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EMBRYO TRANSFER AS A TOOL TO DISSEMINATE K-CASEIN BB GENOTYPE AND IMPROVE MILK CLOTTING PROPERTIES

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Abstract

Dairy industries are asked to be increasingly competitive and efficient. Despite the increasing trend in milk yield and protein content during the last decade genetic selection, milk coagulation ability has diminished and even if the absolute amount of cheese produced has increased, the relative cheese yield from a set amount of milk, has decreased. As casein content and variants, along with milk clotting properties (MCP) are determined to a large extent at DNA level, genetic selection and embryo transfer can provide efficacious tools to reverse this trend and achieve improvements. The aim of the proposed research was to determine how rapidly and to what extent milk coagulation properties could be improved by using embryo transfer (ET) as a tool to increase the frequency of k-casein BB genotype cattle and reducing A and E variants in an Italian Holstein herd with a low prevalence of the favourable genotype. In the effort to optimize superovulation protocols and results, synchronization of wave emergence was performed through manual transrectal ablation of the largest (dominant) ovarian follicle on days 7 or 8 of the cycle (estrus = day 0); different drugs and dosage for the superstimulation protocol were experimented trying to overcome the negative effects of stress and the perturbance of LH secretion in superovulated highly producing lactating cows and the use of SexedULTRA™ sex-sorted semen, for artificial insemination of superovulated cows was reported for the first time. The selection program carried out in this research, gave evidence and gathered empirical data of feasible genetic improvements in cheesemaking ability of milk by means of k-casein BB selection. In conclusion, in this project, selection of k-casein BB genotype markedly enhanced cheese-making properties of milk, providing an impetus to include milk coagulation traits in genetic selection and breeding programs for dairy cattle.

1. Introduction

1.1 Background

Cheesemaking is growing worldwide (International Dairy Federation, 2018): in 2018 in the European Union, cheese yield was about 10 million tonnes with a growing trend in nearly all the Member States. In Italy in the same year the production consisted of nearly half a million tonnes of cheese, mainly high-quality cheese of Protected Designation of Origin produced using traditional methods, whose quality strongly relies on the composition and coagulation traits of milk. The propensity of milk to coagulate on addition of rennet is critical for cheesemaking (Malacarne et al. 2014) due to its effects on cheese yield and cheese quality and its influence on the profitability of the entire process (Pretto et al. 2013). There is universal acknowledgment of the role played by the protein fractions in the cheese-making process (Cipolat et al. 2018) and among milk proteins, there has been much attention on casein and k-casein for their central role in the process. Genetic variants of k-casein have a strong effect on the rennet coagulation ability of milk (Jensen et al. 2012; Toffanin et al. 2012; Perna et al. 2016): the B allele of k-casein has been associated with higher casein and k-casein content, smaller casein micelles, more favourable curd structure and improved overall milk clotting properties (MCP) (Walsh et al. 1998; Bittante et al. 2012). Holstein-Friesian has become the leading breed worldwide and yields poorly and non-coagulating milk much more frequently than many other dairy breeds (Bittante et al. 2011). The cause for this severe, counterproductive and increasing problem has not been fully understood (Malacarne et al. 2014; Troch et al. 2017) but some authors assumed it could involve a low content and proportion of k-casein (Amalfitano et al. 2019). Diffusion of casein alleles could contribute and be part of the problem; in fact although B variants of both κ-casein and β casein apparently promote coagulation (Troch et al. 2017; Cipolat et al. 2018), these alleles have low prevalence in Holstein-Friesians, whereas unfavourable alleles, e.g. β casein A2 and k-casein A and E alleles (Amalfitano et al. 2019) are common (Poulsen et al. 2013; Gustavsson et al. 2014). These could be some of the reasons why in recent years an increase of the proportion of poorly and non-coagulating milk is reported in many countries (Bonfatti, 2010) , including Italy and the Parmigiano-Reggiano cheese production area

(Tedeschi et al. 2010). It is reasonable to assume that the last decades genetic selection of Holstein Friesian cattle has led to set a milk protein composition that has been somehow altered: apparently, improvement of milk yield has simultaneously led to an deterioration of milk in terms of milk coagulation properties and a consequent loss of efficiency in the whole cheesemaking process (Bittante, 2011). The dairy industry needs to balance genetic progress in milk yield and protein content with the need for improving milk coagulation properties. As milk coagulability depends to a large extent on genetic factors, including breed and milk protein polymorphism of the individual animal (Perna et al. 2016), genetic selection is a fundamental tool to pursue productive and economic goals and embryo transfer can boost and accelerate the process.

As k-casein variant B has been constantly reported to have favourable effects on k-casein and casein concentration as well as on milk clotting properties, disseminating this genotype within the herd could be an efficacious strategy to improve dairy industry competitiveness. In Holstein-Friesian which has become the leading breed worldwide and also in the Parmigiano Reggiano area, the B allele of k-casein has a low prevalence (AA 30.1%- AB 36.2%- AE 13.9%- BB 10.6%- BE 7.8%- EE 1.4% ANAFIJ 2019). This situation could be overcome by performing embryo transfer, which allows spreading the rare k-casein BB genotype into the herd in a relatively short time, transferring the embryos of the limited number of k-BB donors to a wide number of recipients. An ET program could markedly shorten the time needed for genetic selection and achieve results that would be unattainable otherwise. Moreover, the effect could be strengthened by inseminating the donors with sex-sorted semen, that would result in 90% of female offspring.

1.2 Description of the studies

Aims

The main aim of the proposed research was to evaluate the effects of genetic selection for k-casein genotype BB on the milk clotting properties in a herd with a relative initial low diffusion of this favourable genotype.

Materials and Methods

Holstein cattle on several dairy farms in Emilia Romagna, Italy, were used for the different parts of the study. All animals had *ad libitum* access to a total mixed ration and with an average daily milk yield between 40 L and 42 L. Lactating cattle were housed in free stall barns with cooling systems such as ventilation and a shower system. Cattle with clinical illness, e.g. mastitis, lameness, respiratory or gastrointestinal disorders with a considerable reduction in milk production and impaired general health after calving, were excluded from the experiments.

The following studies are presented:

- *"Embryo production and quality in dairy cattle is enhanced by manual transrectal ablation of the dominant follicle prior to superovulation"*
- *"Superovulation protocols for dairy cows bred with SexedULTRA™ sex-sorted semen"*
- *"How rapidly and to what extent milk coagulation properties could be improved by using embryo transfer as a tool to disseminate k-casein BB genotype as seen at the herd level"*

Study I

Embryo production and quality in dairy cattle is enhanced by manual transrectal ablation of the dominant follicle prior to superovulation.

The study investigated an "easy to apply in the field" way to synchronize wave emergence before superovulation through manual transrectal ablation of the largest (dominant) ovarian follicle on days 7 or 8 of the cycle (estrus = day 0). Superovulation (SO) was performed in 167 donors, which were randomly allocated into 3 groups: control group, dominant follicle ablation on day 7 and

dominant follicle ablation on day 8. Superstimulatory treatments started on day 10 for control or 1 day after ablation. The study highlighted that manual rupture of the dominant follicle on days 7 or 8 of the cycle and starting SO treatment 1 day later enhanced embryo yield and embryo quality compared to starting SO on day 10. There were no indications that judicious manual ablation had any deleterious effects on subsequent reproductive performance (intervals from superovulation to estrus and to becoming pregnant, percentage pregnant after 1st artificial insemination (AI) and number of AI to achieve pregnancy). Cows culled for reproductive reasons were similar among groups. Data suggest that if a special attention is given to apply only a slight pressure, the procedure could be performed successfully without deleterious long-term reproductive consequences and without increasing the herd culling rate for reproductive reasons.

Study II

Superovulation protocols for dairy cows bred with SexedULTRA™ sex-sorted semen.

Numerous SO protocols have been intensively studied to improve embryo production and embryo quality in cattle. As many donors produce no, few or poor-quality embryos, various FSH treatments have been attempted, including varying total doses of gonadotropins and the FSH:LH ratio. Moreover when embryo transfer is performed to obtain female offspring, inseminating superovulated cows with sex-sorted semen could result in a significantly smaller proportion of transferable embryos and significantly larger proportions of unfertilized oocytes and/or degenerate embryos than heifers or cows inseminated with unsorted semen. With 4M SexedULTRA™ sex-sorted semen, fertility rates between conventional semen and sex-sorted semen in single-ovulating heifers approached equivalence and this study investigated if it may be appropriate even in cows. The objective of this study was to determine whether embryo yield in stressed lactating dairy cows would be altered by changing the FSH:LH ratio and by inseminating cows with SexedULTRA™ sex-sorted semen. Three SO protocols were tested: 1) a total dose of 700 IU of Folltropin 2) a total

dose of 1000 IU of Folltropin 3) 700 IU of Folltropin + 300 IU of Pluset. Donors (n=77) were randomly allocated to receive one of 3 SO protocols. In conclusion, 700 IU of highly purified gonadotropins provided inadequate stimulation for SO of lactating dairy cows in this study. However, a better SO response was achieved by increasing total gonadotropin dose from 700 to 1000 IU, irrespective of increasing primarily FSH or both FSH and LH. The use of SexedULTRA™ sex-sorted semen for insemination of superovulated lactating dairy cows was considered satisfactory in terms of proportion of transferable embryos produced.

Study III

How rapidly and to what extent milk coagulation properties could be improved by using embryo transfer as a tool to disseminate k-casein BB genotype as seen at the herd level.

Poorly and non coagulating milk is a major cause of reductions in cheese quality and value and the problem is of growing importance. Dairy and cheese industries need to balance genetic progress in milk yield and protein content gained in recent decades with the need for improving milk coagulation properties. As the positive role of k-casein B in enhancing MCP was consistently reported among literature an embryo transfer program to improve the frequency of this genotype into the herd was planned and performed. The objective was to determine how rapidly and to what extent MCP could be improved by using embryo transfer (ET) as a tool to increase the frequency of k-casein BB genotype and reducing A and E variants in an Italian Holstein herd with a low prevalence of the favourable genotype. Selection for k-casein BB genotype enhanced cheese-making properties of milk with positive effects on protein, casein and k-casein content along with TA and MCP, confirming previous studies' theoretical expectations. The low prevalence of the k-casein BB genotype can be overcome with ET, using k-casein BB donors and k-casein AA/AE/EE cattle as recipients.

1.3 Milk composition and clotting properties

1.3.1 Use and composition of milk

The proportion of milk produced and processed into other milk-based products has increased worldwide (International Dairy Federation, 2018). As properties of dairy products are influenced to a great extent by the amounts and relative proportions of each of the milk constituents, those that have a major role in determining cheese quantity and quality, such as protein and casein, have progressively obtained more attention from the dairy industry and achieved a higher economic significance. The general process of milk coagulation is based on the formation of an aggregated protein network, where other milk constituents are entrapped. This reticulum mainly consists of a certain group of proteins known as caseins, assembled and organized in the complex structure of casein micelle. Although the fine structure of this entity is still not completely and intimately understood, its fundamental role in cheesemaking is in no doubt. During the past decades the focus of milk production has been kg's of milk protein, whereas the protein composition has been less well investigated. Poorly and non-coagulating milks have puzzled researchers at least since the 1920's (Ikonen et al. 2004; Cassandro et al. 2008) but lately a further general deterioration in cheesemaking aptitude of milk and stagnating cheese yields have been increasingly and worryingly reported in many cheese-producing countries, accentuating the need to reverse this trend to serve the purpose of providing dairies with milk well suited for dairy products manufacture, improving the efficiency of the entire cheese making process.

1.3.2 Protein composition of milk

Milk is a highly diverse fluid whose main components are water, lactose, fat, protein, organic acids, and minerals. Throughout this thesis, the focus will be on the protein fraction of milk, because of its fundamental role in the cheesemaking process. Proteins in cow milk comprise 95 – 97% of the crude protein content, and non-protein nitrogen accounts for 3 – 5%. The protein fraction of milk consists of a heterogeneous group of over 200 different characterized molecules (Ng-Kwai-Hang, 2002), of which the six major ones could be divided into two macro groups defined by their

solubility at pH 4.6: caseins, which precipitate when adjusting milk to pH 4.6 and account for 75-85% of crude protein, and whey proteins, which remain in solution at pH 4.6 and account for 18-20%.

Casein and the casein micelle

Casein consists of 4 protein fractions: αS1, αS2, β and κ, synthetized in the mammary gland and γ which is actually a terminal segment of β-casein after proteolytic cleavage by plasmin. The major part of these phosphoproteins, together with inorganic calcium phosphate (composed by calcium, magnesium, phosphate, and citrate) occur in the form of large colloidal particles, known as casein micelles (Dalgheish et al. 2014). Despite the vast amount of research on the casein micelles, their detailed structure is still not fully known even though several models have been proposed (Holt et al. 2003; Dalgleish et al. 2014). The broad conclusion is that casein micelles are polydisperse, colloidal, roughly spherical particles ranging from 50 to 600 nm in diameter, with an average diameter of around 200 nm (Holt et al. 2003; Fox and Brodkorb, 2008). They result from the aggregation of the αS- and β-casein fractions with calcium phosphate, the whole stabilized by a layer of k-casein bound to their surfaces. The extensively phosphorylated residues of the bovine αS- and β-caseins make them capable of binding considerable amounts of calcium ions, which can lead to their precipitation (calcium-sensitive proteins). The phosphoseryl residues also enable the proteins to bind to calcium phosphate. The k-casein on the other hand is only singly phosphorylated and does not precipitate even in the presence of high concentrations of Ca^{2+} (calcium-insensitive protein); in addition, a fraction of the k-casein molecules is glycosylated. Glycosylated and phosphorylated residues are in the C-terminal third of the molecule (the so-called caseinomacropeptide, CMP) which represents the hydrophilic part of k-casein in contrast to the rather hydrophobic N-terminal region of the molecule (the *para*-*κ*-casein formed on hydrolysis by chymosin) and to the mainly hydrophobic behaviour of the other casein constituents of the micelle.

This part of the k-casein has sufficient hydrophilic amino acids to interact well with water and to protrude 5–10 nm from the surface of the micelle. Extending its CMP moiety (residues 106–169 of the protein) into the surrounding serum, k-casein surface layer manages to stabilize the micelle and determines many physical and chemical properties of the casein micelle itself. One of these characteristics, of great importance in determining the behaviour of milk during processing and the rheological properties of the resulting rennet curd, is micellar size (Amenu and Deeth, 2007; Glantz et al. 2010). The amount of k-casein in a micelle is inversely proportional to its size; the association between content of κ-casein and casein micelle size seems important (Frederiksen et al. 2011; Day et al. 2015), with higher level of κ-casein being associated with smaller casein micelles and more κ-casein at their surface resulting in improved milk coagulation properties (MCP) shorter gelation times and increased gel strength (Glantz et al. 2010; Bijl et al. 2014; Poulsen et al. 2016). The more uniform micelle systems are characterized by increased quantities of small micelles, which form a more dense and compact protein network (Bonfatti, 2010). Hence, a higher κ-casein/casein ratio may favour the bridging of proteins by calcium, resulting in faster coagulation and a firmer gel (Robitaille et al. 1993). Genetic variants A and B of k-casein were correlated significantly with average casein micelle size of milk (Bijl et al. 2014), in particular milks homozygous for the k-casein B variant contain a greater proportion of k-casein in the mixture of caseins and are characterized by smaller micelles and better coagulability.

Whey protein

Whey proteins, or serum proteins, share few common characteristics other than being soluble at pH 4.6. The three main proteins are β-lactoglobulin, α-lactoalbumin and blood serum albumin (BSA), representing approximately 50, 20 and 10 % of total whey proteins, respectively. The remaining part comprises immunoglobulins (Ig) and trace amounts of several other proteins, including enzymes. Whereas α-lactoalbumin and β-lactoglobulin are synthesised in the mammary gland, BSA

and Ig are blood serum components and gain entrance to milk via leakage from blood circulation. From a physiological and biochemical point of view, whey proteins perform different functions such as taking part in the biosynthesis of lactose, providing various types of immune defence to the newborn calf and facilitating the absorption and transportation of vitamin A.

1.3.3 Coagulation of milk

In their native state, casein micelles are very stable, but they can be readily destabilized by acidification or addition of specific proteases or by a combination of the two. The ability of casein micelles to stay in solution at natural milk pH (~6.7) relies on the net negative charge and hydrophilic character of the C-terminal end of κ-casein. The loss of one or both of these fundamental stabilising elements is at the base of milk coagulation and results in the destabilization of the casein micelles, which flocculate and aggregate to form a gel enclosing the soluble milk components. Acid coagulation involves alterations of casein micelle properties by a lowered milk pH, which causes colloidal calcium phosphate to dissociate from the micelles. The H^+ ions released by acidification gradually neutralize the electronegative charges. Electrostatic repulsion decreases and then disappears resulting in the aggregation of casein micelle. Enzymatic coagulation of milk is the modification of casein micelles via hydrolysis of casein by rennet, followed by calcium-induced micelle aggregation (Fox & McSweeney, 1998). Generally, coagulation is divided into two steps that are separated in nature although not entirely in time (Frederiksen et al. 2011): the enzymatic phase in which the κ-casein is degraded by chymosin, and the non-enzymatic phase that corresponds to the formation of a gel through the aggregation of degraded micelles. More in detail enzymatic hydrolysis of κ-casein leads to the dissociation of the molecule in 2 elements: para-κ-casein and the hydrophilic glycomacropeptide (CMP) part, which is released into the whey. Since the glycomacropeptide is highly charged, the electrostatic potential of the casein micelles strongly decreases, weakening the electrostatic repulsions between the micelles. Hydrolysis also removes the

second factor in the stability of casein: steric repulsion due to the hydration layer. These para-casein molecules lack stabilizing elements and will aggregate (Walstra et al. 2006). When a sufficient reduction of the repulsive forces (electrostatic, steric and water) at the origin of the colloidal stability is reached, near or contiguous micelles begin to aggregate. These conditions are achieved for a degree of hydrolysis of the κ-casein of about 80% at pH 6.6 (Croguennec et al. 2008). The destabilised casein micelles will form a three-dimensional network called curd or coagulum, which contains the N-terminal part of κ-casein (para-κ-casein) and entraps other milk constituents. This gel is unstable, and following the contraction of the micelles, it expels the liquid phase out of the curd. This phenomenon, called synaeresis, separates the curd, containing casein and fat, from the whey, containing lactose, minerals and soluble proteins (Vignola, 2002).

