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New technological and nutritional approaches in livestock farming

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Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.

Marie Curie

Table of contents

ABSTRACT

The livestock sector is facing a huge challenge trying to meet the increasing demand of animal products. To this end several efforts have been done to enhance animals' performances, and nutrition provides a valuable asset. Animals' performances can indeed be boosted through nutritional approaches, including both the application of technologies in a context of 'precision nutrition' and the administration of novel feed additives or feedstuffs with beneficial effects on animals' health. The main aim of this thesis was to investigate 1) the proficiency of feeding systems for dairy cows (1st trial) and the suitability of sensor technology to ensure the fulfilment of cows' nutrients requirement (2nd trial), and 2) the effectiveness of feed additives to improve animals' health and consequently their performances (3rd and 4th trial).

The **first study** investigated some aspects connected to the mixer wagon, and their influence on the preparation of the total mixed ration (TMR) for dairy cows. We evaluated how loading levels, cutting time, mixing time and their interaction can affect the homogeneity of the TMR along the feeding alley. The uneven distribution of the TMR along the feeding alley does not allow each animal to receive the same well-balanced diet, with the risk of not satisfying the nutritional requirements, finally impairing productive performances. Even though we identified the most efficient combination of loading levels, cutting, and mixing time, variations in the distribution of DM and NDF were observed, pointing out the necessity to investigate which other factors are influencing TMR preparation and distribution.

In the **second study** we developed a system based on a microwave resonance sensor to optimize TMR preparation. Silages represent a large proportion of feeds included in the TMR, but their nutrient supply (on as fed basis) can vary among time due to moisture content fluctuations. For this reason, if their inclusion rate in the mixer wagon is not adjusted according to the actual dry matter (DM) content, ensuring the proper provision of nutrients, the delivered TMR might be different from the diet formulated by the nutritionist, with the risk of unfulfilling animals' requirement. The sensor performed a real-time measurement of silages DM content during their loading in the mixer wagon and suggested a correction of their inclusion rate. Our hypothesis was that, adjusting the silages as fed inclusion rate according to the real DM content, the final TMR delivered to the animals would have been closer to the target diet. The employment of the sensor reduced the error in silages DM content loads, however the final TMR was not closer to the formulated diet compared to the TMR conventionally prepared. The lack of result was attributed to errors in the loading of dry feedstuffs (concentrates), which can equally affect TMR composition. Our results thus highlight the necessity of systems to improve the accuracy of TMR preparation.

The **third study** was dedicated to the evaluation of the effectiveness of pomegranate rinds and green tea leaves extract on broiler chicken health and performances. We assessed the potential of the product to improve blood antiradical activity, as a way to increase animals' defence against oxidative stress. We further investigated the effect on cecal microbiota, observing a positive modulation of beneficial bacterial population, such as *Lactobacillaceae*. Despite the positive results observed for health-related parameters, growth performances were not enhanced.

Lastly, in the **fourth study** we investigated the effect of nucleotides supplementation to weaning piglets. Nucleotides play a fundamental role in the development of gastrointestinal mucosa and immune system, but due to the high demand in critical moments (i.e. weaning) and the reduced *de novo* synthesis, their supplementation might be beneficial for the animals. However, we did not observe positive effect of nucleotides supplementation on animals' immune response, probably

due to the optimal conditions where the animals were raised, which minimized the stress usually associated with weaning.

Overall, these results contributed to the investigation of technological and nutritional approaches to improve the efficiency of animal feeding. However, further investigations are required to deepen our knowledge about the limitations that have been observed.

SINTESI

Il settore zootecnico sta affrontando una grande sfida nel tentativo di soddisfare la crescente domanda di prodotti di origine animale. Per rispondere a tale richiesta sono stati compiuti numerosi sforzi per incrementare la produttività degli animali e, a tal fine, la nutrizione rappresenta una valida risorsa. La produttività degli animali può infatti essere incrementata attraverso approcci di tipo nutrizionale, i quali includono tanto l'utilizzo di tecnologie in un contesto di 'nutrizione di precisione', quanto l'utilizzo di alimenti o additivi alimentari innovativi con effetti benefici sulla salute degli animali. Gli obiettivi principali di questa tesi sono stati quelli di investigare 1) l'efficienza dei sistemi utilizzati per l'alimentazione delle bovine da latte (1° prova) e l'idoneità di un sistema sensoristico per garantire che le esigenze nutrizionali delle bovine vengano pienamente soddisfatte (2° prova) e 2) l'efficacia di additivi nutrizionali atti a migliorare lo stato di salute degli animali e conseguentemente le loro performance produttive (3° e 4° prova).

Il **primo studio** ha investigato alcuni aspetti strettamente connessi al carro miscelatore utilizzato per la preparazione delle diete per vacche da latte e il loro effetto sulla preparazione della dieta stessa. Sono stati considerati il livello di riempimento del carro miscelatore, i tempi dedicati al taglio dei foraggi e alla fase di miscelazione vera e propria e l'interazione di tali fattori sull'omogeneità della miscelata lungo la corsia di alimentazione. Una distribuzione disomogenea della dieta non assicura infatti che ciascun animale riceva una dieta identica e bene bilanciata, con il rischio che i fabbisogni nutrizionali non vengano correttamente soddisfatti, ripercuotendosi infine sulle performance produttive. Sebbene sia stata identificata la combinazione ideale dei sopracitati fattori che permette la miglior distribuzione della dieta, sono state comunque osservate variazioni nella distribuzione di sostanza secca (SS) e NDF lungo la corsia di alimentazione. Tali risultati suggeriscono dunque la compartecipazione di altri fattori, oltre a quelli già considerati che necessitano quindi si essere ulteriormente investigati.

Nel **secondo studio** è stato sviluppato un sistema sensoristico basato sull'utilizzo di un risonatore a microonde per ottimizzare la preparazione della dieta per bovine da latte. La percentuale di inclusione degli insilati nella dieta è notevole, ma il loro apporto nutrizionale (su base tal quale) può variare nel corso del tempo a causa di fluttuazioni del contenuto di umidità. Per tale ragione, se la quota di insilati caricata nel carro miscelatore non viene corretta in funzione del contenuto effettivo di umidità (e quindi di sostanza secca - SS), assicurando dunque il corretto apporto di nutrienti, la razione finale potrebbe risultare differente da quella formulata dal nutrizionista, con il conseguente rischio di mancato soddisfacimento dei fabbisogni nutrizionali. Il sensore utilizzato operava una valutazione in tempo reale del contenuto di SS degli insilati durante la fase di carico degli ingredienti nel carro miscelatore, suggerendo una correzione del quantitativo tal quale da caricare in funzione dell'umidità rilevata. La nostra ipotesi era che, correggendo l'inclusione degli insilati a seconda del loro reale contenuto di SS, la dieta finale sarebbe stata più simile a quella teorica. Sebbene l'utilizzo del sensore abbia permesso di ridurre l'errore nel carico della SS apportata dagli insilati, la dieta finale non è risultata essere più simile a quella teorica rispetto alla dieta preparata in modo convenzionale. La mancanza del risultato atteso è stata attribuita all'errore nel carico degli alimenti concentrati, i quali possono analogamente influenzare la composizione della razione finale. I nostri risultati evidenziano dunque la necessità di sistemi atti a migliorare l'accuratezza nella preparazione della dieta.

Il **terzo studio** è stato dedicato alla valutazione dell'efficacia di un estratto derivato da bucce di melograno e foglie di thè verde per migliorare la salute e le performance di polli da carne.

È stato valutato il potenziale del prodotto nel migliorare la capacità totale anti-radicalica del sangue, con il fine di incrementare le difese dell'animale verso lo stress ossidativo. Ne è stato inoltre valutato l'effetto a livello di microbioma cecale, evidenziando una modulazione positiva delle popolazioni microbiche benefiche, quali quelle appartenenti alla famiglia delle *Lactobacillaceae*. Nonostante i risultati positivi osservati relativamente i parametri connessi allo stato di salute degli animali, le performance di crescita non sono risultate beneficiate dalla somministrazione dell'estratto naturale.

Infine, nel **quarto studio** abbiamo investigato gli effetti della somministrazione di nucleotidi a suinetti in fase di post-svezzamento. I nucleotidi rivestono un ruolo fondamentale nello sviluppo della mucosa gastroenterica e del sistema immunitario, ma a causa delle elevate richieste che accompagno momenti critici della vita dell'animale (quali lo svezzamento) e della ridotta capacità di sintesi *de novo*, la loro supplementazione potrebbe avere riscontri positivi per l'animale. Ciononostante, nella nostra sperimentazione i parametri relativi a risposta infiammatoria e immunitaria non sono stati influenzati dalla somministrazione di nucleotidi. La mancanza di risultati è stata attribuita alle ottime condizioni ambientali in cui sono stati allevati gli animali, le quali potrebbero aver minimizzato lo stress normalmente associato allo svezzamento.

In conclusione, i risultati riportati a seguito delle sperimentazioni condotte hanno contribuito ad investigare approcci tecnologici e prettamente nutrizionali al fine di migliorare l'efficienza nutrizionale. Tuttavia ulteriori indagini si rendono necessarie per approfondire le conoscenze a riguardo delle limitazioni che sono state osservate.

Chapter 1

General introduction

FORWARD

Innovative solution toward a more efficient and sustainable production

Human population is expected to reach more than 9 billion people by 2050. This increment, along with the increase in both per capita income and health, will lead to a higher demand of animal products, submitting the livestock sector under a remarkable pressure. Whether in the past the high demand for meat, milk and eggs was addressed increasing the number of raised animals, i.e. shifting towards intensive and concentrated production systems, nowadays this approach is no longer pursuable. The growing concern over environmental and societal impact of livestock production is indeed setting boundaries for further intensification in terms of animal number, rising the need for alternative solutions towards a more sustainable production (Steinfeld et al., 2006). Therfore, increasing animal production, while at the same time meeting the demand for a more sustainable livestock farming, represents a huge challenge for the livestock sector.

In this light, it is necessary to improve farm efficiency, i.e. produce more with less, which means maximise the outputs, namely animals' products (meat, milk, eggs) from given inputs. By improving efficiency it is therfore possible to reduce the final costs of production, and at the same time improve the competitiveness. Taking into account that feeding-related costs cover most of the expenses of livestock sector, accounting for up to 70% of total cost of production, feed represents a great opportunity to improve farm efficiency. Indeed, by optimizing feed management it's possible to directly and indirectly improve perfromances, ensuring at the same time a more sustainable production, either from an economic, societal and environmental point of view.

In a context where optimizing the input:output ratio is necessary, the concept of Precision Livestock Farming (PLF) arises. PLF was defined as "the use of technologies which enable the measurement of physiological, behavioral, and production indicators in animals with the aim of improving herd management strategies and farm performance" (Borchers and Bewley, 2015). PLF aim is to enhance farm profitability, efficiency and sustainability improving on-farm data acquisition, management and utilisation (Banhazi et al., 2012). Precision Feeding (PF), also known as Precision Nutrition, is a key component of PLF and has been defined by Makkar as feeding that 'targets individual animals or a group of uniform animals (same stage of production/age/weight) and aims to meet their nutrient requirements with the right amount of the balanced feed' (Makkar, 2001). Through the application of information technology and management process, which differ according to the type of animals and the the production system, PF is a tool to otpimize economic, social, and environmental farm performances (van Empel et al., 2016; Tullo et al., 2019). As a

consequence of a ration that accurately meet each animal's or pens' nutrient requirement, animal productive and reproductive performances are enhanced (Garg et al., 2013), while environmental emissions of N, P and CH_4 can be reduced and the use of available resources optimized (White and Capper, 2014; Lovato et al., 2017). Furthermore, a well-balanced ration prevent feed wastage, leading to decreased feeding costs.

Besides feed management, a second keystone to increase farm efficiency and sustainability, is animal health. Animal health has been defined as a state of complete physical, mental, and social wellbeing and not merely the absence of disease or infirmity (WHO, 1948). Althought strictly related, animal health should not be confused with animal welfare, which is the ability of the animal to express its natural behaviour or its innate 'animalness' within a situation (Goldberg, 2016). Animal health, as well as animal welfare, has a direct impact on animal productivity and efficiency, and plays an essential role in maintaining the balance between the three main components of livestock sustainability, namely economic viability, environmental responsibility, and social acceptability. Optimal health conditions enables animals to fully express their genetic potential, thus increasing economic viability by improving production (milk, meat or eggs) yield, improving fertility and enhancing product quality. In such a way, the maintenance cost are diluted, reducing the cost for each unit of production (Capper et al., 2009; Capper, 2011). For an environmental perspective, healthy animals have also a lower footprint, reducing resource use and greenhouse gases emissions intensity per unit of protein produced (MacLeod et al., 2018; Leinonen, 2019). An additional benefit deriving from healthy animals is the reduction of antibiotic use, which has been severely criticized for the concern over the risk of antimicrobial resistency development, which is estimated to be currently responsible of 50.000 human death across Europe (Barber and Swaden-Lewis, 2017) . The use of antibiotics has already been limited since 2006, when the ban of their use as growth promoter was introduced in Europe. Nevertheless, there is still a large employment of antibiotics in livestock farming, which represents a threat for the society. Improvements in animal health has therfore the potential to to further limit the use of antimicrobials reducing the risk of antibiotic-resistancy development (de Passille and Rushen, 2005; Pinillos et al., 2018).

In oder to pursue improved health of livestock animals, different approaches have been investigated. First of all, farm management plays a fundamental role, encompassing biosecurity, diseases prevention and control, vaccination plans, and the promotion of welfare (Lewerin et al., 2015; Gale et al., 2017). At the same time, health can be promoted through a nutritional approach, which implies not only the fullfilment of nutrient requirements, but also the employment of feed or feed additives with beneficial attribute, such as antimicrobial, immunostimulant or antioxidant properties (Windisch et al., 2008; DeLange et al., 2010).

In conclusion, it is evident the potential of animal feeding to improve livestock efficiency and sustainability, either by optimizing feed management, or by enhancing animal health. The optimization of feed management encompass the precise fullfilment of animals' requirement, which can be attained in a context of PLF/PF, specifically with the employment of technologies that allow to control the process (technological approach). On the other hand, a more pure nutritional approach with the emplyment of specific feeds or feed additived can modulate animal health, thus finally influencing animal performances.

TECHNOLOGICAL APPROACH: THE IMPORTANCE OF PROPER FEEDING PRACTICE IN DAIRY FARMING

Total mixed ration: the importance of consistency

Total mixed ration (TMR) is nowadays the most common feed practice in dairy farms. TMR was introduced for the first time around 1950s, in response to changes in the setup of the farms, namely as a consequence of enlargement of the herds, increased milk production, and more prevalent use of milking parlours (Schingoethe, 2017). By definition, TMR feeding consists in providing all the ingredients in a single and uniform mix, where each bite has the same characteristics, providing the required amount of energy, protein, fiber and minerals to boost and economize the productive performance (Hundal et al., 2004). Thus, TMR provide a consistent supply of nutrients to rumen microbes to optimize rumen function and improve the efficiency of nutrient utilization (Coppock et al., 1981).

Chemical and physical composition of the TMR can be subdued to daily or short-term unintended variations, responsible to produce a ration which do not reflect the theoretical one. These variations might affect animals' health and production, resulting for example in average daily milk yield fluctuation and increased incidence of displaced abomasum (Stone, 2008). The provision of a consistent ration is therefore essential to maximise cow performance and get the best value out of the ration (Sova et al., 2014). Consistency of TMR can be express either as precision or accuracy. ***Precision*** is intended as the degree of variation between ration, both in terms of chemical and physical composition. Factors affecting TMR composition among batches encompass chemical content of the feedstuff included in the ration and the procedures involved in the preparation of the TMR (Buckmaster, 2009). On the other hand, ***accuracy*** refers to the degree to which fed TMR differs from the theoretical ration. Theoretical rations are specifically formulated by the nutritionist through the employment of dedicated software, with the aim to satisfy nutritional requirement of the animals. However, delivered ration may not reflect the theoretical one; indeed, different types of TMR are recognized. The first type is the TMR formulated by the nutritionist; the second one is the TMR loaded in the mixer wagon, which might differ from the theoretical ration due to human error during the loading of the ingredients or due to daily variation of the feedstuff included in the mix. The third type is the TMR delivered in the feeding alley, which might deviate from the previous two because of errors during the mixing and discharging phases. Lastly, there is the TMR effectively eaten by the animals, whose composition can vary according to animal sorting (Sistkova et al., 2015).

Variation between batches: consistency with the formulated ration and consistency among days

Consistency in TMR composition among days is imperative in order to provide consistent rumen function, needed to guarantee high production (Mikus, 2012). Changes in TMR composition may impair animal performance and health. In an observational study, herds fed a TMR with great variability of Net Energy of Lactation (NE_l) and particle size (PD) were found to have reduced milk yield and feed efficiency (Sova et al., 2014). Rossow and Aly (2013) observed a positive correlation between variation in the concentration of certain nutrients (such as lignin) and variation in milk yield and composition. However, other studies conducted in controlled conditions evidenced no or only minor effects on production traits because of TMR variations (McBeth et al., 2013; Weiss et al., 2013; Yoder et al., 2013). Day-to-day TMR variation can result from errors occurred during feed preparation, mainly miscarriages in the amount of each ingredients loaded in the mixer wagon (Sirakaya and Kucuk, 2019), which can be due either to human error or low scale accuracy (Mikus et al., 2012). Beside this, one of the major factors influencing TMR composition among days is the nutrient variability of feeds (St-Pierre and Weiss, 2015). This is a well-recognized problem, hence, in order to reduce the risk of nutrient deficiency in the final ration, it's common practice to formulate diets containing an excess of nutrients, i.e. including a safety factor (Sniffen et al., 1993). However, due to the high cost of feed and the need to reduce nutrient excretion (N and P), farmers are nowadays moving towards a more precise feeding. It is worthy notice that each ingredient doesn't have the same variability. Batch-process feedstuffs, such as concentrates, are characterized by small variation within lots, thus they do not require frequent sampling to adjust their inclusion rate in the ration. On the contrary, feed ingredients such as forages and by-products, can have a moderate to high variability (St.Pierre and Weiss, 2007). Furthermore, it has to be considered that the incidence of each ingredient's variation on the final TMR depends upon the inclusion rate of the same ingredient. Indeed, the effect an ingredient has on the variation of the total diet changes with the square of its inclusion rate (Weiss et al., 2012).

One of the main factors affecting TMR consistency is the moisture content of the ingredients. Ration are indeed formulated on a dry matter (DM) basis, while ingredients are included in the TMR on as as-fed basis, meaning that changes in DM content will affect the amount of nutrient brought by each specific ingredient. Forages, including silages which are characterized by high moisture content, account for more than half of the DM content of the diet fed to dairy cows. Therefore, DM fluctuation of forages can be responsible of a substantial change in the composition of the delivered ration (Stone, 2008). McBeth et al. (2014) measured changes in ingredient DM concentration of alfalfa and corn silage over a 2 months period. Mean, standard

deviation and range were 53.5%, 4.0 and 13.3 percentage units respectively for alfalfa silage; while for corn silage they were 37.1%, 1.6, and 6.3 percentage units, respectively . Day-to-day variability was also speculated to be as great as month-to-month variation (Weiss et al., 2012). Silage DM variability are mainly due to bunk management during the feed-out phase. Borreani et al. (2018), based on previous published literatures, stated that, even with good management practice, 3% DM loss occurs during the feed-out phase. DM loss increases to 21% with non-optimal management practice and reach 31% when no silo covering is applied. Indeed, adverse weather conditions, including rainfall event over uncovered silos are one of the main reasons of short-time bouts in DM content of silages. Then, silages DM variability is reflected in the DM content of the final ration, which can in turn influence DMI of the animals and feed sorting.

Bunk management is however a critical phase not only relatively to DM content variability, but also as regard to the nutrient composition of the silage. Once the silo is opened, phenomena of aerobic deterioration take place, representing a significant problem for farm profitability and feed quality (Borreani and Tabacco, 2010). As air penetrates via the silage face, especially in the peripheral area of the silo, where the density is likely to be lower (Griswold et al., 2010), aerobic microorganisms begin to grow. The initiators of the aerobic deterioration are usually yeasts, which consume sugars and ferment acids, rising silage temperature and pH (Pahlow et al., 2003). As a consequence of increased pH, bacilli and other aerobic bacteria start growing, leading to further temperature increase. Finally, moulds complete the silage deterioration. Losses resulting from this deterioration process, especially in the peripheral areas and near the side walls of the bunkers, could reach 70% of the stored DM (Bernardes et al., 2012), thus finally influencing TMR composition.

Variation within batches: consistency along the feeding alley

TMR feeding aims to provide to each animal the proper amount of nutrients with each mouthful. For this reason, TMR consistency within batches is essential, providing chemical and physical homogeneity of the ration along the feeding alley. When energy density of the TMR was unevenly distributed across the feed bunk, changes in eating behaviour of the animals, including changes in feeding locations and increased competitiveness, were observed (Huzzey et al., 2013). It is generally accepted that a consistent distribution of the ration along the feeding alley should have a coefficient of variation (CV) <3-5%. With special regards to particle size distribution, CV between 5-7% can be indicative of overfilling or undermixing, while CV in the range of 7% to 10% represent a possible need to conduct TMR mixer maintenance (Oelberg, 2011).

TMR homogeneity can be influenced by ingredients properties, including particle size, particle shape, density, hygroscopicity, static charging and adhesiveness. Among them, particle size, shape and density play the major role; for example, too large or too small particles do not mix well and are subjected to directional influence (Behnke, 2005). Besides the characteristics of the feedstuffs, the major factor affecting TMR homogeneity is the mixing equipment employed for the preparation of the ration. Different types of mixer wagon are available, each one with specific and different characteristics. Some mixers are based on tumble action to accomplish the blending. Reel, tumble, chain and slat, and ribbon mixers belong to this category. Other mixers are provided with augers, which can have different configuration according to the position and the number (1,2,3 or 4 horizontal augers, single or twin vertical augers). Different configurations correspond to different material flow paths (Amaral-Phillips et al., 2002; (Buckmaster, 2009).
Numerous factors associated with mixing equipment can influence TMR homogeneity; a brief description will be given below.

Equipment maintenance

Routine maintenance of TMR mixer is important, since malfunctioning due to warn equipment can significantly affect wagon performances. Specifically, the maintenance of two components is fundamental to ensure homogenous mixing of the ration: blades and kicker plates. Blades and knives must be routinely replaced or sharpened to provide a proper cutting, otherwise they will be responsible of improper mixing of the ration or increased mixing time required to achieve the homogeneity (Jarderborg and Di Costanzo, 2012). Even more important is the maintenance of the kicker plates. The kicker plate is a metal component mounted on the lateral aspect of the leading edge of the auger. Its function is to remove feed from along the bottom wall of the mixer. This allows feed from the upper aspect of the mixer to move down the wall (Stone

et al., 2008). Whether the kicker plate is worn, feed will not be accurately removed from the wall of the mixer, resulting in improper feed flow and inadequate mixing.

Mixing time

Adequate mixing time is very important for obtaining consistent TMR. Mixing time should be enough to ensure a proper homogeneity, while at the same time it should not be overlong to avoid excessive reduction of the particle size (Oeldberg, 2011). Under-mixing affects variability of fiber concentration within the bunk. In an experiment conducted by Jarderborg and Di Costanzo (2012), it was observed that a 2 minutes mixing with a 4-auger mixer and a reel mixer was responsible of a CV along the feeding alley of 15.6% and 17.8%, respectively. When mixing time was increased to 4 minutes, the CV was reduced to 5.0% for 4-augers mixer and 5.7% for reel TMR mixer.

