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Research Article
Embryo Transfer for Dissemination of κ-casein BB Genotype and Improvement of Milk Clotting Properties

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Abstract

Background and Objective: Milk production and cheese industries need to balance the genetic progress in milk yield and protein content with the need for improving milk coagulation properties and workability. Our objective was to determine how rapidly and to what extent Milk Clotting Properties (MCP) could be improved by using Embryo Transfer (ET) to increase the frequency of the κ-casein BB genotype and reducing A and E variants in an Italian Holstein herd with a low prevalence of the favorable genotype. **Materials and Methods:** In a herd of 352 cows, with a relatively low distribution of the favorable κ-casein BB genotype (11.0%) and poor milk clotting properties, κ-casein BB animals were superovulated (36 out of 39) and bred with SexedULTRA™ sex-sorted semen of κ-casein BB bulls in order to improve milk workability and cheese yield. Forty-five embryo transfer sessions yielded 203 embryos, 108 pregnancies and 98 females, of which 89 calved and entered the first lactation. **Results:** Milk composition and coagulation properties differed significantly among the groups considered ($p<0.05$) and were more favourable for cheese-making in the groups with a higher percentage of κ-casein BB animals whose tank and pool milk samples consistently showed greater ($p<0.05$) fat, protein, casein and κ-casein B content and improved clotting properties with lower ($p<0.05$) r and k20 and higher ($p<0.05$) a30. **Conclusion:** 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.

Key words: κ-casein B, coagulation traits, milk workability, embryo transfer, genetic selection, breeding program, milk clotting properties

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cheesemaking is growing worldwide[1]: 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 (PDO) produced using traditional methods, whose quality strongly relies on the composition and coagulation traits of milk. The propensity of milk to coagulate on the addition of rennet[2] 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[3].

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[2,4], some authors assumed it could involve a low content and proportion of κ-casein[5]. 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[6]. 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[5,7].

Among milk proteins, there has been much attention on genetic variants of κ-casein for their effect on rennet coagulation ability of milk[6,8]. The B allele of κ-casein has been associated with higher casein and κ-casein content, smaller casein micelles, a more favorable curd structure and improved overall Milk Clotting Properties (MCP)[9,10].

Holstein-Friesian has become the leading breed worldwide and yields poorly and non-coagulating milk much more frequently than many other dairy breeds[11]. Although B variants of both κ-casein and β casein promote coagulation[4,7], these alleles have a low prevalence in Holstein-Friesians, whereas unfavorable alleles, e.g., β casein A2 and κ-casein A and E alleles[5] are common[12,13]. These could be some of the reasons why in recent years an increase in the proportion of poorly coagulating milk is reported in many countries[10,14], including Italy and the Parmigiano-Reggiano cheese production area2. 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[15,16]. The positive role of κ-casein B in enhancing MCP was consistently reported, whereas the effects of other caseins were controversial[17].

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[10,14] but no data about the application in the field of this selection has been reported. Our objective was to determine how rapidly and to what extent MCP could be improved by using Embryo Transfer (ET) to increase the frequency of the κ-casein BB genotype and reducing A and E variants in an Italian Holstein herd with a low prevalence of the favorable genotype.

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-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 κ-casein *locus*. The same analysis, only for κ-casein genetic variants, was then repeated on milk from 364 lactating cows from June-July, 2019.

Superovulation, artificial insemination and embryo transfer: Embryo transfer was performed on 36 out of 39 κ-casein BB genotype animals. Based on the morphological structure and/or productivity parameters three animals were excluded, 37 animals were superovulated once and nine superovulated twice (total of 45 superovulated cycles). Superovulation was done for 12 months, starting in November 2015. Cows were given 9 intramuscular injections of declining doses of Folltropin (Vetoquinol, Bertinoro, Italy). Concurrent with the 7th and 8th injections of gonadotropins, a standard dose of PGF2a analog was administered. Cows were inseminated 3 times, 5 h apart, starting 18 h after the onset of standing estrus. For the first 2 inseminations, 2 straws of SexedULTRA™ sex-sorted semen, each containing 2×10^6 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 κ-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 months of age or primiparous cows, with

known or inferred κ-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-May, 2016 (Gr2015) (n = 14) and then from November, 2018-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 a low frequency of κ-casein BB genotype, pluriparous animals (PLURs), among which the proportion of κ-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 κ-casein BB animals have improved thanks to the embryo transfer program, were separately collected and analyzed. Furthermore, in June 2019, 20 animals for each κ-casein group considered (1. κ-casein AA+AE genotype, 2. κ-casein AB genotype and 3. κ-casein BB genotype) 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 analyzed.