1.3.4 Milk clotting properties

The ability of milk to react to the clotting enzyme in due time and form a curd with an adequate firmness is summarized in the term "technological quality of milk" or "milk clotting properties" (Benedet et al. 2017) and the dairy industry is increasingly focusing on these as they strongly determine the efficiency of cheese production (Cassandro et al. 2008). So traditional milk coagulation properties are used to predict the suitability of milk for cheese-making and are considered good indicators of both the quality and yield of cheese (Bittante, 2011). These milk characteristics are influenced by many factors: temperature, pH, concentration of Ca^{++} ions, SCC and composition of milk, particularly protein and casein content (Troch et al. 2017), which in turn could vary with season, lactation day, parity, feeding, and health status of the cow (Schutz et al. 1990; Bobe et al. 1998) and largely depend on genetic factors encoded in DNA. As the relative proportions of protein fractions and different protein variants have essential roles in coagulum structure and rheological properties of the curd influencing cheese yield and cheesemaking efficiency (Malacarne et al. 2014; Ketto et al. 2017; Cipolat et al. 2018; Amalfitano et al. 2019),

breed and milk protein polymorphism of the individual animal (Perna et al. 2016) have been confirmed by many authors as the principal factor influencing milk coagulation and curd firming processes (Amalfitano et al. 2019). For good cheesemaking, milk must have good reactivity with rennet, provide a firm curd and have good synaeresis ability (allow plenty of whey to be expelled when the curd is contracted) (Cecchinato and Carnier, 2011; Cipolat-Gotet et al. 2012), especially for hard and long ripened cheeses (Aleandri et al. 1989; Malacarne et al. 2006; Pretto et al. 2013), for cheese labelled with Protected Designation of Origin (PDO) (Bertoni et al. 2001; Cassandro et al. 2008) and in the several dairy chains in which these characteristics influence the scores obtained in milk quality payment systems (Bittante et al. 2011).

1.3.5 Determination of milk clotting properties

The methods used to assess MCP explore physicochemical changes in viscosity and elasticity (O'Callaghan et al. 2002) occurring in milk during rennet-induced coagulation. Several techniques have been used to measure MCP and a wide range of mechanical and optical instruments are available (Klandar et al. 2007) to record curd firmness over time. The most common approach is to monitor milk viscosity following the addition of rennet over a period of 30 minutes and for the purpose lactodynamography (LDG) is one of the most widespread mechanical devices customized to analyse the rennet coagulation ability of several milk samples (usually 10) simultaneously (Malacarne et al. 2014; Amalfitano et al. 2019). The milk temperature is held constant during the analysis. The lactodynamograph measures the tiny forces that act on submerged pendula when samples of coagulating milk are oscillated in a linear manner. The outputs are firmness/time graphs from which it is possible to calculate the following rennet coagulation parameters (RCP): rennet coagulation time (RCT, min), curd firmness (a30, mm), and curd-firming time (k20, min), parameters which well describe the duration and the entity of the coagulation process, giving information on coagulation time, gel strength, and gelation rate (Ipsen et al. 1997).

1.3.6 Genetic polymorphism of milk proteins

The major milk protein genes of dairy cattle (αS1, αS2, β and κ casein, β lactoglobulin and α lactalbumin) are polymorphic due to single nucleotide polymorphisms and/or nucleotide deletion or insertion or post-translational modifications (Ketto et al. 2017). The presence of different variants in milk has been associated to milk protein content and relative concentrations of proteins (Bonfatti et al. 2010; Gustavsson et al. 2014a), which in turn affect milk coagulation properties (Bonfatti et al. 2010; Poulsen et al. 2013) thereby the entire cheese-making process. Genetic variants affect both absolute and relative concentrations of the individual milk proteins (Perna et al. 2016), casein micelle size, curd structure, milk clotting properties, cheese yield and cheese quality. Among milk proteins a great deal of attention from dairy science and industry have been drawn to k- casein as it is the most interesting and important casein component centrally involved in the formation and stabilization of casein micelles, in the coagulation of milk by rennet and in many other technologically- important properties of the milk protein system. An extensive literature strongly related it with technological properties of milk (Ng-Kwai-Hang, 1998) as it affects protein content, casein content and k-casein content (Ng-Kwai-Hang, 1998; Heck et al. 2009) as well as clotting time (Mariani et al. 2001), curd firmness (Davoli et al. 1990; Ikonen et al. 1999; Comin et al. 2008) and cheese yield (Cipolat et al. 2018). As cheese consists of a paracasein reticulum in which fat globules and part of the soluble phase of milk are entrapped (Rybak, 2014), the concentration of casein in milk is positively correlated with the quantity of cheese produced per unit of milk (Cipolat et al. 2018). The genotype of κ-casein influences both the casein content in milk and the κ-casein proportion compared with other caseins (Wedholm et al. 2006; Heck et al. 2009). In bovine milk κ-casein exists as variants and the main ones are A, B and E (Hallén et al. 2008). Milk samples containing at least one or both κ-casein B allele have casein micelle diameters less than 200 nm, whereas those with no B allele have micelle diameters greater than 200 nm (Bijl et al. 2014). As mentioned above micelle size is a key element in determining coagulation properties of milk, with

smaller size being more favourable. In general, the B allele of κ-casein has been associated with a higher κ-casein concentration in milk compared to A and also with higher total protein and casein ratio (Hallén et al. 2008). The E allele has been associated with a lower κ-casein content compared to B, possibly also to A (Oloffs et al. 1992; Ikonen et al. 1997; Ikonen et al. 1999). In general κ-CN A and E have been confirmed to have a negative effect on coagulation (Amalfitano et al. 2019) and sometimes they have been linked to poorly and non-coagulating milk samples (Ikonen et al. 1999; Wedholm et al. 2006). Many studies have confirmed that milk containing the BB variant of κ-casein has faster and firmer gelling ability and better suitability for cheese production than other variants (Panthi et al. 2017). According to Ng-Kwai-Hang (1998), milk with the BB variant of κ-casein has a reduced coagulation time by 10%–40%, and increased curd firmness by 20%–140% compared to milk with the AA variant of κ-casein, where, Bittante et al. (2012) while comparing AB and AA milks noted a reduction on average of 9% for coagulation time and of 30% for k20 and an increase of 26% in a30. Furthermore, cheese prepared from milk containing the BB variant of κ-casein has been shown to have higher fat recovery and yield compared to that with the AA variant (Walsh et al. 1998). These effects of the different k-casein variants with coagulation properties of milk are also found regarding cheese yield (Hallén et al. 2008). While the positive role of k-casein B in enhancing MCP was consistently reported across literature, effects of other caseins were often controversial (Bonfatti et al. 2010). However, also the B variant of β-casein and β lactoglobulin have been commonly linked to improved coagulation properties. Although β-lactoglobulin itself is not involved in the enzymatic process of coagulation, it has been shown that the genetic variants of β-lactoglobulin may be affecting coagulation properties (Ng-Kwai-Hang et al. 2002): variant B of β-lactoglobulin is less strongly expressed than variant A, which results in a concomitant increase in casein ratio (Mayer et al. 1997; Braunschweig and Leeb, 2006; Hallén, 2008), improved coagulation properties (Caroli et al. 2009) and higher cheese yield (van den Berg et al. 1992; Lodes et al. 1996). Variant B of β-casein also has more favourable qualities for good coagulation and the

shortest clotting time of all identified variants (Buchberger & Dovč, 2000).

In conclusion cows that produce milk containing the B variant of κ-casein, β-casein and β lactoglobulin are economically important from a cheesemaking perspective, owing to casein and k-casein concentration and to the micelle size-related benefits of these protein types (Panthi et al. 2017). Focusing genetic criteria also on these milk protein variants because of their strong influence on milk clotting properties, cheese yield and cheese quality, could be a valuable strategy for improving the efficiency of the entire cheese making process.

1.3.7 The relationship between milk clotting properties and protein content

A general worsening of MCP, an unfavourable trend over the years at the phenotypic level, has been evidenced in several countries (Bonfatti, 2010). Despite an increased total protein concentration in milk a decreasing trend in coagulation ability has been recorded (Coulon et al. 2001; Lindmark-Månsson et al. 2003; Hallén, 2008). A direct effect of this is that a larger quantity of milk is required to make a set amount of cheese: milk yield has increased but relative cheese yield has decreased, accentuating the necessity to provide dairies with milk better suited for dairy products manufacture (Bonfatti, 2010). The last decades genetic selection has improved milk yield and protein content with limited or no consideration of casein and milk coagulation traits. Even though a strict connection between protein and casein exists, the two parameters can progress differently to the point that today data seem to confirm that protein content has increased and casein content has decreased. It is then evident that the indirect determination of casein content applied sometimes at local level, in some Italian dairy and cheese realities, is misleading. A simple mathematical operation which considers casein content as being constant at 77 % of protein content (casein index of 0,77) is an extreme, if not wrong, simplification. The casein/protein ratio actually shows a wide range of variation when the 2 components are separately analysed and quantified. Milk samples with the same protein content could show very different casein levels. In a study

conducted in more than 7000 farms in and near Lombardia county (Varisco et al. 2004), data about protein and casein content were directly determined and they evidenced that milk samples with the same protein content, for example 3.5, could show a casein content which varies from 2.57 to 2.77 for a casein index of 0.73 and 0.79 respectively. Considering that in the production of long ripened cheese such as Parmigiano Reggiano e Grana Padano a variation as little as 0.1 % in casein index results in a cheese yield difference of about 0.30 kg every 100 Kg of milk processed (Summer et al. 2002), the direct determination of casein content and all actions aimed at improving this parameter assume a major importance. Among breeds, Holstein Friesian resulted to have the lowest casein index (76,92 %) compared to Brown Swiss, Jersey or Modenese breed (79,05%) (Mariani et al. 1998). This could be part of the severe and emerging problem of poorly and non-coagulating milk, which is a major cause of reductions in cheese quality and value. The problem is of growing importance. Holstein-Friesian has become the leading breed worldwide and yields poorly and non-coagulating milk much more frequently than many other dairy breeds (Bittante et al. 2012). This further underlines how the selecting criteria of protein content and milk yield applied for so long in Holstein-Friesian population, probably need to be further investigated and improved.

1.3.8 The problem of non-coagulating milk

Non-coagulating milk is defined as a milk sample that does not clot within 30 min after rennet addition and that is not suitable for the production of cheese. The mixture of just 25% of milk with lower coagulation properties with milk with good properties clearly affects the properties of the entire milk batch resulting in a 20% higher rennet coagulation time and a 30% slower curd firming rate (Frederiksen et al. 2011). The non-coagulation of milk seems to be a fairly common event. Frederiksen et al. (2011) showed that 4% of Holstein-Friesian cows produced non-coagulating milk. The exact cause of this problem has not been determined accurately yet, although the composition of individual milk samples associated with poor coagulation properties has been widely studied

(Jensen et al. 2012). The still elusive structure of the casein micelle and the complexity of the milk coagulation process with its numerous controlling factors make the issue complicated to analyze and to comprehend in its entirety. Milk with low protein and fat content (Tyrisevä et al. 2004) and milk with low content of mineral salts such as calcium, magnesium and inorganic phosphorus (Jensen et al. 2012) could contribute to the occurrence of poorly and non-coagulating milk. Both concentration and genetic variants of milk protein fractions, have been linked with non-coagulating milk (Hallén et al. 2010; Bittante et al. 2012; Poulsen et al. 2016). Ikonen et al. (1999) and Tyriseva et al. (2004) suggested that non-coagulating milk may be partly defined by the genotype. Supporting this hypothesis, Frederiksen et al. (2011) interestingly found that of the 4 cows that produced poorly or non-coagulating milk, 3 of them were genetically related to each other; moreover, they reported that 3 of the 4 cows in the poorly coagulating milk group had the unfavourable haplotype β-casein A2A2/k-casein AA, which is associated with a lower content of κ-casein (Hallén et al. 2008). Also cows with the κ-casein E allele have been associated with non-coagulating milk on several occasions (Ikonen et al. 1999; Wedholm et al. 2006; Caroli et al. 2009). However, κ-casein A and E are only contributing risk factors for the occurrence of poorly and non-coagulating milk, but are not sufficient in themselves. Tight genetic linkage exists between the major milk proteins; therefore, the effect of the composite genotypes, or haplotypes, could be more relevant to consider than the single-locus genotypes (Heck et al. 2009; Amalfitano et al. 2019). Frederikson et al. (2011) observed that poorly and non-coagulating milks were characterized by a low total content of κ-casein relative to total casein and by larger casein micelles, and concluded, in accordance with the study of Hallén et al. (2010), that a low content of κ-casein is a risk factor for non-coagulation, because it is negatively correlated with casein micelle size. Non-coagulating milk has been found to be significantly positively genetically correlated with protein content (Gustavsson et al. 2014b), suggesting that breeding for a high protein content could increase the frequency of non-coagulating milk. Again selecting for high protein content or for high casein content could

make the difference. Disseminating k-casein B variant could provide benefits also in this regard as improving milk k-casein content seems to have a positive impact in preventing too high frequencies of poorly-coagulating milk and in enhancing milk coagulation ability. Dairy cows that consistently produce non-coagulating milk should be precluded from supplying milk to the dairy industry for cheese production and also from producing offspring.

1.3.9 Considerations for improved coagulation of milk

Improving cheese yield and quality, through the direct selection of breeding animals on the basis of milk coagulation property traits, is an option due to genetic variation of MCP traits (Ojala et al. 2005). MCP are heritable, quantitative traits; up to 40% of the variation among animals is caused by genetic factors (Ikonen et al. 2004). Estimates of heritability for MCP traits are from 0.30 to 0.40 (Ikonen et al. 1999; Bittante et al. 2002), and from 0.25 to 0.28 for RCT, and from 0.15 to 0.41 for A30 (Pärna et al. 2012). Milk that coagulates rapidly and has high firmness of the curd is expected to result in more cheese with a more desirable composition and to lose less milk components in the whey compared with milk that coagulates late and has low firmness of the curd (Pretto, 2012). From an economic point of view, to satisfy the increasing cheese demand and to guarantee more products with high added value in the market, it is crucial to valorize the milk suitable for cheese manufacturing (Benedet et al. 2017). Cheese yield and cheese quality are important indicators of profit for the dairy industry, as they reflect the amount of high quality cheese obtained from a given amount of milk (De Marchi et al. 2008; Pretto, 2012). Deploying all possible actions with the aim of improving cheese yield is a main objective in countries where milk is predominantly processed into cheese (Pretto et al. 2013). This is the case of Italy that uses a great amount of available milk to manufacture typical cheeses, in particular Protected Designation of Origin (PDO) products. Genetic selection programs considerably influence milk composition, yield of milk components, and processability, offering possibilities for altering the quality and stability of milk and dairy products

through breeding (Glantz et al. 2009). It could thus be an economical advantage to the dairy industry if more traits on milk quality are considered in the breeding objective. Genetic improvement of coagulating properties of milk by direct selection for these traits has been suggested (Bittante et al. 2002; Cassandro et al. 2008) even though examples of practical implementations are scarce and milk composition traits considered in the breeding objectives are still limited (Glantz et al. 2010). The reason is probably that the laborious task of the several measurements needed for a proper evaluation (Tyrisevä, 2008), limits this possibility at present. To overcome the difficulty of phenotyping large progeny groups, as genetic variants of milk proteins have been shown to be associated with the protein composition and thereby with the technological properties of milk (Buchberger and Dovč, 2000), information on milk protein genotype could be utilized to improve milk protein composition and MCP through marker assisted selection (Hallén, 2008). Indirect selection via milk protein genetic variants associated with increased levels of desirable milk constituents such as casein and k-casein could be a valid tool. Definitively exploring different possibilities for improving casein composition and coagulation properties of milk seems justified and selecting for k-casein BB and against AA and AE genotype could provide an effective tool for this purpose.

1.4 Superovulation and embryo transfer

1.4.1 Synchronization of follicular wave emergence and superovulation treatment

1.4.1.1 Ovarian cycle in cattle

Ovarian follicle growth takes 3-4 months and could be categorized into gonadotropin independent and gonadotropin dependent stages (Crowe and Mullen, 2013a). Gonadotropin dependent growth in cattle occurs in a wave-like pattern (Martinez et al. 2000), typically with 2 or 3 waves in an interovulatory interval, occasionally 4. Each wave of growth involves emergence, selection and dominance, followed by either atresia or ovulation of the dominant follicle. Follicular wave emergence is preceded (1 or 2 days) by a surge in circulating follicle stimulating hormone (FSH), which induces and sustains the synchronous growth of a cohort of follicles (Adams et al. 1992) initially responsive to and then dependent on FSH for their continued growth. The entirety of these follicles could potentially develop and ovulate (Gibbons et al. 1997; Ginther et al. 2000;) if FSH concentrations were sufficient, but within a few days from the detected emergence, FSH concentrations start to decline returning to nadir concentrations and only the future dominant follicle has the ability to keep growing while subordinate follicles have a reduced capacity to survive to low FSH concentrations (Adams et al. 1993; Ginther et al. 2000). An accentuated and abrupt divergence in growing rate between dominant and subordinate follicles becomes evident: the dominant follicle grows at a continuing rate while the subordinate follicles regressed or temporarily grows at a reduced rate and then regresses (Ginther et al. 2001). This portioning has been called deviation (Ginther et al. 1997) and corresponds to the dominant follicle selection which occurs at the end of the common growth phase (Ginther, 2016) on day 1 or 2 of the follicular wave. Selection has been temporally associated with the post-surge decline in circulating FSH (Adam et al. 1992) and occurs when the largest follicle reaches a diameter of about 8-9 mm (Ginther, 2016). As the dominant follicle is selected from the cohort of follicles, an increase in follicular fluid estradiol and inhibin concentrations occur. These endocrine signals are the key elements that suppress FSH concentrations from the anterior pituitary gland via negative feedback reducing FSH to basal

concentrations (Ginther et al. 2000). The selected dominant follicle becomes increasingly responsive to LH. The crucial role of LH pulses in determining ovulation sequence of events is of no doubt. Gong et al. (1996) showed that following the administration of an experimental GnRH agonist in cattle, follicles grew to ~8 mm in diameter, when pulsatile LH release was inhibited, and to ~4 mm in diameter, when both FSH release and LH pulses were inhibited. Crowe et al. (2001a) analyzed the effect of recombinant bovine FSH (rbFSH) administration on serum FSH and follicular growth in GnRH immunized heifers. GnRH immunization prevents pulsatile secretion of LH and prevents recurrent increases in FSH concentrations resulting in anestrous with follicular growth arrested at <4mm in diameter. Administration of rbFSH alone re-stimulated follicle growth, however the pattern of follicular growth was not wave-like as occurs with selection of a single dominant follicle in cyclic heifers, rather a cohort of follicle proceeded to grow to the large follicle size category without selection of a single dominant follicle which was possibly attributed to a lack of LH as a consequence of failure in estradiol concentrations increase (Crowe et al. 2001a). The production of high concentrations of estradiol is a defining characteristic of the dominant follicle and is dependent on sufficient LH pulse frequency (Crowe et al. 2001a; Crowe et al. 2001b). The pattern of secretion of LH at the time when a dominant follicle is selected is responsible for determination of the fate of that dominant follicle (Crowe and Mullen, 2013b). During the follicular phase of the estrous cycle, when progesterone concentrations are basal, this large concentration of estradiol produced by the pre-ovulatory dominant follicle induces a GnRH surge from the hypothalamus and a subsequent LH surge, which leads to dominant follicle final maturation and ovulation (Crowe and Mullen, 2013b). Otherwise luteal phase progesterone concentrations keep LH frequency low and allow dominant follicles to turnover and undergo atresia. Thus, only when the resulting LH surge is of sufficient amplitude and frequency it stimulates final maturation and ovulation of the dominant follicle (Sunderland et al. 1994). Considering the first wave for example, when progesterone concentrations are high, the dominant follicle grows until about day 6 of the

estrous cycle, then enters a plateau or static phase until about day 9 to 11 and eventually undergoes atresia. From selection for all the duration of its growing phase, involving also the initial portion of the static phase, the dominant follicle exerts a continuing negative effect on other subordinate follicles and the emergence of the next wave through the suppression of FSH concentration (Ko et al. 1991; Siddiqui et al. 2015); only when its functional regression begins and it stops its suppressing activity on FSH, a new surge arises and the second follicular wave emerges. The selection process is fundamental to determine the species-specific ovulation rate in females. In monovular species like cattle, it is at the base of the ovulation of a single follicle (Ginther, 2016) and it is actually the mechanism to prevent when performing superovulation whose ultimate aim is to lead several follicles to commonly grow and ovulate. Once selection has occurred and the dominance has established the administration of exogenous FSH does not seem to be able to fully counteract the negative influence exerted by the dominant follicle on the other follicles of the wave.

