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"NEW RESEARCH FRONTIERS IN BUFFALO BREEDING"

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ABSTRACT

The interest around buffalo breeding in Italy has noticeably augmented during the last years, as demonstrated from the increase of buffalo population that passed from about 200,000 heads in 2000 to more than 400,000 in 2020. This is due to the interest around the main product derived from buffalo milk: the mozzarella cheese, also known as Mozzarella di Bufala Campana, that received in 1996 the Protected Designation of Origin (PDO). In Italy buffalo breeding is carried out in intensive and semi-intensive systems, similar to those utilized in dairy cattle: thus, similar issues in terms of productive and reproductive efficiency have been risen in this species. Furthermore, in the Mediterranean region, buffaloes show annual fluctuations in reproduction with distinct breeding and non-breeding seasons. As buffaloes are short-day breeders, the annual peak in fertility coincides with decreasing day length from autumn to winter. Therefore, in order to guarantee milk production throughout the year for mozzarella cheese production, some strategies are applied to increase the reproductive efficiency out of the breeding season, when a greater incidence of anestrus, a decline in the function of the corpus luteum (CL), and an increase in embryonic mortality are usually recorded. Furthermore, natural mating is often preferred to assisted reproduction (estrus synchronization and artificial insemination), because of some peculiarities of buffalo species, such as low estrus behavior and large variability in estrus duration, which makes difficult the individuation of the optimal moment for artificial insemination (AI).

In this scenario, the purpose of this project was to improve reproductive efficiency and management in buffaloes through the application of new methodological approaches, such as synchronization of estrus, eco-color Doppler and metabolomics. In particular, a great attention has been paid to the improvement of AI in buffalo, in both heifers and pluriparous buffaloes.

In the first experiment (Chapter 5), the efficiency of two synchronization protocols for oestrus synchronization and the influence of live body weight (LBW) and age on reproductive performance was evaluated in buffalo heifers. The animals were synchronized by Ovsynch-TAI Program (OVS; n=72) or double prostaglandin administered 12 days apart (PGF; n=74) and all the buffaloes were inseminated twice (24 days apart). Follicle dimensions and ovulation rate (OR) were assessed by ultrasound 24 and 48 h post-insemination. Pregnancy was assessed on day 25, 45 and 90 post-insemination and the incidence of late embryonic (LEM) and fetal (FM) mortality were respectively recorded. Data were analyzed by ANOVA, Chi-square test and multiple logistic regression.

In the second experiment (Chapter 6), a deep study of CL development was carried out in buffaloes out of breeding season through the application of eco-color Doppler technique. Adult Mediterranean buffaloes (n=29) were synchronized by Ovsynch-TAI Program and artificially inseminated. CL B-mode/color Doppler ultrasonography examinations were performed daily from Days 5 to 10 post-synchronization, recording CL dimensions and blood flow parameters. Blood samples were collected on the same days for the progesterone (P_4) assay. Data were grouped into pregnant or non-pregnant and retrospectively analyzed.

In the third experiment (Chapter 7) a metabolomic approach on milk was used on 10 pregnant and 10 non-pregnant buffaloes in order to identify potential biomarkers of early pregnancy. The study was carried out on 10 pregnant and 10 non-pregnant buffaloes that were synchronized by Ovsynch-TAI Program and have undergone the first AI. Milk samples were individually collected ten days before AI (the start of the synchronization treatment), on the day of AI, day 7 and 18 after AI, and were analyzed by LC–MS. Data were analyzed retrospectively by dividing pregnant and non-pregnant subjects. Statistical analysis was carried out by using Mass Profile Professional.

In the fourth experiment (Chapter 8) an advanced GC-MS and metabolite identification approach was also utilized to characterize the metabolome of buffalo milk and mozzarella cheese in a robust and repeatable technology platform. The study utilized eleven dairies located in a protected designation of origin (PDO) region and nine dairies located in non-PDO region in Italy. Samples of raw milk (100 mL) and mozzarella cheese (100 g) were obtained from each dairy and maintained at -80°C until analysis. Metabolomic assay was carried out through gas-chromatography and mass spectroscopy and differently expressed metabolites were identified. Statistical analysis of the results was carried out by ANOVA.

