time on ether extract content, compared to theoretical ration. The differences from theoretical ration are reported in percentage.

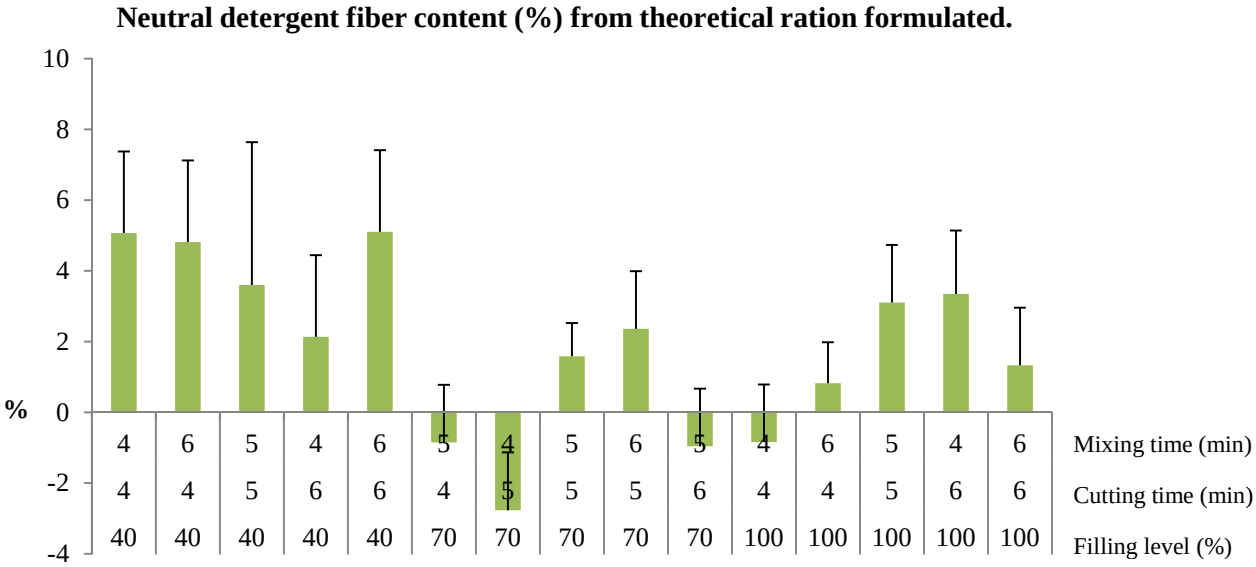

Figure 8: Effect of filling level, mixing time and cutting time on neutral detergent fiber content, compared to theoretical ration. The differences from theoretical ration are reported in percentage.