1.4.1.2 Variability in superovulatory response

Variability in superovulatory response remains probably the most limiting and frustrating factor affecting the success of embryo transfer in the bovine and among the most critical points, status of the follicular wave at the beginning of superstimulatory treatment has been widely investigated in this regard. The negative influence on the superstimulatory response when superovulation (SO) treatment was initiated in the presence of a dominant follicle even just soon after the selection process had begun, was reported by many authors (Guilbault et al. 1991; Huhtinen et al. 1992; Adams et al. 1993). Many researches underlined the importance of starting FSH treatment when a large number of growing follicles is available to respond to gonadotropins, before the occurrence of the dominant follicle-induced suppression of subordinate follicles (Ko et al. 1991; Adams et al. 1994). Initiation of treatment near the time of wave emergence appears critical for maximal superovulatory response (Adams et al. 1994). Moreover the greater the delay from follicular wave emergence the worse the superovulatory response: in fact comparing 2 groups of animal

superovulated 1,2 d and 0.9 d after wave emergence it was stated that a delay in treatment relative to wave emergence of 0.3 day appeared to be responsible for a significant difference in superstimulatory response (Adams, 1994). Early treatment relative to wave emergence appears to be imperative for the rescue of the maximum number of follicles from irrevocable regression, and for optimizing the superovulatory response (Adams et al. 1994). Superovulatory response was lower in animals in which treatments were initiated as little as one day after wave emergence compared to treatments initiated the day before or the day of follicular wave emergence (Nasser et al. 1993, Adams et al. 1994). In particular, a greater superstimulatory response (Nasser et al. 1993), a greater ovulatory response (Adams et al. 1994), more large follicles and significantly more ovulations (Guilbault et al. 1991; Huhntinen et al. 1992; Adams, 1994) were obtained compared with those of later treatments. Even a more restrictive time interval to start SO protocol has been suggested in the study of Nasser et al. (1993) where the maximum response was elicited when treatment was initiated at the expected time of the pre-wave FSH surge (Nasser et al. 1993). Results of the study (Nasser et al. 1993) indicated that selective suppression of subordinate follicles of a wave began within 1 day of wave emergence since treatment initiated after wave emergence (after Day 0) resulted in fewer follicles ≥7mm at the end of treatment and fewer ovulations. Hence treatments initiated near the expected time of the endogenous pre-wave FSH surge, before the manifestation of dominant follicle selection resulted in a greater superstimulatory response (Nasser et al. 1993; Adams et al. 1994). These considerations further highlight how the traditional practice for inducing superovulation in cattle initiating gonadotropin treatments during the middle of the estrus cycle (in the 8-13 day window), a common practice for a long period in the past (Lindsell et al. 1986; Mapletoft and Pierson, 1993; Armstrong, 1993; Nasser et al. 2011) or even today when an exogenous syncronisation of wave emergence is not possible, may result very approximate and often could not correspond to the best moment for optimized superovulation results. The conventional protocol was originally based on anecdotal and experimental information in which a

greater superovulatory response was reported when superstimulatory treatments were initiated 8 to 12 days after estrus (Bo and Mapletoft, 2014). The origin of this tradition is not clear and the reasons of the practice probably rely on the notion that luteolysis could be consistently and reliably induced only after full CL maturation, or that pre-exposure to elevated circulating progesterone is a requisite for normal estrus and ovulation (Adams et al. 1994). Moreover wave emergence was detected, on average, on the day of ovulation (day 0) and day 10 for 2 wave cycles and on day 0, 9 and 16 for 3 wave cycles (Pierson and Ginther, 1988) and this could further justify the 8-13 day window for a lower risk of encountering an active dominant follicle and for a higher chance of matching the initiation of treatments with FSH surge and wave emergence both in 2 and 3 wave cycles. However it is clear how including 6 days, the "8-13 day window", could correspond only approximately and only sometimes, accidentally, to "the day before or the day of wave emergence" and could not fully exploit the potential of superovulation. Because of the great variation in the proportion of animals exhibiting 2 or 3 waves per cycle and in the day of wave emergence, particularly of wave 2, it is difficult to precisely predict when the wave emerges only on the basis of the day of estrus or ovulation and the "8-13 day window". Wave emergence was detected with a great variability among animals. The relatively good responses to "traditional" SO protocol at middle cycle (Goulding et al. 1990) may be due to the relative lower chance compared to earlier or later treatment to encounter the functional dominant follicle of the first or of the second wave at the time of initiation of the gonadotropin treatment, and to the relatively higher chance of synchrony between initiation of treatment and the approximate time of emergence of the second follicular wave. Starting SO treatments before day 8 or after day 13, could entail a greater risk of incurring in dominant follicle negative effects. This "middle cycle" protocol, however, presents the difficulties of estrus detection prior to initiation of gonadotropins administration (Nasser et al. 1993; Bergfelt et al. 1997) and moreover the great individual variation in the day of emergence of the second follicular wave represents a major source of asynchrony between wave emergence and initiation of

SO. The inability to consistently determine the most appropriate time during the cycle in which to administer superovulation treatments has led to attempts to regulate or alter follicle dominance through perturbation of the normal wave pattern (Armstrong, 1993).

1.4.1.3 Manipulation of follicular wave emergence

Several approaches have been developed to synchronize follicular wave emergence, eliminating the suppressive effect of dominant follicle and allow the emergence of a new follicular wave at a specific time after treatment. The basis of this theory was derived from studies in which cauterization of the dominant follicle or its suppression with steroid-free bovine follicular fluid during the growing phase was followed by a premature FSH release and emergence of a new follicular wave (Kastelic et al. 1990; Ko et al. 1991; Adams et al. 1992). To ensure synchrony between superstimulatory treatments and follicular wave emergence several approaches were experimented (Bo et al. 1996) with both hormonal and physical methods (Armstrong, 1993). Hormonal approaches have been directed at causing luteinization or ovulation of the dominant follicle by using hCG or GnRH analogues or its atresia by using progestogens and estradiol (Bo et al. 1995).

GnRH

It has been shown that GnRH could induce ovulation or luteinization of the largest follicle present at the time of treatment and then result in the emergence of a new follicular wave within 2 days (Macmillian and Tatcher, 1991). However, follicular wave emergence occurs only when GnRH induces ovulation (Martinez et al. 1999), and recent studies have shown that the administration of GnRH at random stages of the estrous cycle results in ovulation in 44% to 54% of dairy cows (Bello et al. 2006; Colazo et al. 2009), 56% of beef heifers (Martinez et al. 1999), and 60% of beef cows (Small et al. 2009).

So the effectiveness of the treatment is affected by the stage of follicular development at the time of treatment (Crowe and Mullen, 2013b). Many studies have been carried out to support the hypothesis

that the administration of GnRH at known stages of the follicular wave would consistently induce ovulation/luteinization and thereby induce emergence of a new follicular wave at a predictable interval but the percentage of animals in which the protocol succeeded was never consistent nor too high (Pursley et al. 1995; Twagiramungu et al. 1995). Thus, as GnRH will only have an effect if there is a healthy dominant follicle (≥10 mm) at the time of administration it is not an ideal option to regulate follicle waves before commencement of a superovulatory treatment (Crowe and Mullen, 2013b)

Estradiol and progestin

The most common protocol to synchronize the emergence of a new follicular wave for superstimulation involves the administration of 2.5–5 mg estradiol-17β or 2–2.5 mg estradiol benzoate plus 100 or 50 mg progesterone using intramuscular injection at the time of insertion of an intravaginal progestin device (Bo and Mapletoft, 2014). Administration of estradiol-17β or estradiol benzoate and progesterone induces a high degree of synchrony in the new follicular wave emergence (Bo et al. 1991; Bo et al. 1992). Estradiol treatment suppresses antral follicle growth through suppression of FSH and suppression was found to be more profound when it was given after insertion of a progestogen implant (Bo et al. 1994). When estradiol has been metabolized, FSH surges in the circulation and a new follicular wave consistently emerges on average 4.3 +/- 0.2 days later (Bo et al. 2002). Gonadotropin treatments are initiated at that time. In general, estradiol has been the hormone treatment used most successfully to synchronize follicle wave emergence in cattle, however the use of estradiol is being prohibited in an increasing number of countries and alternative treatments should be considered (Crowe and Mullen, 2013b).

Superstimulation of the first follicular wave

An alternative for the synchronization of follicle wave emergence for superstimulation is to synchronize ovulation and then initiate FSH treatments at the time of emergence of the first follicular wave (Armstrong, 1993; Nasser et al. 1993) rather than the second or third follicular wave

(Goulding et al. 1990; Adams et al. 1992; Nasser et al. 1993; Adams et al. 1994). The day of ovulation (Day 0) could be used as a convenient and consistent point of reference for the emergence of follicular wave, more easily and precisely detectable than wave 2 emergence which is characterized by high variability. Follicles originating from the first follicular wave have the same capacity to respond to exogenous gonadotropins as those from the second follicular wave (Adams et al. 1994). Nasser et al. (2011) induced synchronous ovulations in Nelore (Bos indicus) donors, with an estradiol-based protocol and initiated superstimulatory treatments at the expected time of ovulation correspondent to the emergence of the first follicular wave. Superovulatory response did not differ from a contemporary group superstimulated 4 days after treatment with estradiol. However, the hormonal milieu in which follicles grow differs greatly between the first and the second follicular waves. In particular, follicles of the first follicular wave grow under lower systemic concentration of progesterone due to the presence of the growing CL. Superovulatory responses have been reported to be reduced when gonadotropin treatments were initiated when peripheral progesterone concentrations were low (Nasser et al. 2011) unless the protocol was accompanied by the use of a progestin device (Rivera et al. 2011; Bo and Mapletoft, 2104).

Follicle ablation

An alternative for the synchronization of follicular wave emergence is to eliminate the suppressive effect of the dominant follicle using ultrasound-guided follicle ablation (Bergfelt et al. 1994; Bungartz and Niemann, 1994). Initial studies involved the ablation of all follicles ≥5 mm (Bergfelt et al. 1997), then ablating only the two largest follicles (Baracaldo et al. 2000) has been demonstrated enough to ensure that the dominant follicle was removed. Mechanical removal of the dominant follicle by follicle ablation or in alternative through electro cauterization (Ko et al. 1991) resulted in the emergence of the next follicular wave on average after 1.5 -2 days and provides an accurate and reliable procedure to increase ovarian responses in dairy cattle (Bungartz and Niemann, 1994). Follicle ablation could be performed in any moment of the follicular wave and

offers the advantage of initiating superstimulatory treatment immediately. Removal is followed (within 12 hours) by a surge in FSH (Adams et al. 2008) and by immediate resumption of growth of small follicles (Staigmiller and England, 1982; Ginther et al. 1989). In Ko et al. study (1991) ablation of dominant follicle on day 3 or 5 day (0= ovulation) relieved the suppression with a surge in FSH beginning the day after cautery (Ko et al. 1991; Adams et al. 1992). This FSH surge could lead to resurgence of the largest subordinate follicle, which acts like the new dominant follicle or could result in the emergence of a new follicular wave 1 or 2 days later (Adams et al. 1992; Bergfelt et al. 1994; Baracaldo et al. 2000). What makes a difference in determining the effects of the post ablation induced FSH surge is probably subordinate follicles viability at the moment of dominant follicle ablation. The fate of subordinate follicles seems to become irreversible after day 5 from emergence (Ko et al. 1991; Adams et al. 1993). Ginther et al. (2001) stated that after the rapid establishment of deviation, the subordinate follicles on average remain viable but under the continued suppression of the dominant follicle for about a day. Similarly Siddiqui et al. (2015) found that the largest subordinate follicle can be rescued 18 to 24 hours after beginning of follicle deviation or after regression (diameter decrease) had begun. Follicle ablation, performed when subordinate follicles are not viable anymore, ensures that SOV treatment, starting 24 hours later, coincides with the time of follicular wave emergence so that an optimal response can be achieved. Even though follicle ablation is highly effective, it requires the use of specialized equipment and trained technical staff that makes it difficult to utilize routinely in the field (Bo and Mapletoft, 2014).

Manual rupture of the largest follicle

An easier method applicable in the field in order to eliminate dominant follicle could be manual rupture of the largest follicle. Not much has been written about this procedure especially because preliminary experiments have revealed that manual removal of the dominant follicle cannot be consistently accomplished and may lead to adhesions and subsequent impaired fertility even though

specific data had not been reported (Bungartz and Niemann, 1994). Nevertheless, it was confirmed that manual rupture of the largest follicle was associated with an enhanced superovulatory response to treatment initiated 1 day later (Bungartz and Niemann, 1994).

Manual rupture of the largest follicle can be performed only when a large follicle is liable to break by squeezing. It cannot be performed randomly during the estrous cycle and it needs estrous detection. It actually could help the traditional protocol of initiating superstimulation treatments between day 8 and 13 of the estrous cycle to be more accurate in anticipating or matching follicular wave emergence. When manual rupture of the largest follicle is performed on day 7 or 8 of the cycle, there is a higher probability that subordinate follicles of wave 1 are not viable anymore and cannot be rescued to become the new dominants and that exogenous FSH administered 24 hours after ablation coincides with the endogenous surge and comes the day of or the day before the emergence of the new wave. Later ruptures on day 9 or 10 could most likely lead to asynchronous events as endogenous FSH surge or follicular wave emergence or even selection may already have happened by the time of exogenous FSH administration 24 hours after ablation; on the other hand, earlier ablations (before day 7) could lead to subordinate follicles resurgence and not to a new wave emergence and moreover it is harder to perform probably because of the thickness of the wall of follicles. In literature no data about the real consequences of manual follicle rupture on cow long-term reproductive performance have ever been reported and this highlights the need to gather and register data to evaluate reproductive parameters, as for example intervals from superovulation to estrus and to becoming pregnant, percentage pregnant after 1st insemination, number of AI to achieve pregnancy along with culling rate for reproductive reasons.

1.4.2 Superovulation protocols

The objective of treatments to induce superovulation in embryo transfer programs is to obtain the maximum number of transferable embryos with a high probability of producing pregnancies (Bo

and Mapletoft, 2014). However, wide ranges in superovulatory response and embryo yield in several different species have been reported. As many donors produce no, few or poor-quality embryos, various superovulation treatments have been attempted to improve embryo production and embryo quality in cattle, including varying total doses of gonadotropins and the FSH:LH ratio.

1.4.2.1 Excess of LH in superstimulatory preparations

Most cows nowadays are superstimulated using crude pituitary extracts containing both FSH and LH with a considerable variability in the ratio (Bo and Mapletoft, 2014). Although both FSH and LH are required in physiological reproductive processes, limiting exogenous LH in SO regimens in cattle has been advocated with benefits (Kanitz et al. 2002, Mapletoft et al. 2002), including reduced variability in the SO response (Moor et al. 1984), enhanced embryo production due to higher ovulation rates and improvements in fertilization rate and embryo quality (Donaldson and Ward, 1987; Yamamoto et al. 1993; Quaresma et al. 2003). The role of LH in final follicular maturation is important to maintain oestrogen activity and prevent follicular atresia but when crude pituitary extracts with an elevated content of LH have been used in SOV protocols, the excess of LH seemed to have deleterious effects due to premature oocyte activation (Moor et al. 1984; Hyttel et al. 1991), premature ovulation (Callesen et al. 1987) and luteinization of stimulated follicles (Boland et al. 1991). Purified pituitary extracts with low LH contamination have been reported to improve the superovulatory response in cattle (Bo and Mapletoft, 2014). Goulding et al. (1991) stated that treatment with a purified FSH preparation resulted in greater embryo production than treatment with equine chorionic gonadotropin (eCG), which is known to have a high LH activity (Murphy and Martinuk, 1991). Endocrine studies have revealed that eCG-treated animals more frequently had abnormal profiles of LH and progesterone (Mikel-Jenson et al. 1982; Greve et al. 1983) which were associated with reductions in ovulation and fertilization rates (Callesen et al. 1986). In many studies, increasing LH content in the SO regimen decreased the proportion of transferable embryos: despite a higher ovulation rate, a large proportion of ova/embryos were

unfertilized or degenerate (Donaldson & Ward, 1986; Donaldson et al. 1986; Kelly et al. 1997). Chupin et al. (1984) superstimulated three groups of dairy cows with an equivalent amount of FSH and varying amounts of LH and showed that the mean ovulation rate and the number of recovered and transferable embryos increased as the dose of LH decreased. Excluding LH from SOV preparations seemed to increase the production of transferable embryos (Donaldson & Ward, 1986). Again experiments with an LH-reduced pituitary extract revealed no evidence of detrimental effects on embryo quality when a high dose of the preparation was administered (Gonzalez et al. 1990; Alkemade et al. 1993), while doubling the dose of a crude pituitary extracts containing both FSH and LH resulted in significantly reduced percentages of fertilized ova and transferable embryos (Alkemede et al. 1993). These data confirm the hypothesis that the detrimental effects of high doses of pituitary gonadotropins on ova/embryo quality is because of an excess of LH and that embryo quality might be superior with use of pure FSH (Bo and Mapletoft, 2014).