The results of the first experiment showed that the LBW was significantly ($P<0.05$) higher in inseminated animals, compared to those that did not respond to the treatments (450.0±3.2 vs. 423.2±9.6 kg in inseminated and not inseminated heifers, respectively). Total OR was similar between groups, although OR at 24 h tended to be higher ($P=0.06$) in OVS (86.7 vs. 72.9% in OVS and PGF, respectively). A ($P<0.01$) higher LBW was observed in ovulated heifers of PGF, while no differences were recorded in OVS. LBW affected OR (odds ratio = 1,032; $P < 0.05$) only in PGF, while no effects were recorded in OVS. Total pregnancy rate, LEM and FM were similar between groups. In conclusion, the LBW would be considered before including buffalo heifers in a synchronization program and both synchronization treatments can be useful.

In the second experiment, the total pregnancy rate was 50.0% (13/26) on Day 45. A significant difference between CL average area in pregnant and non-pregnant buffaloes was recorded only on Day 10. Pregnant buffaloes showed a significantly higher mean P_4 concentration and higher mean time average medium velocity (TAMV) values from Day 5 to Day 10 compared to non-pregnant buffaloes. Linear regression analysis showed a significant relationship between P_4 levels and TAMV. Multiple logistic regression highlighted a significant influence of TAMV on pregnancy outcome, particularly on Day 8. This is probably due to the strong relationship between TAMV and P_4 production. Both TAMV and P_4 could be used to predict pregnancy starting on Day 6, although a more reliable result was obtained at Day 10. Thus, the period between Days 5 and 10 is critical for CL development and pregnancy maintenance during the transitional period in buffalo.

During the third experiment, Metabolomic analysis revealed the presence of several metabolites differentially expressed between pregnant and non-pregnant buffaloes. Among these, a total of five metabolites were identified by comparison with an online database and a standard compound as acetylcarnitine (3-Acetoxy-4-(trimethylammonio)butanoate), arginine-succinic acid hydrate, 5'-O-{[3-({4-[(3aminopropyl)amino]butyl}amino)propyl]carbamoyl}-2'-deoxyadenosine, N-(1-Hydroxy-2-hexadecanyl)pentadecanamide, and N-[2,3-Bis(dodecyloxy)propyl]-L-lysinamide). Interestingly, acetylcarnitine was dominant in milk samples collected from non-pregnant buffaloes. The results obtained from milk metabolic profile and hierarchical clustering analysis revealed significant differences between pregnant and non-pregnant buffaloes, as well as in the metabolite expression. Overall, the findings indicate the effectiveness of the metabolomic analysis for the identification of novel potential biomarkers in early prediction of pregnancy in buffaloes after AI, and these findings would give breeders the opportunity to rebreed animals at the next estrus event, saving most of the days as open.

Finally, during the fourth experiment a total of 185 metabolites were consistently detected in both milk and mozzarella cheese. The PLS-DA score plots clearly differentiated PDO and non-PDO milk and mozzarella samples. For milk samples, it was possible to divide metabolites into two classes according to region: those with lower concentrations in PDO samples (galactopyranoside, hydroxybuthyric acid, allose, citric acid) and those with lower concentrations in non-PDO samples (talopyranose, pantothenic acid, mannobiose, etc.). The same was observed for mozzarella samples with the proportion of some metabolites (talopyranose, 2, 3-dihydroxypropyl icosanoate, etc.,) higher in PDO samples while others (tagatose, lactic acid dimer, ribitol, etc.,) higher in non-PDO samples. The findings establish the utility of GC-MS together with mass spectral libraries as a

powerful technology platform to determine the authenticity, and create market protection, for “Mozzarella di Bufala Campana” PDO.