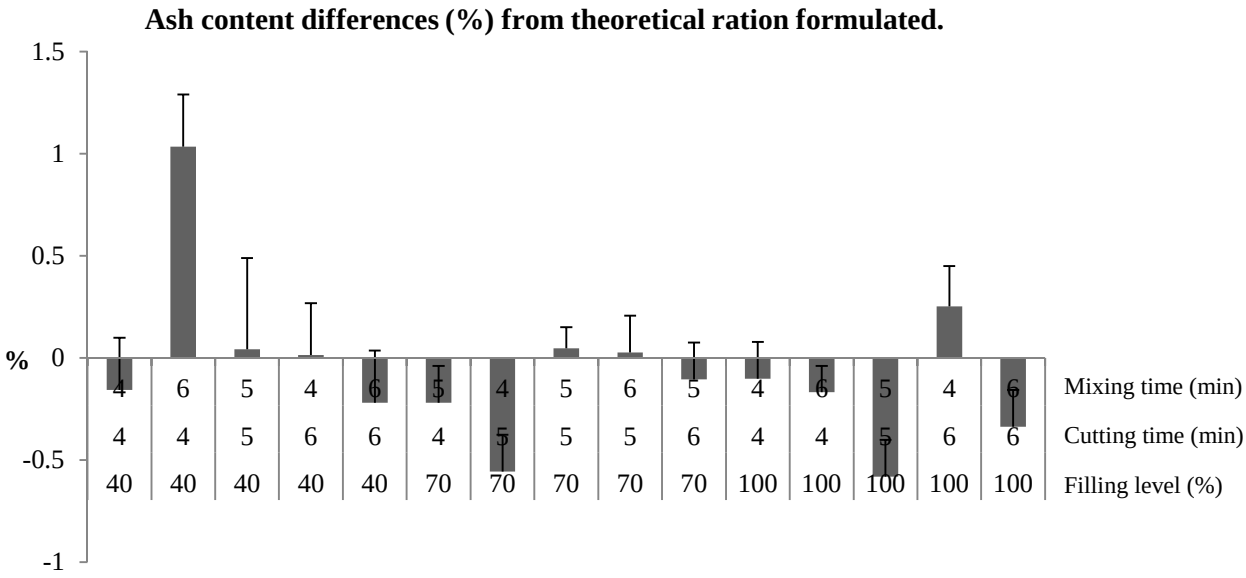

Figure 9: Effect of filling level, mixing time and cutting time on ash content, compared to theoretical ration. The differences from theoretical ration are reported in percentage.

Table 5: Sum of the CV of DM, CP, EE, NDF and ash obtained for each combination of filling level, mixing time and sampling time

Filling level (%)	cutting time (minutes)	mixing time (minutes)	∑ C.V.
70	6	5	2.78
70	5	5	3.77
70	4	5	3.91
100	4	6	4.08
70	5	6	5.12
100	6	6	5.33
100	4	4	5.66
40	6	4	6.15
100	5	5	6.43
40	5	5	7.25
40	4	6	7.3
100	6	4	7.75
70	5	4	8.45
40	4	4	11.98
40	6	6	12.53

CV<5 was considered acceptable.

7.6 Conclusions

No significant differences of particle size distribution were observed at different filling level, mixing time and cutting time, although the best results were obtained at 70% of the filling level compared with 40% and 100%. The data analysis showed overall that the filling level is the main factor affecting ration homogeneity. The best results were obtained with filling at 70% either the DM, CP and NDF. The mixing time had effects on CP and NDF homogeneity overall with the wagon filled at 40% and 100%, no significant effects were observed on DM although the best results were obtained with 6 minutes of mixing. The further data analysis performed with GLM and PCA showed that the filling level is the main factor that affects the differences between the performed rations and the theoretical ration formulated. The filling level at 70% obtained the best results as showed in figure 4 and table 5. The mixing time is the second factor affecting the performed rations. The best results were obtained at 5 minutes. The cutting time is the last factor and showed no significant effect on performed rations.

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Chapter 8

8 General discussion

The nutritional safety is a fundamental aspect for dairy cow nutrition and is a combination of many factors that have to keep in account to achieve the best performance production of dairy cattle.

Provide all dairy cow nutrients to meet the specific requirements for production and reproduction is the first aspect to be considered. From this point of view, formulate the ration with the right amount of forages, concentrates, minerals and vitamins is crucial for dairy cattle health, especially during the periparturient period, as the most metabolic and immunological stressful period of the production cycle.

The forages and overall the silages are the major part of the ration of dairy cattle. Due to the huge amount of this raw material used for dairy cow nutrition, the quality of this product, in terms of amount of nutrients provided and safety is important to support both production and health status of cow. When the silage is not carefully preserved, severe alterations may occur mostly due to contamination of fungi, bacteria and molds with consequent health problems for the dairy cows.

The concentrates are another fundamental components of animal nutrition. They represent the complete ration in swine and poultry nutrition and complementary ration in dairy cow. Their physical quality is an important aspect of animal production.

A correct balanced between the components of the rations is an essential requisite for dairy cows diets to allow suitable rumen conditions and achieve the best production and reproduction performance.