Filling level

A proper capacity of the mixer wagon is fundamental to assure adequate blending of the ration (Buckmaster, 2005). The capacity of the mixer wagon should be chosen according to the herd/feeding group size and the dry matter intake (DMI) per animal. DMI per animal depends upon animals' characteristics (i.e. stage of lactation) and feed composition (Buckmaster, 2009). It is fundamental to account also for the feed characteristics, with especial regard to bulk density of the total ration. Indeed, wetter ration, containing large amounts of wet forages, may have a lower bulk density, thus contributing to the need for larger mixers. (Buckmaster., 2005).

Proper mixing requires that ingredients included in the TMR fall into an open space created by the mixer paddles in a horizontal mixer, or by the screws in a vertical mixer. When the load capacity exceeds the mixer capacity, the TMR will not be properly mixed, leading to a great CV along the feeding alley (Oeldberg, 2011). However, also insufficient filling level can be responsible of non-homogeneous matrix.

Order and location of ingredients loading

Even though to a lower extent compared to the previous aspects, loading sequence can affect TMR uniformity too. Loading sequence depend on several issues, like mixer type, ingredient type, inclusion level and convenience of the feeder relative to ingredient location in the farm. Generally, lower density and large particles feeds, such as straw and hay, are loaded first, followed by dry grains, wet by-products, haylage, corn silage and liquids (Stone et al., 2008). Further, the place where ingredients are loaded in the wagon can influence diet consistency as well. For example,

in two screw vertical mixers, ingredients should be added between the augers, to allow for a proper distribution throughout the tank (Mikus, 2012).

Available technologies for proper preparation of the TMR

As stated in the previous sections, feeding is a key-point to optimize farm profitability. Ensuring that all animals will receive a precise and accurate diet, while at the same time optimizing productive process, is indeed necessary to enhance both animal and farm efficiency. A substantial help to this end can be provided by the employment of technologies, fitting therefore in a context of Precision Livestock Farming and Precision Feeding.

During the last years numerous efforts and progresses have been made to improve TMR feeding, moving toward increased automation of the process, with the development of automatic feeding systems (AFS). AFS allows to reduce errors in the preparation of the ration, avoiding human error thanks to the employment of highly accurate scale and weighing technologies. Furthermore, AFS drastically reduce the time spent by the operator in preparing the ration, which is estimated to be approximately 25% of the total work requirement, being at the second place as the most time-consuming work, after milking (Grothmann, 2010). Most of the AFS for dairy cows are based on the preparation of the TMR for groups of animals, which however represents a limitation, implying that some animals are receiving more nutrients than required, while others are receiving a lower amount than needed. In order to precisely meet animals' requirement, while at the same time avoid over- or underfeeding, the optimal solution would be for each cow to receive a tailored ration, based on her own real requirement. Nowadays, automation is moving in that direction, with the development of individual AFS. However, AFS have some limitations, first the expensive investment required. Moreover, the transition from a conventional system to AFS requires important structural changes, including the reduction of the dimension of the feeding alley, the reduction of specific feeding front, as well as the necessity to simplify feed barrier (Da Borso et al., 2017). For this reason, other possible solutions could be adopted, such as the implementation of technologies to improve the performances of conventional feeding systems (mixer wagons). It is necessary to assist the operator in the preparation of the ration, first aiming to limit as much as possible human error. With this purpose, different technologies have been developed, however scientific literature in this respect is lacking. One of the main objectives of these technologies is to optimize feedstuffs loading in the mixer wagon, avoiding excessive or insufficient loading amount of each ingredient. For this purpose, automatic loading systems have been developed, which control and automatically regulate the discharging phase from silos into feed mixers. Other technologies developed to optimize the loading phase are systems that automatically control the speed of the cutting slasher according to the feedstuff, thus guaranteeing a constant and precise cut for optimal length of the forages. Furthermore, in order to optimize the preparation of the TMR, systems for the management of the mixing time have been developed as well. These systems

automatically adjust the speed of the augers or the paddle according to the loading level, allowing a faster mixing phase and avoiding overmixing or heating of the feed.

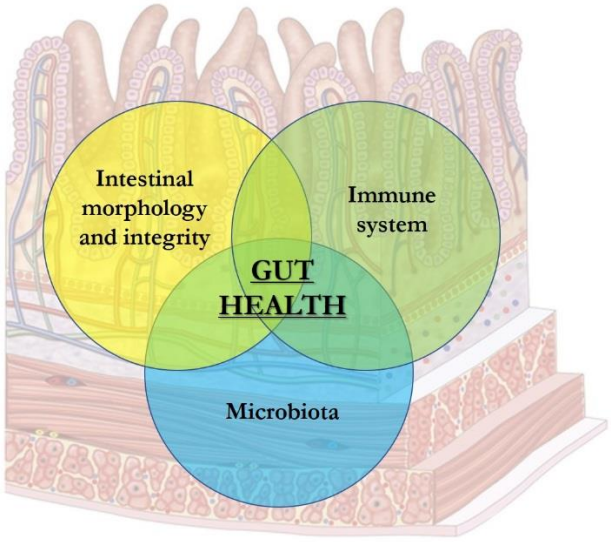
Even though all these systems can be useful for a more precise preparation of the ration, they still do not ensure that the prepared ration actually reflects the ration formulated by the nutritionist. Daily changes in DM content of the ingredients, especially silages, are a well-recognized problem and can be responsible of poor consistency of the diet between days (Stone, 2008). The only solution proposed so far to control daily DM fluctuations is the employment of a sensor relying on Near Infra-Red (NIR) spectroscopy (Barbi et al., 2010). The sensor can be mounted on the front loader of the wagon, where it performs a real-time scan of the material flowing through the bucket, providing the actual values of DM, crude protein, starch, ADF, NDF and Ash. According to the DM content read by the NIR, as-fed weight of each feedstuff to be loaded in the mixer is therefore recalculated, in order to ensure the supply of the right amount of nutrients. In a recent work, Piccioli-Cappelli and colleagues (2019) evaluated the effect of the application of a NIR sensor for real-time adjustment of ingredient load in seven different farms. Their results evidenced a lower deviation between formulated diet and the diet really distributed to the animals, when the sensor was switched on rather than when it was switched off (0.06 vs 0.12 kg, respectively; p=.12). Moreover, they also evaluated the influence of a more consistent ration on metabolic condition and milk production. Milk yield and composition were not affected, but a better feed protein utilization was observed.

NUTRITIONAL APPROACH: FEEDING TO IMPROVE HEALTH AND PERFORMANCES

Gut health in poultry and swine

Optimal animal performances are relying on the effective functionality and health of the gastrointestinal tract (GIT). For this reason, the term "gut health" has started to gain interest over the last years, even though a clear definition is still lacking. In human medicine the term gut health is usually associated with the absence of clinical disease; however, this definition is not suitable in animal science since animals' performances can be impaired even when no clinical signs of illness are manifest. Celi et al. (2017) defined gut health as "a steady state where the microbiome and the intestinal tract exist in symbiotic equilibrium and where the welfare and performance of the animals are not constrained by intestinal dysfunction". The concept of gut health thus encompasses 1) effective digestion and absorption of nutrients; 2) a proper structure and function of the gut barrier; 3) a normal and stable microbial population; 4) an effective function of the immune system. It is therefore possible to state that the main components to consider when referring to gut health are intestinal morphology and integrity, gut microbiota, and immune system (Figure 1), which are interrelated, influencing each other.

Figure 1*. Main components of the gut health.*



Intestinal morphology and integrity

The primary function of the GIT is the digestion of feed and absorption of nutrients; furthermore, the GIT is in charge for the maintenance of fluid and electrolyte balance and elimination of waste products (Celi et al., 2017). Beside these functions, since the GIT is the organ with the largest surface area with constant interaction with the environment, it plays also a crucial role of barrier against the external environments, including pathogens and toxins (Turner, 2009). The integrity of the anatomical structure is essential for the normal function of the gut, both for digestion and absorption of nutrients, and for its barrier role (epithelial lining). Even though there are some differences between poultry and swine, the small intestine (along with ceca in poultry) is the main focus in terms of gut health for both species (Yang and Liao, 2019).

The epithelium of the small intestine is structured in a single layer of tall columnar cells, which are supported by the lamina propria and the muscularis mucosae. Epithelial cells are connected by tight junction complexes, located at the apical end of the lateral membrane (Farquhar and Palade, 1963), helping to form a continuous luminal surface and seal the intercellular space (Kogut et al., 2017The intestinal epithelium, besides enterocytes, is also composed by specialized epithelial cells, including goblet cells, which are responsible for the production of mucin (the main component of the mucous layer), and Paneth cells, which secrete antimicrobial peptides (Bevins and Salzman, 2011). Furthermore, it is also possible to distinguish enteroendocrine cells, secreting hormones, and microfold or M cells, presenting antigens to the underlying lymphoid cells (Yang and Liao, 2019).

The epithelial cells continuously undergo to epithelium turnover. Cells cyclically exfoliate from the tips of the villi and are replaced by cells proliferating in the crypts, which mature during their movement toward the tip of the villi (Simon and Gordon, 1995). Villous height (V), crypt depth (C), and V:C ratio are reliable indicators of the overall health and functions of small intestine (Piva et al., 2001). Changes in V, C and V:C ratio have been reported both for poultry and swine, with diet having a great influence over these changes.

The most critical moment for GIT development for poultry is the post-hatching phase, when animals have to counteract the shift in the source of nutrients, moving from the yolk to an exogenous diet (Uni et al., 1998). This phase is characterized by rapid intestinal development, essential for a proper and efficient growth of the animals. Due to common practice related to hatching (vaccination, debeaking, sexing and transport to the farm), in this phase animals usually experience a period of fasting, commonly comprised between 24 and 72 h. However, the in this phase the GIT is very sensitive to the presence of feed for its proper development. Early access to nutrients and water is indeed fundamental to stimulate the activity of the GIT and the digestive

organs (Sell et al., 1991). Different studies observed depressed small intestinal enterocytes proliferation and migration, as well as reduced crypt and villus development, as a consequence of fasting after hatching (Geyra et al., 2001). The consequences of fasting vary according to the duration of the starving as well as to the intestinal segment considered, with jejunum and duodenum being the most sensitive (Geyra et al., 2001). For this reason, numerous strategies have been proposed to promote GIT development, ranging from specially designed post-hatch diets to *in ovo* feeding (Jha et al., 2019).

Similarly, GIT of pigs experiences drastic changes at weaning. Due to the stressors associated with this phase (e.g. separation from the mother, mixing of the litters), animals undergo a marked reduction of feed intake, which is responsible of insufficient nutrient supply. Consequently, the integrity of the small intestine is compromised, showing villous atrophy and crypt hyperplasia (Pluske et al., 1997), associated with a 20-30% reduction in mucosal weight (Lallès et al., 2004). Beside the insufficient feed or energy intake, alteration in the morphological structure of GIT have also been suggested to be caused by the physical form of the diet (abrasive action) (Hancock and Behnke, 2001), or to local transit hypersensitivity reaction caused by dietary antigens (Li et al., 1991). The changes in intestinal morphology lead to a decline of GIT functionality, reflecting reduced brush-border enzyme activity and absorption ability (Vente Spreeuwenberg et al., 2003), ultimately possibly resulting in diarrhoea and poor performance.

The epithelial cell lining, along with the mucus layer, composes the "upper barrier" of the GIT. They provide a physical barrier, preventing bacterial adhesion and paracellular diffusion to the underlying host tissues (Roda et al., 2010). The mucus layer acts as a fence between the mucosa and the resident microbiota, minimizing both microbial translocation and excessive immune activation by the same microbiota (Kogut and Arsenault, 2016). This structure overlying the epithelial cells can be further divided into two components: an outer layer, with a looser structure, where commensal microorganisms can colonize; and an inner layer, which on the contrary is characterized by a compact structure, repelling most bacteria (Hansson and Johansson, 2010). As reported by Montagne et al. (2003), the mucus layer is in a dynamic balance between physical, chemical, and enzymatic erosion on the luminal side, and mucin synthesis and secretion by epithelial cells. This equilibrium can be strongly influenced by dietary factors, such as fiber, protein and anti-nutritional factors, which are in turn deeply related to GIT microbiome. It is demonstrated that fiber deficient diets are finally responsible of thinning of the mucus layer, thus increasing the vulnerability of the host to pathogen invasion (Desai et al., 2016). The thinning of the mucus layer is due to the degradation and utilization of the mucin by polysaccharide degrading bacteria, as consequence of dietary fiber lack (Desai et al., 2016). In addition, microbial population plays a

fundamental role in the maintenance of mucus layer integrity also thanks to the production of short chain fatty acids (SCFAs). SCFAs, mainly butyrate, are indeed responsible of stimulating goblet cells proliferation and mucus production (Wrzosek et al., 2013).

Immune system

The GIT is considered to be largest immune organ, hosting more than 70% of the cells of the immune system (Vighi et al., 2008). These cells constitute an inner or functional immunological barrier, which cooperate with the aforementioned physical barrier, composed by the epithelial cells and the mucus layer (Pitman and Blumberg, 2000). The GIT mucosal immune system is continuously challenged and one of the major challenges is to regulate the host response to pathogens, while at the same time avoid responding to stimuli derived from commensal bacteria and feed antigens (Celi et al., 2017). With this purpose, several cells, belonging both to innate and acquired immune system, govern the interplay between the GIT microbiome and the same mucosal immune system, to maintain a healthy GIT, while detecting and eliminating pathogens. Besides the mucus layer and the antimicrobial secretion contained within it, other components of the innate response are dendritic cells, macrophages, neutrophils (heterophils in poultry) and NK cells, which are found in the lamina propria and within the epithelium (Broom and Kogut, 2018b). Cells of the innate immunity are the first line of defence and are able to distinguish between harmless and potential pathogens bacteria thanks to specific receptors, named Pattern Recognition Receptors (PRRs). Among PPRs, toll-like receptors (TLR) family plays a major role. TLRs activation is responsible for the initiation of a cascade, which leads to the activation of nuclear factor κB (NF-κB), finally leading to the production of pro-inflammatory cytokines, including tumor necrosis factor α (TNF- α), IL-1 and IL-6 (Bauer et al., 2006). Cytokines act as a linkage between innate and adaptive immune response, affecting distinct cells, such as dendritic cells precursors and B and T cells, to induce an adaptive immune response (Belardelli and Ferrantini, 2002). The adaptive immune response is primarily handled in the gut associated lymphoid tissue (GALT) (Purchiaroini et al., 2013). GALT is the largest collection of lymphoid tissues in the body and consists of lymphoid cells residing in the epithelial lining and in the lamina propria, as well as specialized lymphoid structures located in specific sites along the gut. GALT can be divided into inductive sites, i.e. organized lymphoid tissue, including Peyer's patches and mesenteric lymph node, and effector sites, i.e. diffused lymphoid tissue.

At birth, mucosal immune system of piglets is almost absent or immature, and gradually develops until reaching the complete maturity at around 7 weeks of age (Stokes et al., 2004). Bauer et al. (2006), based on available literature, distinguished 4 major phases in mucosal immune system

development. In the first phase, at birth, only low numbers of macrophages and granulocytes are found in villous and crypt region, and Peyer's patches are present in their primordial form. In the first two weeks of life (i.e. second phase), the intestine is rapidly colonized by lymphoid cells, and Payer's patches begin to adopt a structure, reaching an architecture similar to the adult one at around 10-15 days of age. The third phase takes place between the second and the fourth week after birth, when CD4+ T cells colonize the mucosa, primarily the lamina propria. At the same time, small number of B cells appear, mainly expressing IgM. Finally, from five weeks onwards also CD8+ and IgA+ B cells appear. It is therefore clear that the first 5 to 7 weeks after birth are a crucial moment for immune development and it follows that at the moment of weaning, usually occurring at 21-28 days of age, piglets are still immature from an immunological perspective. Since weaning is a stressful moment for piglets associated with activation of the GIT immune system due to the exposure to environmental and dietary antigens (McCracken et al., 1995; Pié et al., 2004), weaning age can have a strong influence on the development of the immune system (McLamb et al., 2013; Pohl et al., 2017).

As regard to poultry, at the moment of hatch the component of the innate immune response, namely macrophages and heterophils, are already present and fully functional, and their number increases with age (Broom and Kogut, 2018b). On the contrary, the number of lymphoid cells in the mucosa after hatching is very low (Bar-Shira and Friedman, 2006). Thanks to lymphocytes migration from the thymus, starting from 4 days after hatching their number gradually increases, until reaching the peak around approximately 8 weeks post-hatch (Vervelde and Jeurissen, 1993). GALT lymphocytes are functionally immature at the moment of hatching, and the functional maturation of B and T lymphocytes occur during the first and second week after hatching, following a biphasic pattern (Bar-Shira et al., 2003).

There is a great body of evidence that gut immune system functioning and development are largely dependent on microbial population, which in turn is strongly influenced by the diet. Composition and metabolic activity of the microbiota can indeed be affected by dietary changes (Yeoman and White, 2014). The essential role of the microbiome in immune system development was observed in several studies, where germ-free animals were found to have a lower immune response compared with animal with a live microbial community (Sun et al., 2018). Even though the pivotal role of the microbial population is well recognized, the mechanisms of action behind its influence on the immune system are still to be elucidated.

Gut microbiota

The GIT is populated by a complex and diverse community of bacteria, archaea, fungi, protozoa, and viruses. Among them, bacterial population is the most represented; and the strong existing interaction between microbiota and the host lead to their interpretation as a single "superorganism" (Eberl, 2010). Even though some microorganisms are pathogenic and cause diseases, the relationship between the microbial population and the host is usually symbiotic, being either commensal, where the bacteria are benefiting with no consequences for the host, or mutualistic, where both parts are benefiting (Dethlefsen et al., 2007).

Gut microbiota supports the digestive process, providing the breakdown and fermentation of otherwise indigestible nutrients, including indigestible carbohydrates. Non-starch polysaccharides (NSP) and resistant starch are the main sources of carbon and energy for the commensal microbiota, and their fermentation leads to the production of SCFAs, mainly butyrate, acetate, and propionate (Tellez et al., 2006). SCFAs, besides being an important energy source for intestinal epithelial cells, play a wide array of functions, including regulation of the intestinal blood flow, stimulation of enterocytes growth and proliferation, mucin production regulation, as well as modulation of the intestinal immune response (Scheppach, 1994; Cummings et al., 2004). Furthermore, intestinal microbiome is responsible for the production of vitamins, mainly belonging to the B group (LeBlanc et al., 2013), and is also involved in nitrogen metabolism (Davila et al., 2013).

As previously mentioned, the GIT is provided with a physical barrier, composed by epithelial cells, tight junction, mucin, and antimicrobial peptides. Gut microbiota can interact with all these components, thus actively participating to the defence of the GIT (Willing and Van Kessel, 2010). Besides contributing to the physical barrier, gut microbiome can also directly influence the mucosal immune response. Oakley and Kogut (2016), found a significant association between the microbiome and the expression of genes regulating the immune response in broiler chickens. They observed a negative correlation between the phylum *Firmicutes* and the expression of pro-inflammatory cytokine genes; while a positive correlation was observed between pro-inflammatory cytokine and the phylum *Proteobacteria.* Furthermore, the interaction between gut microbiome and host innate immune system can influence the subsequent adaptive immune response. It is speculated that probiotics can be responsible of stimulating Th2 cytokines production, which lead to the enhancement of antibody-mediated immune response (Haghighi et al., 2005; Pan and Yu, 2014).

Gut microbial population is characterized by a dynamic equilibrium between beneficial commensal bacteria and potentially pathogenic bacteria. The first defence mechanism against

pathogens is known as "competitive exclusion" (Gabriel et al., 2006), by which commensal bacteria colonize the intestinal mucosa, forming a layer of bacteria that covers the mucosal surface (Pan and Yu, 2014). The presence of commensal bacteria therefore impedes pathogens to attach and entry into the cells. Furthermore, indigenous bacteria compete with exogenous microbes both by competing for the nutrients (e.g. Zinc, Gielda and DiRita, 2012), and by secreting bacteriostatic and bactericidal substances, such as SCFAs (Ciarlo et al., 2016). In addition, some bacteria also produce bacteriocins, to selectively inhibit the growth of other bacteria (Teo and Tan, 2005; Shin et al., 2008).

Gut microbiome is distinct in each different region of the GIT, varying according to the function and the physiological characteristics of that specific tract. The composition and diversity of gut microbial population evolves over time, starting from the first day of life, until reaching a final equilibrium (although still dynamic), in adult animals (Isaacson and Kim, 2012).

The development of poultry intestinal microbiome begins at the moment of hatching, which usually takes place in commercial facilities. Differently from other livestock species, young animals are separated from the parents, therefore the first microbial population encountered by these animals is derived from environmental sources (Stanley et al., 2014). Gut microbial population rapidly grow and fluctuate after hatching. Apajalahti et al. (2004) reported a bacterial density in the ileum and cecum of 10^8-10^{10} cells/g at 1 day of age, which further grew to 10^{11} cells/g digesta by day 3. Gut microbiota undergoes continuous changes throughout the maturation, being influenced by several factors, such as genetics, sex, diet, and the environment (Zhu et al., 2002). Initially, avian gut is colonized by *Enterobacteriaceae, Lactobacillus,* and *Streptococcus* (Wise et al., 2007), while in adult animals the most predominant genera found are *Clostridium, Ruminococcus, Lactobacillus* and *Bacteroides* (Wei et al., 2013).

As regard to swine, the situation is slightly different. New-born piglets are in strict contact with the mother, therefore the first microbial population encountered is not only deriving from the environment, but also from the sow (Thompson et al., 2008). Firstly, gut is colonized by aerobic and facultative anaerobes, which create an environment suitable for subsequent colonizer, including *Bacteroides, Bifidobacterium, Clostridium,* and *Lactobacillus* (Konstantinov et al., 2006; Petri et al., 2010). This microbial population, which is strongly influenced by the milk of the mother (Frese et al., 2015), remains almost stable until the moment of the weaning. At this moment, the shift toward a cereal-based diet causes major perturbation in gut microbiota, which experience a profound decrease in population biodiversity, and a decrease in *Lactobacillus* spp. (Mach et al., 2015). On the contrary, *Clostridium* spp., *Prevotella* spp., or other facultative anaerobes such as *Proteobacteriaceae,* including *E. Coli,* are positively impacted (Gresse et al., 2017).

Several factors, including stress, changes in feeding practice, imbalanced diets and antibiotics can impact gut microbiome (Bauer et al., 2006; Burkholder et al., 2008). However, even though the microbial population of adult animals can be temporally modified thanks to the retain of a certain degree of plasticity, there is a growing evidence that the first microbial population with which animals get in contact can have long-lasting effect on animals' life (Ballou et al., 2016; Broom and Kogut, 2018a). For this reason, there is a growing interest in the investigation of early nutrition programming, including for example *in ovo* and post-hatch feeding for poultry, to shape the intestinal microbiota, aiming to enhance animals' health and performances (Jha et al., 2019; Warne et al., 2019).

Feed additives

One of the main challenges in poultry and livestock production is to maintain optimal gut condition to enhance animal health and performances. In the past, this objective was met with the employment of antibiotics, whose administration at sub-therapeutic dosage was responsible of growth promoting effects. Even though the mechanism of action residing behind the growth promoting effect of antibiotics is still not completely elucidated, it is speculated that their efficacy is due to a reduction in the overall number or diversity of the gut microbiota (Francois, 1961; Visek, 1978). This reduction results in decreased nutrient competitions and reduced production of microbial metabolites, which can affect animal's growth (Knarreborg et al., 2004). Another theory was proposed by Niewold (2007), by which the beneficial effects of antibiotic growth promoters (AGPs) are due to the reduction of proinflammatory cytokines production. This way, by lowering the inflammatory response, reduced appetite and muscle catabolism fade away, allowing all the energy to be directed towards production. However, the general concern about the risk of antibiotic-resistance development led to the ban of AGPs by the European Union in 2006. Starting from this moment, increasing interest towards possible alternatives began to grow, leading to the investigation of numerous additives. Feed additives, among which probiotics, prebiotics, exogenous enzymes, and organic acids are the most employed, aim to mimic the beneficial effect of AGPs in the gut, therefore they aim to 1) enhance the immune response; 2) reduce pathogen load in the gut; 3) stimulate the establishment of a beneficial gut microflora; and 4) stimulate digestive function (De Lange et al., 2010).