1.4.2.2 Inadequate exogenous LH

Notwithstanding the deleterious effects of excessive exogenous LH, many studies reported that also inadequate exogenous LH seemed to have negative effects on SO and to be responsible for a great variation in responses including a complete lack of ovulation (Herrler et al. 1991; Martinez et al. 1999; Ree et al. 2009; Rosa et al. 2010), a normal ovulation rate but with a small number of embryos (Schmidt et al. 1988), a reduced embryo yield (Chupin et al. 1984) and a reduced ovulation rate (Chupin et al. 1987; Ereno et al. 1988). In many studies involving non superovulated cows, lack of ability to achieve normal dominant follicle selection and ovulation has often been linked to an abnormal LH pattern, correlated to perturbed estradiol and progesterone concentrations as a cause or a consequence of altered feedback mechanisms involved. During post-partum anestrous in beef cows for example, failure of ovulation of the dominant follicles has been strongly correlated to inadequate secretion of LH, in particular inadequate LH pulse frequency to stimulate androgen precursor for FSH stimulated estradiol synthesis and subsequent pre-ovulatory

gonadotropin surge and ovulation (Crowe et al. 1993; Crowe and Mullen, 2013b). In these animals FSH secretion result unaltered while the pre-ovulatory LH surge is blocked, thus, the major cause of a persistent dominant follicle in long-term anestrous beef cows has been attributed to a failure of ovulation due to a lack of sustained increased estrogen secretion (Stagg et al. 1995) rather than a lack of development of dominant follicles. Also in cystic ovarian disease the major factor involved in the dominant follicle persistence seems to be the absence of a preovulatory LH surge often caused by a disturbance in the positive feedback effects of estradiol on the hypothalamus and/or abnormal circulating progesterone concentrations or both. Progesterone concentration is the major factor that affects LH pulse frequency in cyclic animals. Generally lactating Holstein dairy cows tend to have lower progesterone concentrations during the cycle than cyclic heifers and these lower progesterone concentrations tend to allow a subtle increase in LH pulse frequency and allows for prolonged growth of each dominant follicle rather than the faster atresia that occur in cyclic heifers (Crowe and Mullen, 2013a). Again failure of ovulation has been linked to lack of positive feedback induced by estradiol and thus failure of the LH/FSH pre-ovulatory surge, despite increased LH pulse frequency to an intermediate level (Crowe and Mullen, 2013a). Endocrine patterns correlated to follicular cyst and/or persistent dominant follicle are subluteal/intermediate level of progesterone and inadequate LH pulse frequency and amplitude. Risk factors that predispose to abnormal LH patterns and to delayed or lack of ovulation are metabolic disorders associated with negative energy balance and poor BCS, quite frequent situations in highly productive dairy cattle. Moreover increased steroid liver metabolism characteristic of highly productive dairy cows can perturbate both estrogen and progesterone concentrations and along with stress, adrenocorticotropic hormone, cortisol and endotoxins are all predisposing or determining factors that abolish LH pre-ovulatory surge. Some analogies could be found with studies involving superovulated cows in which perturbations of the ovulation process as failure or asynchrony have been often attributed to an altered LH pattern. A notable lack of synchrony in ovulation was noted in dairy heifers given 5.0

mg pLH (Ambrose et al. 2005), dose that was considered inadequate to consistently synchronize ovulation; in subsequent experiments the authors used a higher doses (12.5 and 25.0 mg) of pLH and determined that synchronization of ovulation was satisfactory with at least 12.5 mg of pLH. A tendency toward a decreased ovulation rate in the group where LH dose administered was 2.0 mg compared to 4.0 mg and eCG groups was reported also in the study of Oliveira et al. (2014), despite no significant difference in average number of viable embryos recovered. The authors had 2 potential explanations: the LH dose was too low to enhance ovulation rate or a small quantity of pFSH on the last day of superstimulatory treatment may be necessary (in that study, on the last day of the SO, only pLH and no FSH were given) (Oliveira et al. 2014).

The conflicting results of studies involving the use of crude or highly purified gonadotropins might be due to variations in the content of contaminating LH in the FSH preparations. Crude or highly purified pituitary extracts both contain LH; purified preparations obviously contain a lower amount of LH which anyway is never equal to zero. Moreover, it is often difficult to quantify the two hormones separately and consequently to investigate the effects of each distinctly.

1.4.2.3 Recombinant gonadotropins

Recombinant DNA technology, which enables production of FSH in the complete absence of contaminating LH (Takagi et al. 2001) could resolve this problem allowing the study of the different actions of the 2 gonadotropins separately and more specifically. Since the first studies about the use of recombinant FSH in superovulation protocol for cows, most investigators sustained that FSH induced high superovulatory responses without adding exogenous LH as endogenous levels were adequate to support the final follicle growth (Looney et al. 1988; Wilson et al. 1993). However more recently impaired follicular maturation in heifers superovulated with a recombinant human FSH preparation (Takagi et al. 2001) was reported and was attributed to a lack of exogenous LH activity and a severe suppression of LH pulsatility. It was found that exogenous LH is not necessary for stimulation of growth of follicles beyond the stage of 8 mm in diameter and that these

follicles should be competent to induce an LH surge, however a higher incidence of aberrations in final follicular maturation was registered (Takagi et al. 2001).

1.4.2.4 LH suppression during the superovulation protocol

The role of LH in SO protocols is controversial; outcomes could depend on many factors, including subspecies, breed or genetic differences, general health condition and energy balance, acute or chronic stress and various management or environmental conditions. Even though the mechanism by which gonadotropin stimulation suppresses the LH pulsatility is not known, it is well established that SO regimens alter LH secretion, including reductions in pulse amplitude and frequency, a reduced basal secretion of LH and an altered (absent, inhibited, premature or late) preovulatory LH surge which reduces ovulation rate, fertilization rates, egg/embryo quality and embryo production (Roberge et al. 1995; Price et al. 1999; Gosselin et al. 2000). This downregulation was found to be more apparent when relatively pure FSH preparations were used instead of preparations with a high LH activity like eCG for exemple (Ben Jabara et al. 1994; Takagi et al. 2001) and it was correlated with failures in ovulation (Roche et al. 2000). Lesser amounts of exogenous LH administered throughout the entire SO protocol resulted in the greatest suppression of endogenous LH (Ben Jabara et al. 1994). This is at the base of the assumption that in gonadotropin preparations the downregulation of the LH pulsatility might be compensated for by administration of exogenous LH (Takagi et al. 2001).

1.4.2.5 Administering LH at the end of the superovulation protocol

The need for FSH and LH at different times during superstimulation has been long debated. Basic studies on follicular development have shown that FSH is required for follicle recruitment and growth until dominant follicle selection when it acquires LH receptors and become LH-dependent (Bo and Mapletoft, 2014); that's why follicles of superstimulated cattle might benefit from the inclusion of LH near the end of the treatment protocol as endogenous LH pulse pattern could not be sufficient to stimulate maturation and development of so many follicles in the superovulated animal.

Equine chorionic gonadotropin is a gonadotropin with high LH activity (Murphy and Martinuk, 1991) and could provide a constant stimulus to the LH receptors of the growing follicles near the end of a conventional FSH superstimulation treatment protocol (Bo and Mapletoft, 2014). Barros et al. (2008) conducted an experiment where the last two injections of FSH were replaced with eCG and results were a significant improvement in the number of ova/embryos and of transferable embryos. Also Barcelos et al. (2006) and Cifuentes et al. (2009) found beneficial effects by administering eCG at the end of the superstimulation protocol. Barros et al. (2013) reported a similar improvement in embryo production when 1 mg pLH was added to each of the last two Folltropin-V administrations. Moreover, LH suppression has been linked not only to SO treatment, but also to stress and/or negative energy balance conditions, which were considered responsible for attenuating or suppressing LH surge (Matteri and Moberg, 1982; Butler, 2003). In lactating cows, high production and consequent diseases are important sources of stress for cows, with negative impacts on feed intake, BCS, and reproductive performance. Overcrowding and competition are not uncommon conditions and exacerbate the situation. It is noteworthy that the high hepatic blood flow and metabolism in dairy cows promoted clearance of steroid hormones (Sangsritavong et al. 2002; Wiltbank et al. 2006), thereby diminishing the effect not only of LH but of both gonadotropins, which is why a higher total dose may be necessary to induce a good SO response in high producing lactating cows. An increasing number of authors support the thesis that an optimum superovulatory protocol may include the incorporation of balanced amounts of both FSH and LH in varying but known concentration (Takagi et al. 2001; Algriangy, 2007; Hesser et al. 2011). Stimulation with a combination of FSH and pulsatile LH would be an appropriate strategy for superovulation (Crowe and mullen, 2013b) and in the near future an increasing use of recombinant gonadotropins would provide a valid tool for further knowledge and improvement.

1.4.3 Sexed sorted semen in superovulated cows

1.4.3.1 Sex-sorted semen and technique of sperm sorting

Sexed semen has brought a revolution to the dairy industry. It can be used to produce offspring of the desired sex from a particular mating. The biological basis of the technique lies on the 4.2% difference in the length of chromosomes in sperm bearing X- or Y-chromosomes (Moruzzi, 1979). Among several methods for semen sexing, flow cytometry based sorting has emerged as most efficient. The technology has been refined through the last decades and finally sex sorting is possible at the purity of more than 90%. To briefly describe the technique which is well detailed in Garner et al. (2012), the initial step is the staining of sperm DNA with a nucleic acid-specific fluorophore. The process relies on the Hoechst 33342 dye diffusing through an intact cell membrane and selectively binding to the DNA. Flow cytometry measures the intensity of the signal from the H33342 bound to the DNA when excited by a laser. The difference in intensity of the fluorescence corresponds to the difference in the amount of DNA. To do that the fluorescently stained sperm are directed as a stream, one by one, to pass through two fluorescence detectors, positioned at a 90 degrees angle to each other. The stream of sperm is broken into droplets using a crystal vibrator. Opposing charges are placed for droplets containing X- or Y-sperm by a differentiating droplet charge which allows for the two separated populations of spermatozoa to be deflected into opposite streams for collection: one containing X- and the other Y-sperm, which is then collected into a biologically supportive media prior to be concentrated by centrifugation, added of an extender including the cryoprotectant glycerol, and freezed. The sorted populations are distinguished by fluorescence histograms on the flow cytometer and the software also allows for gating out the dead and the moribund sperm (Vishwanath and Moreno, 2018). The sperm concentration in the sexed semen straw is approximately 2 million which is lower than the ordinary non-sexed semen straw which contains approximately 20 million (Sharpe and Evans, 2009). The most critical aspect of preparing sperm for use in sperm sexing is that the less insult imposed on the

sperm, the greater the likelihood that the ultimate population of sorted sperm will keep and maintain their fertilizing capacity (Johnson et al., 1989; Johnson and Welch, 1999). In contrast to conventional semen processing which has minimal intervention points (about three or four depending on the processing method), the processing of sex-sorted semen involves in excess of 20 steps before it is subjected to cryopreservation (Vishwanath et al. 2014). Examples of insults imposed are: extended holding time before staining, adding stain to the sperm, rewarming the sperm by incubation of the stained sperm, exposing the sperm to a laser beam and to an electrical field, subjecting the sperm to pressure changes in the sorting system, centrifugation of sorted sperm and freezing (Johnson, 2000; Seidel, 2014). Even though the process has undergone refinement since the initial method, each of the processing steps remains physically and bio-chemically challenging and could compromise the function of the sperm cell (Vishwanath and Moreno, 2018). As a result of both sperm damage during the sorting process and lower sperm numbers included in each straw, use of sexed semen generally results in lower fertility and conception rate (DeJarnette et al. 2009; Borchersen and Peacock, 2009; Norman et al. 2010) and consequently lower embryo transfer rate if utilized in superovulated animals, compared with conventional semen.

1.4.3.2 Artificial insemination with sex-sorted semen

AI of single ovulating cows

The fertility of sexed semen in cattle has always lagged behind that of conventional semen and dealing with compensable elements that normally would lift the fertility of a sub-fertile sire, such as higher sperm numbers or a higher proportion of better morphological features, did not yield better results with sexed sperm (DeJarnette et al. 2011; Vishwanath and Moreno, 2018). The relative fertility of sex-sorted semen was usually around 70% of that of conventional semen (DeJarnette et al. 2011; Vishwanath et al 2014) even though in well-managed farms could reach a maximum of 90% (Seidel, 2014). Frijters et al. (2009) estimated that two-thirds of the decrease is due to the fewer sperm, and one third to the damage caused by the sorting process. The study of Schenk et al.

(2009) confirmed the importance of sperm dose as pregnancy rates in heifers were virtually identical with 10 million sexed or unsexed sperm per dose. Nevertheless, in contrast to these findings the damage appears to be only partially compensated by increasing sperm numbers per dose in the studies of DeJarnette et al. (2008, 2010 and 2011). Although a few other experiments have been done to identify the causes of low fertility, the consensus is that for most bulls, both factors, dose and damage, are involved in lowered fertility (Seidel, 2014). The main application of sex-sorted semen is breeding dairy heifers because the base fertility level in lactating dairy cows is much lower than in heifers and makes the cost of using sexed semen per female calf produced too high. Moreover sex-sorted semen in heifers enables to reduce dystocia rate because female calves average about 2.5 kg lighter at birth than male calves (Tubman et al. 2004) so heifers having heifer calves are less likely to present problems at calving. Again sexed semen decreases the generation interval: with any rational breeding program for genetic improvement, on average, the youngest animals in the herd are the best genetically, so calves resulting from first calf heifers are ideal replacements (Seidel, 2014). Furthermore, Hinde et al. (2014) demonstrated that milk synthesis is the result of in utero programming of mammary function by the offspring and that Holsteins favor heifers. Analysis of a large database of lactation records revealed that the sex of the fetus of the first parity dam affects her milk production. Heifers carrying a heifer calf produce approximately 445 kg more milk over the first two lactations than when carrying a bull calf. In addition to the milk production of the dam, epigenetic programming of the milk production of the offspring has been demonstrated. Female calves gestated and born in the absence of maternal lactation produce more milk than heifers born to lactating mothers (Gonzalez-Recio et al. 2012).

AI of superovulated cows

Sex-sorted semen is used for superovulated donors to enhance production of heifer calves from genetically superior females. However embryo yield is generally compromised in superovulated animals inseminated with sex sorted semen compared with those inseminated with conventional

semen. Despite the pregnancy rate of single ovulating females being nearly comparable with that using conventional semen, the impaired fertilization rate in superovulated animals has hindered its use in embryo transfer programs (Mikkola and Taponen, 2017). Yields of transferable quality embryos can be reduced by half in cows and somewhat less in heifers (Schenk et al., 2006; Kaimio et al., 2013). Again reduced fertilization with sexed semen may be due to low doses of sperm, an abnormal uterine environment or atypical sperm transport in superovulated cows, damage to sperm during sex sorting, or some combination. Superovulated cows inseminated with sex-sorted semen produced a significantly larger proportions of unfertilized oocytes and/or degenerate embryos than superovulated heifers or cows inseminated with unsorted semen (Peippo et al. 2009, Monteiro et al. 2016, Mikkola and Taponen, 2017). Several studies have clearly demonstrated a decrease in the numbers or proportions of transferable or good quality embryos and higher proportions of unfertilized ova for cows inseminated with low numbers of sex-sorted semen (Baruselli et al. 2007; Schenk et al. 2006; Hayakawa et al. 2009; Peippo et al. 2009; Soares et al. 2011), while for heifers, the adverse effect of sexed semen on embryo production has been moderate or negligible (Hayakawa et al. 2009; Peippo et al. 2009; An et al. 2010). As stress and damage of the sperm is inevitable as intrinsic in the process of sorting, dose, site and time of insemination have been widely investigated in order to enhance fertility of sexed semen in superovulated animals however with no consistency of results.

Dose of semen, site of insemination and time of insemination

Panarace et al. (2003) reported successful embryo production when superstimulated heifers and cows were inseminated using 10 $x10^6$ sexed frozen-thawed sperm per dose at 0, 12 and 24 h after estrus detection. Sartori et al. (2004) reported no difference in transferable embryo rates between superovulated Holstein heifers inseminated with 20 $x10^6$ sperm once (12 h after estrus detection) or 10 $x10^6$ sperm twice (12 and 24 h after estrus detection) using sexed frozen-thawed sperm, but the rates were lower than that of unsexed sperm. Schenk et al. (2006) suggested multiple inseminations

totaling >20 $x10^6$ sexed frozen-thawed sperm might be advantageous in Holstein heifer donors but results in cows were more unpredictable and inconsistent than in heifers possibly because of postpartum uterine conditions and/or physiological transitions (Peippo et al. 2009). Hayakawa et al. (2009) confirmed that using heifers as donors, with two inseminations of 10 $x10^6$ total sexed sperm is the best approach to maximize embryo production, as a single insemination likely provided insufficient numbers of intact sperm at the site and time of fertilization in superovulated cattle, especially in cows. Peippo et al. (2009) have recently reported that the fertilization rate was significantly decreased in cows with sexed sperm compared with unsexed sperm. Sperm transport in superovulated cattle is challenging for limited numbers of sexed, viable, frozen-thawed sperm that have been affected by the sperm sorting process (Schenk et al. 2006; Seidel, 2014). Increased numbers of sperm per dose or multiple inseminations may be beneficial for embryo production when using a single fixed time insemination protocol, even though the optimal dosage and insemination time remains unknown (Schenk et al. 2006).

Deep uterine insemination (DUI) is a common approach that is believed to ensure sufficient numbers of sorted spermatozoa at the fertilization site of the donor. However, there is no clear evidence of the superiority of this technique compared with insemination in the uterine body and again no consistency of results has been reported. Some have inseminated both semen types in the uterine body (Schenk et al. 2006; Hayakawa et al. 2009; Larson et al. 2010; Soares et al. 2011), some have used deep uterine horn insemination for both semen types (Sartori et al. 2004) and others have deposited sex-sorted semen deep in uterine horns and conventional semen in the body (An et al. 2010; Peippo et al. 2010). Sartori et al. (2004) compared deep uterine horn AI of equal doses of sexed or conventional semen (10 million sperm/dose) in superovulated Holstein heifers. There was a reduction in the fertilization rate when sexed semen was used. The number and proportion of viable embryos collected from heifers inseminated with sexed semen was compromised compared with unsexed semen, suggesting that sexing per se reduces the fertilization potential of semen.