OVERVIEW

The buffalo has raised great interest in recent years, in many countries including Italy. It is sufficient to consider that while the world cattle population has increased by 37% over the past forty years, the buffalo population has seen 98% growth over that same time. These increases are particularly considerable in Venezuela, Brazil, and above all in Italy, where, between 1991 and 2020, the species' numbers grew from about 112,000 to more than 400,000 heads (Data: Anagrafe Nazionale, IZS Teramo 2020).

From the technological standpoint, buffalo husbandry in Italy is considered among the most advanced and, with a national average of 2,356 kg (with fat and protein levels equal respectively to 8.01% and 4.63% – ANASB 2019), there are farms that exceed 3,000 kg of milk/lactation on average, with production/lactation peaks topping 6,000 kg.

Between 1990 and 2019, average domestic production rose by more than 860 kg/lactation, and fat and protein levels saw a variation from 8.10% and 4.39% to 8.01% and 4.63% respectively. These productive levels were achieved thanks to selective criteria pursued thus far with little incisiveness, especially in consideration of the species' considerable numerical increase, new rationing schemes, improved environmental conditions for buffalo breeding, and optimized farm management in general. Food rationing, which in the past was done by slavishly echoing what was done for dairy cattle, is increasingly taking on its own features, thanks also to the numerous studies performed to clarify the real needs and potential of the species.

A not insignificant part of the productive improvement in Italy is due in particular to breeders that have implemented a systemic selective process by maternal transmission, also aided by careful use of functional controls. This selection was made thanks to the increasingly frequent use of Mature Buffalo Equivalent (MBE) data, through the elimination of the less productive animals and, at the same time, by selecting for culling – both male and female – the buffalo offspring considered the best from the productive standpoint.

In favourable years, the breeder can count on an adequate number of animals for making the selection but, in many cases and depending on the year, neonatal mortality is just enough to satisfy the mandatory replacement. Moreover, quite often, the need to allocate all pregnant heifers to production, in order to increase the number of heads at the farm, or also the possibility of selling at market the replacement at prices that not even the finest bovine heifers can demand, conditions the breeder's choice which is almost always dictated by the economic convenience of the moment rather than by the need to replace the less productive animals.

The technical and management levels attained in raising dairy buffalos have nothing to envy of the levels that characterize cattle raising. One need merely recall that the first mechanical milking of a buffalo took place in Italy, and that ours is the first country to have instituted the species herd book (Ministerial Decree of 23 June 1980). However, in spite of the considerably better situation, buffalo breeding still has many margins for improvement, especially as concerns certain breeding techniques too often rigidly patterned after the experiences and parameters gained for cattle.

Chapter 1

The buffalo species

CHAPTER 1

1.1. The buffalo: origins and characteristics

The buffalo is a mammal in the order *Artiodactyla*, belonging to the ruminants group and the bovine family. Buffalo and cattle are similar in general appearance and share the skeletal feature of 13 pairs of ribs. The former, however, appear stockier, with a broader, taller trunk. Unlike cattle, they lack a dewlap on the lower portion of the neck, and have a convex-shaped forehead and differently shaped horns, first pointing outward and then curving back with converging tips. The coat is sparse and the hide (nearly bare) is thicker and tougher than a bovine's, richer in sebaceous glands (skin oily to the touch), but with a limited number of sweat glands. With this last feature, buffaloes protect themselves from the heat, wallowing in the water and covering themselves with mud. The buffalo prefers hot and humid environments (it is quite widespread in tropical zones, in some cases almost entirely replacing bovines) rich with vegetation and water, in which it spends much of its day immersed: this allows it to keep parasites away, and keeps its skin from drying out.