Phytobiotics administration in broiler chickens: the case of green tea and pomegranate

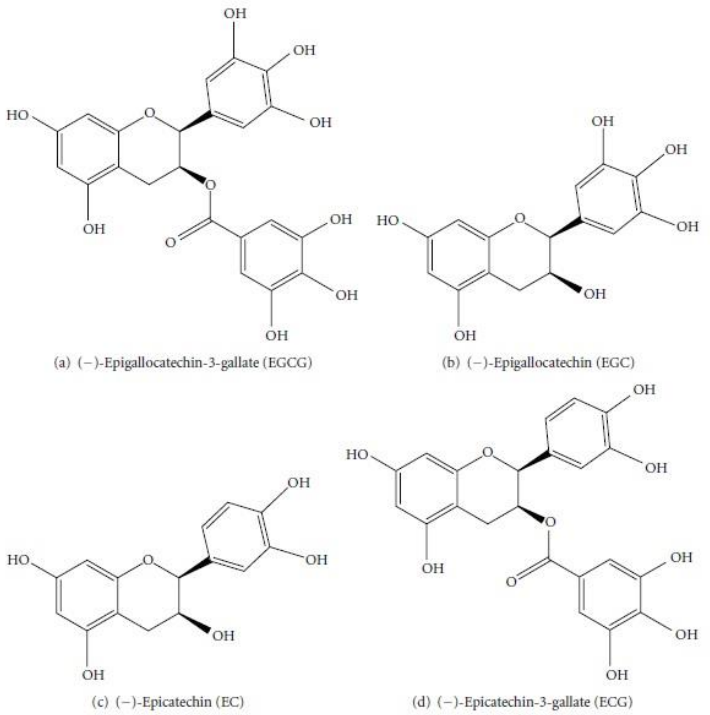
Phytobiotics (PH) or botanicals, are a diverse group of natural ingredients derived from herbs, spices, fruit, or other plants, which can exert positive effects when supplemented in the diet of livestock animals, improving health and production efficiency (Windisch et al., 2008). According to their biological origin, formulation, chemical description and purity, they can be classified in 4 groups: 1) herbs (products from flowering, non-woody and non-persistent plants); 2) botanicals (entire or processed parts of a plant, such as roots or leaves); 3) essential oils (hydro distilled extracts of volatile plant compounds); and 4) oleoresins (extracts based on non-aqueous solvents) (Windish and Kroismayr, 2006). The beneficial effects of PH administration can be reconducted to the modulation of gut health components. PH have been reported to maintain and improve gut morphology, increasing villi height and thus expand absorptive surface of the intestine (Ghazanfari et al., 2015). Furthermore, PH can modulate digestive enzyme secretion, e.g. improving trypsin, maltase, and pancreatic amylase (Jang et al. 2004).

PH, mainly essential oils containing phenolic structures (e.g. carvacrol and thymol), can modulate gut microbiota equilibrium, exerting antimicrobial activity (Dorman and Deans, 2000). It was suggested that the antimicrobial activity of hydrophobic essential oils is attributable to their ability to intrude into the cell membrane of pathogen, damaging the membrane and therefore leading to ion leakage (Burt, 2004). PH also take part in the modulation of the host defence. Some studies demonstrated their ability to stimulate intestinal mucus production, thus contributing to inhibit pathogens adherence to the mucosa (Mohammadi Gheisar and Ho Kim, 2018). Furthermore, PH can have immunomodulatory effects, including increased proliferation of immune cells, elevated expression of cytokines, and increased antibody titres (Pourhossein et al., 2015). Besides the aforementioned properties, PH show also antioxidant properties, which are mainly attributable to polyphenolic compounds, which prevent lipid peroxidation of the membranes, while maintaining the correct level of glutathione in the cells (Ognik et al., 2016). PH are therefore employed both for improving antioxidant defence of the animals, while at the same time improving quality of animals' derived product.

PH found a large interest in poultry sector, which was indeed one of the most affected by the ban of AGPs. Several herbs, spices (e.g. thyme, oregano, rosemary, marjoram, garlic, ginger, black cumin, coriander, and cinnamon) and essential oils (e.g. carvacrol, thymol) have been tested over years for their potential application as AGPs alternatives, assessing their effectiveness in improving productive performance and animal health. Some work has been made also on green tea and pomegranate.

Green tea is the dried, unfermented product of leaves from the *Camelia sinensis* species of the *Theaceae* family. Numerous beneficial properties have been attributed to green tea, including antioxidant, anticoccidial, antiviral, and immunomodulatory properties, as well as the ability to modulate gut microflora and enhance animal performances (Cabrera et al., 2006). The aforementioned beneficial effects of green tea are ascribed to its high content of polyphenols, among which catechins are the most represented (Singh et al., 2011; Figure 2). However, the administration of green tea products to broiler chickens showed contrasting results, which can be related to the composition, type, and origin of the employed products, the production process, the way and dosage of administration, as well as animal and environmental condition during the experiment (Franz et al., 2010).

Figure 2*. Structure of green tea polyphenols (Source: OyetakinWhite et al., 2012)*



Several authors reported improved animal performances due to green tea powder administration in broilers diet (Kaneko et al., 2001; Sarker et al., 2010; Liu et al., 2018). Improved body weight and feed efficiency were also reported due to dietary administration of green tea in the form of extract (Erener et al., 2011). Rowghani and colleagues (2016) reported similar results when green tea was administered via drinking water. On the contrary, other researchers observed no effect on growth performances with either green tea powder or extract (Cao et al., 2005; Afsharmanesh and Sadaghi, 2014; Farahat et al., 2016). Chen et al. (2019a) observed reduced body weight in the first

21 days of administration of green tea powder included in the diet at 1%, even though the opposite trend (increased body weight) was observed in the last half of the trial (21-42d). Similarly, reduced feed intake and body weight gain was reported by Jelveh and colleagues (2018) with the administration of 10, 20, 30 or 40 g/kg of green tea powder. However, the same authors reported no negative effect when green tea was administered at the same inclusion rate in the form of extract. Positive effects of green tea administration were also reported for carcass weight and dressing percentage (Erener et al., 2011; Liu et al., 2018; Chen et al., 2019a). However, in other experimental trials reduced relative weight of breast, carcass and drumstick were reported with the administration of green tea powder (Jalveh et al., 2018).

In terms of carcass quality, beneficial effects of green tea were reported as regard to fat content. Several authors observed reduced abdominal and subcutaneous fat associated with the administration of both green tea powder and extract (Huang et al., 2013; Liu et al., 2018; Chen et al., 2019a). Equally, improvement of carcass quality traits was reported as well (Sarker et al., 2010; Erener et al., 2011). It is evident a role of green tea in the modulation of lipid metabolism, which has been corroborated at plasmatic levels, with reduced level of total cholesterol, triglycerides and HDL following green tea administration (Huang et al., 2013; Afsharmanesh and Sadaghi, 2014). Huang and colleagues (2013) reported reduced gene expression of lipid anabolism genes, while the expression levels of fat transportation and catabolism-related genes were notably upregulated.

Farahat et al. (2016) confirmed antioxidant properties of green tea, reporting reduced glutathione-reduced levels in the liver and reduced malondialdehyde (MDA) in the meat. Analogously, reduced TBARs levels and increased oxidative stability were reported for green tea by-product supplementation (Yang et al., 2003).

Immunomodulatory properties have been recognized to green tea as well. The administration of green tea extract in the diet of broiler chickens increased specific antibody titre against Newcastle disease virus vaccines (Farahat et al., 2016). Equally, increased antibody titre against avian influenza were reported by Lee et al. (2012) and Rowghani et al. (2016), following the supplementation of green tea extract either in the feed or in the water, respectively. It was indeed reported the ability of catechins to alter the infectivity of influenza virus, both by specific interaction with viral hemagglutinin (HA), and with viral RNA synthesis in the cells (Song et al., 2005; Lee et al., 2012). As regard to catechin, the supplementation of L-theanine (an amino acid extracted from green tea) reduced mRNA expression of TLR-2 and TLR-4 and some cytokines (TNF-α, INF-γ and IL-2) (Saeed et al., 2018).

A beneficial modulatory effect of green tea on intestinal microbial population was reported by several authors, with increased *Lactobacillus* and decreased coliform counts (Erener et al., 2011;

Saeed et al., 2018; Chen et al., 2019b). On the contrary, other researchers observed a negative effect on gut microbiota. Green tea polyphenols administration reduced bifidobacteria, bacteroidaceae, Peptococcaceae, lactobacilli, Eubacteria, and lecithinase-positive bacteria (Cao et al., 2005). Chen et al. (2019b) recorded higher abundance of potentially pathogenic *Gallibacterium* in gut of animals supplemented with green tea powder.

Finally, a potential role of green tea in the modulation of coccidiosis was highlighted as well (Jang et al., 2007), with a dose-dependent increased cellular and humoral immunity against coccidia (Abbas et al., 2017).

Pomegranate, *Punica granatum*, is a fruit belonging to the Punicaceae family and has been used for years in traditional medicine. Several beneficial properties are recognized to pomegranate fruits and products, which are attributed to the presence of considerable amounts of polyphenols, including punicalagin, ellagic acid and anthocyanins, predominantly found in the peel (Reddy et al. 2007). Pomegranate has been tested as feed additive for poultry in different forms, including seed or peel extract, seed oil, peel meal of fermented by-products.

Numerous studies reported beneficial effect over animal performances, independently from the form of supplementation. Ahmed et al. (2017a, 2017b) reported linear increase of animal performances (ADG, ADFI, F:G) with fermented pomegranate by-products supplementation included in the diet at the rate of 0.5, 1 or 2 %. Similarly, enhanced weight gain, feed intake and feed efficiency were observed following the supplementation of either pomegranate peel extract or pulp (Hosseini et al., 2014; Hamady et al., 2015). On the contrary, other studies reported no influence of pomegranate on growth performances (Rajani et al., 2011; Rao et al., 2019). Contrasting results are reported also for slaughtering parameters. Hamady et al. (2015) and Hosseini et al. (2014) observed improved carcass weight and dressing percentage with pomegranate peel and pulp supplementation, while other studies showed no effect (Ahmed et al., 2017a).

Thanks to its bioactive compounds, pomegranate can be considered a functional ingredient, and several studies attested its role in improving meat quality. The supplementation of fermented by-products increased nutritional quality and acidic profile of poultry meat (Ahmed et al., 2017a). Crude protein, Fe, Mg and Na linearly increased in breast meat, while cholesterol was found to be reduced. In the same study, lower amount of saturated fatty acids and increased amounts of monounsaturated fatty acids in breast, and n-3 fatty acids in breast and tight, were observed (Ahmed et al., 2017a). Similarly, higher level of n-3 fatty acids were reported with pomegranate peel extract administration (Saleh et al., 2018). Pomegranate seed oil administration increased deposition of c9,t11 conjugated linoleic acid (CLA) in adipose tissue (with a concentration-dependent pathway, Manterys et al., 2016) and in breast meat (Szymczyk, B. and Szczurek, 2016).

Banaszkiewicz et al. (2018) reported deposition in the meat of punicic acid, then converted in rumenic acid, thus enhancing health-promoting properties of the meat. Modification of the lipid metabolic profile were also reported, with reduced level of cholesterol, triglycerides, and LDL, both in blood, liver, and heart (Manterys et al., 2016; Saleh et al., 2018).

Antioxidant properties of pomegranate were confirmed by reduced TBARs levels and increased total phenolic content and antioxidant activity in breast meat of animals supplemented with pomegranate fermented by-products or peel extract, respectively (Ahmed et al., 2017a; Saleh et al., 2018). Pomegranate peel meal was found to reduce lipid peroxidation level and increased glutathione-peroxidase (GSPHx) activity in the liver; while glutathione-reductase and superoxide dismutase were not affected (Rao et al., 2019).

Immunomodulatory effects were observed with fermented by-products and peel extract administration, enhancing the immune response, with increased IgG and IgA levels (Ahmed et al., 2017b; Saleh et al., 2018). Rao et al. (2019) also reported an increased titre against Newcastle disease virus, even though cell-mediated immune response versus phytohemagglutinin phosphate was not influenced.

Finally, pomegranate also showed potential gut microflora modulation effects. Ahmed et al. (2017b) observed positive effects in the modulation of *E. Coli* and *Salmonella* at different levels. In ileal digesta both pathogens were reduced, independently from the additive inclusion level. Differently, at cecum level only *E. Coli* was found to be reduced, and only when the product was included at 1% in the diet. Other studies reported increased *Lactobacillus* count in the cecum (Rezvani et al., 2018), and inhibitory activity against Gram+ and Gram-, comparable to ampicillin effect (Hamady et al., 2015).

Nucleotides administration to piglets

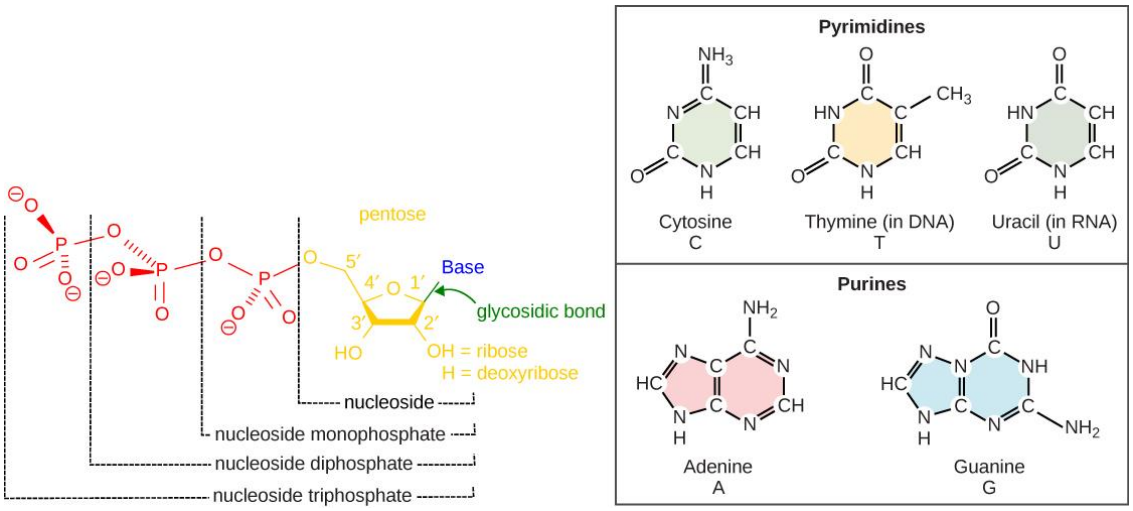
Nucleotides are a group of bioactive compounds playing several roles in biochemical processes. They are composed by a nitrogenous base, a pentose sugar and one or more phosphates (Figure 3). Nitrogenous bases can be either pyrimidine, which include cytosine (C), thymine (T) and uridine (U), or purine, which include adenine (A), guanine (G) and hypoxanthine (I). A molecule composed by a base and a pentose sugar (ribose or deoxyribose) is defined nucleoside. Phosphate can be bonded either to C3 or C5.

Animals' nucleotides requirement can be met by three different sources: *de novo* synthesis, salvage pathway and diet. *De novo* synthesis is a metabolically costly process, which requires substantial amount of energy, specifically 6 molecules of ATP for purine synthesis and 4 molecules of ATP for pyrimidine synthesis (Carver and Walker, 1995). Salvage pathway is characterized by linkage of a ribose phosphate moiety to free bases formed by hydrolytic degradation of nucleic acid and nucleotide (Sauer et al., 2011). This mechanism allows recycling more than 90% of purine bases. Nucleotides are also introduced with the diet, being present in most feed ingredients of both animal and plant origin (Mateo and Stein, 2004). Dietary nucleotides become essential nutrients in stressful moment. At weaning, due to the high intestinal epithelial turnover and the development of the immune system, nucleotides requirement is strongly marked. However, their *de novo* synthesis is limited due to weaning anorexia, which is responsible of energy and glutamine supply depletion (Waititu et al., 2016). Dietary nucleotides requirements at the moment of weaning are further increased due to the switch from maternal milk to weaning diet. Sow's milk is indeed a rich source of nucleotides (around 2715 ppm), while weaning diets are usually reduced in nucleotides content (Mateo et al., 2004).

Nucleotides have several biochemical functions, first of all being precursor of nucleic acids (DNA and RNA). They are the common currency of metabolic energy transfer in the form of ATP; they act as co-enzyme, and they participate in biological regulations, such as cyclic adenosine monophosphate (cAMP), which is a second messenger for intracellular signal communication. Besides biochemical functions, nucleotides also participate to the regulation of various biological reactions.

A beneficial effect of nucleotides administration in post-weaning piglets have been observed for intestinal development and function. Several authors observed a positive effect on gut morphology, recording higher villous high and crypt depth compared to animals receiving a control diet (Martinez-Puig et a., 2007; Moore et al., 2011; de Andrade et al., 2016). The trophic action of nucleotides in the GIT was recently confirmed by the observed accelerated carbon

Figure 3. *Schematic representation of nucleotides composition*



turnover, both in the stomach and intestine of weaning piglet receiving a diet supplemented with 1% nucleotides (Amorim et al., 2017; Assoni et al., 2017). In addition, higher expression of tight junction proteins (Claudin-1 and ZO-1) was reported in the ileum of piglets receiving nucleotides (Che et al., 2016). Positive effects of nucleotides were reported for intestinal function as well, including higher lactose and maltase enzymatic activity in jejunum of weaned piglets (Che et al., 2016). On the contrary, other authors observed no significant effect on intestinal morphology (Andrés-Elias et al., 2007; Sauer et al., 2012b; de Andrade et al., 2016) or enzyme activity (Sauer et al., 2012c; Sauer et al., 2012a). Lee et al. (2007) reported decreased ileal maltase, sucrase and aminopeptidase activity, even though increased gastric pepsin and jejunal alkaline phosphatase activity were enhanced.

Dietary nucleotides may also affect immune function in pigs. Domeneghini et al. (2004) reported higher percentages of macrophages in the intestine and higher intra-epithelial lymphocytes in the mucosa of weaning piglets receiving nucleotides at 0.05% in the diet. An increase in the peripheral count of leukocyte was observed as well (Che et al., 2016). An immunomodulating effect indicated by increased levels of plasma immunoglobulins was reported by several authors, even though with some differences. Li et al. (2015) observed increased serum IgA and IgM in piglets receiving nucleotides at the dosage of either 150, 220 or 275 mg/kg. On the contrary, when nucleotides were supplemented in the water, Sauer et al. (2012b) reported a positive modulation only for IgA, while no differences were recorded for IgG and IgM. According to Cameron et al. (2001), the effect of nucleotides on cell function and proliferation is strongly dependent on duration of the supplementation, since the immune-enhancing effect seems to require at least 14 days of administration to be effective.

Even though the important role played by nucleotides in modulating pigs' gut health, only few studies are available as regard to their modulation over intestinal microbiota. Waititu et al. (2017), after supplementation of nucleotide-rich yeast extract, observed positive modulation of gut microbiota in piglets under a sanitary challenge. Piglets raised in cleaned room, reported reduced Enterobacteriaceae in the cecum, and *Enterococcus* in the colon, while *Lactobacillus* spp. and *Clostridium* cluster IV and XVI members were increased in cecum and colon, respectively. Similarly, in challenged animals (i.e. raised in unclean room), cecal *Clostridium* cluster IV was increased, while Enterococcus was reduced in the colon (Waititu et al., 2017). Similar beneficial effects were observed by Li and colleagues (2015), with reduced fecal *E. Coli* counts and fecal score. Conversely, other authors observed no effect of nucleotides on gut microflora (Sauer et al., 2012b; Sauer et al., 2012c).

As result of the aforementioned functions played by nucleotides, beneficial effects could be expected also on animals' growth performances, even though results are inconsistent. Zomborszky-Kovacs et al. (2000) observed increased body weight and feed intake after nucleotides supplementation. Similarly, Superchi et al. (2012) reported increased body weight, but no effect on feed intake. Nucleotides supplementation in the water increased ADFI, but no effects were observed on ADG (Sauer et al., 2012b). At the same time, in several studies nucleotides supplementation failed to improve growth performances (Domeneghini et al., 2004; Lee et al., 2007; Martinez-Puig et al., 2007; De Andrade et al., 2016).

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Chapter 2

Study objectives

The world growing population along with the shift in the consumption pattern is challenging the livestock sector to meet the increasing demand of animal products. Whether in the past this request was addressed increasing the number of raised animals, nowadays this approach is no longer pursuable. Therefore, the interest has been moved to improve animals' performances, and to this end nutrition provides a valuable asset. Animals' performances can indeed be enhanced through nutritional approaches, including both the application of technologies in a context of 'Precision Nutrition' and the administration of novel feed additives or feedstuffs with beneficial effects on animals' health.

The objectives of this thesis were to evaluate:

- a **technological approach** to improve the efficiency of feeding systems in dairy cows farming. The diet for dairy cows is usually provided as total mixed ration (TMR), whose preparation relies on the employment of dedicated mixer wagons. To obtain the beneficial effects associated with TMR feeding, it is essential for the diet to be homogeneous. Indeed, non-uniform diets can impair nutrient supply, finally affecting cows' performances and health. However, the homogeneity of the ration is often subdued to the efficiency of the mixer wagons. Therefore, the aim of the *first trial* was to assess how loading levels of the mixer wagon, cutting time and mixing time can influence the homogeneity of the ration along the feeding alley, and to identify the ideal combination of these factors to ensure a homogeneous distribution.

  Besides ensuring the homogeneity of the TMR, it is also essential to guarantee that the diet provided to the cows actually reflects the diet formulated by the nutritionist, satisfying animals' nutrients requirements. Since nutrient supply of the delivered TMR might be affected by the daily variations of feedstuffs (mainly silages), the aim of the *second trial* was to develop a sensory system to provide a real-time adjustment of the silages inclusion rate according to their actual dry matter content during the preparation of the ration;
- a **nutritional approach** to improve animals' health and performances. With the aim to improve farm profitability, swine and poultry production cycle have been progressively shortened, pushing the animals to achieve the commercial weight in shorter time. However, this approach is responsible of health-related problems, mainly concerning the gastrointestinal tract. In order to overcome these problems, antibiotics have been largely employed over the years, but due to the risk of antibiotic resistance development their use has been prohibited since 2006. From that moment onward the interest of the research has been focused to identify suitable alternatives, mainly aiming to improve gut health. For this

purpose, we evaluated the effectiveness of a natural product based on green tea and pomegranate extract (*third trial*) and nucleotides (*fourth trial*) to improve animals' health and performances.

Chapter 3

Influence of different loading levels, cutting and mixing times on total mixed ration (TMR) homogeneity in a vertical mixing wagon during distribution: a case study

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ABSTRACT

The present study investigated the influence of different loading levels, cutting and mixing times on total mixed ration (TMR) homogeneity delivered along the feeding alley. The TMR homogeneity along the alley was investigated according to three mixing wagon loads (40, 70 and 100% of the nominal capacity), three cutting times (4, 5 and 6 min), and three mixing times (4, 5 and 6 min). The diet (70:30 forage/concentrate ratio) was supplied by a two-screws vertical mixing wagon (maximum nominal capacity, 21m^3). A preliminary variance analysis of chemical parameters was performed on samples collected in the mixing wagon. Samples of released TMR were taken at the beginning, in the middle and at the end of the feeding alley (50 m long). The chemical parameters of the diet revealed no significant effects on the homogeneity of the samples for cutting time ($P>0.05$). Mixing wagon loading affected CP ($P<0.05$) and NDF content ($P<0.01$), while mixing time influenced DM of the diet ($P<0.05$). The loading level of the mixing wagon affected the difference of the collected samples from the formulated diet for ash content. The 70 % of the nominal capacity load of the mixing wagon showed the lower difference values between expected and chemically determined NDF ($P<0.01$), CP ($P<0.05$) and Ash ($P<0.05$) content in the diet. The DM and NDF differences along the alley, affected by mixing times ($P<0.05$), showed a poor uniformity of ingredients during distribution, although samples uniformity at wagon level.