Some studies applying conventional semen on superovulated donors did not report an advantage in depositing the semen in the uterine horn compared with in the body (Pallares et al. 1986; Gatius et al. 1988), while others established a higher fertilization rate in heifers inseminated in the uterine horns compared with in the body, but there was no effect on superovulated cows (Carvalho et al. 2013). Carvalho et al. (2013) concluded that deep uterine insemination in superovulated cows using conventional semen was not preferable compared with insemination in the uterine body, while in heifers the technique resulted in a higher number of fertilized structures recovered and an increased fertilization rate. Therefore no evidence of the benefits of deep uterine insemination have been consistently reported. The timing of AI with sexed semen on superovulated animals affects the fertilization rate and subsequent embryo yield. The sorting process shorten life span of sperm (Maxwell et al. 2004) as it causes "precapacitation" of sperm, shortening the time required for capacitation in the genital tract and subsequent life span (Lu and Seidel, 2004; Schenk et al. 2009). Many in vitro studies on different species have confirmed that sex-sorted semen shows physiological and functional differences from conventional unsorted semen and reported a more advanced membrane state which resembles in vitro capacitation (Bucci et al. 2012), an altered motility, velocity and amplitude of lateral head displacement (Suh et al 2005; de Graaf et al 2006) and an impaired ability to penetrate cervical mucus (de Graaf et al. 2006) along with a distorted way to bind to and detach from oviduct epithelial cells (Hollinshead et al. 2003; de Graaf et al. 2006). It could be concluded that sex-sorted semen shows changes which reflect a partial capacitation and consequently it may need less time to complete capacitation in the oviduct and be functional for fertilization compared to unsorted semen (Winters et al. 2017). Thomas et al. (2017) noted that changing the timing of insemination, delaying it closer to the time of ovulation, improved conception rates with sex-sorted semen obtaining levels almost equal to those of conventional unsorted semen. Schenk and Seidel (2008) suggested that the optimal fixed time for inseminating single ovulating heifers with sexed, frozen-thawed sperm might be later than for unsexed sperm.

Baruselli et al. (2008) have recently reported increased transferable embryos using sexed sperm inseminated at 18 and 30 h after estrus detection/GnRH treatment in Nelore and Holstein cattle compared to a protocol inseminating at 12 and 24 h after estrus/GnRH. Another important aspect which could affect the fertility of sex-sorted semen is the physiological heterogeneity within the sperm population of an ejaculate. Semen sample contains distinct sub-populations of sperm that would capacitate and be physiologically ready for fertilization at different times post insemination. This diversity allows some flexibility from the time of insemination to the time of ovulation and fertilization compared to samples where this heterogeneity has been altered. In any ejaculate a competent population of sperm are available and functional for fertilization at different times, covering a relatively long period of time, allowing a quite large divergence between the time the sperm enters the female reproductive tract to the time ovulation occurs. In sex-sorted sperm the majority of spermatozoa is fertile in a narrow time window (Vishwanath and Moreno, 2018).

All these considerations together lead to the necessity of accurately timing the artificial insemination with sex-sorted semen in relation to ovulation. There is evidence in single-ovulating animals that delaying the insemination with sexed semen close to ovulation, i.e. 24 hours after the onset of estrus, increases pregnancy rates (Schenk et al. 2009) and it is logical to deduce that the same principle is true also in superovulated animals where in addition the longer time needed for multiple ovulations has to be considered.

1.4.3.3 Embryo quality and pregnancy rate with sex-sorted semen

In addition to the increase in the proportion of unfertilized oocytes and degenerated embryos, evidence has emerged that sex sorting alters embryo quality (Mikkola and Taponen, 2017). Deviations in the gene expression of embryos produced with sex-sorted semen were reported (Morton et al. 2007). Along with impaired embryonic development during the 7-day period from insemination to embryo recovery, Mikkola et al. (2015) found that the pregnancy rate after transfer of in vivo embryos produced with sex-sorted semen was lower than that after transfer of embryos

produced with conventional semen: the difference was 5.0 percentage points when frozen embryos were used (conventional 42.3% vs. sexed 37.3%), corresponding to a total decrease in pregnancy rate of 12% and the difference in pregnancy rate was even bigger (8.7 percentage points) when fresh embryos were transferred. Mikkola and Taponen (2017) revealed a statistically significant delay in the developmental stage of the embryos when heifers were inseminated with sexed sorted semen and a tendency to delay in development in cows. A decrease in the proportion of quality grade 1 embryos (Baruselli et al. 2008; Hayakawa et al. 2009; Larson et al. 2010; Soares et al. 2011) and an increase in the proportion of quality grade 2 and 3 embryos along with a higher probability of recovering no transferable embryos (Mikkola and Taponen, 2017) were reported when donors were inseminated with sexed sorted semen. Moreover calf mortality was higher for the "wrong" sex calves, male calves born from sexed semen than for male calves born from conventional semen (DeJarnette et al 2009; Norman et al. 2010; Healy et al. 2013; Mikkola et al. 2015). Nevertheless, as the final outcome of embryo production does not culminate in embryos but the goal is to produce calves as efficiently and economically as possible, when evaluating the entire process of calf production by embryo transfer, sex-sorted semen still makes excellent economic sense to produce progeny of the desired sex, without wasting recipient resources to produce calves of the less valuable sex (Seidel, 2014; Mikkola and Taponen 2017). To evaluate the efficency of calf production, the reduced number of recipients when using sex-sorted semen also needs to be considered and efficiently using this limited resources is a startegic point in breeding schemes (Mikkola and Taponen, 2017). The whole issue has been facilitated by genomic breeding which favors young heifers as embryo donors rather than cows so that sex-sorted semen can be used in the category of animals which have shown the lowest negative "side-effects". Historically, there was very little or no selection on the female side in commercial dairies as at replacement of 40%, considering calf losses and a sex ratio of 50/50, dairy farmers need to keep every single born female simply to maintain herd size (Vishwanath and Moreno, 2018). Sex-sorted semen offers the chance

to introduce selection on the female population by skewing the sex ratio. Moreover the advent of 4M SexedULTRA™ sex-sorted semen could further fill the gap between sexed and conventional semen providing further impetus to its routine application for insemination in all female categories both single and multiple ovulating.

1.4.3.4 SexedULTRA™ sex-sorted semen

For many years the challenge has been to minimize the effect of the multiple steps that sperm have to go through during the sorting process in order to raise the fertility of sex-sorted semen. With the advent of SexedULTRA™ sex-sorted semen, compared to the traditional hereinafter called "XY method", a complete revamp of the all media used for the process from the initial holding and preparation of sperm for staining, the sheath fluid and the subsequent collection and freezing of sperm have been introduced so that the major sources of stress were attenuated (Vishwanath and Moreno, 2018). The SexedULTRA™ process has been designed to be more benign to sperm during the various stages of the sorting process (Gonzalez-Marin et al. 2016; Lenz et al. 2016) and in vitro tests have revealed an improved sperm motility as well as acrosome integrity compared with the XY method at the same sperm concentration (Gonzalez-Marin et al. 2016). The first small-scale field trial with SexedULTRA™ semen registred an improvement of 7.4 percentage points in heifer conception rates compared with the XY technology and subsequent larger field trials confirmed that SexedULTRA™ method resulted in a 4.5 percentage points improvement in conception rate compared with XY method (46,1% vs 41,6%) (Vishwanath et al. 2014; Lenz et al. 2016). All together these trial results seemed to confirm that the deleterious effect of the XY method for semen processing was partly alleviated through the use of the SexedULTRA™ technology (Vishwanath and Moreno, 2018). Determining whether the corresponding sperm fertility was compensable through increasing dose rates was the next logical step, even though in the past increasing sperm dosage did not compensate for lower fertility of XY method sorted semen (DeJarnette et al. 2010). A field trial was conducted to investigate dose rate effects and compare against conventional semen.

Five dairy bulls located at German Genetics International GmbH in Cloppenburg (Germany) were used. Each ejaculate was split 4 ways, sex-sorted, and frozen in 0.25-mL straws as follows: XY 2.1 million/straw, SexedULTRA™ 2.1 million/straw, SexedULTRA™ 3.0 million/straw, and SexedULTRA™ 4.0 million/straw. The 56-day non-return rate was the parameter taken into consideration to identify a positive outcome. Overall the 2.1 million XY method resulted in lower non-return compared with all the SexedULTRA™ treatments and conventional. The 2.1 and 3 million SexedULTRA™ treatments were similar and lower than conventional but increasing the dose rate to 4 million resulted in a non-return rate comparable to conventional (Lenz et al. 2016). For the first time a dose response effect with sex-sorted bovine sperm and parity in conception rates with conventional semen was demonstrated (Vishwanath and Moreno, 2018). Analysing the performance of sex-sorted semen over the last decade Hutchison and Bickhart (2016) observed a positive improvement in overall fertility of sex-sorted semen soon after the introduction of SexedULTRA™ technology. Mean conception rates have increased and the differences from conventional semen have decreased consistently in all female category. Conception rates of >90% of that obtained with conventional semen seem accessible. However, no data have been published regarding superovulated cattle yet. Although the economic impact of using sexed semen in an embryo transfer program was deemed profitable only in heifers (Hayakawa et al. 2009; Mikkola and Taponen, 2017), 4M SexedULTRA™ sex-sorted semen may be appropriate in cows and this needs to be further confirmed with field trials.

2. Aims of the studies

The aim of the proposed research was to determine whether milk coagulation properties could be improved by using embryo transfer to increase the frequency of k-casein BB genotype cattle and reducing A and E variants in an Italian Holstein herd with a low prevalence of the favourable genotype. In the effort to optimize superovulation protocols and results, beside the main objective, other experiments were carried out:

1) efficacy of synchronization of wave emergence before superovulation through manual transrectal ablation of the largest (dominant) ovarian follicle on days 7 or 8 of the cycle (estrus = day 0);
2) the effect on embryo production and quality of different drugs and dosage for the SO protocol were experimented: Folltropin 700 I.U.; Folltropin 1000 I.U. and Folltropin 700 I.U. + Pluset 300 I.U.;
3) the use of ULTRA sexed sorted semen for AI of superovulated cows was reported for the first time.

3. Experimental studies

3.1 Experimental study I

Embryo production and quality in dairy cattle is enhanced by manual transrectal ablation of the dominant follicle prior to superovulation

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Embryo production and quality in dairy cattle is enhanced by manual transrectal ablation of the dominant follicle prior to superovulation

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SUMMARY

The objective was to determine effects of manual transrectal ablation of the largest (dominant) ovarian follicle on days 7 or 8 of the cycle (estrus = day 0) to synchronize wave emergence before superovulation. Superovulation was performed in 167 donors, 32 nulliparous, 36 primiparous and 99 multiparous Holsteins. They were randomly allocated into 3 groups: control group (n=64, including 15 cows where the follicle failed to rupture by light digital pressure), dominant follicle ablation on day 7 (n=57) and dominant follicle ablation on day 8 (n=46). Superstimulatory treatments started on day 10 for control or 1 day after ablation. Superovulation was induced by 9 im injections of decreasing dosage of gonadotrophins at 12-hour intervals over 4.5 days. PGF2α was administered in 2 im injections at the time of the seventh and the eighth injections of gonadotrophins. All females were artificially inseminated with frozen-thawed semen. Seven days after estrus embryos/ova were recovered by flushing the uterine horns, classified for stage and quality according to the IETS guidelines, and transferred fresh or frozen. Treated cows were subsequently monitored for adverse effects that could affect reproductive parameters or reproductive culling rate at 270 days post-partum. Total numbers of recovered structures and transferable embryos were lower ($P<0.05$) in control (7.8 ± 4.5 and 5.1 ± 3.3, respectively; mean ± SD) compared to ablation 7 (9.0 ± 2.5 and 7.0 ± 1.9) and ablation 8 (9.6 ± 3.4 and 6.9 ± 2.5) groups. There was no difference ($P>0.05$) among groups in mean grade of transferable embryos, but percentage of Grade-I embryos were higher in ablation 8 group (47.3%) than in control (37.9%), whereas Grade-II embryos were lower in ablation 8 group (37.5%) compared to control (45.6%) ($P<0.05$). Percentage of unfertilized oocytes was higher ($P<0.05$) in control (14.4%) than in ablation 8 group (9.8%), whereas degenerating embryos were lower ($P<0.05$) in ablation 7 group (12.2%) than in control (20.0%). Conversely, percentage of morulae was higher ($P<0.05$) in ablation 7 group (32.8%) than control (25.5%). Reproductive parameters and culling rates were similar among groups ($P>0.05$). In conclusion, improvements in embryo number and quality were achieved by manually ablating the dominant ovarian follicle on days 7 or 8. Furthermore, there was no evidence that judicious manual ablation could have any detrimental effect on subsequent reproductive performance and culling rate.

KEY WORDS

Dairy cow, superovulation, follicle ablation, follicular wave synchronization.

INTRODUCTION

Much of the variation in response to superstimulation has been attributed to the status of the follicular wave at the start of superstimulatory treatment[1]. Ovarian follicles in cattle develop in a wave-like pattern, typically with 2 or 3 waves in an interovulatory interval. Each wave is preceded (1 or 2 days) by a follicle stimulating hormone (FSH) surge, which induces and sustains synchronous growth of a cohort of follicles. A few days after wave emergence, FSH concentrations start to decline and selection occurs: subordinate follicles regress, due to a reduced capacity to survive when FSH concentrations are low[2], whereas the dominant follicle keeps growing[3]. In the absence of luteal regression, the dominant follicle enters a plateau or static phase ~6 days after wave emergence and usually regresses[4]. During the growing phase and initial portion of the static phase, a dominant follicle supresses other subordinate follicles and emergence of the next wave[4,5]. Many authors have emphasized the importance of starting superstimulation when a large number of growing follicles is available, prior to dominant follicle-induced suppression of subordinate follicles[6] or just after the onset of selection[3]. The rationale for starting a "traditional" superovulation (SOV) protocol at mid-cycle (8-13 days post ovulation)[7] is expected absence of a functional dominant follicle, concurrent with the approximate time of emergence of the second follicular wave in both 2- and 3-wave cycles. However, due to variations in day of emergence of the second follicular wave, mid-cycle regimens have yielded highly variable superovulatory responses[8].

The best superstimulatory responses, i.e. more large follicles and significantly more ovulations[9,10] were obtained when treatments were initiated on the day of, or the day before, wave emergence. Instead of predicting spontaneous wave emergence, various approaches have been used to synchro-

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nize it, by eliminating the dominant follicle, with predictable emergence of a new follicular wave[11]. Hormonal and physical approaches have been used, including exogenous estradiol-17 , estradiol benzoate and progesterone, GnRH, LH or hCG, or mechanical removal of the dominant follicle. Transvaginal ultrasound-guided ablation is highly effective, but requires specialized equipment and trained technical staff, limiting routine use in the field[12,13].

A less complicated and laborious way to eliminate dominant follicle could be manual rupture. The study of Bartmann[14] confirmed that manual rupture of the largest follicle was associated with an enhanced superovulatory response to treatment initiated 1 day later. Bartmann[14] reported no complications after removal of the dominant follicle in donor cows. Furthermore, a similar procedure for manual rupture of ovarian cysts has been widely used[15] without long-term damage if excessive ovarian manipulation was avoided[16,17].

The objective was to determine: 1) if under field conditions, manual rupture of the dominant follicle on days 7 or 8 of the cycle could enhance embryo yield compared to starting superovulation treatment on day 10; and 2) if manual rupture of the dominant follicle could be performed without detrimental effects on subsequent reproductive function.

MATERIALS AND METHODS

Donors

Holstein cattle on several dairy farms in Emilia Romagna, Italy, were used. Cattle had *ad libitum* access to a total mixed ration. Lactating cattle were housed in free stall barns with cooling systems such as ventilation and a shower system, with average daily milk production of 41 litres. Superovulation and embryo recovery were done during the spring or late autumn/winter, avoiding high ambient temperatures in July, August and September.

There were 32 nulliparous, 36 primiparous and 99 multiparous cattle. Nulliparous ranged from 14 to 15.5 months. Lactating cows had a body condition score between 2.75 and 3.5 (scale of 1-5), had at least 2 or 3 apparently physiologic estrus cycles after calving and were subjected to the superovulation treatment on average 72 ± 12 days after calving.

Cattle with clinical illness, e.g. mastitis, lameness, respiratory or gastrointestinal disorders with a considerable reduction in milk production and impaired general health after calving, were not used.

To detect negative consequences of manual follicle ablation, reproductive end points and culling for reproductive reasons (i.e. cow not pregnant at 270 days post-partum) were evaluated after treatments. To detect abnormalities in reproductive function, transrectal palpation and ultrasonography were done after SOV procedure every 14 days until cattle became pregnant and then at 60, 150 and 210 days of pregnancy.

Estrus was detected both with pedometers and visually by the herdsman who recorded behavioral estrous signs. Cattle were then randomly assigned to treatments: ablation of dominant follicle on day 7 (ablation 7 group, n=57; day 0 = standing estrus), ablation of dominant follicle on day 8 (ablation 8 group, n=46), and control group (n=49+15 ablation failure=64). Only in ablation groups an ultrasound examination was performed the day of ablation to identify the dominant follicle. Cattle designated for manual follicle ablation were given caudal epidural anesthesia, 5 ml of 2% procaine (Procamidor, Izo s.r.l., Brescia, Italy) injected into the epidural space at the sacro-coccygeal or first intercoccygeal junction. Manual ablation was done by squeezing the largest follicle by transrectal manipulation. The cows in which the follicle did not readily rupture in response to modest pressure were re-assigned to the control group.

Superovulation

Nine gonadotrophic treatments, in decreasing doses at 12-hour intervals over 4.5 days, were started on days 8 (ablation 7), 9 (ablation 8) or 10 (control). Total gonadotrophin doses were 700 IU for heifers (Folltropin, Vetoquinol, Bertinoro, Italy) and 1000 IU for cows (Pluset,Calier Italia, Milan Italy), given im. To induce luteolysis, PGF2α (Dinolytic, Pfizer, Milan, Italy) was given concurrent with seventh and the eighth gonadotrophin injections (each treatment was 25 mg im).

Artificial insemination

Estrus was detected both with pedometers and visually by the herdsman who recorded behavioral estrous signs, including cow standing and being mounted or mounting other cows and vulvar discharge. All cattle detected in estrus within 36 h after the second PGF2α injection were artificially inseminated with frozen-thawed bull semen. Inseminations were initiated 12 h after the onset of standing estrus and repeated 6 h later. Transrectal ultrasonography was used to evaluate ovaries 6 hours after the second insemination; if >3 follicles ≥ 10 mm were present, the cattle were inseminated a third time.

Embryo collection

At 7 days after the onset of estrus, transcervical uterine flushing was done using a standard protocol. Embryos were identified and evaluated under a stereomicroscope and classified according to IETS guidelines[18] for quality (Grade 1: excellent or good; Grade 2: fair; Grade 3: poor; Grade 4: dead or degenerating) and developmental stage (1: 1-cell; 2: 2 to 16-cell; 3: early morula; 4: morula; 5: early blastocyst; 6: blastocyst; 7: expanded blastocyst; 8: hatched blastocyst; and 9: expanded hatched blastocyst).