All tank and pooled milk samples were analyzed 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 κ-casein B was determined by the ELISA-test kappa (Bender Med system). 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, a 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 analysis: Data were expressed as mean±standard deviation (SD). All data were checked for normality using a Shapiro Wilk test. Depending on the 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 κ-casein genotype groups, one-way ANOVA or a Kruskal Wallis test was 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 κ-casein in cows genotyped by IEF in 2015/2016 and 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 κ-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%). 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 κ-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, 45 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 the first lactation.

Basic milk composition, Somatic Cell Count (SCC), Total Bacterial Count (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, κ-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 analyzed for pooled milk samples from Primiparous (PRIMs) and Pluriparous (PLURs) (Table 3). These 2 groups were different

Table 1: Distribution of κ-casein genotypes (%) before (2015/2016) and after (2019) the embryo transfer program and in the Italian Holstein-Friesian population in 2019

Distribution (%)	AA	AB	k-casein AE	Genotype BB	BE	EE
In the herd considered in 2015/2016 (%)	37.5	35.5	13.3	11.0	1.9	0.5
In the herd considered in 2019 (%)	16.2	45.8	6.5	29.6	1.3	0.2
In Italian Holstein-Friesian population in 2019 (%) (National data-ANAFIJ)	30.1	36.2	13.9	10.6	7.8	1.4

Table 2: Mean±SD comparisons of tank milk samples and relevant data between Gr2015 and Gr2018 (respectively before and after the embryo transfer program)

Parameters	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 (×1000 mL^{-1})	263.00±60.82	236.00±51.91
Total bacterial count (CFU mL^{-1})	21.50±10.85	16.43±09.01
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±0.09[a]	4.92±1.28[b]
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]

Gr 2015: milk tank samples and data collected from November, 2015 to May, 2016 before the ET program, Gr 2018: Milk tank samples and data collected from November, 2018 to May, 2019 after ET-derived heifers calved and entered lactation, 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 and relevant data between pluriparous (PLURs) and primiparous (PRIMs) cattle in June 2019

Parameters	PLURs	PRIMs
Parity	2.65±0.11[a]	1.00±0.00[b]
Days in milk	143.00±27.95	138.66±14.95
Milk production (L)	31.76±0.82[a]	33.16±18.14[b]
Somatic cell count (×1000 mL^{-1})	186.33±67.57[a]	108.17±19.69[b]
Total bacterial count (CFU mL^{-1})	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]

PLURs: Pooled milk samples and data collected from the group of pluriparous cows where the proportion of k-casein BB animals has remained very similar to the initial situation of 2015, before the embryo transfer program, PRIMs: Pooled milk samples and data collected from the group of primiparous cows where κ-casein BB animals have improved thanks to the embryo transfer program, r: Milk clotting time, K20: Curd firming time, a30: Curd firmness, [a,b]Within a row, means without a common superscript differed ($p<0.05$)

($p<0.05$) in SCC, with PRIMs having a lower level and in fat, protein, casein, κ-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 κ-casein genotypes considered, there were differences ($p<0.05$) in protein, casein, κ-casein B content and titratable acidity between k-AA/AE and k-BB groups, with the k-AB group being intermediate (Table 4). In a comparison of coagulation parameters, r of the 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 κ-casein markedly increased milk protein, casein and κ-casein B contents and led to improved overall MCP in a relatively short time.

Data regarding κ-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 and EE 1.4% (ANAFIJ 2019) (Table 1) with an evident low diffusion of the B variant. Embryo transfer is useful to hasten the genetic improvement of the herd. In the present study, 98 K-BB females were produced in a short interval.