INTRODUCTION

The total mixed ration (TMR) system for dairy cows nutrition was introduced to provide a consistent supply of nutrients to rumen microbes, to optimize rumen function, and improve the efficiency of nutrient utilization although in some circumstances it may not accurately reflect the formulated diet, slightly varying day by day (Sova et al. 2014). Together with the balance between the components of the diet to allow suitable rumen condition, the feed particle size distribution and the physical effectiveness of the diet lead to adequate rumination stimuli and intensity of rumen fermentation (Zebeli et al., 2011). In this view, TMR homogeneity and feed particle size distribution are of primary importance in dairy cow nutrition, but these elements are often subdued to the efficiency of the mixing wagon that actually can be limited by the loading level, cutting and mixing times among other factors (Buckmaster, 2009).

TMR is formulated to obtain a homogeneous and balanced ration for all the components in a single solution (Baumgard et al., 2017). This type of diet forecasts the carrying out of an array of operating machines with the mixing wagon as most important machinery. The modern cutter-mixer wagons derive from the evolution of the first mixer wagons, from which they differ for the equipment for trimming long stemmed products (i.e. hay and straw) and mixing them with the other feedstuffs of the ration.

The simultaneous administration of all nutrients allows for greater stability of ruminal pH, hence increasing productive performance and health of dairy cattle, avoiding rumen acidosis (Mäntysaari et al., 2006; DeVries et al., 2005).

The basic assumption for obtaining the benefits associated with TMR rationing is the homogeneous mixing of feeds. Non-uniform diets, from the nutritional and physical points of view, can affect feed intake and can give the animals the possibility to select, with possible negative effects on their performance (Kmicikewycz et al., 2015). The characteristics of the cutter–mixer wagon and its rational use are the basis for a correct preparation of the ration.

The available literature reports studies about the efficiency of mixing wagons (Šístkov et al., 2015; Vegricht et al., 2007), mainly describing the procedure of loading, cutting and mixing adopted in the farms.

Nowadays, information about an 'ideal combination' of loading level, cutting and mixing times are still missing.

For the above described reasons, the aim of this trial was to determine the influence of different combinations of loading levels, cutting and mixing times on homogeneity of dairy cows total mixed ration (TMR) along the feeding alley.

MATERIAL AND METHODS

Experimental design

The present trial was run in a dairy Friesian Holstein cows farm, 400 animals, with 180 lactating cows, and 220 dry cows, heifers and calves. The dairy farm is located in Lodi area (Northern Italy), an area strongly addressed to intensive animal production.

The study was carried out using a two-augers vertical type TMR mixing wagon (Grizzly 8100 model 8122/2, Sgariboldi, Italy), with a nominal maximum capacity of 21 m^3. The technical characteristics of the mixing wagon were the following: driveline reduction ratio 1:16, clockwise augers rotation, auger speed of 24 rpm (cutting) and 38 rpm (mixing).

The mixing wagon distributed TMR on the feeding alley at a uniform speed of 1.5 km/h. The experimental design was set up to account for the loading level of the mixing wagon (40%, 70% or 100% of maximum nominal capacity), the cutting time of roughage after the load of long stemmed hay (4, 5 or 6 min.), and the mixing time of TMR after the load of the last ingredient (4, 5 or 6 min). Factors were combined during the 15-day trial, as reported in Table 1.

The feed loading sequence was the following: long-stem hay (cutting), corn meal, concentrate, silage and molasses. During the trial ingredients were drawn from the same bulks, and silage humidity varied in a not significant way (CV less than 3 %).

Every day of the trial, the weight of every feed introduced was checked by positioning the mixing wagon on the weighbridge scale of the farm (± 0.02 - 0.04 % precision) to make a double check with wagon weigh precision.

The TMR was formulated for 60 late lactating (more than 200 days in milk) Friesian Holstein cows, see Table 2.

Five TMR samples (200 g each) were collected for each day of the trial in five different points of the wagon hopper at the end of the cutting-mixing procedure, according to CE rule N. 152/2009, modified by CEE n. 691/2013 rule. Five TMR samples (200 g each) were collected from the beginning (0 m), in the middle (25 m) and at the end (50 m) of the feeding trough, as released diet and for every combination. For each of the 15 days of the experimental study, 20 TMR samples (five in the wagon, and 15 in the three points along the alley) were collected, for a total of 300 samples to be analysed.

The chemical composition of each TMR samples was analysed to determine dry matter DM (method 930.15), crude protein CP (method 984.13), ether extract EE (method 920.39A), ash (method 942.05) content following the relative Association of Analytical Communities official methods of analysis (AOAC, 2005). Neutral detergent fiber NDF was measured through Van Soest

method (AOAC. Official Methods of Analysis. 18th ed. Gaithersburg: Association of Official Analytical Chemists, International; 2005).

The research was performed in full compliance with all relevant codes of experimentation and legislation.

Table 1*. Experimental design*

Day	Loading level*	Cutting time (min)	Mixing time (min)
1	40	4	4
2	40	4	6
3	40	5	5
4	40	6	4
5	40	6	6
6	70	4	4
7	70	4	6
8	70	5	5
9	70	6	4
10	70	6	6
11	100	4	4
12	100	4	6
13	100	5	5
14	100	6	4
15	100	6	6

* % of the nominal capacity of the mixing wagon

Statistical analysis

A preliminary analysis of variance, a General Linear Model (GLM) procedure of SAS statistical package (SAS v. 9.2, 2016), was performed on the samples collected in the mixing wagon, to highlight potential de-mixing occurring in the wagon, after cutting and mixing, before feed release, for each day of the study. The sample was the experimental unit, for each sampling day. The obtained mean value was considered the reference for the TMR samples collected along the feeding alley, for the variance analysis described as follows.

The uniformity of the diet released along the alley was evaluated through variance analysis, General Linear Model (GLM) procedure of SAS statistical package.

The following items, i) loading level of the mixing wagon (100%, 70%, 40% of maximum nominal capacity); ii) cutting time of roughage (4, 5 or 6 min.); iii) mixing time of TMR (4, 5 or 6 min) were

considered independent variables in the model, affecting TMR uniformity, or DM, CP, EE, NDF, and ashes values differences of the samples collected at the feeding trough (5 samples at the beginning 0 m; five samples in middle 25 m; 5 samples at the end, 50 m) from respective chemical parameters of the diet collected in the wagon.

The interaction loading level*cutting time*mixing time*sample point was considered in the model to evaluate the 'ideal combination' of the three parameters.

Level of significance was adopted for $P<0.05$.

Table 2. *Composition of the diet and chemical composition analysis of the TMR*

Feed	kg
Corn silage	21.5
Concentrate	8.2
Meadow hay	2
Alfalfa hay	2
Ryegrass hay	1.7
Corn meal	1.4
Molasses	0.7
Total amount of TMR/cow	37.5
Chemical composition	% DM
DM (% as fed)	54.3
CP	12.8
EE	2.6
NDF	35.1
Ash	6.8

RESULTS AND DISCUSSIONS

TMR samples collected in five different points of the wagon hopper at the end of the cutting-mixing procedure did not show significant differences for chemical parameters content, per each sampling day.

In table 3, the Least Square mean values and the Standard Error of the Means (SEM), calculated through variance analysis, of chemical parameters of samples diet released to cows, according to the loading level of the mixing wagon (100 %, 70 %, 40 %), cutting and mixing times are reported.

Table 3. *Chemical values (LS means and SEM) of samples collected on the alley according to loading level, cutting and mixing time (n= 225)*

Loading level*	Cutting time (min)	Mixing time (min)	DM		Ash		CP		EE		NDF	
			% as fed	SEM	%DM	SEM	%DM	SEM	%DM	SEM	%DM	SEM
40	4	4	52.15	1.01	6.64	0.26	10.98	0.61	2.38	0.14	40.17	1.97
40	4	6	54.03	1.01	7.83	0.26	12.27	0.61	2.73	0.14	39.92	1.97
40	5	5	55.47	1.01	6.87	0.26	14.97	0.61	2.79	0.14	38.15	1.97
40	6	4	55.81	1.01	6.81	0.26	12.55	0.61	2.47	0.14	37.24	1.97
40	6	6	56.82	1.01	6.58	0.26	12.19	0.61	2.46	0.14	40.21	1.97
70	4	4	53.91	1.01	6.84	0.26	13.42	0.61	2.69	0.14	33.35	1.97
70	4	6	54.00	1.01	6.89	0.26	13.48	0.61	2.65	0.14	35.47	1.97
70	5	5	55.4	0.58	6.92	0.15	12.45	0.35	2.45	0.08	36.19	1.14
70	6	4	56.09	1.01	6.23	0.26	11.99	0.61	2.46	0.14	35.54	1.97
70	6	6	52.04	1.01	7.05	0.26	12.51	0.61	2.58	0.14	34.74	1.97
100	4	4	56.27	1.01	7.20	0.26	11.82	0.61	2.26	0.14	35.56	1.97
100	4	6	54.00	0.71	6.94	0.18	14.09	0.43	2.86	0.10	37.67	1.39
100	5	5	53.33	1.01	6.29	0.26	12.51	0.61	2.57	0.14	39.21	1.97
100	6	4	55.12	1.24	7.09	0.32	13.67	0.75	2.71	0.17	39.89	2.43
100	6	6	54.87	1.01	6.52	0.26	11.22	0.61	2.70	0.14	36.77	1.97

* % of the nominal capacity of the mixing wagon

Figure 1 shows the mean values (and SEM) of the difference between the diet sampled in the wagon and the diet collected along the feeding alley, according to the different combinations of loading level, cutting and mixing time

Figure 1. *Chemical parameters difference between the diet sampled in the wagon and the collected samples along the feeding alley (on the x axis, from the bottom, loading level, cutting and mixing times combinations, as reported in the experimental design*

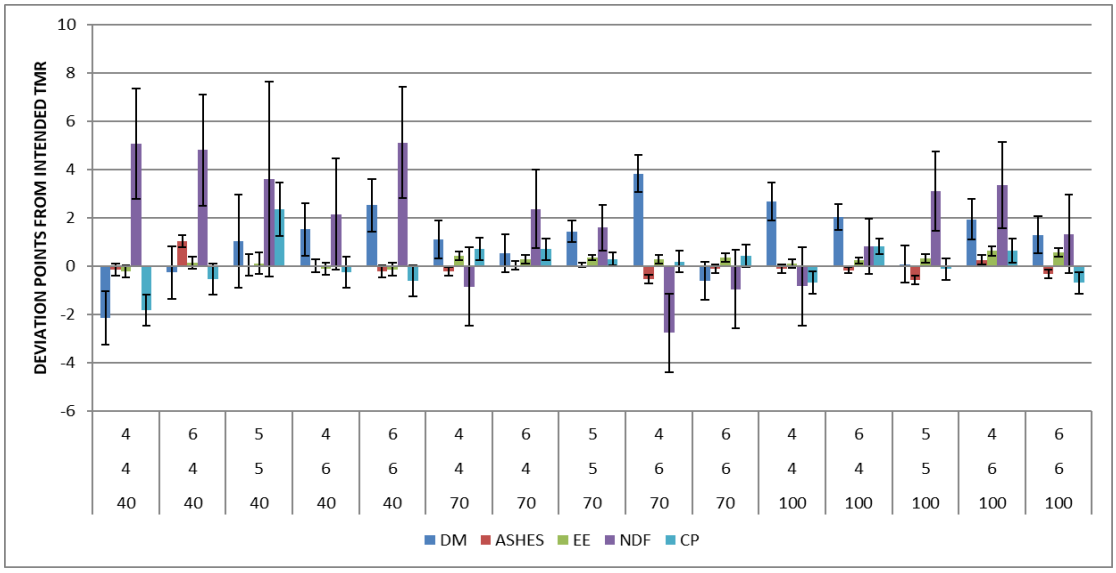


Table 4 reports the statistical significance of data analysis related to the difference of chemical characteristics of each collected sample compared to the chemical characteristics of the diet collected in the wagon, kept as reference.

Mixing wagon loading affected CP content (P<0.05), ash (P<0.05), EE (P<0.05) and NDF content (P<0.01), while mixing time influenced DM content of the diet (P<0.05), see Table 4.

Table 4. *Statistical significance of data analysis related to the difference of chemical characteristics of each collected sample compared to the chemical characteristics of the diet sampled in the mixer*

Chemical parameters[1]	**Effect**	**Loading level of the wagon***		
		40	**70**	**100**
DM content	Mixing time	0.19^{a}	1.68	1.57^{b}
Ash content (% DM)	Loading level	0.13^{a}	-0.05	-0.23^{b}
EE content (% DM)	Loading level	-0.07^{aA}	0.33^{b}	0.34^{bB}
NDF content (% DM)	Loading level	4.88^{Aa}	-0.59^{B}	0.72^{b}
CP content (% DM)	Loading level	-0.54^{a}	0.38	0.7^{b}

* % of the nominal capacity of the mixing wagon

[1] Difference between the analysed diets collected in the alley and in the mixing wagon.

[A,B] Values in the same row with different superscripts differ significantly (p<.01).

[a,b] Values in the same row with different superscripts differ significantly (p<.05).

The results related to chemical parameters of the diet did not show significant effects on the homogeneity of the collected samples for cutting time (P>0.05).

The main effects on difference of analysed samples were mixing time for DM content, with a minimum difference of 0.19 points when the wagon is loaded at 40 % of nominal capacity, and 1.57 with a 100 % loading level (P<0.05). The loading level of the mixing wagon affected the difference of the diet sampled in the wagon vs the collected samples for ash content (-0.05 for 70 %; +0.13 vs -.23 respectively for 40 % and 100 %; P<0.05). The loading of mixing wagon for 70 % of the nominal capacity gave the lower difference values between NDF (P<0.01), CP (P<0.05) and Ash (P<0.05) of the formulated and the TMR distributed along the alley.

The choice of the ideal combination

Table 5 reports the summation of differences (Σ) of each considered chemical parameter. It confirms the previous exposed results, that 70 % of loading for the mixing wagon, together with 6 min of cutting time and 6 min of mixing gives the lowest summation, 2.78 units.

The loading of 40 % gives the greatest difference of the distributed TMR. Considering a cut-off level of 5 % (Buckmaster et al., 2014), the 70 % of loading level seems to guarantee a lower deviation of nutrients in the TMR distributed to cows along the alley. The loading of 100 % could be acceptable, when cutting is done for 4 min and mixing for 6 min (Σ = 4.08).

Table 5. *List of the most efficient combination for loading cutting and mixing times expressed by sums of the differences of each chemical parameter from collected to formulated TMR.*

Loading level	Cutting time (min)	Mixing time (min)	Σ items
70	6	6	2.78
70	5	5	3.77
70	4	4	3.91
100	4	6	4.08
70	4	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	6	4	8.45
40	4	4	11.98
40	6	6	12.53

Uniformity of samples along the alley

Variance analysis evidenced significant differences in the three distribution points for the considered chemical parameters for DM and NDF content, as shown in Figure 2, for all combinations of loading, mixing and cutting times.

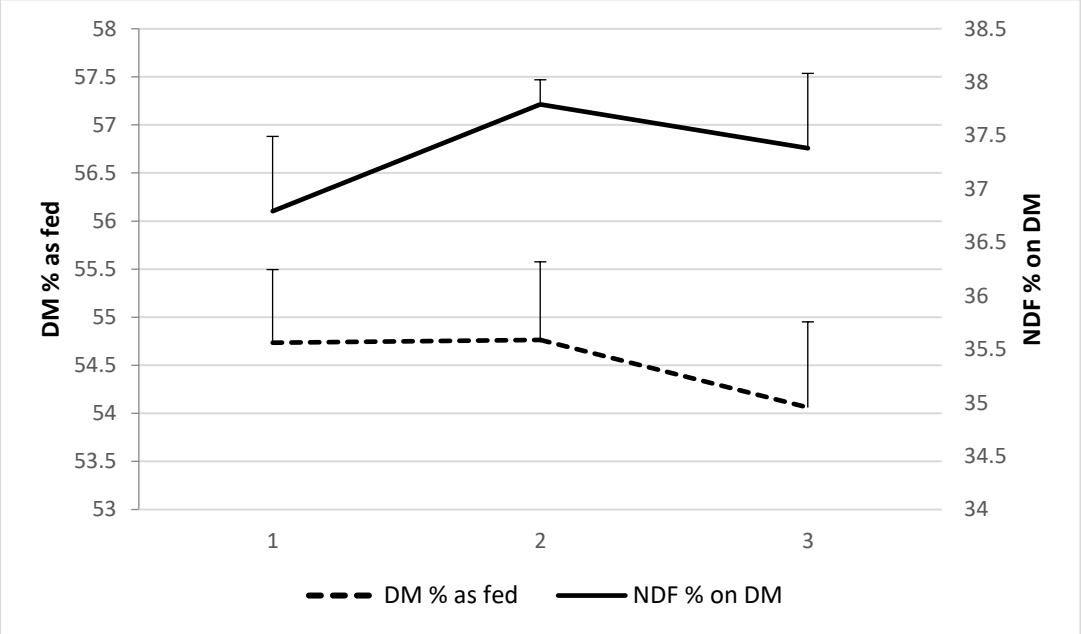
DM was different at point 1 and 2 from point 3 of the alley ($P<0.01$); NDF differed at points 1 and 2 ($P<0.01$), and at points 1 and 3 for ($P<0.05$).

The less amount of DM, released at the end of the alley, together with the NDF trend at the different sampling points, suggests that a stratification of the material in the mixing wagon may occur during the last time of distribution. This aspect seems to highlight the need to adjust the distribution time along the alley.

Although this trial revealed that this mixing wagon can perform a good TMR preparation at a 70 % of loading level for 6 minutes of cutting and for 6 minutes of mixing time, a difference in DM and NDF content was measured along the feeding alley: the difference of chemical contents of collected samples along the alley, in a particular way for DM and NDF, together with the behaviour

habits of dairy cow to choose the same position at the feeding trough, highlight the different TMR composition for the various cows at the distribution.

Figure 2. *DM and NDF content variation of the diet samples collected along the alley*



1, 2 and 3 represent beginning, middle and end of the feeding alley, respectively.

Many reasons can affect the homogeneity of the delivered ration, which may be different than the intended ration (Buckmaster, 2009; Baumgard et al., 2017), given that the ration is formulated properly from the start, with the right order of loaded ingredients, from forages to concentrates.
In the present study, DM and NDF differed in the samples collected along the alley, highlighting a non-uniform TMR distribution. Mostly, NDF content and its digestibility are the main factors affecting feed intake and the TMR digestibility (Mertens, 2009). Although Yoder et al. (2013) found that extreme daily fluctuations in Forage NDF had no cumulative negative effect on milk production over a 21-d period, a continuous variation in time of NDF, during lactation, can affect TMR intake, since when TMR has high fibre content and low energy, cows limit feed assumption in dependence of the ruminal filling.
Undoubtedly, the homogeneity and uniformity of NDF in TMR is closely related to its particle size, that is the result of mixer type, makeup of the ration, and mixing protocol (Heinrichs, 2013), and NDF concentration is linked to particle size distribution (Buckmaster, 2009; Yang and Beauchemin, 2007; Dahlke and Strohbehn, 2009).

CONCLUSIONS

The DM and NDF differences along the feeding alley revealed a different TMR composition for the various cows at the distribution, although sampling after the cutting–mixing procedure showed a good uniformity at wagon level.

These preliminary results on the efficiency of a mixing wagon for dairy cows TMR put in evidence the necessity of further studies addressed to a potential de-mixing of ingredients during TMR release.

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Chapter 4

Application of a microwave resonance sensor for real-time measurement of silages dry matter content during the preparation of the total mixed ration

Presented at:

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Perricone V., Agazzi A., Costa A., Lazzari M., Calcalnte A., Baiocchi M., Sesan E., Savoini G and Tangorra F. M. *Development of a microwave sensor application for online detection of corn silage dry matter content.*

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Perricone V., Costa A., Calcante A., Agazzi A., Savoini G., Sesan E., Chiara M. and Tangorra F.M. 2019 October. *TMR mixer wagon real time moisture measurement of animal forages.* In 2019 IEEE International Workshop on Metrology for Agriculture and Forestry (MetroAgriFor): 247-250 IEEE.

ABSTRACT

Silages are usually the main components of the diet for dairy cows, with the highest inclusion rate compared to the other components of the ration. Unlike hay and concentrate, whose composition remains almost stable throughout the time, silages are more variable given their high moisture content. Due to fermentative phenomena, as well as to inappropriate silo management, silages moisture and consequently their DM, can undergo fluctuations among time, which in turn can affect the nutritional value on as fed basis. Because of silages DM fluctuation, the TMR delivered to the animals might differ from the formulated ration, with the risk of not satisfying the nutritional requirement of the animals, finally affecting their health and performances. For the aforementioned reasons (i.e. high inclusion rate and high variability among time), it is necessary to daily adjust the as fed amount of each silage to be included in the TMR according to their actual DM content. With this aim we developed a system based on microwave resonance technology for real-time silage DM determination during the loading of the ingredients in the mixer wagon. The microwave sensor (MS) was calibrated in static condition for corn, high moisture corn and grass silage, with the best curve fitting observed for corn silage (R^2=0.73). MS was installed behind the loading drum of the conveyor arm of a horizontal self-propelled mixer wagon and the calibrated MS was tested in dynamic conditions. In the farm were the dynamic test was carried out, only corn silage and high moisture corn silage were available, thus grass silage calibration was not tested. However, grass silage calibration was tested over soybean and barley silages, to assess whether the calibration for one silage could be suitable also for other silages. Underestimation of the real moisture content was observed for all the silages, with the greatest discrepancy observed for soybean silage. However, given the strong relation highlighted between sensor readings and the real moisture (R^2 between 0.87 and 0.98), and a minor standard deviation, a correction coefficient was applied to improve MS performance. Following, the prototype was tested at the experimental farm of the University of Milan (Landriano). For one month the prototype was used for the preparation of the ration for lactating cows. The herd, composed by 100 animals, was divided in two groups, each one of 50 animals. One group (OFF) was fed without applying the correction suggested by MS; while as fed amount inclusion rate of each silage was adjusted according the moisture content recorded by the sensor for ON group. MS reduced the deviation from the target DM of each silage loaded in the wagon, reducing the percentage error by more than 50%. Our major hypothesis was that, adjusting the as fed silages inclusion rate according to the real DM content, the final TMR delivered to the animals would have been closer to the TMR formulated by the nutritionist. Both diets prepared with or without MS employment were found to be different from the formulated ration, however TMR prepared with MS support was closer to the target diet, except for DM, Ash and starch. Although MS reduced the error from silages, the lack of expected result on the final TMR might be attributable to operator error in the loading of ingredients other than silages. In conclusion, MS could be a valuable tool for supporting TMR preparation, however sensor performances can be improved. Furthermore, besides silages DM variation, error in the loads of concentrate ingredients can affect TMR composition as well, therefore greater attention should be paid during the loading phase.