Among the necessary nutrients, vitamins definitely play an important role. Vitamins have different functions including involvement in many metabolic pathways, immune cell function, and gene regulation (NRC 2001). Particularly α-tocopherol showed critical role in the decline of immune function during peripaturient period. The studies conducted on α-tocopherol demonstrated its potential role as an antioxidant and the ability to prevent free-radical mediated tissue damage, and delay the development of certain degenerative and inflammatory diseases (Baldi, 2005). Weiss et al. (2009) reported that all-rac α-tocopherol form administered to dairy cows increased neutrophil activity with an increased phagocytosis and number of bacteria killed. The experimental studies reported by Bourne et al. (2007), LeBlanc et al. (2002) and Kolb and Seehawer (2002) showed that an increase in plasma α-tocopherol was associated with a reduction in the risk for retained fetal membranes. The effect of α-tocopherol supplementation on reduction of somatic cell count and consequently on incidence of mastitis is widely reported (Hogan et al., 1993; Smith et al., 1997; Weiss et al., 1997). As previously reported in this thesis, during the transition period cows are usually in severe

negative energy balance (NEB) conditions that increase the risk of diseases. Non-esterified fatty acid (NEFA) and β-hydroxybutyrate (BHB) are used as marker of the NEB condition during the transition period (Ospina et al., 2010a,b) but to date limited information's concerning the relationship between indicators of NEB and α-tocopherol are available. For this reason, the objectives of the first experimental study that I performed were to determine the relationship between fatty acids and BHB and examine the relationship between blood concentrations of NEFA, BHB, and α-tocopherol during the periparturient period. The data obtained showed that NEFA and BHB were not correlated during the transition period ($P > 0.05$) they followed similar trends in the prepartum period but exactly opposite in the postpartum. Our data have many similarities with those of McCarthy et al. (2015) that showed that the concentrations of NEFA and BHB are not well correlated during the transition period.

A weak correlation with statistical tendency between NEFA and α-tocopherol was observed at dry-off ($P=0.06$) instead negative correlation between NEFA and blood α-tocopherol was observed at 30 d postpartum ($P<0.001$). In contrast the α-tocopherol was negatively correlated with BHB at dry-off ($P<0.001$) but that those correlations were nonsignificant or extremely weak at calving and 30 d postpartum ($P < 0.05$). Our results show similarities with those of Qu et al. (2014), who reported that elevated BHB concentrations before calving coincided with low α-tocopherol concentrations. Another support to our results come from Bouwstra et al. (2008) that reported that vitamin E supplementation in heifers during the periparturient period reduced oxidative damage in the liver.

Not only the quantity but also the quality of the raw materials used for animal nutrition is important.

As previously said silage represent the main forage used in the ration of dairy cattle. It is contains several potential hazards for dairy cattle health which can affect the dry matter intake, nutrient digestibility and the milk production (Driehuis, 2013; Scudamore, 1998; Whitlock et al., 2000). Thus, his nutritional safety and quality is fundamental. Bacteria inoculation is used to improve and speed up the process of acidification of the silage mass. This acidification promotes the development of acid-tolerant lactic acid bacteria (LAB) species (Holzer et al., 2003), which convert water-soluble carbohydrates mainly into lactic acid creating an unfavorable environment for harmful substances such as micotoxins. Silage deterioration is usually correlated with increase of temperature (Borreani and Tabacco, 2010) and changes in volatile compounds. For these reasons, the thermal camera and electronic nose can be used to evaluate the silage quality (Addah et al., 2012; Borreani et al., 2007).

With the objective to assess the efficacy of uncommon inoculated bacteria to improve silage fermentations on ryegrass and alfalfa silages and the use of thermal camera and electronic nose to rapid control of silage quality on all the silages used, I performed two experimental study, one with ryegrass and alfalfa silage packaged on micro-fermenters and one with corn silage on field.