Statistical analyses

Data were analysed for normality using a Shapiro-Wilk test. Depending on distribution, comparisons of data between heifers and cows was made using a Student's *t*-test or a Mann-Whitney test. Statistical differences in total recovery, transferable and non-transferable embryos, mean embryo grade and reproductive parameters were assessed by one way-ANOVA or Kruskal-Wallis ANOVA, with post-hoc comparisons done with a Tukey HSD or a Wilcoxon-Mann-Whitney test, respectively. Chi square was used for analysis of embryo grade, embryo stage, embryo collection yielding and reproductive culling rate. All statistical analyses were done with IBM SPSS Statistics 23 (IBM Corporation, Milan, Italy). For all analyses, $P<0.05$ was considered significant.

RESULTS

Light pressure on the dominant follicle did not cause its rupture in 8/65 (12.3%) and in 7/53 (13.2%) cows in ablation 7

Table 1 - Mean ± SD number of structures recovered, transferable and non-transferable embryos after superovulation in control cattle and those subjected to manual dominant follicle ablation.

	Control (n=64)	Ablation 7 (n=57)	Ablation 8 (n=46)
All structures	7.8 ± 4.5[b]	9.0 ± 2.5[a]	9.6 ± 3.4[a]
Transferable embryos (%)	5.1 ± 3.3[b] (65.5)	7.0 ± 1.9[a] (77.5)	6.9 ± 2.5[a] (71.9)
Non-transferable embryos (%)	2.7 ± 2.3 (34.5)	2.1 ± 1.4 (22.5)	2.7 ± 2.4 (28.1)

Control: no follicle ablation; Ablation 7: ablation at day 7 (estrus = day 0); and Ablation 8: ablation at day 8. Superovulation was started on day 10 in the Control group or at 1 day after ablation.
[a,b] Within a row, means without a common superscript differed (P<0.05).

Table 2 - Quality grade (IETS 1-3) of transferable bovine embryos recovered after superovulation in control cattle and those subjected to manual dominant follicle ablation.

	Control (n=64)	Ablation 7 (n=57)	Ablation 8 (n=46)
Grade 1 (%)	124/327[b] (37.9)	170/399[a,b] (42.6)	150/317[a] (47.3)
Grade 2 (%)	149/327[a] (45.6)	157/399[a,b] (39.3)	119/317[b] (37.5)
Grade 3 (%)	54/327 (16.5)	72/399 (18.1)	48/317 (15.1)
Mean grade	1.8 ± 0.7	1.8 ± 0.7	1.7 ± 0.7

Control: no follicle ablation; Ablation 7: ablation at day 7 (estrus = day 0); and Ablation 8: ablation at day 8. Superovulation was started on day 10 in the Control group or at 1 day after ablation.
[a,b] Within a row, proportions without a common superscript differed (P<0.05).

and ablation 8 groups, respectively. Data from heifers and cows were not different (P>0.05), so all were considered together. Data regarding all structures recovered, transferable and non-transferable embryos are shown (Table 1). Total number of recovered structures and transferable embryos were lower in control group compared to ablation groups (P<0.05).

Effects of group on quality of transferable embryos are shown (Table 2). There was no difference (P>0.05) among groups in mean grade of transferable embryos. However, there was a higher proportion (P<0.05) of Grade-I embryos in ablation 8 group than in control group, whereas there was a higher proportion (P<0.05) of Grade-II embryos in control versus ablation 8 group.

Data regarding embryo developmental stage are summarized (Table 3). There was a lower percentage (P<0.05) of unfertilized oocytes in ablation 8 group than in control group, whereas there was lower percentage of degenerating embryos were lower (P<0.05) in ablation 7 group than in ablation 8 and control group. Percentage of morulae was higher in ablation 7 group as compared to control (P<0.05).

There were no differences (P>0.05) among groups in culling rate for reproductive reasons (control: 2/64 - 3.1%; ablation 7: 2/57 - 3.5%; ablation 8: 1/46 - 2.2%). Days from SOV to estrus, days from SOV to conception, percentage conceiving at 1st insemination and number of AI to conception were not significantly different among groups (P>0.05) (Table 4). No abnormalities were detected during pregnancy monitoring.

Table 3 - Developmental stages of bovine embryos recovered after superovulation in control cattle and those subjected to manual dominant follicle ablation.

	Control (n=64)	Ablation 7 (n=57)	Ablation 8 (n=46)
Unfertilized oocytes (%)	72/499 (14.4)[b]	53/515 (10.3)[a,b]	43/441 (9.8)[a]
Early morula (%)	100/499 (20.0)[b]	63/515 (12.2)[a]	81/441 (18.4)[b]
Morula (%)	127/499 (25.5)[b]	169/515 (32.8)[a]	127/441 (28.8)[a,b]
Early blastocyst (%)	134/499 (26.9)	146/515 (28.3)	122/441 (27.7)
Blastocyst (%)	55/499 (11.0)	67/515 (13.0)	60/441 (13.6)
Expanded blastocyst (%)	11/499 (2.2)	17/515 (3.3)	8/441 (1.8)

Control: no follicle ablation; Ablation 7: ablation at day 7 (estrus = day 0); and Ablation 8: ablation at day 8. Superovulation was started on day 10 in the Control group or at 1 day after ablation.
[a,b] Within a row, proportions without a common superscript differed (P<0.05).

Table 4 - Reproductive performance after superovulation in control cows and in cows treated by dominant follicle manual ablation to synchronize wave emergence.

	Control (n=64)	Ablation 7 (n=57)	Ablation 8 (n=46)
Days from SOV to estrus	28.2 ± 11.0	26.3 ± 10.1	26.6 ± 9.4
Days from SOV to conceiving	55.6 ± 24.4	52.6 ± 28.7	1.9 ± 25.2
Pregnant after 1st AI (%)	35.9	38.6	37.0
No. AI to become pregnant	2.2 ± 1.1	2.2 ± 1.3	2.4 ± 1.3

Control: no follicle ablation; Ablation 7: ablation at day 7 (estrus = day 0); and Ablation 8: ablation at day 8. Superovulation was started on day 10 in the Control group or 1 day after ablation.

DISCUSSION

In the present study, manual rupture of the dominant follicle on days 7 or 8 of the cycle and initiating superstimulation treatment 1 day later enhanced embryo yield compared to the control group, with superstimulation initiated on day 10 (in the absence of any manipulation of follicular wave emergence). Based on previous reports and current results, we inferred that follicular wave emergence was more synchronous in cattle in which ablation was performed. In previous studies[19,20], superovulatory responses were lower in cattle in which gonadotrophin treatments were initiated as soon as 1 day after wave emergence compared to treatments initiated the day before or the day of follicular wave emergence. A delay in treatment from 0.9 to 1.2 day appeared to be responsible for a difference in superstimulatory response[20]. These considerations further highlight how the "8-13 day window" is often not an optimal moment to start superovulation. Due to considerable variation in proportion of cattle with 2 versus 3 waves per cycle and in the day of wave emergence, particularly of wave 2, it is difficult to predict the optimal day to initiate SOV. Consequently, methods were developed to synchronize follicular wave emergence to optimize initiation of gonadotropin treatment. It is widely accepted that before start-

ing a superovulation protocol, it is useful to eliminate the dominant follicle. When superstimulation treatments were initiated in the absence of a dominant follicle, there was greater follicular growth and ovulation rate[21], an enhanced number of recovered structures[22], and an enhanced number of transferable embryos[9,10,23]. Follicle ablation eliminates risk of premature ovulation of the largest follicle present at start of superstimulation[24] and offers the advantage of initiating superstimulatory treatment with minimal delay. Removal of a dominant follicle is followed (within 12 hours) by a surge in FSH and by immediate resumption of growth of small follicles[25]. For example, ablation of dominant follicle on days 3 or 5 (day 0 = ovulation) resulted in a surge in FSH beginning the day after cautery[4]. This FSH surge could lead to resurgence of the largest subordinate follicle which becomes the new dominant follicle or could result in emergence of a new follicular wave 1 or 2 days later[26,27,28]. Effects of the post-ablation FSH surge probably depend on viability of subordinate follicles at the moment of dominant follicle ablation. Subordinate follicles seem to become nonviable by 5 days after emergence[2,3,4,5]. At this time, follicle ablation, with superstimulatioin starting 24 hours later, coincides follicular wave emergence so that an optimal response can be achieved. In this study, ablation of the largest follicle was performed on days 7 or 8 of the cycle to ensure that in cattle with either 2- or 3-wave cycles, there was a high probability that: 1) an active dominant follicle was present and its ablation would eliminate the major source of FSH suppression[29]; 2) subordinate follicles of wave 1 are not viable and cannot be rescued to become the new dominants; 3) exogenous gonadotropins administered 24 hours after ablation coincided with the endogenous surge and were given the day before emergence of the new wave. We concluded that ablation of the dominant follicle on days 7 or 8 enhanced results due to predictable emergence of a new follicular wave, with optimal initiation of superstimulation treatments. However, delaying ablations to days 9 or 10 could lead to asynchronous events due to an endogenous FSH surge or follicular wave emergence or even selection by the time of exogenous FSH administration, 24 hours after ablation. Conversely, earlier ablations (before day 7) could lead to resurgence of subordinate follicles in lieu of emergence of a new wave[30].

Regarding stages of recovered embryos, in the present study, percentage of morulae was higher in ablation 7 group as compared to control. Furthermore, rate of degenerated embryos was lower in ablation 7 compared to control. We inferred that these differences were due to more synchronous follicular emergence and ovulation. Ablating at day 7 could have had more cattle in a pre-wave situation and could better correspond to "the day before or the day of wave emergence". Also, embryo quality was influenced by ablation of the dominant follicle, as demonstrated by the higher percentage of Grade-I embryos in ablation 8 group compared to control, and vice versa, the higher percentage of Grade-II embryos in control compared to ablation 8 group. It further supports the hypothesis that a more synchronous timing of events when exogenous control of follicular wave emergence is performed has consequences not only on embryo yield, but also on embryo quality.

The reproductive career of all cattle was evaluated after supervovulation and embryo recovery. Reproductive performance (intervals from superovulation to estrus and to becoming pregnant, percentage pregnant after 1st insemination and number of AI to achieve pregnancy) and cows culled for reproductive reasons were similar among groups. Therefore, there were no indications that manual follicle ablation had deleterious long-term consequences.

CONCLUSIONS

In conclusion, manual rupture of the dominant follicle on days 7 or 8 of the cycle and starting SOV treatment 1 day later enhanced embryo yield and embryo quality compared to starting SOV on day 10. There were no indications that judicious manual ablation had any deleterious effects on subsequent reproductive performance.

DECLARATION OF CONFLICT OF INTEREST

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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3. Experimental studies

3.2 Experimental study II

Superovulation protocols for dairy cows bred with SexedULTRA™ sex-sorted semen

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Superovulation protocols for dairy cows bred with SexedULTRA™ sex-sorted semen

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Summary

The objective was to compare embryo yield and quality in lactating dairy cows superovulated (SO) with varying amounts of gonadotropins and FSH:LH ratios and inseminated with SexedULTRA™ sex- sorted semen. The SO treatments (n = 77) involved 3 protocols: groups F700 and F1000 were given total doses of 700 and 1,000 IU of Folltropin (FSH:LH ratio 49:1), respectively, whereas group F700P300 was given 700 IU of Folltropin + 300 IU of Pluset (FSH:LH ratio 1:1). Cows were artificially inseminated 3 times over a 10- hr interval with frozen- thawed SexedULTRA™ sex sorted semen (total of 10 Å~ 106 sex- sorted sperm), starting 18 hr after onset of oestrus, with embryos/ova recovered 7 d after oestrus. Total number of recovered structures and transferable embryos were lower ($p < 0.05$) in F700 (4.7 ± 3.0 and 1.9 ± 1.7, respectively; mean ± SD) compared to F1000 (8.1 ± 3.8 and 4.4 ± 2.6) and F700P300 (8.5 ± 6.4 and 4.5 ± 3.3). Percentage of cows ovulating >50% of follicles ≥0.8 cm in diameter was lower ($p < 0.05$) in F700 (35.5%) than in F1000 (82.4%) and F700P300 (73.1%). Percentage of unfertilized oocytes was higher ($p < 0.05$) in F700 (45.0% vs. 27.7% for F1000 and 29.0% for F700P300) whereas percentage of morulae was higher ($p < 0.05$) in F1000 (19.3% vs. 8.7% for F700 and 12.2% for F700P300). Embryo quality was similar among groups ($p > 0.05$). In conclusion, embryo production in lactating dairy cows was improved by increasing total dose of gonadotropins from 700 to 1,000 IU, with SexedULTRA™ sex- sorted semen yielding satisfactory fertilization rates and embryo quality.

Keywords: dairy cow, gonadotropins, sexedUltra, sex-sorted semen, superovulation

1. Introduction

Numerous superovulation (SO) protocols have been intensively studied to improve embryo production and embryo quality in cattle. As many donors produce no, few or poor-quality embryos, various FSH treatments have been attempted, including varying total doses of gonadotropins and the FSH:LH ratio. Although both FSH and LH are required in physiologic reproductive processes, limiting exogenous LH in SO regimens in cattle has been advocated (Kanitz et al. 2002, Mapletoft et al. 2002), with benefits including reduced variability in the SO response (Moor et al. 1984), enhanced embryo production due to higher ovulation rates and improvements in fertilization rate and embryo quality (Donaldson and Ward 1987, Yamamoto et al. 1993, Quaresma et al. 2003). Excess LH seemed to have deleterious effects due to premature oocyte activation (Hyttel et al. 1991), premature ovulation (Callesen et al. 1987) and luteinization of FSH-stimulated follicles (Boland et al. 1991). In many studies, increasing LH content in the SO regimen decreased proportion of transferable embryos; despite a higher ovulation rate, a large proportion of ova/embryos were unfertilized or degenerate (Donaldson and Ward 1986, Donaldson et al. 1986, Kelly et al. 1997). Treatment with a purified FSH preparation resulted in greater embryo production than treatment with equine chorionic gonadotropin (eCG), which has high LH activity (Goulding et al. 1991).

Notwithstanding the deleterious effects of excessive exogenous LH, inadequate exogenous LH reduced embryo yield (Chupin et al. 1984), as highly purified FSH preparations significantly reduced ovulation rates compared to FSH supplemented with LH (Chupin et al. 1987, Schmidt et al. 1988, Herrler 1991, Ereno et al. 2010). Impaired follicular maturation in heifers superovulated with a recombinant human FSH preparation (Takagi et al. 2001) was attributed to a lack of exogenous LH activity and severe suppression of LH pulsatility.

The role of LH in SO protocols is controversial; outcomes could depend on many factors, including subspecies, breed or genetic differences, general health condition and energy balance, acute or

chronic stress and various management or environmental conditions. It is well established that SO regimens alter LH secretion, including reductions in pulse amplitude and frequency, a reduced basal secretion of LH and an altered (absent, inhibited, premature or late) pre-ovulatory LH surge which reduces ovulation rate, fertilization rates, egg/embryo quality and embryo production (Greve et al. 1984, Roberge et al. 1995, Price et al. 1999, Gosselin et al. 2000). In 1 study (Ben Jabara et al. 1994), lesser amounts of exogenous LH administered throughout the entire SO protocol resulted in greatest suppression of endogenous LH. However, providing more exogenous LH, only near the end of the SO regimen, increased transferable embryos (Barcelos et al. 2006, Cifuentes et al. 2009).

Fertilization rates with sexed semen have always been severely reduced, particularly in superovulated cows versus single-ovulating females or superovulated heifers. Reduced fertilization with sexed semen may be due to low doses of sperm, an abnormal uterine environment or atypical sperm transport in superovulated cows, damage to sperm during sex sorting, or some combination. Superovulated cows inseminated with sex-sorted semen produced a significantly smaller proportion of transferable embryos and significantly larger proportions of unfertilized oocytes and/or degenerate embryos than heifers or cows inseminated with unsorted semen (Peippo et al. 2009, Monteiro et al. 2016, Mikkola and Taponen, 2017). With 4M SexedULTRA™ sex-sorted semen, fertility rates between conventional semen and sex-sorted semen in single-ovulating heifers approached equivalence (Vishwanath and Moreno, 2018); however, no data have been published regarding superovulated cattle. Although the economic impact of using sexed semen in an embryo transfer programme was deemed profitable only in heifers (Mikkola and Taponen, 2017, Hayakawa et al. 2009), 4M SexedULTRA™ sex-sorted semen may be appropriate in cows.

The objective was to determine whether embryo yield in stressed lactating dairy cows would be altered by changing the FSH:LH ratio and inseminating with SexedULTRA™ sex-sorted semen. Three SO protocols were tested using the 2 products commercially available in Italy: Folltropin, with a high FSH:LH ratio (49:1) (Henderson et al. 1990) and Pluset, with a low FSH:LH ratio (1:1)

(Kelly et al. 1995).

2. Materials and methods

2.1 Donors

The study was conducted on a dairy farm in Emilia Romagna, Italy, with the consent of the owner. No ethical approval was needed for the routine veterinary procedures and drugs used. This herd had 300 lactating Holstein cows, housed in a free-stall barn with cooling systems (ventilation and shower system), fed *ad libitum* and with an average daily milk yield of 40 L. The SO protocols were performed during spring or late autumn/winter to avoid the hottest part of the year.

Donor cattle (36 primiparous and 41 multiparous cows) had a body condition score between 2.75 and 3.5 (scale, 1-5) and had at least 2 physiologic estrous cycles after calving (the SO treatment was initiated 78 ± 15 d after calving). Cows with clinical illness, for example mastitis, lameness or gastrointestinal disorders with a considerable reduction in milk production and impaired general health after calving, were not used.

2.2 Superovulation protocols

Potential donor cows were observed at least twice daily for behavioural signs of oestrus and SO was induced by 9 im injections of decreasing dosages of gonadotropins at 12-h intervals over 4.5 days, beginning 9 to 11 days after the onset of standing oestrus (Day 0). Concurrent with the seventh and eighth injections of gonadotropins, 150 μg d-cloprostenol (Dalmazin, Fatro, Ozzano dell'Emilia, Italy), a PGF2a analog, was given im.