INTRODUCTION

Dairy producers are increasingly mindful of factors affecting efficiency of milk production, and among them feeding is one of the most important. Significant time and effort are spent in diet formulation to ensure that animals' requirements are fulfilled. It is indeed important that nutrient availability does not limit animals' performances and health (Naas et al., 2001). However, the nutritional value of TMR received by the animals do not always reflect the diet formulated by the nutritionist. Several factors can contribute to TMR inconsistency (Mikus et al., 2012), but variations in nutrient composition of feeds play the major role (St-Pierre and Weiss, 2015). Nutrient concentrations of feedstuffs can vary substantially over short periods of time (Weiss et al., 2012), and transient changes in ingredients dry matter (DM) can be responsible of daily variations (Sova et al., 2014). More than half of the DM content of the diet fed to dairy cows is provided by forages (Shaver and Keiser, 2004), mainly silages, whose DM and quality undergo reductions among time. Not even a good management of the silo can prevent from DM losses during storage and feed-out phase (Borreani et al., 2018). These losses, along with other occurrences responsible of DM variations, such as rainfall event over uncovered silos, can modify the nutrient supply of the TMR. Diets are indeed formulated on a DM basis, but ingredients are included in the mixture on as-fed basis. Therefore, if as-fed inclusion rate in not adjusted for changes in DM concentration, the nutrient composition of the TMR would change, possibly affecting cows' health and performances, with subsequent consequences on farm profitability (Fadel et al., 2006). Sova et al. (2014) reported reduced milk yield and feed efficiency in herds receiving TMR with great variability of Net Energy of Lactation (NE_l). Similarly, a positive correlation between variation in concentration of certain nutrients, such as lining, and variation in milk yield and composition were reported (Rossow and Aly, 2013). However, other studies conducted in controlled conditions evidenced no or only minor effects on production traits because of TMR variations (McBeth et al., 2013; Yoder et al., 2013).

In view of the significant impact of silages DM fluctuation over TMR composition, daily sampling of the trench would be required. The official method for DM determination is oven-drying under controlled condition, but this process is time-consuming and usually carried out in the laboratory, thus it's not suitable for field application. Alternative and more practical methods have been proposed, including portable instrument such as the Koster moisture tester or Near Infra-Red (NIR) devices, or even sample-drying with microwave oven. However, these methods could be time-consuming as well, and they require samples collection over the trench. If sample collection in not performed properly, samples might be not representative of the entire batch, leading to a wrong estimation of the moisture content, and finally affecting the formulation of the TMR (Weiss and St.Pierre, 2015). Therefore, real-time evaluation of silages DM during their

loading in the mixer wagon, and subsequent correction of their as fed inclusion rate, could represent a valuable tool to improve TMR consistency. In this regard, a system based on NIR technology mounted on the scraper of the TMR wagon has been recently proposed, with encouraging results (Piccioli-Cappelli et al., 2019). However, NIR technology presents some limitations, first of all the cost of the instrument. A potential alternative to NIR systems can be provided by microwave resonance technology, which is based on the interaction between water molecules and changing electromagnetic fields. If a product containing water is passed over a microwave resonance sensor, its resonance frequency decreases, and the half-width of the resonance curve increases. The magnitude of these changes can be correlated to the water content of the sample (Buschmüller et al., 2008; Kocsis et al., 2008).

The aim of this trial was to develop a system based on microwave resonance technology for real-time measurement of silages moisture content (and consequently DM) during the loading phase of the TMR mixer wagon. The objective is to adjust the amount (kg as fed) of silages to be loaded in the mixer wagon according to their actual DM content to meet the target DM defined by the nutritionist. The hypothesis is that by correcting the inclusion rate of silages, the nutrient supply provided by them will be accordingly adjusted, finally leading to a TMR delivered to the animals closer as possible to the TMR formulated by the nutritionist.

MATERIALS AND METHODS

Microwave sensor

A microwave sensor (MS) for bulk materials (FL-Wapp, Mainz, Germany), with a 75-mm diameter ceramic planar sensing face, was employed. The main technical characteristics of the sensor are summarized in Table 1.

Table 1*. Main technical data of the microwave sensor (MS).*

Parameter	Value
Power supply	12-24 V DC
Outputs	0-20 mA or 4-20mA
Measuring range	0-100 %, 0-80 °C
Depth	75-100 mm
Accuracy	± 0.3%
Frequency	433.92 MHz
Dimensions	Ø: 75 mm, Length: 100 mm
Weight	1.8 kg

Calibration of the sensor, installation on the mixer wagon and dynamic test of the calibrations

The sensor was calibrated in static condition over different types of silages, namely corn, high moisture corn and grass silage. For the construction of the calibration curves, samples of each silage were collected from different farms located in Lombardy. Samples were collected from the entire front of the trench through the employment of a silage corer (Master forage probe, Dairy One, Ithaca, NY, USA), trying to gather as much as DM content variability as possible within them. Each sample was applied to the sensors using a 150-mm diameter x 50-mm tall polyvinyl chloride (PVC) container. The silage was poured into the container, filling it completely and ensuring a minimum sample thickness of 5 cm, as specified by the manufacturer of the sensor for the calibration procedure. To avoid electrical interference effects, the sensor was flanged with a 5-cm thick holder metal ring. Four readings, achieved by a 90° rotation of the container, were performed for each sample. A raw signal (Q), function of frequency shift and amplitude attenuation of the resonance curves, was recorded for each reading. After performing the readings, silage samples were weighed and carried to the laboratory for moisture content assessment according to the official method (Commission Regulation 152/2009). The relationship between the sensor reading values and the real moisture content, defined with a linear regression, was established as the calibration equation. Three calibration equations, respectively for corn, high moisture corn and grass silage were obtained.

After the calibration curves were developed, the sensor was mounted on the mixer wagon. MS was positioned behind the loading drum of the conveyor arm of a horizontal self-propelled mixer wagon (Gulliver 6014, Sgariboldi srl, Codogno, Italy). The position was chosen in accordance with the manufacturer of the wagon, in order to ensure a constant flux of material flowing over the sensor during the milling, while at the same time offering the least resistance to the same flux. The position of the sensor is shown in Figure 1.

MS was further integrated in an on-line measurement system, designed to continuously record real-time silages moisture content during the loading phase. For this purpose, filtering procedures and identification algorithms have been developed by a commercial partner involved in the project (PTM srl, Isorella, Italy). Following, a brief description of the integrated measurement system will be provided, and an explicative block diagram is given in Figure 2. The output signal (4-20 mA) from the sensor is acquired through front-end electronics, which convert the raw signal into a digital signal, then transferred to the control unit through a PTM proprietary protocol. The following devices compose the system:

- DRIVE 8 DRY: through this I/O board the raw signal of the sensor is conditioned, converted in digital and then filtered. At this stage, the conditioning has the goal of providing reliable raw signal to the control unit.
- ADVANCED SUPER USB (CONTROL UNIT): the control unit is responsible for estimating the moisture value of the loaded silage, performing a real-time evaluation of the validity and goodness of the data coming from the sensor. First, it converts the signal in moisture values, according to the calibration curve applied. Then, specific filtering algorithms considering the physical and mechanical phenomena that can potentially affect the measurement during the silage loading are applied, in order to further filter the data, providing reliable results. One algorithm verifies whether the sample is consistent or not, thus if it can contribute to the measure or it has to be discarded. A first information used for this purpose is the activation of the cutting slasher. It is indeed a prerequisite for the elaboration of the signal that the cutting slasher is operating, meaning that the silage is being actually loaded in the wagon. Then, it is evaluated whether the sample is within the moisture range considered acceptable for that specific ingredient. A second algorithm is used to discard data generated by the persistency of the same material over the probe. When the moisture content of the silage is very high, it is possible that, near to the sensor, an area of compressed material can persist for few seconds. This event can be observed in the recording trace, when the normal and continuous fluctuations of the signal become steady on a constant value. In order to avoid this event to interfere in the proper measurement of the moisture content, when the signal persists on the same value it is discarded until a minimum defined size variation is recorded.

Figure 1*. Positioning of the sensor behind the loading drum of the conveyor arm of the wagon. The sensor is indicated with a red arrow. MS was flanged with a 5-cm thick holder metal ring to avoid electrical interference effects.*

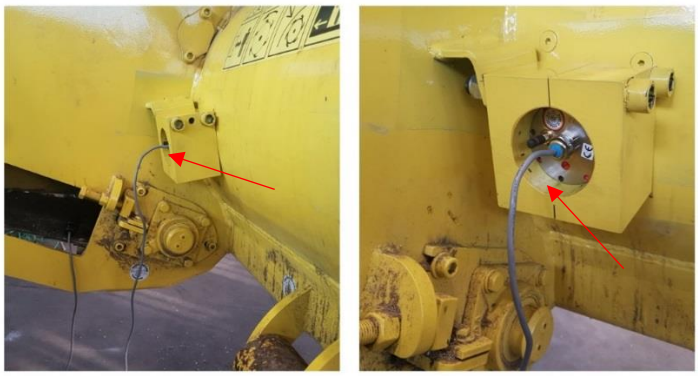
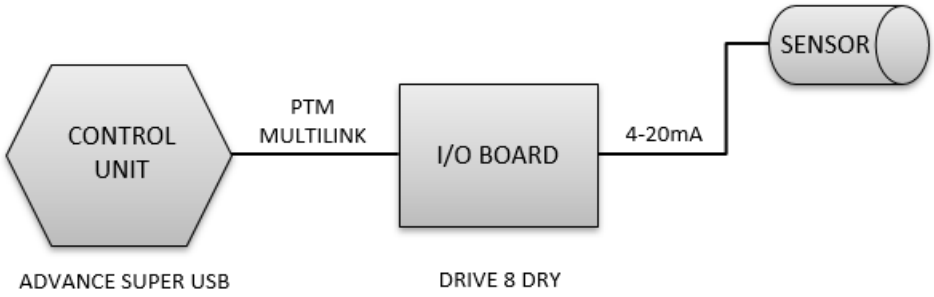
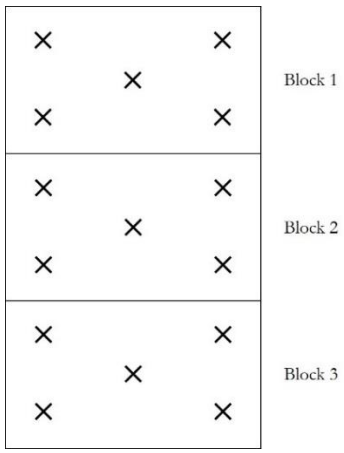


Figure 2*. Block diagram of the integrated measurement system*



Following the implementation of the sensor on the wagon and the development of the integrated measurement system, dynamic tests of the calibration curves were performed for one week. The test was conducted at the University of Milan experimental farm (Landriano, Italy), where only corn and high moisture corn silage were available; thus, grass silage calibration was not tested. However, grass silage calibration was tested over two other silages available in the farm, namely soybean and barley silages, to assess whether a calibration for one silage could be suitable also for other silages. The front of each silage trench was divided in three equal blocks along the entire height of the trench. Prior to milling, five samples were collected for each block as described in figure 3. Each sample was weighed and carried to the laboratory for moisture content assessment according to the official method (Commission Regulation 152/2009). The average moisture of the five samples belonging to the same block was considered as the representative moisture of the corresponding block. After samples collection, each block was individually milled and moisture values of the material running over the probe were read and recorded. MS moisture values were then compared to the real moisture.

Figure 3. *Schematic representation of silage sample collection. The trench was divided in three equal blocks, and five samples (represented by a cross) were collected for each block prior to milling.*



Field test

The goal of the MS was to include in the TMR the right DM amount from each silage. The final objective was to prepare a TMR closer as possible to the diet formulated by the nutritionist. Therefore, the last step of the experiment was dedicated to test in field the mixer wagon prototype and its effect on the final TMR received by the animals.

The final prototype was composed by the horizontal self-propelled mixer wagon (Gulliver 6014, Sgariboldi srl, Codogno, Italy), equipped with MS and integrated measurement system. Furthermore, the system was equipped with PTM management software for feeding management. The software provided the amount and sequence of loading of each ingredient, and stored the records of the loading process, the suggested adjustment for silages loads based on the recorded moisture, and the amount actually loaded.

The test in field was carried out at the experimental farm of the University of Milan (Landriano, Italy). For one month the prototype was used for the preparation of the TMR for lactating cows, whose herd was composed by 100 Holstein-Friesian cows, housed in a free stall barn. The herd was divided in two subgroups of 50 animals each. One group (ON) was fed with the sensor switched ON, meaning that the amount of each silage to be included in the ration was adjusted according the moisture content recorded by the sensor. The second group (OFF) was fed with the sensor switched OFF, meaning that no correction was suggested, even though the moisture content of the silages during the loading phase was still recorded. Cows were fed *ad libitum*, with TMR distributed once a day in the morning. Each group received the same diet, whose composition is reported in Table 2.

Table 2. *Composition of the ration formulated by the nutritionist.*

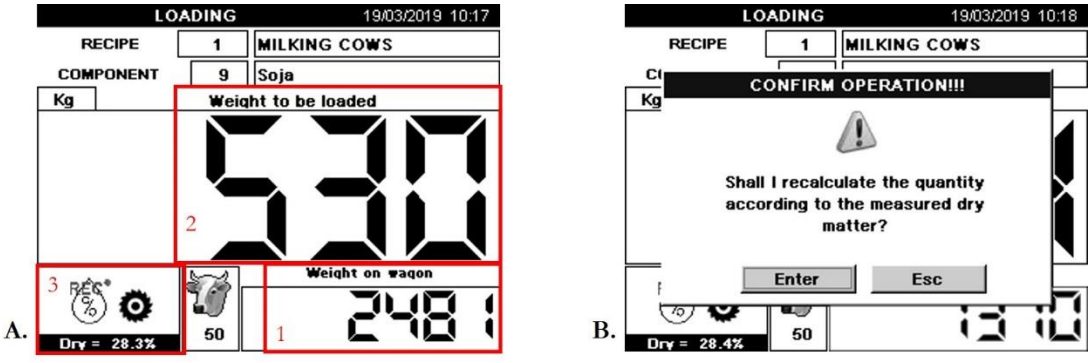
Ingredient	kg as fed/cow/day
Corn silage	15
Soybean silage	12
Corn meal	5
Protein feed	5
Barley silage	5
Flaked corn	2.5
Alfalfa hay	2.5
Molasses	1.9
Soybean meal	1.6
Wheat straw	0.9
Bicarbonate	0.24
Total	51.64

Functioning of the system

The control unit (Advance Super USB), through the presence of a display in the driver's cab, guides the operator during the loading of each ingredient. During the loading phase, the operator can see in the display: 1) the total amount of feed in the mixer wagon, 2) the residual amount of the ingredient to be loaded (target amount, based on the ration formulated by the nutritionist), and 3) the real-time value of dry matter (only for silages). When the TMR is prepared with the aid of the sensor (ON group), after 80% of the target amount of that specific silage has been loaded, the system adjusts the remaining amount to be loaded (20%) based on the average moisture content recorded on the previous 80% (Figure 4).

To compare the TMR daily delivered to the animals (ON and OFF group) with the formulated ration, chemical composition of TMR was analysed daily. For this purpose and to avoid the risk of collecting from the feeding alley samples not representative of the entire TMR, a NIR analyser was installed in the tank of the mixer wagon. The selected NIR analyser (Corona Extreme, Carl Zeiss AG, Oberkochen, Germany) has been specifically developed for agricultural application and it is employed for real-time measurement to optimize process control, while minimizing time, waste and cost. The NIR was purchased along with a specific calibration for TMR, which provided the analysis for DM, CP, EE, Ash, NDF, ADF and Starch.

Figure 4. *A) Information provided to the operator in the display located in the driver's cab. Red boxes indicated 1. the total amount of feed in the wagon; 2. the residual amount of the current ingredient to be loaded; and 3. the moisture content. B) When the sensor is switched ON, after loading the 80% of the theoretic amount, the software suggests a correction for the remaining 20% to be loaded, based on the moisture content recorded in the previous phase of loading.*



Calculations and statistical analysis

For the determination of the calibration curves, linear regression analysis was performed over data obtained from sensor readings against moisture content determined in the laboratory, evaluating the accuracy of the sensor by means of the coefficient of determination (R^2). For the evaluation of the goodness of the calibrations tested in dynamic condition, coefficient of determination (R^2) and mean difference (Md) between the moisture content read by the sensor and the real moisture of the silages were determined. Furthermore, the coefficient of variation (CV), obtained as 'standard deviation between the readings/average recorded moisture value', was calculated for precision.

Deviations from the target amount of silage DM to be loaded in the mixer wagon were calculated by subtracting the target value (formulated by the nutritionist) to the DM amount actually loaded, both when the correction suggested by the MS was applied (ON) or not (OFF). The deviation was expressed both as kg of DM and as percentage error, which was calculated as |expected value – observed value|/expected value*100. Similarly, deviation from the target amount (kg as fed) was calculated for the components of the ration other than silages (corn meal, protein feed, flaked corn, alfalfa hay, soybean meal, wheat straw and molasses).

Mean difference and percentage error was calculated also to determine the degree of agreement between chemical composition of the formulated and delivered TMR.

Data were analysed by means of a GLM procedure of SAS 9.2 (SAS Inst. Inc., Cary, NC) to compare silages DM inclusion rate and TMR chemical composition between the diets (formulated, OFF and ON).

RESULTS AND DISCUSSION

Silages are characterized by high moisture content, which is prone to fluctuation among time. The variability of moisture content, and therefore of DM content, influences silages nutrient composition, which can in turn be reflected in the final TMR composition. Even though the effect of silages variation on TMR composition is well-recognized, little has been done to minimize its impact, and literature on this topic is lacking. To the best of our knowledge, only a recent work (Piccioli-Cappelli et al., 2019), investigated the employment of a NIR sensor with our same goal, i.e. real-time correction of silages inclusion rate in the mixer wagon based on the real DM content, and its effect on TMR, reflected on animal performance and health.

Corn silage is the main ingredient of diets fed to lactating cows in North-Italy, except for those farms producing milk processed into some Protected Denomination of Origin cheeses (i.e.

Parmigiano-Reggiano) (Cattani et al., 2017). For this reason, the first calibration for MS was developed for corn silage, and samples were collected from different farms located in Lombardy (Italy). High moisture corn and grass silage are quite common as well, however their inclusion in the diet is significantly lower compared to corn silage. Moisture content of samples collected for the calibrations ranged between 59% and 68% for corn silage, 22% and 30% for high moisture corn silage, and 60% and 70% for grass silage. The best fitting was reported for corn silage (R^2 = 0.73), followed by high moisture corn silage and grass silage, both with R^2=0.69.

Soybean and barley silages are rather uncommon, therefore after discovering that the farm where the dynamic test of the calibration would have been carried out was feeding soybean and barley silage, we decided not to develop a specific calibration curve for those silages. We decided rather to test the calibration of grass silage for their moisture estimation. Our objective was to verify whether a generic calibration could be suitable for moisture content estimation of different silages. If that is the case, it would be possible to employ the sensor also for uncommon silages, each of which will otherwise require an *ad hoc* calibration. Results of the test of the calibrations in dynamic conditions are reported in Table 3. Grass silage was not available in the experimental farm; thus no results are presented. The three calibrations underestimated the real moisture content of the silages. The best results were observed for high moisture corn silage, whose difference from the real value was lower than one percentage point. Soybean and barley silages reported the highest difference, outlining the poor adaptability of grass silage calibration for their moisture evaluation. We hypothesized that the observed differences, at least for corn silage and high moisture corn, could be referred to the pressure exerted by the sample over the probe in dynamic condition, caused by the action of the scraper. Indeed, for the development of the calibrations no pressure was applied over the probe other than the one generated by the weight of the same sample. However, for all the silages, a strong relation between sensor readings and the real moisture of the silages was highlighted by the coefficient of determination (R^2), between 0.87 and 0.98. Based on the good R^2 and the minor standard deviation, a correction coefficient was defined to ameliorate MS performance. Furthermore, the low coefficient of variation (CV) highlighted a good repeatability of the measurements, meaning a good precision of the instrument, with the only exception of high moisture corn silage.

Table 3. *Performance of the calibration developed in static condition applied in dynamic condition.*

	Md[1] ± SD	**[2]R[2]**	**CV[3] (%)**
Corn silage	-2.67 ± 0.26	0.98	2.63
Barley silage	-5.17 ± 1.12	0.97	1.65
Soybean silage	-6.55 ± 0.32	0.87	2.39
High moisture corn silage	-0.70 ± 0.49	0.96	4.15

[1]Md: mean difference between the moisture content recorded by the sensors and the real moisture content determined in the laboratory. Values are expressed as percentage
[2]R[2]: coefficient of determination between the moisture content recorded by the sensors and the real moisture content determined in the laboratory
[3]CV (%): coefficient of variation. Calculated as standard deviation of the readings/average value of the readings

The diet fed in the farm where the field trial was carried out was slightly different from the diet usually fed in Northern Italy (Table 2). Even though silages were the main component of the ration, accounting for 61.97% of the diet (as fed), the diet was not based only on corn silage, as in common practice (Mantovi et al., 2015). Corn silage represented indeed less than half of total amount of silages (46.88%), followed by soybean silage (37.50%) and a minor proportion (15.62%) of barley silage.

Silage DM amount according to the diet formulated by the nutritionist and silage DM amount actually loaded in the mixer wagon without (OFF) or with (ON) the correction suggested by MS are reported in table 4. Average amount of corn silage (kg DM) loaded in the mixer wagon was not different from the formulated amount, both when the correction suggested by MS was applied (ON) or not (OFF). Even though corn silage DM variability among days and within the same trench is well-documented (Weiss et al., 2012), our result might be indicative of low DM corn silage variability. On the contrary, DM amount of barley and soybean silage loaded in the wagon was different from the theoretical value for both groups. This result might suggest that the correction coefficient based on the results obtained with the dynamic test is still improvable. An additional explanation for this result is the variability within the same trench. If there is a strong DM variability within the trench, the average moisture content read by MS during the loading of the first 80% might not be representative of the remaining 20% still to be loaded.

Average differences between loaded and formulated DM, expressed as kg of DM and percentage error, are presented in table 5. On average, regardless of MS employment, less corn silage DM and more barley and soybean silage DM were loaded in the mixer wagon. Even though corn silage DM actually loaded was not different from the formulated value in both groups (Table 4), the deviation from the target value was lower when MS was employed. In terms of kg of DM,

the difference between the two groups was not significant; while the % error was significantly lower in ON group($p<0.05$). Similarly, the deviation from the target value of soybean and barley silages was lower when the correction suggested by MS was applied ($p<0.001$ and $p<0.0001$, respectively). MS allowed to reduce the deviation from the target DM for all silages, reducing the percentage error by more than 50%. In practical terms, when applied to the total herd of the experimental farm (100 animals) and considering the same diet used in this trial, MS employment will be reflected in saving approximately 53 kg/day of barley silage and 50 kg/day of soybean silage.

***Table 4**. Silages DM formulated and actually loaded in the mixer wagon, without (OFF) or with (ON) the correction suggested by the microwave sensor.*

	kg DM formulated	kg DM fed		*p-value* †	
		OFF	**ON**	**OFF**	**ON**
Corn silage	4.50	4.34 ± 0.49	4.43 ± 0.24	n.s.	n.s.
Barley silage	1.40	1.65 ± 0.15	1.50 ± 0.06	***	*
Soybean silage	3.36	3.66 ± 0.23	3.52 ± 0.08	***	**

* p<0.05
** p<0.001
*** p<0.0001
† kg DM formulated vs fed (OFF or ON)

***Table 5**. Mean differences between DM actually loaded and formulated, expressed either as kg of DM or error %*

	kg DM			error %		
	OFF	**ON**	*p-value* †	**OFF**	**ON**	*p-value* †
Corn silage	-0.16 ± 0.49	-0.07 ± 0.24	n.s.	8.64 ± 7.37	4.14 ± 3.51	*
Barley silage	0.25 ± 0.15	0.10 ± 0.06	**	18.19 ± 10.79	7.39 ± 4.31	**
Soybean silage	0.3 ± 0.23	0.16 ± 0.08	*	9.34 ± 6.06	4.77 ± 2.29	*

* p<0.05
** p<0.001
*** p<0.0001
† OFF vs ON

Our hypothesis was that, adjusting the as fed silages inclusion rate according to the real DM content, the final TMR delivered to the animals would have been closer to the TMR formulated by the nutritionist. Chemical composition of formulated and fed diet is shown in table 6. Both diets prepared with or without the correction for silages inclusion rate differed from the formulated TMR, except for DM, Ash and Starch content. No differences were observed between ON and OFF diet, apart for NDF and ADF ($p<0.05$).