The research performed with LAB inoculation (*Lactobacillus farciminis and Lactobacillus rhamnosus*) showed that treated samples on ryegrass silage had lower pH value with significant difference compare to untreated samples ($P \leq 0.01$), but this difference was probably due to the higher content of ammonia and ammonia nitrogen in untreated samples ($P \leq 0.01$). In fact, the lactic acid and acetic acid had no differences between treatments as expected by data reported by literature (Holzer et al., 2003; Jatkauskas et al., 2015; Rodríguez Amado et al., 2012) although the correlation analysis showed significant negative correlation of pH value with lactic acid (71%; $P \leq 0.01$) and acetic acid (40%; $P \leq 0.01$). The untreated samples showed higher content of ethanol compare to treated silage ($P \leq 0.01$). The results of ammonia, ammonia nitrogen and ethanol content are very important because as reported by Weinberg and Muck (1996) and Li and Nishino (2011) these parameters have to be low for well-preserved silage. The dry matter content decreased during the trial ($P \leq 0.01$) in both treatment and was observed significant difference between them ($P \leq 0.01$). The untreated samples lost more dry matter content. This is an important aspect because one purpose of the LAB inoculation is improve dry matter recovery (Muck and Kung, 1997; McAllister et al., 1998). The LAB concentration showed a significant difference between the treatments ($P \leq 0.01$). The difference is probably due to the inoculation with *Lactobacillus farciminis and Lactobacillus rhamnosus,* which decreased the pH, might have created not favorable environmental conditions for indigenous bacteria communities as reported in literature (Li and Nishino, 2011; McDonald et al., 1991).

The treated alfalfa silage had lower pH value ($P \leq 0.01$) with higher lactic acid content than untreated samples. These results are in according with Wang et al. 2006 although they used different lactobacilli inoculation. The unexpected results were observed on ammonia and ammonia nitrogen content. The two parameters were constantly higher in treated silage during all the research with significant differences ($P \leq 0.01$). Kung et al. (2003) reported similar results of ammonia content in silage treated with *Lactobacillus buchneri.* Although these are not good results, they did not affect the acidification and preservation of silage, probably due to the higher content of lactic acid in the treated silage.

The results obtained, showed the possibility of using *Lactobacillus farciminis and Lactobacillus rhamnosus* for the silage preservation, especially if we consider that the experimental test was carried out on alfalfa silage. This forage crop can be difficult to ensiling because has a low content of soluble carbohydrates, with high contents of organic acids, salts, proteins, and minerals result in a high buffering capacity which may limit the ensiling process (McDonald et al., 1991; Sheperd at al., 1995).

To date, the chemical analysis of raw materials for animal nutrition is definitely the best and most accurate method to control their safety and quality. As previously reported, provide a good silage quality is very important for dairy cattle nutrition. Unfortunately, chemical analysis are very expensive and take a long time to run and then provide the necessary information.

The studies performed with the use of thermal camera and electronic nose had the aim of providing a rapid assessment and low costs of the silage quality. In literature are reported few information's about the use of these technologies to evaluate the silage quality and furthermore no study on their combined use. Novinsky et al. (2012) reported a study on the use of thermal camera to detect the presence of mycotoxins on corn silage, suggesting that temperature of silo face is not a good indicator of these compounds. Completely different results were published by Addah et al. (2012), on the use of the thermal imaging to assess the impact of an inoculant with feruloyl esterase activity on the aerobic stability and digestibility of barley silage. Masoero et al. (2007) reported that electronic nose offer interesting possibilities of providing quick, easy and lowcost information relevant to the stocking and to the monitoring of the main quality parameters in farm silages.

In our trial performed on alfalfa and ryegrass silage using micro-fermenters no differences of temperature were detected between treated and untreated samples. This result is probably due to the good quality of silage, in fact was not detected the presence of propionic acid, butyric acid, isobutyric acid and valeric acid, which are markers of wrong fermentations. The use of electronic nose showed two different results. In ryegrass silage the data obtained showed significant difference ($P \leq 0.01$) between treated and untreated samples, instead no differences were observed in alfalfa silage. This result is probably due to the greater acidification and pH value differences between treated and untreated samples of ryegrass compare to alfalfa silage.

In the trial performed on field on corn silage the results obtained showed that the samples with higher temperature detected with the thermal camera have had higher trend of pH value and lower trend of lactic acid during the trial, although without significant difference. Someone of them had an abnormal content of lactic acid and pH (Kung and Shaver, 2001).