Donors were randomly allocated to receive one of 3 SO protocols: 1) F700 (n=17), a total dose of 700 IU of Folltropin (Vetoquinol, Bertinoro, Italy) administered morning and evening as follows:1st day 150 IU and 127 IU, 2nd day 108 IU and 87 IU, 3rd day 74 IU and 63 IU, 4th day 45 IU and 29 IU, 5th day 17 IU; 2) F1000 (n=34), a total dose of 1,000 IU of Folltropin administered morning and evening as follows: 1st day 170 IU and 155 IU, 2nd day 135 IU and 125 IU, 3rd day 115 IU and 100

IU, 4th day 88 IU and 77 IU, 5th day 35 IU; and 3) F700P300 (n=26) cows received 700 IU of Folltropin for the first 5 injections administered as follows: 1st day 170 IU + 155 IU, 2nd day 135 IU + 125 IU, 3rd day morning 115 IU + 300 IU of Pluset (Calier Italia, Milan Italy), for the last 4 injections administered as follows: 3rd day evening 100 IU, 4th day 88 IU + 77 IU, 5th day 35 IU.

2.3 Artificial insemination and semen

At the time of the first insemination, a transrectal ultrasonographic examination was done to determine number and size of ovarian follicles present. Only cows with at least 3 ovarian follicles ≥ 0.8 cm in diameter were included in the study. Oestrus was detected both with pedometers and visually by the herdsman who recorded behavioural oestrous signs, including cow standing and being mounted or mounting other cows and vulvar discharge. On the basis of these observations, cows with signs of oestrus within 36 hr after the last PGF 2α analog injection were artificially inseminated. Inseminations were initiated 18 hr after the onset of standing oestrus and consisted of 3 inseminations, 5 h apart. For the first 2 inseminations, 2 straws of SexedULTRA™ sex-sorted semen, containing 2 x 10^6 sperm each, were used, whereas on the third and final insemination, only 1 straw was used (total of 5 straws and 10 x 10^6 sex-sorted sperm). SexedULTRA™ sex-sorted semen from 4 bulls were randomly used so that each cow received semen from at least 3 bulls. At 5 hr after the last insemination, ultrasound examinations were conducted to determine number of follicles ≥0.8 cm in diameter persistent on the ovaries and, based on this data, it was calculated if the cow was ovulating at least 50% of the follicles ≥0.8 cm in diameter present on the ovaries at the time of the first insemination.

2.4 Embryo collection

Transcervical uterine flushing was done 7 days after onset of oestrus. Embryos were evaluated under a stereomicroscope and classified according to the IETS classification guidelines (Robertson and Nelson, 2010) for quality (Grade 1: excellent or good; Grade 2: fair; Grade 3: poor; Grade 4: dead or degenerating) and developmental stage (1: 1-cell; 2: 2 to 16-cell, 3: early morula; 4: morula;

5: early blastocyst; 6: blastocyst; 7: expanded blastocyst; 8: hatched blastocyst; and 9: expanded hatched blastocyst).

2.5 Statistical analyses

Data were analyzed for normality using a Shapiro-Wilk test. Homogeneity of groups for parity, days in milk (DIM), milk production and BCS was evaluated using a one-way ANOVA or a Kruskall-Wallis ANOVA. Statistical differences in total recovery, transferable and non-transferable embryos and mean embryo grade were assessed by one way-ANOVA or Kruskal- Wallis ANOVA, using a Tukey HSD test for *post hoc* comparison or a Wilcoxon-Mann-Whitney test. A Chi-square test was used for analysis of embryo grade, embryo stage, embryo collection yield and ovulation rate. All statistical analyses were performed using IBM SPSS Statistics 23 (IBM Corporation, Milan, Italy). For all analyses, $P<0.05$ was considered significant.

3. Results

Groups were homogeneous for parity, DIM, milk production and BCS ($P>0.05$). Data regarding embryo collection are summarized in Table 1. Total number of recovered structures and transferable embryos were lower ($P<0.05$) in F700 versus F1000 and F700P300; however, there was no difference ($P>0.05$) for mean number of non-transferable structures.

Although the proportion of collections yielding no transferable embryos was not different ($P>0.05$) among groups (3/17 – 17.6% F700; 1/34 – 2.9% F1000; 2/26 – 7.7% F700P300), low embryo collections yielding <3 transferable embryos per flushing were higher ($P<0.05$) in F700 group (11/17 – 64.7%) than in F1000 (9/34 – 26.5%) or F700P300 (7/26 – 26.9%) groups. Percentage of cows ovulating ≥50 % of follicles ≥0.8 cm in diameter present at the first insemination was lower ($P<0.05$) in F700 (35.5%) than in F1000 (82.4%) and F700P300 (73.1%) groups.

Quality grades were similar among groups, with no difference ($P<0.05$) in mean grade of transferable embryos (Table 2). Unfertilized oocytes were higher ($P<0.05$) in F700 than in F1000

and F700P300 (Table 3). Regarding transferable embryo stages, the only significant difference was percentage of morulae, which was highest in the F1000 group ($P<0.05$).

4. **Discussion**

In the present study, embryo yield was better in cows given 1,000 versus 700 IU of gonadotropins. Group F700 was given 700 IU, the dose recommended by the manufacturer of Folltropin, a drug with a high FSH:LH ratio (49:1) (Henderson et al. 1990), purified to avoid detrimental effects of excessive LH. The other 2 groups received 1,000 IU of gonadotropins, using the same drug (1000 IU of Folltropin), without modifying FSH:LH ratio throughout the entire SO treatment, or reducing the FSH:LH ratio in the final part of the SO treatment, switching from Folltropin (700 IU) to Pluset (last 300 IU), a drug with a 1:1 FSH:LH ratio. The rationale for keeping LH low from the beginning to the end of the protocol was based on the premise that exogenous LH is not needed in the SO process, as endogenous LH would support growth of ovarian follicles (Kanitz et al. 2002, Mapletoft et al. 2002). Adding an extra dose of gonadotropin (300 IU) was prompted by reports that high hepatic blood flow and metabolism in dairy cows promoted clearance of steroid hormones (Sangsritavong et al. 2002, Wiltbank et al. 2006), thereby diminishing gonadotropin effect and the SO response. The rationale for an extra dose of gonadotropin with a higher LH content was not only the LH suppression linked to SO treatment, but that LH seems to be required, especially if there is stress and/or negative energy balance, which attenuate or suppress the LH surge (Butler, 2003, Matteri and Moberg, 1982). The farm where the study was conducted had greater than average overcrowding and competition, which along with high production and consequent diseases, are important sources of stress for cows, with negative impacts on feed intake, BCS, and reproductive performance.

In the F700 group, the number of recovered structures and transferable embryos were the lowest among all 3 protocols. Cows in the F700 group had a good superovulatory response with several

follicles reaching pre-ovulatory size by first insemination, although many failed to ovulate, despite displaying oestrous behaviours. In F700 group there were very few ovulations and many anovulatory follicles persisting on the ovary 5 hr after the last insemination. It is likely that, for the few follicles which reached ovulation, that was a very asynchronous and prolonged process that yielded poor quality, aged oocytes, resulting in the higher percentage of unfertilized eggs and degenerate embryos in the F700 group.

The success rate of SO did not differ between 1,000 IU groups; the only significant difference was that the F1000 group had more embryos at the morula stage, attributed to a more synchronous and slightly delayed ovulation compared to the F700P300 group. These findings supported the notion that less exogenous LH reduces premature ovulations.

We inferred that the better outcome of F700P300 and F1000 groups compared to F700 was due to an increased total amount of gonadotropins administered, irrespective of the FSH:LH ratio in the last part of the SO treatment. Further investigations are needed to clarify why a relatively high dose of gonadotropins was needed to produce a satisfactory SO response. In F700 group, multiple ovarian follicles developed until ovulatory size, so the first part of the SO process was considered efficacious; however, ovulation was either delayed or failed to occur. Based on ovarian follicular dynamics in this group, an altered LH release from the hypothalamus-pituitary axis most likely caused development of these anovulatory structures (VanHolder et al. 2006). We inferred that disturbed LH secretion in stressed cows was a critical point; this must be considered, along with choosing an appropriate FSH dosage. It is noteworthy that a low dose of exogenous LH has been believed responsible for a lack of ovulation rate improvement in previous studies (Martinez et al. 1999, Ree et al. 2009, Rosa et al. 2010). A notable lack of synchrony in ovulation was noted in dairy heifers given 5.0 mg pLH (Ambrose et al. 2005); therefore, as 5.0 mg of pLH was considered inadequate to consistently synchronize ovulation, in subsequent experiments using higher doses (12.5 and 25.0 mg) of pLH, it was determined that synchronization of ovulation was satisfactory

with at least 12.5 mg of pLH. A tendency toward a decreased ovulation rate in the group where LH dose administered was 2.0 mg compared to 4.0 mg and eCG groups was reported (Oliveira et al. 2014), despite no significant difference among the protocols in average number of viable embryos recovered. The authors had 2 potential explanations: the LH dose was too low to enhance ovulation rate or a small quantity of pFSH on the last day of superstimulatory treatment may be necessary (in that study, on the last day of the SO, only pLH and no FSH were given; Oliveira et al. 2014). In the present study, we inferred that: 1) superior results in both 1,000 IU protocols were due to more FSH; 2) the amount of LH administered in the F1000 protocol (least amount) was adequate to increase ovulation rate, thus additional LH was not warranted; 3) additional LH in the F700P300 protocol (highest amount) induced more precocious ovulations as compared to the F1000 group, as confirmed by a higher percentage of morula-stage embryos in this group.

In superovulated cows, SexedULTRA™ sex-sorted semen yielded acceptable results (average of 4.5 embryos per flush), which seemed better than most studies involving traditional XY sex-sorted semen in superovulated lactating dairy cows (3.1 embryos Schenk et al. 2006; 2.4 embryos Hayawaka et al. 2009; 2.1 embryos Peippo et al. 2009; and 2.4 embryos Monteiro et al. 2016).

To our knowledge, only 2 studies with XY sorted semen had better results, with 6.4 (Soares et al. 2011) and 5.4 (Mikkola & Taponen, 2017) embryos per flush. It is noteworthy that cows used in both studies responded successfully to the SO treatment with a standard dose of Folltropin. In Soares' study SO protocol included also P4 and pLH, and the best results were achieved inseminating 18 and 30 hr versus 12 and 24 hr after pLH (6.4 vs 4.6 embryos; Soares et al. 2011). Comparisons of these studies are challenging and must be done with caution, as there were many differences and many critical points difficult to analyze and consider. It is noteworthy that in the present study, although environmental and management conditions were somewhat stressful, the mean number of DIM was quite low and semen quality was not assessed before insemination, we considered that the SexedULTRA™ sex-sorted semen yielded satisfactory outcomes.

5. Conclusions

In conclusion, 700 IU of highly purified gonadotropins provided inadequate stimulation for SO of lactating dairy cows in this study. However, a better SO response was achieved by increasing total gonadotropin dose from 700 to 1,000 IU, irrespective of increasing only FSH or both FSH and LH. The use of SexedULTRA™ sex-sorted semen for insemination of superovulated lactating dairy cows was considered satisfactory in terms of proportion of transferable embryos produced.

6. Acknowledgements

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7. Conflict of Interest Statement

There was no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Table 1

Mean ± SD number of all structures recovered, transferable and non-transferable embryos recovered from lactating dairy cows superovulated with 3 protocols.

	F700 (n=17)	F1000 (n=34)	F700P300 (n=26)
All structures	4.7 ± 3.0[a]	8.1 ± 3.8[b]	8.5 ± 6.4[b]
Transferable embryos (%)	1.9 ± 1.7[a] (41.2)	4.4 ± 2.6[b] (54.7)	4.5 ± 3.3[b] (52.9)
Non-transferable embryos (%)	2.8 ± 3.2 (58.8)	3.6 ± 2.9 (45.3)	4.0 ± 5.4 (47.1)

F700: Folltropin 700 IU, F1000: Folltropin 1,000 IU, F700P300: Folltropin 700 IU + Pluset 300IU.

[a,b]Within a line, means without a common superscript differed ($P<0.05$).

Table 2

Quality grade (IETS 1-3) of transferable bovine embryos recovered from lactating dairy cows superovulated with 3 protocols.

	F700	F1000	F700P300
Grade 1	19/33 (57.6%)	96/150 (64.0%)	66/117 (56.4%)
Grade 2	13/33 (39.4%)	46/150 (30.7%)	43/117 (36.8%)
Grade 3	1/33 (3.0%)	8/150 (5.3%)	8/117 (6.8%)
Mean grade	1.45 ± 0.55	1.41 ± 0.59	1.50 ± 0.62

F700: Folltropin 700 IU, F1000: Folltropin 1000 IU, F700P300: Folltropin 700 IU + Pluset 300IU.

Table 3

Developmental stages of bovine embryos recovered after 3 superovulation protocols.

	F700	F1000	F700P300
Unfertilized oocytes	36/80 (45.0%)[a]	76/274 (27.7%)[b]	64/221 (29.0%)[b]
Early morula	11/80 (13.8%)	48/274 (17.5%)	40/221 (18.1%)
Morula	7/80 (8.7%)[a]	53/274 (19.3%)[b]	27/221 (12.2%)[a]
Early blasocyst	18/80 (22.5%)	72/274 (26.3%)	63/221 (28.5%)
Blastocyst	7/80 (8.7%)	23/274 (8.4 %)	26/221 (11.7%)
Expanded blastocyst	1/80 (1.3%)	2/274 (0.7%)	1/221 (0.5%)

F700: Folltropin 700 IU, F1000: Folltropin 1000 IU, F700P300: Folltropin 700 IU + Pluset 300IU.

[a,b]Within a line, percentages without a common superscript differed ($P<0.05$).

3. Experimental studies

3.3 Experimental study III

How rapidly and to what extent milk coagulation properties could be improved by using embryo transfer as a tool to disseminate k-casein BB genotype as seen at the herd level.

Submitted article

How rapidly and to what extent milk coagulation properties could be improved by using embryo transfer as a tool to disseminate k-casein BB genotype as seen at the herd level

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Running title: K-casein BB genotype dairy cows selection by embryo transfer

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Abstract

In a herd of 352 cows, with a relatively low distribution of the favourable k-casein BB genotype (11.0%), k-casein BB animals were supervovulated (36 out of 39) and bred with SexedULTRA™ sex-sorted semen of k-casein BB bulls. Forty-five embryo transfer sessions yielded 203 embryos, 108 pregnancies and 98 females, of which 89 calved and entered first lactation. Milk fat, protein, casein, k-casein B and clotting properties differed significantly among the groups considered ($P<0.05$). In the relatively short period of the project, the embryo transfer program allowed the establishment of a herd whose milk has considerably improved composition and clotting properties.

Keywords: k-casein B, coagulation traits, embryo transfer, genetic selection, breeding program

Introduction

Cheesemaking is growing worldwide (International Dairy Federation 2018): in 2018 in the European Union cheese yield was about 10 million tonnes with a growing trend for mainly all the Member States. In Italy in the same year the production consists of nearly half a million tonnes of cheese, mainly high-quality cheese of Protected Designation of Origin produced using traditional methods, whose quality strongly relies on the composition and coagulation traits of milk. Propensity of milk to coagulate on addition of rennet (Malacarne *et al.* 2014) is critical in cheesemaking, due to its effects on cheese yield and quality and its influence on the efficiency and the profitability of the entire process (Pretto *et al.* 2013).

Poorly coagulating milk is a major cause of reductions in cheese quality and value and the problem is of growing importance. Although several studies have been conducted recently, the cause for poorly or non-coagulating milk is not fully understood (Malacarne *et al.* 2014; Troch *et al.* 2017); some authors assumed it could involve a low content and proportion of k-casein (Amalfitano *et al.* 2019). Reactivity to rennet, curd-firming capacity, syneresis ability and whey drainage are influenced by several environmental and management factors but to a large extent they depend on genetic factors, including breed and milk protein polymorphism of the individual animal (Perna *et al.* 2016). There are six major milk proteins: α and β lactoglobulin and caseins αS1, αS2, β, and κ and the relative proportions of these protein fractions and their genetic variants have essential roles on milk coagulation, coagulum structure and rheological properties, as well as cheese yield and cheese-making efficiency (Cipolat *et al.* 2018; Amalfitano *et al.* 2019).

Among milk proteins, there has been much attention on genetic variants of k-casein for their effect on rennet coagulation ability of milk (Toffanin *et al.* 2012; Perna *et al.* 2016). The B allele of k-casein has been associated with higher casein and k-casein content, smaller casein micelles, more favourable curd structure and improved overall milk clotting properties (MCP) (Walsh *et al.* 1998a; Bittante *et al.* 2012).

Holstein-Friesian has become the leading breed worldwide and yields poorly and non-coagulating milk much more frequently than many other dairy breeds (Bittante *et al.* 2011). Although B variants of both κ-casein and β casein apparently promote coagulation (Cipolat *et al.* 2018; Troch *et al.* 2017), these alleles have low prevalence in Holstein-Friesians, whereas unfavourable alleles, e.g. β casein A2 and k-casein A and E alleles (Amalfitano *et al.* 2019) are common (Gustavsson *et al.* 2014; Poulsen *et al.* 2013). These could be some of the reasons why in recent years an increase of the proportion of poorly coagulating milk is reported in many countries (Bittante *et al.* 2012; Pretto 2012), including Italy and the Parmigiano-Reggiano cheese production area (Tedeschi *et al.* 2010). Total milk protein not always seems to be a consistent indicator of MCP: both non-coagulating milk and well-coagulating milk were observed among milk samples with high protein and casein content (Ikonen *et al.* 2004; Hallèn *et al.* 2007). The positive role of k-casein B in enhancing MCP was consistently reported, whereas effects of other caseins were controversial (Bonfatti *et al.* 2010).

Our objective was to determine how rapidly and to what extent MCP could be improved by using embryo transfer (ET) to increase frequency of the k-casein BB genotype and reducing A and E variants in an Italian Holstein herd with a low prevalence of the favourable genotype. Many previous studies already revealed a strong correlation between casein genotypes and MCP, suggesting that β and κ-casein were major genes for r and a30 in Italian Holstein Friesian cows (Pretto 2012; Bittante *et al.* 2012) but no data about the application in the field of this selection has been reported.

Materials and Methods

Selection of donor cows

This study was conducted on an intensively managed dairy farm in the Parmigiano Reggiano region with a herd of >350 Italian Holstein cows. From November 2015 to January 2016, milk from 352 lactating cows was characterized by isoelectric focusing (IEF) to define their genetic variants for

αS1, β and κ-casein and to select those that were homozygous for the B allele at the k-casein *locus* . The same analysis, only for k-casein genetic variants, was then repeated on milk from 364 lactating cows from June to July 2019.