Table 4: Mean±SD comparisons of pooled milk samples and relevant data among groups of cattle characterized by the k-casein genotype AA+AE, AB and BB in June 2019

	k-casein genotype		
Parameters	AA/AE	AB	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 (L)	31.50±1.50	30.50±1.50	33.00±1.00
Somatic cell count (×1000 mL^{-1})	213.00±65.00	205.50±41.50	158.50±28,50
Total bacterial count (CFU mL^{-1})	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}

κ-casein AA+AE: Pooled milk samples and data collected from 2 groups of 10 animals each, characterized by the k-casine AA or AE genotype, κ-casein AB: Pooled milk samples and data collected from 2 groups of 10 animals each, characterized by the k-casein AB genotype, κ-casein BB: Pooled milk samples and data collected from 2 groups of 10 animals each, characterized by the k-casein BB genotype, r: Milk clotting time, K20: Curd firming time, a30: Curd firmness, ^{a,b}Within a row, means without a common superscript differed ($p<0.05$)

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 the first lactation. Similar results were achieved in a Finnish study, with 219 female calves from 100 collections[18]. 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[2], milk samples from Gr2015, PLURs and k-AA groups were classified as "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 reported as optimal for cheese-making by Malacarne *et al*., 2014[2] but this seems to be consistent with the worsening trend of MCP[10]. Gr2015 and Gr2018 differed for fat, protein, casein, κ-casein B and all other technical traits analyzed. It was not possible to determine whether the improvement was due solely 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 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 κ-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[3]. Confirmation of the superiority of the k-BB milk for fat, protein, casein, κ-casein and MCP were apparent from the comparison of pooled milk samples of various genotype groups and agreed with other reports[19,20]. 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 milk, but k20 and curd firmness at 60 min for κ-casein AB and BB milk were significantly lower and higher respectively than those for the κ-casein AA milks[19]. 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[10]. Regarding other parameters involved in the analysis, it was noteworthy that parity did not differ between Gr2015 and Gr2018 nor between κ-casein genotype groups, although it differed between PRIMs and PLURs. In the study of Ikonen *et al*.[15] 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 the coagulation properties of individual milk samples nor the proportion

of non-coagulating samples. However, in contrast to Ikonen *et al.*[15], we recorded a 12% increase in a30 for PRIMs and that could be due in whole or in part to a higher content of κ-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[21]. 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.*[22] 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 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. The total bacterial count was similar among the experimental groups, whereas PLURs had a higher SCC than PRIMs (186×10^3 mL^{-1} versus 108×10^3 mL^{-1}). Although all samples were far below the legal limit of 400×10^3 mL^{-1}, the higher SCC could have unfavorably affected MCP, which along with cheese yield and quality are strongly and negatively influenced by SCC[2,4].

Titratable Acidity (TA) is another important parameter to evaluate, its association with MCP is due to the capacity of TA to influence the aggregation rate of para-casein micelles, the reactivity of rennet and rate of syneresis[23]. There are associations among TA, MCP[8] and cheese yield[3], with increasing TA favoring cheesemaking. In this study, milk composition and coagulation were better in groups with higher percentages of κ-casein BB animals.

CONCLUSION

Milk production and cheese industries need to balance genetic progress in milk yield and protein content with the need for improving milk coagulation properties. The selection of κ-casein BB genotype markedly enhanced the 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 κ-casein B allele can be overcome with ET, using κ-casein BB donors and κ-casein AA/AE/EE cattle as recipients.

SIGNIFICANCE STATEMENT

This study reveals how rapidly and to what extent milk clotting properties can be improved by using embryo transfer as a tool to disseminate κ-casein BB genotype as seen at the herd level. The research discovers the strong synergy between a breeding program with a focus on κ-casein genotypes and the main milk characteristics and cheese processing parameters and it presents a new strategy to improve the herd milk workability in a short time. Furthermore, it offers practical information on the real and tangible advantages that can be obtained by applying a κ-casein BB genotype genetic selection leading to beneficial effects on cheese yield and thus the profitability of the herd.

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