Furthermore, in one sample with higher temperature was detected the presence of butyric acid, propionic acid, molds and yeasts. The data obtained with electronic nose analysis showed that the samples in which were detected wrong fermentations with highest pH value and lowest lactic acid content had significant differences compare to good silage. Although further studies are needed, these results confirm the possible use of electronic nose to detect the silage quality as reported by Masoero et al. (2007).

Besides the forages, also the concentrates are an important component in animal nutrition. Their physical characteristics have been shown to have significant effects on livestock production, especially in swine and poultry. Several studies investigated the effects of feed form on swine nutrition. Wondra et al. (1995) reported that reducing particle size improve growth performance and nutrient digestibility of finishing pigs. Mavromichalis et al. (2000) reported same results on nursery pigs. The pelleted diet improve growth performance and diet digestibility compare to mash form (De Jong et al., 2016; Lahaye et al., 2008; Murphy et al., 2009).

As in swine also in poultry nutrition the pellet form showed an increase of growth performance compare to mash form (Amerah et al., 2007; Chewning et al., 2012).

The feed form has also an important aspect for swine and poultry health. In fact finely grinding increases the risk of stomach keratinization and ulceration in pigs (Mavromichalis et al., 2000; Wondra et al., 1995). Reduction of particle size has negative effect on gizzard weight and consequently on gut function when diets are fed as mash in poultry (Amerah et al., 2007).

Although for dairy cattle the physical characteristics of the feed are not so important as for swine and poultry, the concentrates have a fundamental role in the nutrition of dairy cows. In fact, they complete the supply of nutrients required.

Is important that the feed does not only contain all the nutrients to meet the needs of the animal, but also that it maintains its characteristics from production to animal administration.

For example poor quality pellet, with low values of hardness and durability, will break very easily with an increase of dustiness and wastage, will have effects on the productive performance.

Starting from these reasons, I performed an experimental study with the aim to investigate the physical quality/properties of a selected lot of samples of animal feed collected in Italy and Serbia.

The results obtained showed coarser structure of Italian poultry feed samples comparing cattle and pig feed which is in line with animals' requirements. The pig feed pellets had lowest

value of hardness. Samples of cattle feed had higher values of hardness, than samples of pig and poultry feed. Results of durability test showed that almost all collected pellets had good durability and therefore good resistance against the stresses exerted on them during transportation and distribution to the animals.

Besides the right amount of nutrients, safety of forages and physical feed quality, in dairy cows nutrition is fundamental the correct administration of total mixed ration (TMR).

TMR is composed of forages, byproducts, grains, protein, minerals, and vitamins that have been mixed together. The aim of TMR is to provide a balance ration that contain the same proportion of forages and concentrates in order to provide the same meal to each cow. A uniform TMR ration is very important for cattle health, production and reproduction performance. First of all is important provide energy, it is necessary for supporting milk production, with adequate amounts of dietary physically effective fiber, which is needed to prevent rumen disorders (Zebeli et al., 2011). Finding an optimal balance between physically effective fiber and degradable carbohydrates in the diet is difficult but crucial not only for maintaining proper rumen metabolism (Zebeli et al., 2006; Plaizier et al., 2008), but also for maintaining a stable metabolic health status and enhancing the productivity of dairy cattle (Ametaj et al., 2010; Zebeli et al., 2011). Failure of these balancing contributes to the occurrence several pathologic conditions as acidosis, laminitis, systemic inflammation and milk fat depression syndrome (Ametaj et al., 2010). The management of the techniques and technology used to prepare and provide the TMR play an important role to ensure proper uniformity of the ration. Factors such as fill order, mixing time, mixing protocol, moisture levels of feedstuffs, and scale maintenance and calibration can all affect mix uniformity (Buckmaster, 2009). James and Cox (2008) reported a great variation in feed delivered if feeding programs are not well managed with reduction of milk production. In the same study they reported a variability in crude protein concentration of TMR with inadequate feeding management.