Superovulation, artificial insemination and embryo transfer

Embryo transfer was performed on 36 out of 39 k-casein BB genotype animals. On the basis of morphological structure and/or productivity parameters three animals were excluded, twenty-seven animals were superovulated once and nine superovulated twice (total of 45 superovulated cycles). Superovulation was done for a period of 12 months, starting in November, 2015. Cows were given nine intramuscular injections of declining doses of Folltropin (Vetoquinol, Bertinoro, Italy). Concurrent with the seventh and eighth injections of gonadotropins, a standard dose of PGF2a analogue was administered. Cows were inseminated 3 times, 5 hours apart, starting 18 hours after the onset of standing estrus. For the first 2 inseminations, 2 straws of SexedULTRA™ sex-sorted semen, each containing $2x10^6$ sperm, were used, whereas on the third and final insemination, only 1 straw was used (total of 5 straws and 10×10^6 sex-sorted sperm). SexedULTRA™ sex-sorted semen only from k-casein BB genotype bulls was used. Transcervical flushing was done 7 days after onset of estrus, with embryos evaluated under a stereomicroscope, classified (IETS manual) and then transferred fresh or frozen. Recipients were heifers between 14 and 16 month of age or primiparous cows, with known or inferred k-casein genotype containing no B allele. If the first transfer failed to produce a pregnancy, a maximum of 1 additional transfer was done.

Tank milk collection and analysis

Tank milk samples were collected twice a month in the morning from November 2015 to May 2016 (Gr2015) (n=14) and then from November 2018 to May 2019 (Gr2018) (n=14) when ET-derived heifers calved and entered lactation. In June 2019, pooled milk samples (n=6) from the 2 groups of cows that best represented the original herd with low frequency of k-casein BB genotype, pluriparous animals (PLURs), among which the proportion of k-casein BB animals has remained

very similar to the initial situation of 2015 and then the "altered" herd with a higher k-BB prevalence, primiparous cows (PRIMs), where k-casein BB animals has improved thanks to the embryo transfer program, were separately collected and analysed. Furthermore, in June 2019, 20 animals for each k-casein group considered (1. k-AA+AE, 2. k-AB and 3. k-BB) were selected to be as homogeneous as possible for parity, days in milk (DIM), production, total bacterial count (TBC) and somatic cell count (SCC) and divided randomly into 2 groups of 10 animals each; pooled milk samples from each group were collected (n=2) and analysed.

All tank and pooled milk samples were analysed to assess composition and coagulation properties. Fat, protein and casein were determined by infrared analysis with a Milko-Scan FT 6000 (Foss Electric, DK-3400 Hillerød, Denmark). Titratable acidity was measured by titration with 0·25 M-NaOH using the Soxhlet-Henkel method. The content of k-casein B was determined by the ELISA-test kappa (Bender Medsystem). Somatic cell count and total bacterial count were determined with Fossomatic and BactoScan 8000 instruments (Foss Electric, DK-3400 Hillerød, Denmark), respectively. Milk coagulation properties (MCP) were determined within a few hours after sample collection. For this, 0.2 ml rennet solution (1:19 000; Chr. Hansen,Corsico MI, Italy) was added to milk samples (10 ml). Coagulation characteristics, milk clotting time (r), curd firming time (k20) and curd firmness (a30) were measured at 35 °C using a Formagraph (Foss Electric). The value of MCP for each sample was the average of 3 measures.

Statistical analyses

Data were expressed as mean ± standard deviation (SD). All data were checked for normality using a Shapiro Wilk test. Depending on distribution, a Student's *t*-test or a Mann-Whitney U test were used to compare data between Gr2015/Gr2018 and PLURs/PRIMs, whereas for comparison of data from the 3 K-casein genotype groups, one-way ANOVA or a Kruskall Wallis test were used. Tukey HSD test was used for post-hoc comparison and $P<0.05$ considered significant. All statistical analyses were done using IBM SPSS Statistics 25 (IBM Corporation, Milan, Italy).

Results

The initial and final frequencies of genetic variants of k-casein in cows genotyped by IEF in 2015/2016 and in 2019 are reported in Table 1. Initial distribution (n=352) was αS1-casein: 4 BC and 348 BB; β-casein: 16 AB and 336 AA and k-casein: 132 AA (37.5%), 125 AB (35.5%), 47 AE (13.3%), 39 BB (11.0%), 7 BE (1.9%) and 2 EE (0.5%). Clearly, there was an essentially monomorphic situation of this herd regarding the αS1-casein and virtual lack of the B variant of β-casein. Final distribution (n=364) was considered only for k-casein genetic variants and was 59 AA (16,2%), 167 AB (45,8%), 24 AE (6,5%), 108 BB (29,6%), 5 BE (1,3%) and 1 EE (0,2%). During the ET program, forty-five ET sessions were performed, obtaining 203 embryos (average of 4.5 embryos per session), which were transferred obtaining 124 pregnancies (61.1%) on days 28-30 and 108 (53.2%) on days 60-70. Of these 108 pregnancies, 10 were male (9.3%) calves and 98 females (90.7%), of which 89 became pregnant, calved and entered first lactation.

Basic milk composition, SCC, TBC and coagulation traits of Gr2015 (before ET sessions) and Gr2018 (after cows born from ET entered lactation) tank milk samples are shown in Table 2. There were no differences ($P<0.05$) for parity, days in milk, production, SSC and TBC. However, Gr2018 milk samples had greater ($P<0.05$) fat, protein, casein, K-casein B content and titratable acidity. Regarding coagulation parameters, Gr2018 samples had lower ($P<0.05$) r and k20 and higher ($P<0.05$) a30 compared to Gr2015 samples. The same parameters were analysed for pooled milk samples from PRIMs and PLURs (Table 3). These 2 groups were different ($P<0.05$) in SCC, with PRIMs having a lower level, and in fat, protein, casein, K-casein B content and titratable acidity with PRIMs having higher values. Coagulation properties differed ($P<0.05$) between the 2 groups, with PRIMs having better MCP with a shorter r and k20 and a higher a30. Regarding pooled milk samples of the different k-casein genotypes considered, there were differences ($P<0.05$) in protein, casein, K-casein B content and titratable acidity between k-AA/AE and K-BB groups, with k-AB group being intermediate (Table 4). In a comparison of coagulation parameters, r of k-BB group

was shorter (P<0.05) than r of k-AA/AE and k-AB groups, whereas k20 and a30 of k-AA/AE group were respectively longer and lower (P<0.05) than those of k-BB and k-AB groups.

Discussion

In this study, increasing the frequency of the B allele of k-casein markedly increased milk protein, casein and k-casein B contents and led to improved overall MCP in a relatively short period of time. Data regarding k-casein genotype distribution in the herd before embryo transfer project was consistent with Italian Holstein Friesian population data: AA 30.1%- AB 36.2%- AE 13.9%- BB 10.6%- BE 7.8%- EE 1.4% (ANAFIJ 2019) (Table 1) with an evident low diffusion of the B variant. Embryo transfer is useful to hasten genetic improvement of the herd. In the present study, 98 k-BB females were produced in a short interval. Using dams and sires with the desired genotype and ET with sex-sorted semen, we obtained 2.17 female calves born alive per ET and 1.97 female calves entering first lactation. Similar results were achieved in a Finnish study, with 219 female calves from 100 collections (Mikkola and Taponen 2017). Tank milk samples before and after k-BB cows entered lactation and milk samples from groups of animals with distinct k-BB distributions were compared. Based on rennet coagulation properties (Malacarne *et al.* 2014), milk samples from Gr2015, PLURs and k-AA groups were classified “Poor” whereas samples from Gr2018, PRIMs, k-AB and k-BB were “Suboptimal.” Mean values for r, k20 and a30 were not close to those recommended for cheese-making by Zannoni and Annibaldi (1981) who reported optimal values of 13 min, 9 min and 35 mm for, k20 and a30, respectively, but this seems to be consistent with the worsening trend of MCP (Bittante *et al.* 2012). Gr2015 and Gr2018 differed for fat, protein, casein, k-casein B and all other technical traits analysed. It was not possible to determine whether improvement was due soley to k-BB selection or to other affiliated genetic factors. Regardless, Gr2018 milk had marked improvements in suitability for cheese-making. Environmental and management factors were as homogeneous as possible throughout the period of the study and were

not considered important sources of differences. Furthermore, the vertical temporal comparison between the legacy 2015/2016 herd and the improved 2018/2019 herd was supported by a horizontal contemporary comparison between pluriparous cows (PLURs) that better represented what the herd would have been without the k-casein BB program and primiparous cows (PRIMs) represented what the herd will be in the future with increased prevalence of this genotype. It was noteworthy that milk from PRIMs had improved MCP. It is reasonable to assume that milk from Gr2018 and PRIMs will produce more and better quality cheese, as cheese yield is positively and strongly correlated with fat, protein, casein content and a30, but negatively associated with r and k20 (Pretto *et al*. 2013). Confirmation of the superiority of the k-BB milks for fat, protein, casein, k-casein and MCP were apparent from comparison of pooled milk samples of various genotype groups and were in agreement with other reports (Walsh *et al.* 1998b; Jakob and Puhan 1992). Rennet coagulation time and k20 were shorter and a30 was higher in milk from k-BB cows. In a previous study, there were no significant differences in rennet coagulation times between milks, but k20 and curd firmness at 60 min for k-casein AB and BB milks were significantly lower and higher respectively than those for the k-casein AA milks (Walsh *et al.* 1998b). Furthermore, there was a reduction of 9 and 30% in RCT and k20, respectively, and an increase of 26% in cows with the heterozygous AB genotype compared to cattle with AA genotype (Bittante *et al.* 2012). Regarding other parameters involved in the analysis, it was noteworthy that parity did not differ between Gr2015 and Gr2018 nor between k-casein genotype groups, although it obviously differed between PRIMs and PLURs. In the study of Ikonen *et al.* (2004) curd firmness was lower for milk from primiparous versus pluriparous cows and was attributed to a higher proportion of non-coagulating milk samples from primiparous versus other cows (17 versus 9%, respectively). In this study no data were recorded regarding coagulation properties of individual milk samples nor proportion of non-coagulating samples. However, in contrast to Ikonen *et al.* (2004), we recorded a 12% increase in a30 for PRIMs, and that could be due in whole or in part to a higher content of k-casein BB.

Even though data are not always consistent in literature, it seems like milk coagulation properties are optimal at the onset of lactation, deteriorate rather quickly and are at their worst during mid-lactation (Bittante *et al.* 2015). However, in the present research, as DIM did not differ between compared groups, it was not expected to have affected our data. Cassandro *et al.* (2008) demonstrated that there were no correlations between MCP and milk yield in Friesian cows; in the present study, milk yield differed among groups, with higher milk production in Gr2018 and in PRIMs and it was attributed to general genetic improvement of the herd. Somatic cell count and total bacterial count strongly influence milk processing and cheese quality. Total bacterial count was similar among the experimental groups, whereas PLURs had a higher SCC than PRIMs (186 x 10^3/mL versus 108 x 10^3/mL). Although all samples were far below the legal limit of 400 x 10^3/mL, the higher SCC could have unfavourably affected MCP, which along with cheese yield and quality are strongly and negatively influenced by SCC (Malacarne *et al.* 2014; Troch *et al.* 2017).

Titratable acidity (TA) is another important parameter to evaluate; its association with MCP is due to the capacity of TA to influence aggregation rate of para-casein micelles, reactivity of rennet and rate of syneresis (Penasa *et al*. 2016). There are associations among TA, MCP (Toffanin *et al*. 2012) and cheese yield (Pretto *et al.* 2013), with increasing TA favouring cheesemaking. In this study, milk composition and coagulation were better in groups with higher percentages of k-casein BB animals.

Conclusions

Milk production and cheese industries need to balance genetic progress in milk yield and protein content with the need for improving milk coagulation properties. Selection of k-casein BB genotype markedly enhanced cheese-making properties of milk in the relatively short period of this ET program, providing an impetus to include milk coagulation traits in genetic selection for dairy cattle. A low prevalence for the k-casein B allele can be overcome with ET, using k-casein BB

donors and k-casein AA/AE/EE cattle as recipients.

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Conflict of interest

There was no conflict of interest that could be perceived as prejudicing impartiality of the research reported.

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Table 1

Initial (2015/2016) and final (2019) distribution of K-casein genotypes (%) in the herd considered, before and after the ET program and in the Italian Holstein-Friesian population in 2019.

K-casein genotype	AA	AB	AE	BB	BE	EE
Distribution in Italian Holstein-Friesian population in 2019 (%) (ANAFIJ)	30,1	36,2	13,9	10,6	7,8	1,4
Distribution in the herd considered in 2015/2016 (%)	37,5	35,5	13,3	11,0	1,9	0,5
Distribution in the herd considered in 2019 (%)	16,2	45,8	6,5	29,6	1,3	0,2

Table 2

Mean±SD comparisons of milk tank samples from November 2015 to May 2016 (Gr2015) and from November 2018 and May 2019 (Gr2018) and mean±SD comparisons of parity, days in milk and milk yield of animals constituting the 2 groups considered.

	Gr2015	Gr2018
Parity	2.33±0.07	2.19±0.12
Days in milk	191.50±11.25	186,64±14.95
Milk production (l)	31.76±0.82[a]	33.16±1.16[b]
Somatic cell count (x 1000/ml)	263.00±60.82	236.00±51.91
Total bacterial count (cfu/ml)	21.50±10.85	16.43±9.01
Fat (g/100 g)	3.50±0.10[a]	3.58±0.08[b]
Protein (g/100 g)	3.39±0.03[a]	3.47±0.02[b]
Casein (g/100 g)	2.58±0.03[a]	2.69±0.02[b]
Casein number (%)	6.04±0.40[a]	77.71±0.23[b]
K-casein B (mg/100 mL)	0.061±0.003[a]	0.132±0.034[b]
K-casein B/casein (%)	2.37±0.09a	4.92±1.28b
Titratable acidity (°SH/50 ml)	3.41±0.03[a]	3.48±0.06[b]
r (min)	22.79±1.24[a]	20.32±2.15[b]
k20 (min)	5.36±0.60[a]	3.83±0.69[b]
a30 (mm)	31.78±1.96[a]	38.30±2.22[b]

r: milk clotting time; K20: curd firming time; a30: curd firmness.

[a,b]Within a row, means without a common superscript differed ($P<0.05$).

Table 3

Mean±SD comparisons of pooled milk samples collected in June 2019 from pluriparous versus primiparous cattle and mean±SD comparisons of parity, days in milk and milk yield of animals constituting the 2 groups considered.

	Pluriparous	Primiparous
Parity	2.65±0.11[a]	1.00±0.00[b]
Days in milk	143.00±27.95	138.66±14.95
Milk production (liters)	31.76±0.82[a]	33.16±18.14[b]
Somatic cell count (x 1000/ml)	186.33±67.57[a]	108.17±19.69[b]
Total bacterial count (cfu/ml)	18.00±6.88	14.33±4.46
Fat (g/100 g)	3.49±0.02[a]	3.60±0.05[b]
Protein (g/100 g)	3.41±0.02[a]	3.49±0.02[b]
Casein (g/100 g)	2.61±0.02[a]	2.72±0.02[b]
Casein number (%)	76.27±0.44[a]	77.60±0.21[b]
K-casein B (mg/100 mL)	0,065±0,007[a]	0,149±0,020[b]
K-casein B/casein (%)	2.50±0.26[a]	5.47±0.73[b]
Titratable acidity (°SH/50 ml)	3.54±0.04[a]	3.67±0.05[b]
r (min)	23.19±0,93[a]	19.66±1.16[b]
k20 (min)	5.54±0.70[a]	3.14±0.56[b]
a30 (mm)	32.07±1.05[a]	39.06±1.34[b]

r: milk clotting time; K20: curd firming time; a30: curd firmness.

[a,b]Within a row, means without a common superscript differed (P<0.05).

Table 4

Mean±SD comparisons of pooled milk samples from k-AA/AE, AB and BB cows and mean±SD comparisons of parity, days in milk and milk yield of animals constituting the 3 groups considered.

	k-AA/AE	k-AB	K-BB
Parity (number)	2.15±0.05	1.95±0.25	1.90±0.20
Days in milk	124.00±3.00	115.50±7.50	119.50±18.50
Production (liters)	31.50±1.50	30.50±1.50	33.00±1.00
Somatic cell count (x 1000/ml)	213.00±65.00	205.50±41.50	158.50±28,50
Total bacterial count (cfu/ml)	17.50±5.50	10.50±3.50	13.00±4.00
Fat (g/100 g)	3.51±0.06	3.56±0.08	3.65±0.01
Protein (g/100 g)	3.40±0.01[a]	3.49±0.01[a,b]	3.51±0.02[b]
Casein (g/100 g)	2.58±0.03[a]	2.69±0.00[a,b]	2.72±0.02[b]
Casein number (%)	75.55±0.45[a]	77.05±0.19[a,b]	77.60±0.24[b]
K-casein B (mg/100 mL)	0.077±0.006[a]	0.124±0.011[a,b]	0.166±0.008[b]
K-casein B/casein (%)	3.02±0.30[a]	4.31±0.70[a,b]	6.10±0.25[b]
Titratable acidity (°SH/50 ml)	3.45±0.01[a]	3.52±0,04[a,b]	3.68±0.05[b]
r (min)	22.93±0.81[a]	21.61±0.83[b]	19.76±0.93[b]
k20 (min)	5.62±0.60[a]	3.85±0.65[b]	3.36±0.65[b]
a30 (mm)	33.82±1,36[a]	37.46±2.06[b]	38.82±1.34[b]

r: milk clotting time; K20: curd firming time; a30: curd firmness.

[a,b]Within a row, means without a common superscript differed ($P<0.05$).

4. Conclusions

The dairy industry is asked to be increasingly competitive and efficient. Despite the increasing trend in milk yield and protein content during the last decades genetic selection, milk coagulation ability has diminished and even if the absolute amount of cheese produced has increased, the relative cheese yield from a set amount of milk, has decreased. As casein content and variants, along with MCP are determined to a large extent at the DNA level, genetic selection and embryo transfer can provide efficacious tools to reverse this trend and achieve improvements. The present thesis contributes to define a valid way of wave synchronization before superovulation, improves our understanding of the SO response in stressed lactating dairy cows treated with different total gonadotrophin dosage and different FSH:LH ratio during the protocol, reports for the first time the use of ULTRA sexed sorted semen in superovulated cows and applies in the field a selection model that so far was only hypothesized and suggested theoretically. The selection program carried out in this research, gave evidence and gathered empirical data of feasible genetic improvements in the cheesemaking ability of milk by means of k-casein BB selection. In the present study, 98 k-BB females were produced in a short interval. Using dams and sires with the desired genotype and ET with sex-sorted semen, we obtained 2.17 female calves born alive per ET and 1.97 female calves entering first lactation. In conclusion, in this project, selection of k-casein BB genotype enhanced the cheese-making properties of milk, providing an impetus to include milk coagulation traits in genetic selection for dairy cattle.

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