Since both fed diets (OFF and ON) differed from the formulated TMR, but were similar among them, it is likely that the nutritional value of the ingredients included in the TMR did not reflect the nutritional value used for the formulation of the ration. Concentrate feeds are usually

stable over time, while nutritional value of silages can undergo variations, e.g. due to silo respiration and fermentation, effluent production, and oxygen exposure during feed-out phase (Borreani et al., 2018). Furthermore, even though diet formulation is based on chemical analysis of the ingredients included in the ration (especially for forages), samples collected might have been not representative of the entire silo. Weiss and St.Pierre (2015) reported that sampling is a great source of variation within farm, meaning that inaccurate sampling could lead to error in the formulation of the ration. Finally, in the present study the chemical composition of TMR has been assessed by means of a NIR analyser mounted in the tank of the wagon. The instrument has been purchased with specific calibration for TMR, however we hypothesized that the analytical method could have partially contributed to the observed variation.

Although both diets (OFF and ON) differed from the formulated ration, TMR prepared adjusting silages inclusion rate was closer to the target diet. When MS was employed, the average mean difference from the formulated ration was numerically lower for all chemical parameters, except for DM and Ash (Table 7).

Although the error for silage DM inclusion rate was reduced by means of MS correction, the total DM from silages (formulated ration) expressed on total DM of the TMR accounted for 35.5%, meaning that the remaining 64.5% DM was from other ingredients. The other ingredients included in the TMR, except for molasses, are characterized by high DM content (>80%); thus, loading in the wagon the wrong amount of these ingredients could have affected the final DM of the ration. Errors in the loads of the ingredients are recognized as one of the major sources of TMR variation (Sirakaya and Kucuk, 2019). They can be referred to improper functioning of the instruments, such as scales, or to human error.

Analogous results were observed by Piccioli-Cappelli et al. (2019). Average difference between DM of formulated and fed diet did not differ when the sensor for correcting silages inclusion rate according to the real moisture was employed or not. The deviation from the target diet, expressed as kg of DM, was numerically greater when the sensors was switched ON (0.100 kg/d) rather than OFF (0.070 kg/die). However, the same authors evidenced how loading the wrong amount of dry feedstuff can influence the final composition of TMR. Indeed, when the operator errors in the loads of dry feed were computed, the discrepancy between TMR fed and formulated was reduced, resulting in lower difference for the group fed with the employment of the sensor.

Table 8 shows the deviation from target amount of ingredients other than silages. Even though no MS correction was applied for these ingredients, data are divided according to the group (ON or OFF) to investigate whether the error was similar between the two groups. All the ingredients tended to be loaded in greater amount compared to the target amount. In both ON and OFF group

the greatest variability was observed for wheat straw, while the lowest was observed for protein feed, followed by molasses. No differences were observed between the two groups, except for soybean meal, whose error in ON group was greater than in OFF. Overall, the total variability was numerically greater for ON group (average % error 6.39%, corresponding to 1240g more than the formulated amount) compared to OFF group (average % error 5.67%, corresponding to 1100g more than the formulated amount). We could have therefore expected the TMR of ON group to be more different from the formulated ration; however, it is not what we observed, except for DM (Table 6). The greater inclusion of concentrate in ON group could have been responsible of increasing the total DM of the diet; however, no effect on the final composition of the TMR was observed. This can be explained by the low inclusion rate of these ingredients in the diet, or by the fact that the error from the wrong amount of concentrate might have been mitigate by the reduced error from silages.

CONCLUSIONS

Our results have shown that MS reduced the error in the loads of silages DM, lowering its deviation from the target amount. However, discrepancies form the theoretical amount were still observed; therefore, calibration curves should be strengthened. The greater accuracy in silage DM loads when MS was employed was however insufficient to prepare a diet closer to the formulated one, compared to the diet prepared without MS employment. Errors in the loads of ingredients other than silages, mainly concentrates, might have contributed to the lack of results. Furthermore, correcting silages inclusion rate according to their DM content could be not enough to ensure the proper nutrient supply. Silages can indeed undergo nutrient variability among time, therefore regular analysis should be performed. In conclusion, MS could be a valuable tool for supporting TMR preparation, but sensor performances have to be improved. In addition, for a proper TMR preparation, more accuracy is also required in the loads of concentrate ingredients.

Table 6*. Chemical composition of formulated and fed diet, prepared without (OFF) or with (ON) the correction for silages inclusion rate suggested by the microwave sensor. Values are expressed as % of DM, unless otherwise specified.*

		Fed		*p-value*		
	Formulated	**OFF**	**ON**	**formulated vs OFF**	**formulated vs ON**	**OFF vs ON**
DM (kg)	50.53	50.85 ± 1.79	51.68 ±1.98	n.s.	n.s.	n.s.
CP	14.55	15.87 ± 0.63	15.64 ±0.65	***	***	n.s.
EE	4.00	3.03 ± 0.22	3.13 ±0.32	***	***	n.s.
Ash	6.94	6.90 ± 0.31	6.85 ±0.23	n.s.	n.s.	n.s.
NDF	29.41	34.92 ± 1.46	34.03 ±1.49	***	***	*
ADF	19.50	22.99 ± 1.30	22.24 ±1.35	***	***	*
Starch	25.57	25.68 ± 0.42	25.87 ±0.37	n.s.	n.s.	n.s.

* $p<0.05$
** $p<0.001$
*** $p<0.0001$

Table 7. *Mean difference and error % between fed diet, prepared without (OFF) or with (ON) the correction for silages inclusion rate suggested by the microwave sensor, and formulated diet.*

	kg DM[1]						error %		*p-value*	
	OFF			ON			*OFF*	*ON*	kg DM	error %
	mean ± SD	min	max	mean ± SD	min	max	mean ± SD	mean ± SD		
DM	0.32 ± 1.79	-2.69	4.38	1.15 ± 1.98	-2.20	5.93	2.79 ± 2.18	3.36 ± 2.98	n.s.	n.s.
CP	1.32 ± 0.63	0.24	2.56	1.09 ± 0.65	-0.31	2.07	3.53 ± 2.66	2.83 ± 2.10	n.s.	n.s.
EE	-0.97 ± 0.22	-1.42	-0.54	-0.87 ± 0.32	-1.56	-0.34	9.09 ± 4.34	7.91 ± 3.65	n.s.	n.s.
Ash	-0.04 ± 0.31	-0.65	0.58	-0.09 ± 0.23	-0.59	0.24	24.32 ± 5.47	21.73 ± 7.94	n.s.	n.s.
NDF	5.51 ± 1.46	2.25	8.36	4.62 ± 1.49	0.81	8.02	18.74 ± 4.96	15.72 ± 5.06	n.s.	n.s.
ADF	3.49 ± 1.30	0.30	5.53	2.74 ± 1.35	-0.95	5.67	17.88 ± 6.65	14.61 ± 5.64	n.s.	n.s.
Starch	-5.01 ± 0.42	-5.78	-4.25	-4.82 ± 0.37	-5.40	-4.08	16.32 ± 1.37	15.71 ± 1.20	n.s.	n.s.

[1]Difference calculated as 'nutrient composition of the fed ration – formulated nutrient composition'; negative values indicate deficiencies compared with the TMR formulated by the nutritionist

Table 8. *Degree of agreement between the formulated amount of ingredients other than silages and the real amount actually loaded in the wagon. For these ingredients no correction according to the DM was operated, therefore differences are attributed to operator error. Values are expressed as kg of as fed.*

	Formulated	OFF			ON			*p-value*				
		Mean	Md[1]	error (%)	Mean	Md	error (%)	form vs OFF	form vs ON	OFF vs ON	Md	error(%)
Corn meal	5.00	5.28	0.28 ± 0.20	5.56	5.26	0.26 ± 0.19	5.26	***	***	n.s.	n.s.	n.s.
Protein feed	5.00	5.11	0.11 ± 0.03	2.28	5.12	0.12 ± 0.03	2.32	***	***	n.s.	n.s.	n.s.
Flaked corn	2.50	2.65	0.15 ± 0.19	6.11	2.72	0.22 ± 0.18	8.93	*	***	n.s.	n.s.	n.s.
Alfalfa hay	2.50	2.68	0.18 ± 0.11	7.38	2.65	0.15 ± 0.10	5.94	***	***	n.s.	n.s.	n.s.
Molasses	1.9	1.97	0.07 ± 0.05	3.68	1.97	0.07 ± 0.04	3.94	***	***	n.s.	n.s.	n.s.
Soybean meal	1.60	1.77	0.17 ± 0.10	10.32	1.84	0.24 ± 0.12	15.26	***	***	n.s.	*	*
Wheat straw	0.90	1.04	0.14 ± 0.06	15.52	1.08	0.18 ± 0.10	19.50	***	***	n.s.	n.s.	n.s.

[1]Md: Mean difference between the amount (kg of as fed) loaded in the mixing wagon and the formulated value

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Chapter 5

Green tea and pomegranate extract provided via drinking water to broilers in critical moments improves blood antiradical activity and alters cecal microbial ecology

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ABSTRACT

The present study evaluated the effect of a commercial plant extract (PE) based on green tea *(Camelia sinensis)* and pomegranate *(Punica granatum)* on performance, blood antiradical activity and cecal microbiome of broiler chickens. A total of 480 one-day old male broiler chicks (ROSS 308) were randomly assigned to two experimental groups of 12 replicates each (n = 20/replicate) in a 50-days trial. Broilers received drinking water (C) or drinking water plus PE (T) at a rate of 0.2 mL/L. The innovative aspect of this study is that PE was included only during critical moments of the production cycle, namely in the post-hatching phase (day 0 to day 4), and during the transition between feeding phases (days 10-11 and 20-21). Growth parameters were determined on days 0, 10, 20 and 50. At the end of the trial, one representative chick per pen was sacrificed. Slaughtering performance were determined, and samples of blood and ceca content were collected. Blood was analysed for total antiradical activity, while 16S amplicon sequencing was performed on caeca content. PE supplementation did not affect growth and slaughtering parameters. Total antiradical activity was significantly improved by PE administration, both in the whole blood ($p < 0.01$) and red blood cells ($p < 0.05$). In T broilers cecal microbiome assay revealed a greater abundance of lactic acid bacteria (FDR < 0.05); this result was confirmed at lower taxonomic level with a higher *Lactobacillaceae* abundance (FDR < 0.05). Our findings suggest that PE administration during critical moments of the production cycle of broiler may exert beneficial effects both at systemic level and on gut health of adult birds.

INTRODUCTION

Phytobiotics, also known as botanicals, are plant-derived products which constitute a natural source of bioactive compounds (Lillehoj et al., 2018). During the last decade, the interest toward phytobiotics increased thanks to their potential as substitute to antibiotic growth promoters (Huyghebaert et al., 2011). The supplementation of phytobiotics to broiler chickens have shown beneficial effects on animal production and on the quality of animal derived products (Windisch et al., 2008). However, their mechanisms of action are still to be elucidated and different hypotheses have been proposed. Among them, the antioxidant properties of phytobiotics seems to exert positive effect on broiler's health and performances (Brenes and Roura, 2010). Phytobiotics are indeed rich in polyphenolic compounds (flavonoids, tannins, phenolic acids and terpenes), which can support the antioxidative capacity by counteracting the harmful effect of free radicals generated during stressful situations, such as hatching and changes in the diet, finally resulting in improved general health and better performance in broilers (Ognik et al., 2016).

Furthermore, phytobiotics showed to be able to modulate the gut microflora (Lillehoj et al., 2018; Saleh et al., 2018). The chance to modulate the gut microflora in chicken via a nutritional approach is of particular interest during critical moments of broiler's life, such as post-hatching phase. It is indeed recognized that the first microbial population colonizing the gut could impact the entire life-span of the animals (Ballou et al., 2016). In the same way nutrition can contribute to the sharpening of the gut microbiota by changes in the physical form and the nutrients content of the diet during the different rearing phases (Gabriel et al., 2006).

Specifically, green tea *(Camelia sinensis)* has been widely studied in human and animal due to its numerous bio functional properties, including antioxidant, antiviral and anticoccidial activities (Chacko et al., 2010; Khan, 2014). Most of these properties are ascribed to the high levels of polyphenolic compounds, among which catechins are the most represented group (Singh et al., 2011). Similarly, pomegranate *(Punica granatum)* also possess similar bifunctional properties mainly due to the presence anthocyanins, gallotannins, ellagitannins, gallagyl esters, hydroxybenzoic acids, hydroxycinnamic acids and dihydroflavonol (Negi et al., 2003; Aviram et al., 2008).

At the present moment the available literature considered the supplementation of these two phytobiotics in the feed, while the effects of their combined inclusion in water were not evaluated. The supplementation of these compounds via drinking water could represent a chance to perform treatments at specific critical timepoints, such as post-hatching or during the transition between feeding phases, avoiding their inclusion in the feed during the whole rearing cycle.

This experiment hypothesized that the inclusion of a blend of green tea (*Camelia sinensis*) and pomegranate (*Punica granatum*) in the drinking water during the most stressful rearing periods of

broiler could positively impact antiradical activity and cecal microbial ecology, leading to improved performance.

MATERIAL AND METHODS

Ethical statement

The present study was performed at the facility of Animal Production Research and Teaching Centre of the Polo Veterinario, Università degli Studi di Milano (Lodi, Italy). All procedures were reviewed and approved by the Animal Care and Use Committee of the University of Milan.

Broilers, diets and experimental treatments

A total of 480 one-day old male broiler chicks ROSS 308 were assigned to two experimental groups of 12 replicates each (n = 20/replicate) in a 50-days trial according to a completely randomised design. Animals were housed in two rooms, with similar microclimate conditions. The photoperiod was 24 h of light from day 0 to day 7, and 23 h of light from day 7 to the end of the trial. Room temperature was 35 °C for the first three days and was then weekly decreased by 2 °C, to a final temperature of 21 °C at the end of the trial. Pens (2.93 m^2) were bedded with shavings of white wood. At hatching all chickens were vaccinated against Marek's disease, Newcastle disease, infectious bronchitis and coccidiosis.

All animals received the same diets (Table 1), offered *ad libitum* and formulated to meet the nutrient requirements established by the National Research Council (NRC, 1994).

Diets were provided according to a three-phase feeding program, in crumbled form for starter- and grower-phase (0-10 and 11-20 days, respectively), and pelleted form for finisher phase (21-50 days). All the experimental diets were formulated and manufactured using the same lots of ingredients and without the inclusion of antibiotics or coccidiostats. Collected feed samples were analysed before the beginning of the trial to determine dry matter (method 930.15), crude protein (method 984.13), ether extract (method 920.39A), ash (method 942.05), Ca (method 968.08) and P (method 946.06) content following the relative Association of Official Analytical Chemists methods of analysis (Association of Official Analytical Chemists, 2005).

Experimental treatments consisted on the inclusion (T) or not (C) of a plant extract (PE) in the drinking water at the dosage of 0.2 mL/L for five consecutive days at the beginning of the trial (0-4 days), and for two consecutive days during the transition phase between diets (10-11 days and 20-21 days, respectively). Plant extract was composed of green tea leaves (*Camellia sinensis*) and

pomegranate rinds (*Punica granatum*) extract (Grazix™, InQpharm Group Sdn Bhd, Kuala Lumpur, Malaysia). During the entire length of the trial water was provided *ad libitum*, via automatic nipple cup drinker, except during the three treatment periods, when it was provided in graduated plastic tanks placed in each pen.

Growth performance and carcass characteristics

The body weight (BW) and the feed intake (FI) of the broilers were monitored on a replicate basis at 0, 10, 20 and 50 days of age. Mortality was recorded daily, and the BW of death broilers was used to adjust feed conversion ratio (FCR; feed/gain) accordingly. At the end of the trial, one animal was selected from each pen on the basis of the average weight and sacrificed.

The dressing percentage was calculated dividing the eviscerated weight by the live weight. Breast muscle was then removed and weighed, and the breast muscle yield was calculated as the percentage of eviscerated weight.

Total antiradical activity

Blood samples were collected on day 50 in 10 mL vacutainer tubes containing EDTA (Venoject®, Terumo Europe NV, Leuven, Belgium) and stored at 4°C for the determination of the total blood antiradical activity. Blood samples for KRL test were processed within 3 h from sampling and analysed in the next 24 hrs after collection by biological test Kit Radicaux Libres (KRL, Laboratories Spiral, Dijon, France) following users' protocol. The results were expressed as the time (minutes) required to achieve 50% of maximal haemolysis (half-haemolysis time – HT50 – in min), which reference to the whole blood and red blood cells (RBC) resistance to free-radical attack.

Cecal microbiota

Cecal contents were collected from the sacrificed birds to perform 16S rRNA gene sequencing. Cecal contents were removed into a sterile tube (Sarstedt, Nümbrecht, Germany) and immediately snap-frozen in liquid nitrogen and stored at -80°C. Bacterial DNA was isolated from cecal contents using the Exgene™ Stool DNA mini kit (Geneall Biotechnology Co., LTD, South Korea) starting with 200 μg of samples following the manufacturers' procedure. The extracted DNA was quantified using *Synergy HTX (*Biotek, United States*)* with a final concentration ranging from 3-10 ng/ul. Variable regions V3-V4 of the 16S rRNA were amplified by PCR with universal primers for prokaryotic (Takahashi et al., 2014). Amplicon sequencing was carried out on Illumina MiSeq 300PE platform in order to obtain raw paired-end reads 2x300 bp (BMR Genomics, Pavia, Italy).

The 16S sequencing data was processed and analysed using CLC Genomics Workbench version 12.0 and CLC Microbial Genomics Module version 4.1 (CLC bio, Arhus, Denmark). The paired end reads were merged into one high quality representative by default settings of CLC Workbench (Mismatch cost = 1, Minimum score = 40, Gap Cost = 4, Maximum unaligned end mismatches = 5). The CLC pipeline was used for primer and quality trimming (Trim using quality scores = 0.05; Trim ambiguous nucleotides: maximum number of ambiguities = 2; Discard reads below length = 5). SILVA reference database (https://www.arb-silva.de/) (Quast et al., 2013) was used for sequence alignment and sequences were binned into Operational Taxonomic Unit (OTUs) based on 97% similarity. The OTU table was further filtered by removing OTUs with low abundance (Minimum combined abundance = 10), to get a final abundance table for each sample. The phylogenetic tree was constructed using maximum likelihood phylogeny tool based on a multiple sequence alignment of the OTU sequences (100 most abundant OTUs) generated by MUSCLE (Multiple Sequence Comparison by Log- Expectation) tool (Edgar, 2004) in the workbench. The maximum likelihood phylogeny tool determines the probability of the sequences in the tree, using Neighbor Joining as construction method and Jukes Cantor as Nucleotide substitution model. The OTU table was used to calculate alpha diversity indices like Chao1 and Shannon's indices.

Statistical analysis

A completely randomized design was used. Growth performance were analysed using the Statistical Analysis System software (SAS version 9.4; SAS Institute Inc., Cary, NC, USA) applying a MIXED procedure for repeated measurements and accounting for the effects of treatment, time and treatment x time interaction. Total weight gain (TWG), total feed intake (TFI), total feed conversion rate (TFCR), carcass characteristics and KRL measurements were analysed using one-way analysis of variance (ANOVA) to compare the means of the two groups using a GLM procedure of SAS. Mortality rate was analysed by a PROC FREQ of SAS overall the trial period.
The pen represented the experimental unit for growth performances parameters, while the broiler represented the experimental unit for the carcass characteristics and KRL measurements.
All the numerical data in tables are presented as average values and accompanied by *SEM* (standard error of the mean) values.
Differences between groups were considered statistically significant at $p < 0.05$, whereas a trend for a treatment effect was noted for $p < 0.10$.

To determine diversity shared among the communities in the caecal microbiome of the samples, beta diversity (both weighted and unweighted UniFrac) was calculated in CLC workbench (Aarhus, Denmark) and significance was measured by PERMANOVA analysis (PERmutational

Multivariate ANalysis Of VAriance). MicrobiomeAnalyst tool, available at www.microbiomeanalyst.ca (Dhariwal et al., 2017), was used for further relevant statistical analysis. During the analysis, abundance of the OTU that did not meet the following parameter were removed: minimum number of counts 1, 5% prevalence in the sample and 1% of the samples below the standard deviation. Log transformation was used as a normalization method for downstream analyses, that also includes differential abundant analysis at different taxon level, performed by metagenomeSeq package (Paulson et al., 2013).

RESULTS

Growth performance, carcass characteristics, and total antiradical activity

Body weight, gain, FI, and FCR are shown in Table 2. The administration of PE via drinking water did not affect growing performance of treated broilers during the different rearing phases ($p > 0.05$). In the same way no significant differences were outlined for mortality rate, dressing and breast percentage.

The effects of PE on total antioxidant activity are showed in table 3. The inclusion of PE in drinking water during critical moments of the rearing cycle of broiler significantly improved the total antiradical activity, both in the whole blood (HT50 blood, $p < 0.01$) and RBC (HT50 RBC, $p < 0.05$).

Cecal microbiota

Sequencing of the amplicons resulted from the amplification product of PCR for the variable regions V3-V4 of the 16S rRNA by PCR was performed to investigate the treatment effect on cecal microbiota. Detail of sequence reads count and the OTU counts are provided in supporting material (Figure S1).

No statistical differences ($p > 0.05$) were evidenced in alpha diversity measured by Chao 1 bias-corrected and Shannon's index between C and T groups. Similarly, for beta-diversity, no statistical differences ($p > 0.05$) were observed in PERMANOVA (both unweighted and weighted UniFrac) between the experimental groups.

The relative abundance at different taxon level (phylum, order ad class) is shown in Figure 1. Firmicutes was found to be the most abundant phylum in both experimental groups, accounting for 69.47% in C group and 68.65% in T group. Bacteroidetes emerged as the second abundant

phyla with 20.94% in C group and 25.55% in T group. Proteobacteria resulted the third phylum, with 8.49% in C group and 4.84% in T group. At class level, Clostridia was the most abundant taxon in both experimental groups, followed by Bacteroidia, Gammaproteobacteria and Bacilli (Figure 1).

Differential abundant analysis was performed to find the significantly different (FDR < 0.05) taxon among the two groups (i.e. C and T). No significant differences were found at phylum level. At class level, Bacilli were significantly higher in T group with respect to C group. Similarly, at order level, Lactobacillales have shown a significantly (FDR < 0.05) greater abundance in T animals compared to C animals. At family level, *Lactobacillaceae* and *Peptococcaceae* were significantly more abundant in T group compared to C group (FDR < 0.05). *Clostridiaceae_1* tended (FDR = 0.06) to be higher in abundance for T group compared to C group. At genus level, *Roseburia* was found to be significantly higher in T group compared to C group (FDR < 0.05). On the contrary, *Shuttleworthia* was found to be significantly (FDR = 0.04) higher in C group. However, *Lactobacillus_ambiguous_taxa*, *Christensenellaceae_R7_ambiguous_taxa* and *Tyzzerella_3* tended (FDR = 0.06) to be higher in T group compared to C group. Further, a list of significantly differentially abundant taxon based on *p-value* ($p < 0.05$) is given in supplementary table S1.