Sexual dimorphism is not particularly accentuated: the bull is stockier, with a broader, taller neck, and can weigh up to 700-800 kg, while cows weigh about 600-700 kg. Puberty comes late: in females, it is observed at about 2 years, and in bulls at about 2-2.5 years of age. From a reproductive perspective, it is by tendency a photoperiod-negative seasonally polyoestrous species, and therefore calving in Italy is concentrated from August to December. This raises a considerable problem for the breeder, since the animal's physiology is unable to face the main demand for milk (and mozzarella), which is concentrated in the spring-summer period. In fact, the "***herd out of breeding season mating***" (OBMS) technique is adopted: sexual promiscuity is interrupted (or artificial insemination is avoided) when mating is undesired (generally from late September to February), and is restored from February to September (see below). The average gestation period is 315 days, and the average age at first calving is about 30 months (28-42 months). At birth, calves weigh 38-39 kg on average for males, and 35-36 kg for females.

The buffaloes' productive career is exceptionally long: they can reach up to 20 years peaking at 15 lactations, which is why the replacement rate is quite low. Lactation has an average duration of 270 days, with productions averaging about 23,500 kg.

1.2. Origin of the species and its introduction in Italy

The buffalo (*Bubalus bubalis*) was present in the Pleistocene both in Europe and in Southern Asia. The climate changes that took place during this period confined the species to the current territory that comprises India, Indochina, and Southeast Asia. Subsequently, it spread to Mesopotamia, Eastern Europe, Syria, and Egypt. Primitive man during the Pleistocene had already depicted the *Bubalus antiquus* (*Duvernoy*). Among the most ancient depictions are those discovered in Mesopotamia on a cylinder seal of King Sirgulla from 5000 BC, when the buffalo was also present in Europe, as demonstrated by the portions of skull discovered in the Gdansk *Diluvium* and the fossil remains found in Lazio and on the island of Pianosa in the Tuscan Archipelago (*Maymone, 1942*). More recent depictions are those found on a cylinder seal dedicated to *Ibnicharru,* scribe of King Sargon of Akkad, who lived about 3,000 years before the Common Era (3500-3800 BC): it depicts a man giving water to a buffalo, bearing witness to the familiarity that existed between man and this animal. This is said to be the earliest representation bearing witness to the domestication of the species, although most scholars date this event to the third millennium BC in the Indus Valley, and only shortly thereafter (about 2500 BC) in Mesopotamia and China.

The water buffalo originates from the Asian buffalo which is phylogenetically distinct from the African one (*Syncerus caffer*). The Asian buffalo originally comprised three different wild species: the *Sulawesi Anoa*, the Mindoro Tamaraw, and the Arni or wild Asian buffalo. Around 2000 BC, the wild Asian buffalo (*Bubalus arnee*) began to be domesticated, giving rise over the centuries to the water buffalo (*Bubalus bubalis*). In the latter species, *Macgregor* (1939) distinguishes two groups based on the different number of chromosomes: 48 for the *Swamp buffalo,* present in the countries of Southeast Asia (live weight of adults: 350 and 650 kg for females and males, respectively) and 50 for the *River buffalo,* large in size (live weight of adults: 500 – 700 kg in females and 700 – 1,000 kg in males), raised in India and in the Western countries. The different breeding conditions (which used increasingly rational criteria for river buffalo, while tending to remain archaic for swamp buffalo) contributed to the differences between the two groups in terms of production. Also different is the capacity to use food for growth, as reported by Moran (1986); in fact, food rations being equal, the weight increase of young swamp buffalo specimens does not exceed 700 grams/day, while river buffalo can exceed 900 grams/day.

The buffalo's introduction into Italy is still the object of dispute, since it is not easy to glean from the existing documentation whether the species was present in our territory prior to the Lombard

invasion. Ulisse Aldrovandi (1642) in his *Quadrupedum omnium bisulcorum historia*, emphasized the confusion that was made in attributing names: in the Roman Age, in fact, wild oxen were usually referred to as "buffalo." Further confusion may be induced in the erroneous translation into Italian of the word "*buffalo*" used by people in the English-speaking world to refer to the bison. In fact, these speakers refer to our buffalo with terms like "*river buffalo*" or "*swamp buffalo*".