For these reasons the last trial presented in this thesis had the aim to determine the influence of cutting and mixing time on homogeneity and particle size distribution of a dairy cow total mixed ration (TMR), considering different loading levels of the mixing wagon.

The results obtained showed that the filling level was the main factor affecting the ration uniformity. To evaluate the uniformity the percentages of dry matter (DM), crude protein (CP) and neutral detergent fiber (NDF) of TMR provided to dairy cattle were used. The best results were obtained with filling level to 70% of the maximum nominal load capacity of the equipment used to prepare and provide the ration. The mixing time has had minor effects with

differences between DM, CP and NDF but always dependent on the filling level. No effect was observed with different cutting time. Furthermore, was evaluated the effect of filling level, mixing time and cutting time on TMR obtained compared to theoretical ration. The filling level was the main statistical significant effect for CP ($P \leq 0.05$), EE ($P \leq 0.05$) and NDF ($P \leq 0.05$), with a tendency for ash ($P = 0.08$). Instead, the mixing time has had significant effect on DM ($P \leq 0.05$). As for ration uniformity, also in this case the filling level to 70% showed the best results with mixing time to 5 minutes.

The aim of the Precision Feeding approach is to ensure full effectiveness of the diet in terms of chemical quality, nutrient content, physical characteristics and homogeneity of the supplied diet. The studies reported in this thesis represents a contribution to this approach, accounting for the all the different aspects concerning the animal nutrition starting from the importance of right amount of nutrients provided with the diet, which is important to keep the animal in good health condition, especially during the transition period, and obtain the best production performance. To provide the correct amount of nutrients the quality of raw materials used in animal nutrition is very important. In this thesis, two experimental studies were performed on ensiled forages, which are the most raw materials used in dairy cattle nutrition, and for this reason, their quality play a fundamental role. The last aspect considered in this thesis is the manufacturing procedures of the feeds and diet. Besides the chemical and organoleptic qualities, even the physical characteristics of the diet are very important because provide all necessary nutrients is not enough if they are not properly balanced.

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Chapter 9

9 Summary

In this thesis five experimental studies were reported with the objective to improve the knowledge and provide new solutions for different and important aspects of dairy cow nutrition. The first trial was performed to examine the relationships between blood concentrations of fatty acids (NEFA), β-hydroxybutyrate (BHB) and α-tocopherol during the periparturient period in dairy cows. For the study 131 cows from 4 different farms, 2 in Italy and 2 in Greece were used. We determined blood concentrations of NEFA, BHB, and α-tocopherol at dry-off, at calving, and 30d postpartum. We found a weak correlation between NEFA and BHB throughout the periparturient period. Negative correlations between NEFA and α-tocopherol were highly significant at 30d postpartum and approached the level of significance at dry-off. However, both correlations became nonsignificant following the adjustment of α-tocopherol with cholesterol, indicating that the correlations were a reflection of changes in lipid transport. We found significant negative correlations between BHB and α-tocopherol after adjustment with cholesterol.

The aims of the second research presented was to understand the effects of lactic acid bacteria (LAB) inoculation on fermentation products and quality of alfalfa and ryegrass silage and the use of thermal camera and electronic nose to evaluate the silage quality. Wilted ryegrass and alfalfa silage were inoculated with a commercial product containing *Lactobacillus Rhamnosus* and *Lactobacillus Farciminis* and stored in triplicate micro fermenters for each treatment. The silos were opened after 2, 5, 30, 60, 90 and 120 days and silages were collected and analyzed. The results obtained showed better acidification of treated silages with pH value lower in both alfalfa and ryegrass silages ($P \leq 0.01$). Alfalfa silage has had higher content of lactic acid ($P \leq 0.01$) with unexpected higher values of NH_4 ($P \leq 0.01$). In ryegrass silage the LAB concentration was lower in treated compared to control silage ($P \leq 0.01$). Instead no significant difference of LAB concentration was observed between control and treated alfalfa silages. Strong negative correlation between pH and lactic acid 97% ($P \leq 0.01$) and positive correlation between pH and LAB concentration 93% ($P \leq 0.01$) were observed in alfalfa silage. The LAB concentration was positive correlated with pH value 94% ($P \leq 0.01$). Significant but not strong negative correlation was observed between pH and NH_4 33% ($P \leq 0.05$). Probably due to the good quality of both treated and control silages no differences of temperature were observed. Although, different results were obtained between ryegrass and silage analysis the electronic nose showed promising ability to evaluate the silage quality but further studies will be needed to understand his ability to evaluate the silage quality.