DISCUSSION

During the last years phytobiotics gained increasing attention as replacement for antibiotics to enhance growth performances and improve animal health (Ri et al., 2017; Ahasan et al., 2019). The positive effect of phytobiotics is reported to be related both to the high polyphenolic content that can counteract the effect of free radicals generation (Brenes and Roura, 2010), and their modulation capacity of the gut microflora (Lillehoj et al., 2018; Saleh et al., 2018) leading to increased performance.

Among phytobiotics, green tea (Jelveh et al., 2018) and pomegranate (Hamady et al., 2015; Saleh et al., 2018) have been tested in poultry by different authors, with positive results on growth. All the studies performed with these two phytobiotics were conducted including the compounds in the feed and for all the rearing period. To the best of our knowledge, at the present moment very few studies were conducted including green tea or pomegranate in the drinking water and, among them, none considered the supplementation only in critical phases.

The lack of positive results on performance in our study could be both attribute to the way of administration or to the use of PE only for few days rather than for the total length of the trial. However, the same experimental design was previously used to evaluate the efficacy of PE in post-

weaning piglets by our group. In that study, we observed enhanced average daily gain during the last week of the trial, but no effect during the overall period (Bontempo et al., 2014).

As for growth parameters, PE did not affect dressing and breast percentages in the present study. Previous trials showed contrasting results when including phytobiotics alone or blended in the diet of poultry. Farahat et al. (2016) observed no effect on carcass characteristics with different inclusion rates of green tea extract, while Erener et al. (2011) and Hamady et al. (2015) found improved carcass characteristics after green tea or pomegranate extracts supplementation, confirming a lack of consistency among results. This variability in research findings can be explained by the different biological potential of the phytoadditives tested, which is subdued to numerous factors, such as extraction procedure, part of the plant used, geographical origins, and harvesting season (Bakkali et al., 2008).

In the current study, PE significantly increased the total antiradical activity of whole blood and RBC, which was measured through the KRL test. To the best of our knowledge this test was previously employed in chickens only once (De Marco et al., 2015). Our results confirm the beneficial effect of PE in improving antioxidant defences of the animals, in accordance with previous findings. Although the same parameters were not evaluated in the present study, the antioxidant effects of PE were recently confirmed by Rao et al. (2019) who observed reduced lipid peroxidation and increased glutathione peroxidase activity after supplementation of pomegranate peel meal to broiler chickens. Similarly, the inclusion of green tea extract in poultry diet increased glutathione-reduced level in the liver and significantly decreased malondialdehyde level of meat tissue (Farahat et al., 2016). The positive effects observed were attributed to the high polyphenols content of both green tea and pomegranate extracts, able to prevent reactive oxygen species (ROS) generation or the damage induced by the same ROS. The proposed mechanism of action for polyphenols is that after being absorbed in the gut, they can be bound by blood cells, mainly erythrocytes, leading to enhanced total antioxidant-scavenging capacity of the blood (Koren et al., 2010; Ginsburg et al., 2011).

To the best of our knowledge, there are no indication in the literature on the effects of phytobiotics supplementation only during the post-hatching phase and the transition between feeding phases, and their consequent effect on intestinal microbiota in later stages of life. It is recognized that the sharpening of the gut microbiota in critical moments of life could have an impact on the entire life-span of the animals (Ballou et al., 2016). Among the critical moments, the post-hatching phase is one of the most important, since it is when the first colonization of the gut occurs (Apajalahti et al., 2004). Several studies have shown that early gut microflora modulation can affect health and productivity in later stages of broilers' life (Yin et al., 2010; Schokker et al.,

2017). The post-hatching phase is not however the only critical moment in the sharpening of the microbiota. The gut microbial population can indeed be affected also by changes in the diet, referable for example to the form of the feed (grounded or pelleted), the kind of cereal, the sources of fat, starch and proteins, as well as the quantity of water-insoluble non-starch polysaccharides (Gabriel et al., 2006).

In our study PE did not impact cecal microbiota composition, keeping the microbial profile in line with the diet used for general practice. Gut microbial population observed in this study was indeed aligned with what was reported by Wei and colleagues (2013). In this review, authors described the cecal microbial composition of adult birds, reporting Firmicutes as the most abundant phylum, followed by Bacteroidetes and then Proteobacteria. Although in our study no differences at differential abundant analysis were observed at phylum taxonomy level, a higher presence of Bacilli was observed at class level in broilers receiving PE. Analogous results were observed in the ileum and jejunum of broiler chickens following the supplementation of L-theanine, an amino acid extracted from green tea (Saeed et al., 2019). At lower taxonomic level, animals receiving PE showed greater abundance of lactic acid bacteria compared to control group. This result was confirmed at family level, where *Lactobacillaceae* and *Enterococcaceae* were found to be more abundant in T broilers. Also, at genus level, *Lactobacillus* showed a tendency to be higher in T group. Lactic acid bacteria are well recognized for their beneficial effect at intestinal level, regulating the composition of intestinal microflora, developing the immunity of the intestine and promoting gut health (Muir et al., 2000). Lactobacilli can indeed protect against the colonization of pathogenic bacteria through the acidification of the lumen and the production of bacteriocins (Messaoudi et al., 2012; Cao et al., 2013)

At genus level, *Roseburia_ambiguous_taxa* were found to be significantly higher in animals receiving PE. *Roseburia* genus is a commensal saccharolytic bacteria, producing SCFAs, which has been proposed in human medicine as probiotic for restoration of beneficial flora (Duncan et al., 2002; Tamanai-Shacoori et al., 2017). In contrast with our results, the supplementation of green tea polyphenols in mice model was responsible of a reduction of *Roseburia* in the colon (Zhang et al., 2018). We observed significantly lower abundance of *Shuttleworthia* in T group. Information about this genus are limited, but a study reported that the enrichment of *Shuttleworthia* in the ceca of male broiler chicken was associated with high body weight (Lee et al., 2017), that was not evidenced in our study.

CONCLUSIONS

The present study confirmed the beneficial effects of phytobiotics in animal nutrition. Our results demonstrated that the administration of 0.2mL/L green tea and pomegranate extract in drinking water during the post-hatching and at the changes between feeding phase can improve total blood antiradical activity and may positively affect gut microbial ecology of adult broiler chickens.

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TABLES

Table 1 *Feed ingredients and nutrient composition of the basal diets (as-fed basis).*

Item	Starter	Grower	Finisher
Ingredients, (g/kg)			
Corn	550.5	574	616.7
Soybean meal (480 g crude protein)	373	341	292
Soybean oil	30	43	53
Dicalcium phosphate	25	25	21
Calcium carbonate	7	4.5	5
Mineral + Vitamin Premix†	5	5	5
NaCl	4	4	4
DL-Methionine	3.2	1.8	1.6
L-Lysine-HCl	2.3	1.7	1.7
Nutrient values of the mixtures, calculated, per kg			
ME, MJ	3,002.54	3,099.86	3,200.07
Crude protein, g	224	212.3	198
Calcium, g	9.8	9.3	7.9
Phosphorus, g	8.5	8.2	7.2
Lys, g	10	8.3	7.6
Met + Cys, g	6.4	4.9	4.4
Nutrient values of the mixtures, analysed, per kg			
Dry matter, g	877.7	878.2	878
Crude protein, g	229.7	215.1	195
Ether extract, g	56.3	69.4	79.8
Crude ash, g	68.2	64.04	58.6
Calcium, g	10	9.1	8.1
Phosphorus, g	8.7	8.5	7.6

† Provided the following per kg of diet: vitamin A, 11,250 IU; vitamin D_3, 5000 IU; vitamin E, 60 mg; $MnSO_4 \cdot 1H_2O$, 308 mg; $ZnSO_4 \cdot 1H_2O$, 246 mg; $FeSO_4 \cdot 1H_2O$, 136 mg; $CuSO_4 \cdot 5H_2O$, 39 mg; KI, 2.4 mg; Na_2SeO_3, 657 µg; 6-Phytase EC 3.1.3.26, 750 FTU; Endo-1, 4-beta-xylanase EC 3.2.1.8, 2250 U.

Table 2. *Effects of plant extract supplementation on growth performance parameters and carcass characteristics of broilers.*

	Experimental groups[1]		SEM	*p-Value*		
Item	C	T		Treatment	Time	Treatment X Time
Body weight, kg						
0 day	0.883	0.872	0.84	0.469	<0.001	0.638
10 days	6.195	6.215				
20 days	18.312	18.332				
50 days	74.892	73.106				
Gain, kg						
0-10 days	5.312	5.342	1.08	0.445	<0.001	0.533
11-20 days	12.117	12.117				
21-49 days	56.58	54.774				
TWG	74.008	72.233	1.67	0.46		
Feed intake, kg						
0-10 days	6.393	6.343	0.82	0.276	<0.001	0.294
11-20 days	18.102	18.158				
21-49 days	122.808	120.863				
TFI	147.302	145.363	1.26	0.287		
Feed conversion ratio, kg/kg						
0-10 days	1.2	1.19	0.04	0.721	<0.001	0.689
11-20 days	1.5	1.5				
21-49 days	2.18	2.23				
TFCR	2	2.02	0.04	0.613		
Mortality, %	8.75	10.42		0.535		
Carcass characteristics						
Dressing, %	75.59	76.83	0.56	0.133		
Breast, %	21.41	22.41	0.66	0.293		

Note: $p < 0.05$ were considered significantly different, $p < 0.1$ were considered tendency
Abbreviation(s): SEM = standard error of the mean. TWG = total weight gain; TFI = total feed intake; TFCR = total feed conversion rate.
[1] C = animals receiving no supplementation; T = animals receiving 0.2mL/L green tea and pomegranate extract via drinking water at days 0-4, 10-11 and 20-21.

Table 3. *Effects of plant extract supplementation on total antioxidant activity. Data are shown as means ± SEM*

	Experimental groups[1]		***p-value***
Item	**C**	**T**	**Treatment**
HT50 whole blood, min.	69.17 (± 2.77)	76.52 (± 7.05)	<0.001
HT50 RBC, min.	56.72 (± 3.03)	61.28 (± 3.87)	0.023

Note: $p < 0.05$ were considered significantly different.
Abbreviation(s): HT_{50} = time (minutes) required to achieve 50% of maximal haemolysis; RBC = red blood cells. [1] C = animals receiving no supplementation; T = animals receiving 0.2mL/L green tea and pomegranate extract via drinking water at days 0-4, 10-11 and 20-21.

Table S1. *Significantly different taxon according to p-value (P ≤ 0.05) shown by differential abundant analysis among the two experimental groups.*

TAXON	*p-value*
Class	
Bacilli	<0.01
Order	
Lactobacillales	<0.01
Family	
Lactobacillaceae	<0.01
Peptococcaceae	<0.01
Clostridiaceae_1	<0.01
Enterococcaceae	0.05
Genus	
Roseburia[1]	<0.01
Shuttleworthia	<0.01
Tyzzerella 3	<0.01
Lactobacillus[1]	<0.01
Christensenellaceae R7 [1]	<0.01
Anaerostipes	<0.01
Harryflintia	<0.01
Clostridium sensu stricto1	<0.01
Ruminococcaceae UCG014 [1]	0.01
Butyricicoccus	0.01
Ruminococcus gauvreauii group [1]	0.02
Lachnospiraceae UCG006	0.03
Anaerotruncus [1]	0.03
Merdibacter	0.04
Blautia [1]	0.04
Enterococcus [1]	0.04
Ruminiclostridium 5	0.04
Candidatus Arthromitus	0.05
Species	
Gut metagenome	<0.01
Merdibacter massiliensis	0.04
uncultured Clostridiaceae	0.05

Bolded taxa have showed a significant difference at FDR ≤ 0.05
[1]Ambiguous taxa

Figure 1. *Relative abundance at different taxon level: phylum (A), class (B) and order (C) in control and treated group. Class and Order having counts <10 are merged together and are reported as 'others'*

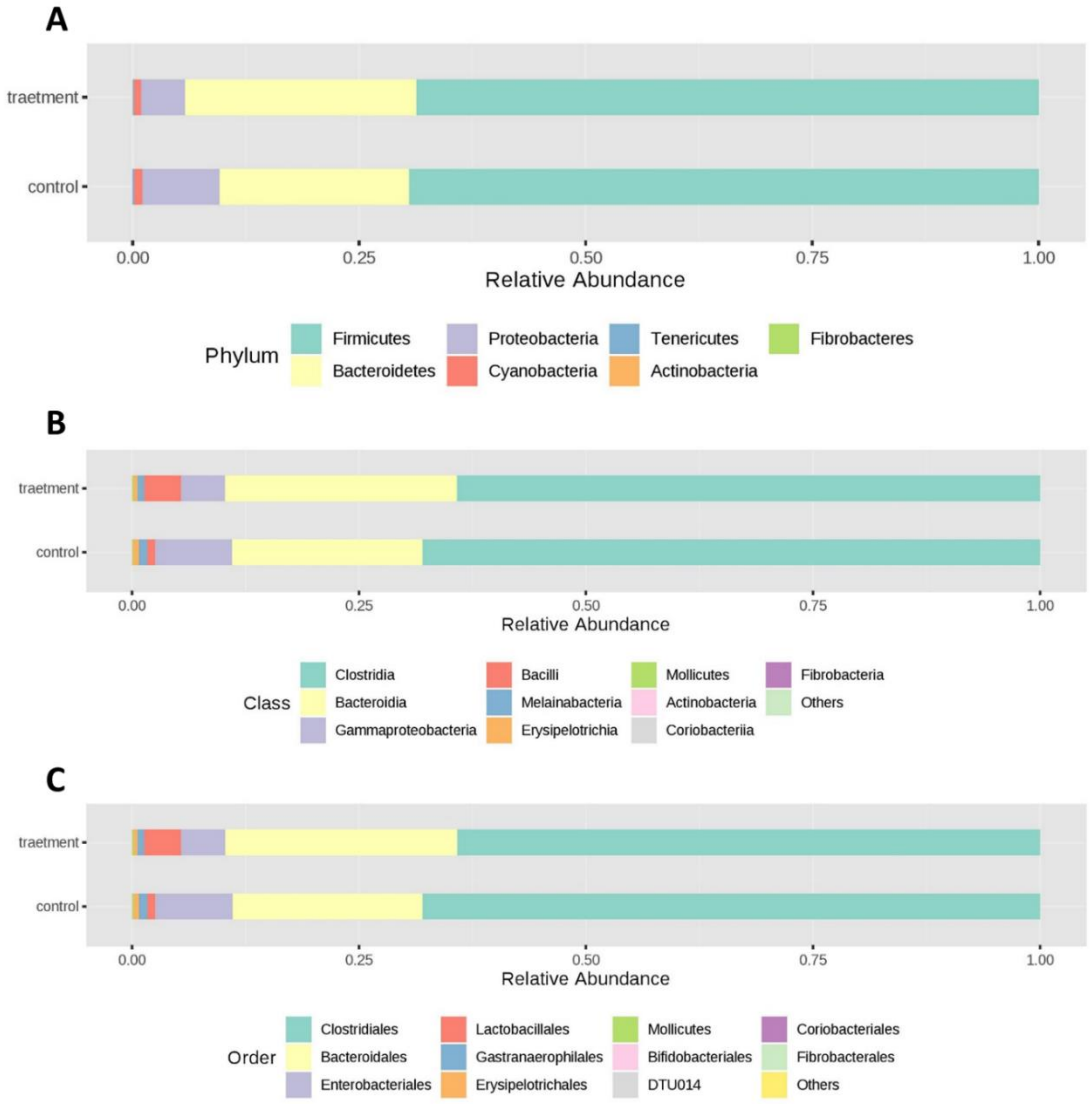
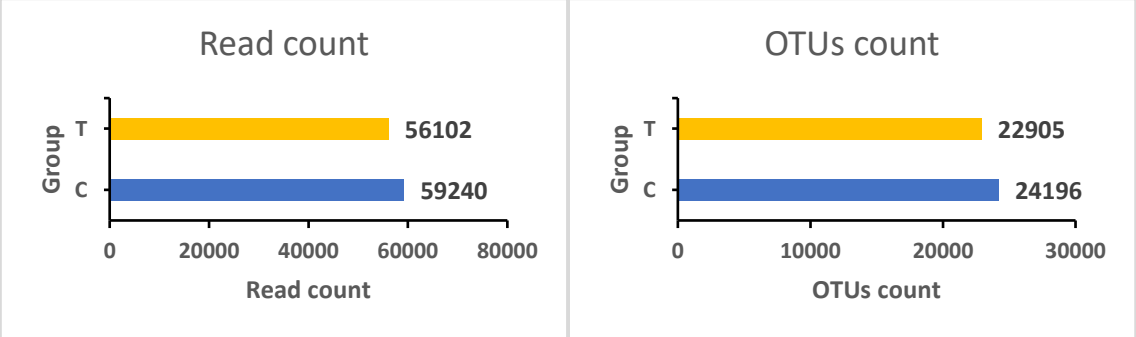


Figure S1. *Mean number of 16S rRNA sequence reads (A) and the number of OTU counts (B) detected in the cecal samples of broilers in treated (T) and control (C) groups.*



Chapter 6

Effects of nucleotides administration on growth performance and immune response of post-weaning piglets

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ABSTRACT

Nucleotides are essential for the proper development of the gastrointestinal tract, but their endogenous production can be insufficient to meet the high demand of rapid turnover cells, such as enterocytes and lymphocytes. Under certain stressful conditions, such as weaning, dietary nucleotides become essential to optimize intestinal and immunological function. The aim of this study was to assess the effect of nucleotides administration on growth performance, and inflammatory and immune response in post-weaning piglets. Twenty-eight weaned piglets, homogeneous for age and weight were randomly allocated to two experimental treatments. Treated group (T) received every morning 0.8g/head of nucleotides suspended in 2.1 ml water solution; while control group (C) received 2.1 ml saline solution. The nucleotides composition of the product was: 88.05% uridine-monophosphate (UMP), 5.51% guanosine-monophosphate (GMP), 3.82% adenosine-monophosphate (AMP), 1.94% cytidine-monophosphate (CMP) and 0.68% inosine-monophosphate (IMP). Body weight (BW), feed intake (FI), average daily gain (ADG) and gain:feed (G:F) ratio were recorded weekly to determine growth performances. Fecal scores was recorded weekly, on a pen basis. On day 0, 9, 18 and 27 blood samples were collected for the determination of IgA, IgG and haptoglobin concentration. At the end of the trial (d28) all piglets were sacrificed, and slaughtering performance recorded. Furthermore, ileal Peyer's patches were collected for the evaluation of IL1α, IL1β, IL6, IL10, TNFα, TLR2, TLR4 and PPARγ gene expression. Nucleotides supplementation had a significant effect on BW, ADG, and FI overall the period of the trial, and at slaughtering T group was heavier than C group ($p < 0.01$). Nutrient utilization tended to be improved in T group in the first week of the trial (G:F ratio, $p = 0.07$). Faeces tended to be softer in T group ($p = 0.076$), however no occurrence of diarrhoea was reported. Nucleotides supplementation did not affect inflammatory and immune response ($p > 0.05$). The lack of results for inflammatory response and the expression of immune related genes is likely due to the optimal rising condition, which were not challenging for the animals, minimizing the stress usually induced by weaning. The absence of stressful event was corroborated by haptoglobin concentrations, which remained steady overall the period of the trail. Further studies are required to investigate the effect of nucleotides supplementation in stressful situation.

INTRODUCTION

Over the last years efficiency and quality of commercial swine production have been significantly improved thanks to breeding and nutritional programs, as well as management practices. With the aim to further accelerate the production cycle, weaning age have been progressively reduced, and piglets are weaned at 3 to 4 weeks of age. Weaning is a stressful moment in pigs' life, accompanied by physiological changes in the gastrointestinal tract (GIT), which become more pronounced when early-weaning practices are adopted. Those changes, along with reduced feed intake due to the shift from a liquid to a solid diet and the social stress derived from the separation from the mother, are ultimately responsible of growth check at weaning, which can adversely affect pigs' long-term performance (Dirkzwager et al., 2005; Moore et al., 2011). A fast recovery of the intestine is therefore essential for proper growth of weaned piglets, and to this end antimicrobials have been widely used in the past to counteract the adverse effects of weaning. Their beneficial impact on growth performance have been ascribed to their modulation of the gut microflora (Visek, 1978; Knarreborg et al., 2004), or to the reduction of proinflammatory cytokines production (Niewold, 2007). However, the general concern about the risk of antibiotic-resistance development led to the ban of their use as growth promoter by the European Union in 2006. In this light, the interest towards alternative substances has strongly increased, including the investigation of feed additives that stimulate growth and cell differentiation of intestinal tract and immune system of piglets (Assoni et al., 2017; Li et al., 2019).

Nucleotides are a group of bioactive compounds, composed by a nitrogenous base, a pentose sugar and one or more phosphates. Nucleotides play several roles in biochemical processes; they serve as nucleic acid precursors, physiological mediators, components of coenzymes and sources of cellular energy (Grimble and Westwood 2001; Sauer et al. 2011). Nucleotides have also a key role for the maturation of enterocytes and lymphoid cells. Despite intestinal epithelial cells can provide endogenous nucleotides, either via *de novo* synthesis or via salvage pathway (Craver and Walker, 1995), dietary supply might become 'conditionally essential' in stressful moments, such as weaning. At this stage, the requirement of nucleotides strongly increases to promote the growth of intestinal epithelium and lymphoid cells (Sato et al., 1999). Until the moment of weaning, the huge demand of nucleotides for piglets' GIT development is supplied by sow's milk. Mammalian milk is characterized by a high concentration of nucleotides, which account for up to 20 % of the non-protein fraction of milk (Uauy, 1989). At the moment of weaning, the nucleotide contribution of milk fails, and post-weaning diet may be not suitable for proper intestinal maturation because of its low nucleotides content (Martinez-Puig et al., 2007). Thus, dietary supplementation of nucleotides may positively contribute to the post-weaning phase.

Several authors observed positive effect of dietary nucleotides supplementation on piglet's gut morphology (Moore et al., 2011; de Andrade et al., 2016) and immune system development, including lymphocyte maturation, activation and proliferation (Gil et al., 2002). Domeneghini et al. (2004) observed higher percentages of macrophages in the intestine, and higher intra-epithelial lymphocytes in the mucosa of weaning piglets receiving nucleotides. Furthermore, since nucleotides enhance enterocytes maturation which in turn are involved in processes related to the immune response, such as cytokine production and antigen presentation, it is likely that dietary nucleotides can contribute to the maturation of the gut associated lymphoid tissue (Gil et al., 2002). However, the effect of nucleotides supplementation on cell function and proliferation seems to be strongly dependent on the duration of the treatment (Cameron et al., 2001).

The aim of the present study was to investigate the effect of nucleotides administration to post-weaning piglets on growth performances and immune response, both at local and systemic level.

MATERIALS AND METHODS

Ethical statement

The present study was performed at the facility of Animal Production Research and Teaching Centre of the Polo Veterinario, Università degli Studi di Milano (Lodi, Italy). All experimental procedures were reviewed and approved by the Ethics Committee of the University of Milano

Animals, housing, diet and experimental treatment

A total of twenty-eight weaned pigs (Topig40 x Topigs Fomeva), homogeneous for age (28 ± 1.6 days) and initial body weight (7.68 ± 0.31 kg) were provided from a commercial farm (Az. Agricola Arioli e Sangalli, Genzone, Italy). According to their initial body weight, piglets were assigned to two experimental groups (control and treated) of 7 replicates each (2 animals/replicate) in a completely randomized design

Animals were housed in one room, with computer-controlled heating and mechanical ventilation systems. Room temperature was maintained at 28°C at the beginning of the experimental period, and decreased by 1°C every 3 days, to a final temperature of 24°C at the end of the trial. Each pen was provided with plastic slatted floor, a feeding trough and two drinking nipples. Two stainless steel chains per pen were provided as environmental enrichment. At day 11 and 19 post-partum,

piglets were vaccinated against *Mycoplasma Hyopneumoniae* (Ingelvac Mycoflex, Boehringer Ingelheim) and *Porcine Circovirus type 2* (Ingelvac Circoflex, Boehringer Ingelheim), respectively.
All piglets were fed a standard commercial diet (meal form) formulated to meet or exceed nutrient requirements for post-weaning piglets (NRC, 2012) (Table 1). Water and feed were provided *ad libitum*.
For 28 consecutive days, every morning at 0800, control animals (C) were orally administrated 2.1 ml of saline solution, while treated animals (T) received 0.8g/head/day of nucleotides (Prosol spa, Madone, Italy) in a 2.1 ml water solution. The nucleotides mix contained uridine-monophosphate (UMP), guanosine-monophosphate (GMP), adenosine-monophosphate (AMP), cytidine-monophosphate (CMP) and inosine-monophosphate (IMP).
The chemical composition of the basal diet was analysed at the beginning of the trial following the Association of Official Analytical Chemists methods of analysis (AOAC, 2005).