In the third experimental trial the combined use of thermal camera and electronic nose was evaluated for rapid assessment of unfavorable fermentations that can occur in a corn silage

mass. From April to September, five sampling times were performed to collect silage samples. The thermal camera was used to detect the temperature. For all samples chemical analysis were performed to evaluate the silage quality and was used electronic nose to perform the off-flavors analysis. The silage quality remained constant and no differences were observed throughout the trial. Significant statistical differences were observed between samples with normal temperature and that with higher temperature for pH value ($P<0.01$), lactic acid ($P<0.05$). The off-flavors analysis showed the ability of electronic nose to distinguish the strong wrong fermentation. The results obtained showed that the use thermal camera and electronic nose can provide an indication of silage quality in short time and with low cost. Further studies aimed to study the accuracy of both instruments are necessary.

The aim of the fourth experimental study was to investigate the physical quality of a selected lot of twenty animal feed samples collected in Italy and Serbia. The samples belonged to different categories and analyzed to assess the flowability, particle size distribution, hardness and durability. Granulation of cattle and pig feed was finer in Italian than in Serbian samples. Flowability of samples from both countries in mash form were rated from fair to good (angle of repose >30°) while granulated samples (pellets and extrudates) had improved flowability (angle of repose < 20°). Extruded products had higher hardness (>10 kg) than pelleted products (<8 kg). Durability of most of the extruded and pelleted products was higher than 95%. Most of the physical characteristics of the samples responded to recommendations, which showed that the similar practices are in use in both countries.

The last research reported in this thesis had the aim to determine the influence of filling level, cutting and mixing time on homogeneity and particle size distribution of a dairy cow total mixed ration (TMR). The mixing wagon was loaded to 40%, 70% and 100% of maximum nominal load ($21 m^3$). The cutting time and mixing time were 4, 5 and 6 minutes. Samples of TMR were collected at the beginning, in the middle and at the end of the feeding alley and analysed to evaluate the dry matter (DM), ether extract (EE), crude protein (CP), neutral detergent fibre (NDF), and ash content. At the same time TMR particle size distribution was determined in triplicate for each sampling point by Penn State Particle Separator. The coefficient of variation (CV %) of DM, CP, NDF and EE was used to evaluate the multiple effect of filling level, cutting and mixing time on ration uniformity and differences between the theoretical ration formulated and that provided to dairy cattle.

The filling level was the main factor affecting uniformity and differences from theoretical ration, the best results were obtained to 70%. The mixing time had less and different effects uniformity and ration composition. The cutting time showed no effects.

Glossary

ADF: Acid detergent fiber.

ADG: Average daily gain.

ADL: Acid detergent lignin.

ANOVA: Analysis of variance.

ASAE: American society of agricultural engineer.

BHB: β-hydroxybutyrate.

CCD: Central composite design.

CF: Crude fiber.

CP: Crude protein.

CV: coefficient of variation.

DIM: Days in milk.

DM: Dry matter.

DMI: Dry matter intake.

EE: Ether extract.

EFSA: European food safety authority.

GLM: generalized linear model.

HPLC: High performance liquid chromatography.

LAB: Lactic acid bacteria.

MOS: metal oxide semiconductor.

NEB: Negative energy balance.

NEFA: Non-esterified fatty acids.

NDF: Neutral detergent fiber.

PCA: Principal component analysis

PDI: Pellet durability index.

PKC: Protein kinase C.

PSPS: Penn state particle separator.

ROS: Reactive oxygen species.

SCC: Somatic cell count.

TG: Triglyceride.

TMR: Total mixed ration.

TTP: α-tocopherol transfer protein.