Samples and measurements

Individual body weight (BW) and pen feed consumption were recorded on weekly basis (at day 0, 7, 14, 21 and 28) by electronic scale (Ohaus ES100L, Pine Brook, New Jersey; sensitivity ± 0.02 kg). Growth performance, including average daily gain (ADG), feed intake (FI), and gain:feed ratio (G:F ratio), were subsequently determined for each pen.
Faecal score was recorded once a week on pen basis by subjective four-point scale, where 1=firm and 4=watery, according to Wellock et al. (2007).
Blood samples were collected from the same one piglet per pen at day 0, 9, 18 and 27. Animals were manually contained, and blood samples were collected from cranial vena cava into a 10ml vacuum tubes with ethylenediaminetetraacetic acid (VT100STK, 0.1ml EDTA) as anticoagulant. Samples were subsequently analysed for haptoglobin (Hp), IgA, and IgG. The concentrations of haptoglobin were determined by colorimetric assay (Tridelta Phaserange serum haptoglobin assay, cat. no. TP-801) and expressed on the basis of a standard curve (Cooke and Arthington, 2013). Intra-assay CV and inter-assay CV were 7.41% and 6.18%, respectively. IgA and IgG were measured by porcine-specific ELISA kit according to the recommendations of the manufacturer (Bethyl Laboratories, Montgomery, TX, USA). All samples were assayed in duplicate.
Piglets were sacrificed at day 28, and dressing percentage was calculated. Furthermore, 10mg samples of ileal Peyer's patches were obtained from each animal. Ileal segments containing Peyer's patches were collected approximately 5 cm before the ileocecal valve. The pieces of tissue were cut longitudinally along the side of the intestine opposite the Peyer's patches, gently rinsed with saline solution and stripped of the underlying smooth muscle layer. Peyer's patches were then excised

from the tissue samples with a lancet and immediately stored in 1.5ml cryovials with 0.9ml RNAlater solution (Invitrogen, Life Technologies Ltd, Paisley, UK), and frozen at -80°C for further analyses.

Immune-related genes quantification by RT-qPCR

Total RNA was extracted with TRIzol Reagent® (Invitrogen, Life Technologies Ltd, Paisley, UK) and purified with a commercial kit (Macherey-Nagel, Oensingen, Switzerland), according to the manufacturer's recommendations. The RNA concentration was quantified by use of the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The purity of RNA (A_{260}/A_{280}) was ~2. Specific mRNAs were amplified and quantified using the iScriptTM One Step RT-PCR for Probes reagent (Bio-Rad, CA, USA), according to the manufacturer's instructions. RT-q-PCR was performed with CFX384 Real-Time System (Bio-Rad, CA, USA). Thermal protocol was: 50 °C for 10 minutes for reverse transcription and then 95 °C for 10 seconds/60°C 30seconds for 40 cycles. For assessment of melting curves, PCR products were incubated at 55 °C for 60 s then the temperature was increased to 95 °C at 0.5 °C increments for 10 s.

Samples from ileal Peyer's patches were analysed for the expression of *interleukin 1a* (*IL1a*), *interleukin 1β* (*IL1β*), *interleukin 6* (*IL6*), *interleukin 10* (*IL10*), *peroxisome proliferator-activated receptor γ* (*PPARγ*), *tumor necrosis factor a* (*TNFa*), *toll-like receptor 2* (*TLR2*), *toll-like receptor 4* (*TLR4*), and *β-actin* as reference gene.

Primers and probes for real-time qPCR were purchased from Applied Biosystems (Carlsbad, California, USA) except the set for β -actin quantification (forward primer 5'-ACTCGATCATGAAGTGCGAC-3', reverse primer 5'-GTGATCTCCTTCTGCATCCTG-3', Taqman probe 5'-CGTGTTGGCGTAGAGGTCCTTCC-3'), which were designed with IDT software available online, optimized to work in a one-step protocol and were synthesized by Eurofin MWG Operon (Huntsville, AL, USA). The relative expression levels were determined by normalizing the C_t of the indicated target with the C_t of *S. scrofa* β-actin, as the reference gene for normalization, using the $\Delta\Delta C_t$ method.

Statistical analysis

A multivariate ANOVA for repeated measurements (SAS version 9.4, SAS Institute Inc., Cary, NC, USA.) was applied to analyse growth performance, fecal scores, haptoglobin, and immunoglobulin. Effects of treatment, time and treatment x time interaction were included in the statistical model.

Gene expression levels, carcass weight and dressing percentage were analysed by a General Linear Model (GLM). The experimental unit for growth performance was represented by the pen, while the piglet represented the experimental unit for IgA, IgG and Hp plasma content and gene expression. Significance level was declared at $p \leq 0.05$, while trends were considered at $p < 0.1$.

RESULTS

Growth performance and fecal score

Growth performances are shown in Table 2. Overall the period of the trial, animals receiving nucleotides supplementation showed increased ADG and FI than control animals ($p < 0.05$ and $p < 0.01$, respectively), while treatment x time interaction evidenced a trend ($p = 0.07$) for improved G:F ratio in T group than C, especially in the first week of trial. At slaughtering, carcass weight of T group was higher than C group ($p < 0.01$), but dressing percentage did not differ between groups. Nucleotides supplementation significantly affected fecal score during the trial ($p = 0.01$) and the interaction between treatment and time outlined a trend ($p = 0.08$) of T group to have softer faeces in the first two weeks of the experiment although no diarrhoea incidence was reported throughout the whole period.

Inflammatory and immune response

Hp concentration was not affected by treatment ($p = 0.477$), time ($p = 0.422$) and their interaction ($p = 0.514$). Analogously, nucleotides didn't affect IgA and IgG plasma concentration ($p > 0.05$) and no interaction of treatment x time was observed ($p = 0.514$).
Results of immune-related gene expression are summarized in Table 4. No effect of nucleotides supplementation was observed for interleukins, *TNFa, TLR2, TLR4* and *PPARγ* gene-expression.

DISCUSSIONS

Nucleotides are essential for the proper development of the gastrointestinal tract, but in young animals their *de novo* synthesis can be inadequate. Until the moment of weaning, nucleotides requirements are fulfilled by sow's milk supply (Mateo et al., 2004), which is then replaced by commercial diets, whose nucleotides content is usually poor. It is therefore hypothesized that nucleotides supplementation might be useful to support the GIT development in the post-weaning phase, finally leading to better piglets' health and performance.

Overall the period of the trial, animals receiving nucleotides supplementation showed better ADG and FI than control animals. In the first week after weaning, nucleotides tended to improve nutrient utilization, as indicated by the greater G:F ratio to parity of feed intake with control group. Accordingly, even though body weight at day 7 was not significantly different between the two groups, T animals were heavier than C animals. After the first week, growth performance were similar between the two groups, but body weight difference between C and T animals increased, however still remaining not significant. At the end of the trial, animals receiving nucleotides were weighing on average 1.69 kg more than C animals, further confirmed by the significant heavier weight at slaughtering. Over the years, several studies investigated the effect of nucleotides on animals' performances, but results are still contrasting or, at least, variable. In agreement with our findings, Weaver and Kim (2014) observed beneficial effects of nucleotides supplementation in the first week after weaning, but not further in the trial. On the contrary, Superchi et al. (2012) reported improved body weight and ADG in post-weaning piglets starting from day 35 of administration. Finally, in several other studies, nucleotides failed to improve growth performances (Lee et al., 2007; Martinez-Puig et al., 2007; de Andrade et al., 2016). The discrepancy in the findings may possibly be related to the source and dosages of nucleotides used, the biological differences between the animals used in the different trials, and the experimental conditions applied. The first week after weaning is probably the most challenging for the animals, accompanied by significant alterations of the GIT (Pluske et al., 1997). Nucleotides are recognized for their trophic effect on enterocytes (Amorim et al., 2017), and their supplementation may contribute to reduce the damage of intestinal mucosa and/or to a faster restoration of its integrity, as previously observed (Domeneghini et al., 2004; Che et al., 2016).

Better intestinal health condition accompanied by improved growth performance have been reported to be associated with reduced incidence of diarrhoea, as observed by Martinez-Puig et al. (2007). Even though we observed softer faeces in treated animals, the difference was minimal, and no occurrence of diarrhoea was reported. Similar results were reported in other studies (Superchi et al., 2012; Weaver et al., 2014).

In our study immunoglobulin production was not affected by nucleotides supplementation, in accordance with Moore et al. (2011). On the contrary, a general positive effect of nucleotides supplementation on the immune system, which is supposed to be mainly related to their interaction with T cells has been previously reported (Carver and Walker, 1995). In particular, Lee and colleagues (2007) observed an increase of plasma IgA concentration following the supplementation of 0.1% nucleotides in the diet of post-weaning piglets, and similar results were reported by Sauer et al. (2012); however, in both studies no effect was recorded for IgG. Based on this variability

between results, we hypothesized that the experimental conditions, which did not exactly reflect the situation of commercial farms, may have influenced the results on immune-related parameters (Mateo, 2005). More pronounced stressful rearing conditions can be useful to further exacerbate the effects of nucleotides administration. In our trial, this hypothesis was corroborated by observed haptoglobin concentrations. Haptoglobin is a positive acute-phase protein, whose concentration has been found to increase after weaning due to chronic stress or inflammatory stimuli exposure, and to reach again basal levels starting from the third week post-weaning (Sauerwein et al., 2005). In the present trial the lack of a significant time effect on haptoglobin levels during the whole experimental period suggests minimal stress of our piglets associated with weaning. Analogously, optimal raising conditions might have been also responsible for the lack of difference in cytokine gene expression at ileum level. Waititu et al. (2017) highlighted beneficial effect of nucleotide-rich yeast extract supplementation in enhancing the expression of proinflammatory cytokines, which was however observed only when piglets were raised in unclean room. This result emphasises the requirement of immune stimuli to evaluate the immunomodulatory properties of the product.

CONCLUSIONS

In conclusion, our results showed a positive effect of nucleotides supplementation on FI and ADG overall the period of the trial, finally leading to greater carcass weight. However, no nucleotides effect was observed on the immune response. The absence of stressful input due to the optimal conditions under which the animals were kept might have been responsible for the lack of difference on the immune response, therefore further studies in stressful situations are warranted to better investigate nucleotides' role in enhancing animal health and performance.

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TABLES

***Table 1**: Ingredient and composition of the diet administered to the animals (as fed basis)*

Components	**(% as fed)**
Barley flaked	21.35
Micronized wheat	18.20
Micronized barley	16.00
Soybean protein concentrate	9.00
Soybean meal (48%CP)	8.20
Flaked Corn	6.00
Micronized Corn meal	5.00
Lactose	5.00
Minvit premix *	4.00
Soybean oil	3.60
Potato protein concentrate	2.00
Calcium carbonate	0.90
Monocalcium Phosphate	0.40
Sodium cloride	0.30
Flavor	0.05
Total	**100**

Composition**	(% as fed)
CP	18.56
CF	3.17
Ca	0.69
P	0.62
Ca/P ratio	1:1
ME (Kcal/kg)	3,370.00

*Provided per kg of complete diet: vitamin A, 10,000 IU; vitamin D3, 1,000 IU; vitamin E, 50 mg; vitamin B1 1.0 mg; vitamin B2 3.0 mg; vitamin B6 3.0 mg; vitamin B12, 0.03 mg; riboflavin, 9 mg; pantothenic acid, 14 mg; nicotinic acid, 15 mg; biotin, 0.06 mg; vitamin PP, 0.35 mg; folic acid, 0.97 mg; vitamin K3, 3 mg; choline, 300 mg; Fe, 100 mg; Cu, 20 mg; Co, 0.75 mg; Zn, 100 mg; Mn, 10 mg; I, 0.85 mg; Se, 0.4 mg; ethoxyquin, 150 mg.
**CP: Crude Protein; CF: Crude Fiber; Ca: Calcium; P: Phosphorus; ME: Metabolized Energy

Table 2. *Effect of nucleotides supplementation on growth and slaughtering performances*

Item	Time	Treatment		SEM	*P*-value		
		C	T		Treatment	Time	Treatment*time
BODY WEIGHT							
kg	0 d	15.08	15.59	0.75	<0.002	<0.001	0.376
	7 d	16.73	18.14				
	14 d	21.84	23.41				
	21 d	28.03	30.82				
	28 d	34.74	37.99				
Feed intake							
kg/box/week	0-7 d	3.53	3.83	0.38	<0.007	<0.001	0.636
	7-14 d	7.84	8.13				
	14-21 d	10.96	11.69				
	21-28 d	11.06	11.51				
	0-28 d	7.92^{B}	8.79^{A}				
ADG							
g/box/d	0-7 d	236	363	46.83	0.012	<0.001	0.390
	7-14 d	731	753				
	14-21	883	1058				
	21-28 d	959	1025				
	0-28 d	702^{b}	800^{a}	23.41			
G:F							
	0-7 d	0.50^{b}	0.66^{a}	0.04	0.279	0.236	0.070
	7-14 d	0.69	0.64				
	14-21 d	0.61	0.63				
	21-28 d	0.64	0.62				
	0-28 d	0.61	0.64	0.02			
CARCASS WEIGHT		13.66^{B}	15.14^{A}	0.36	<0.007		
kg							
DRESSING %		78.93	79.62	0.45	0.294		

^{A,B} $P < 0.01$; ^{a,b} $P < 0.05$

Table 3. *Faecal score of C (control) and T (treated) groups*

Item	Time	Treatment		SEM	*P*-value		
		C	T		Treatment	Time	Treatment*time
FAECAL SCORE							
	7 d	3.14^{B}	3.43^{A}	0.07	<0.01	<0.001	0.076
	14 d	3.00^{B}	3.29^{A}				
	18 d	3.00	3.00				
	21 d	3.00	3.07				
	26 d	3.00	3.00	0.03			

^{A,B} $P < 0.01$; ^{a,b} $P < 0.05$

Table 4. *Effect of administration of dietary nucleotides on gene expression at ileal Peyer's patches level. Values are expressed as Relative Arbitrary Unit (RAU).*

Item	Treatment		SEM	P-value
	C	T		
IL1a	0.799	0.902	0.23	0.663
IL1b	0.829	0.637	0.26	0.479
IL6	0.996	1.256	0.21	0.245
IL 10	0.789	1.105	0.20	0.144
TNF	0.869	1.417	0.47	0.286
TLR2	1.124	1.004	0.17	0.459
TLR4	0.931	1.147	0.23	0.358
PPARG	0.903	1.185	0.19	0.172

^{A,B} $P < 0.01$; ^{a,b} $P < 0.05$

Chapter 7

General discussions and conclusion

The livestock sector is facing a huge challenge trying to meet the increasing demand of animal products. To this end several efforts have been done to enhance animals' performances, and nutrition provides a valuable asset. First of all, it is essential to make sure that nutrients requirements are utterly fulfilled, so that animals can optimally express their genetic potential. In this light a great work has been done over the years to accurately define livestock's nutrients requirement, and refinements are still underway. In addition, through a nutritional approach is being sought to improve animal heath, finally leading to improved productive and reproductive performances (Verstegen and Williams, 2002).

Dairy cows feeding is based on forages and concentrates, which are supplied in different proportions according to the lactation stage. In intensive production systems the diet is usually provided as total mixed ration (TMR) as a mean to supply a consistent provision of nutrients to rumen microbes. This allows to optimize rumen function and improve the efficiency of nutrient utilization (Coppock et al., 1981), hence increasing productive performances and health of dairy cattle, avoiding the incidence of diseases such as rumen acidosis (DeVries et al., 2005). The basic assumption for obtaining the benefits associated with TMR feeding is its homogeneity. TMR composition has to be homogeneous along the feeding alley in order to ensure each animal is receiving the same well-balanced diet. However, the achievement of the homogeneity is often subdued to the mixer wagons used for the preparation of the ration. The available literature reports studies about the efficiency of mixer wagons (Vegricht et al. 2007; Sıstkova et al. 2015), mainly describing the procedure of loading, cutting and mixing adopted in the farms. However, information about the effect of their combination is not available so far. For this reason, one of the aims of this thesis was to investigate the effect of cutting times, mixing times, loading levels and their interaction on TMR consistency along the feeding alley (1st trial). The results showed a significant effect of the mixing time for DM, and an effect of loading level for CP, Ash, EE and NDF. On the contrary, cutting time did not affect TMR nutrient distribution. The most efficient combination was observed with 70% loading of the nominal capacity, together with 6 minutes of cutting and 6 minutes of mixing. Nevertheless, a variation of DM and NDF along the feeding alley was observed, suggesting a stratification of the material in the mixer wagon during the unloading phase. Despite factors related to the preparation of the diet might be controlled, it is clear that there are other factors influencing TMR homogeneity along the feeding alley. Further studies are required to identify these factors, since the uneven distribution of the diet can affect nutrient supply, impairing animal health and performances (Barmore and Bethard, 2005), and might also be responsible of increased competitive interaction between the animals (Esmaeili et al., 2016).

Besides homogeneous distribution along the feeding alley, it is also necessary to guarantee that the delivered ration actually reflects the ration formulated by the nutritionist (Sova et al., 2014). Based on chemical composition analysis and/or tabular values, rations are indeed formulated to fulfil animals' nutrients requirements. However, feedstuffs included in the TMR can undergo variations among time, with special regards to silages, impairing the nutritional supply of the delivered TMR, which might differ from the theoretical ration (Weiss et al., 2012). The fluctuations of feedstuffs' composition, and accordingly of the delivered TMR, do have an impact on farm economy, as result of both reduced animals' performances (Mertens and Berzaghi, 2009) and feedstuff wastage (Stockdale, 2010). As previously mentioned, silages are greatly liable to variations over time because of their high moisture content. Due to fermentative phenomena, as well as to improper silo management, silages moisture and consequently their DM, can undergo fluctuations among time, which in turn can affect the nutritional value on as fed basis, finally modifying TMR composition. Diets are indeed formulated on a DM basis, but ingredients are included in the mixture on as-fed basis. Therefore, if as-fed inclusion rate in not adjusted for changes in DM concentration, the nutrient composition of the delivered TMR might differ from the formulated diet. For this reason, we decided to develop a sensor for real-time silages DM determination during the loading of the ingredients in the mixer wagon (2nd trial). The final goal was to adjust silages inclusion rate according to their actual DM content, in order to prepare a TMR closer as possible to the diet formulated by the nutritionist. The employment of the sensor enabled to load silages DM amount closer to the target value, however for some silages (namely soybean and barley silages) the difference from the theoretical value was still significant. Even though the error in the silage DM amount loaded in the wagon was halved thanks to the employment of the sensor, prepared diet was not closer to the formulated diet compared to the TMR prepared conventionally (without the employment of the sensor). The lack of positive results on the final TMR has been attributed to the error in the loads of dry feedstuffs (concentrate), which can equally contribute to affect TMR nutritional composition (Piccioli-Cappelli et al., 2019).

Therefore, based on our results (1st and 2nd trial), we can assert that numerous factors influence TMR consistency of the delivered TMR. Although the use of aids, i.e. sensors and indications about mixing time and loading levels, allows to reduce the error associated with TMR preparation, it does not guarantee the consistency of the TMR. Therefore, the investigation to identify valid solutions for optimal TMR preparation is still open.

Unlike TMR for dairy cows, the risk of daily variation in diets for pigs and poultry is strongly reduced. The feeding is indeed exclusively based on standardized commercial feeds, whose moisture content is minimum, reducing the risk of nutrient supply fluctuations over time.

Therefore, whether the feed is well formulated, animals' requirements are completely fulfilled. In poultry and swine, however, nutrition can be used as a mean to improve animals' health (Verstegen and Williams, 2002), with a focus on specific critical moments of the production cycle (e.g. post-hatching phase and weaning). In these moments animals experience a considerable stress, due to both physiological and exogenous factors, which determines a growth check. In order to minimize the impairment of animal's growth, antimicrobials have been widely used until 2006 when their use as growth promoter was prohibited in the European Union. Their ban was a consequence of the risk of antibiotic-resistance development, along with consumers' request for healthier and more natural food. Antimicrobials limitation opened therefore the interest toward alternative solutions, with particular interest for those substances whose action is similar to the antimicrobials. Among these substances, great interest has been attributed to phytobiotics, plant-derived products which constitute a natural source of bioactive compounds (Lillehoj et al., 2018). Their use in pigs and swine highlighted beneficial effects for the animals, influencing the three major players of gut health, namely gut morphology, immune system and gut microbial population (Celi et al., 2017). However, available literature about their effect is discordant (Windisch et al., 2008; Mohammadi Gheisar and Kim, 2018). Such discrepancies in the observed results have been attributed to the different biological potential of the phytoadditives tested (Bakkali et al., 2008), as well as to the route of administration, the dosage used and the length of the treatment. In our experiment (3rd trial), the administration of pomegranate rinds and green tea leaves extract to broilers chickens did not affect animals' growth performances. However, the product evidenced beneficial effects, enhancing blood total antiradical activity (thus the defence to counteract oxidative stress) and increasing the abundance of beneficial bacterial population, such as *Lactobacillaceae*. The most interesting aspect of this study was the experimental design. The product was indeed administered via drinking water and only in critical moments of the production cycle (post-hatching and switch between feeding phase). Such supplementation might enable the farmer to carry out targeted treatments, avoiding the inclusion of the products in the feed during the whole rearing cycle, finally minimizing treatment-related costs.

Lastly, among the additives employed to ameliorate animals' health and performances, there are also nucleotides. Nucleotides play a fundamental role for the proper and complete development of the intestinal mucosa and the immune system, but in stressful and critical moments, such as weaning, their *de novo* synthesis is inadequate to meet the high demand for GIT development (Waititu et al., 2016). Therefore, nucleotides supplementation is deemed to benefit the animals. As for phytobiotics, data reported in literature are discordant (Sauer et al., 2011). In our study (4th trial), the administration of nucleotides to weaning piglets improved nutrient

utilization in the first week after weaning, even though no major improvement of the growth performance were observed. In addition, no beneficial effect of the treatment was observed for inflammatory and immune response, in contrast with other studies (Li et al., 2015; Che et al., 2016). The lack of results has been attributed to the optimal environmental condition in which the animals were raised. Trials conducted in experimental facilities under controlled environment do not actually reflect commercial farms' conditions, limiting the stress usually associated with weaning, and consequently potentially covering the beneficial effect of the tested product. It could be interesting replicate the trial in commercial facilities, or in the experimental facilities but performing an acute (e.g. *E.Coli* LPS) or chronic (unclean room conditions) challenge.

In conclusion, in this thesis a nutritional and a technological approach have been investigated to improve the efficiency of animal feeding, and consequently its effect on animals' performance and health. Despite positive results have been pointed out in both areas, some limitations have been highlighted, requiring further investigations.

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Chapter 8

Appendices

LIST OF TABLES

LIST OF FIGURES