In any event, of the theories explaining the buffalo's introduction into Italy, the most accredited ones are three in number. The native origin of the Italian buffalo is supported by the discovery of fossils dating to the Quaternary Period in Lazio and on the island of Pianosa, and by the studies done by Balestrieri and Colonna. The latter have demonstrated that the primary structure of fibrinopeptides A and B in the Mediterranean buffalo raised in Italy differs from that of other buffalo populations: the Indian buffalo shows a difference in fibrinopeptides A, in particular the replacement of an amino acid residue of serine with one of glycene, in position 8.

The paleontological data on buffalo species do not allow the taxonomic and phylogenetic correlations to be traced in detail; buffaloes were grouped only in a few species, since hybrids have produced a series of more or less stable variations in relation to particular environmental conditions. Therefore, they cannot be considered properly distinct species: both Italian buffaloes and water buffaloes have always been considered as belonging to the same species (*Bos bubalus L.*). However, with respect to this premise, the difference found in the sequence of fibrinopeptides is considerable. In support of this, it bears emphasizing that animals that are morphological quite similar (for example the horse/ass, dog/fox, zebra I/zebra II pairs), also show differences in their sequences of fibrinopeptides. It also bears noting that, before the last Ice Age, the species was widespread throughout Europe and in Asia, and that, immediately thereafter, it migrated to the hotter areas of Asia's tropical and subtropical zones (*Zicarelli, 1997*). However, the species' physiological characteristics to be discussed below do not support this hypothesis.

There are currently two main theories on the introduction of buffaloes into Italy: one considers the Lombards, and the other the Arabs. According to Cimmino (*Cimmino, 1982*), the introduction of the buffalo took place in the eighth century AD with the invasion by the Arabs, who presumably transported some animals from Egypt. This supposition would be supported by the custom, established in the fifteenth century, of calling the buffalo in the "terra di lavoro" – the current province of Caserta and the area of Mazzoni in particular – by the term "*vacca africana*" or "African cow." On the other hand, from a morphological perspective, Italian buffaloes are quite similar to Egyptian ones and extremely different from those of the Asian areas – places from which our buffaloes appear to have arrived with the Lombards. Many (*Cristin, 1862*) agree in asserting

that the introduction of the buffalo into Italy took place precisely during the Lombard invasions: after his victory over the Gepids, Agilulf was given a buffalo bull by the king of the Avars, as a sign of friendship for the aid that was received. The entry of the Lombards into Italy is described in Paolo Diacono's "*Historia Longobardorum*," which reports information learned from an old man and occurring two centuries earlier. According to the old man, the inhabitants of the Po Valley, upon seeing the hordes of Barbarians, were quite struck not only by the wild horses, but above all by the "*buffaloes with large horns*," that followed the army. These were quite likely the progenitors of the modern-day Podolica cattle, the Maremmana in particular, both because buffaloes do not have large horns, and because it is highly unlikely that all those buffaloes could have descended from that single buffalo bull received as gift from the king of the Avars (parthenogenesis?). It bears recalling that during Latin's decline, that language used the term "*bubalus*" when referring to cattle as well.

Moreover, in the *Leges longobardorum* promulgated in the edict of Rothari, the Lombards, relying on the principles of Roman Law, in quantifying the damage caused by many animal species, do not cite the buffalo, which creates more damage than cattle does. It is to be borne in mind that in this code, the Lombards were quite meticulous, going so far as to estimate even the damage caused by the falcon, used in hunting; according to Cantalupo (*Cantalupo, 1990*), this is a clear sign that the species was wholly unknown to the Lombards. Cimmino (*1982*) reported that the historic excavations carried out in Capaccio and Altavilla did not bring buffalo bones to light, nor is the buffalo mentioned in documents dating back to the 1000s. He therefore hypothesized that its introduction dated to the Norman period with the invasions by the Saracens and Moors in the late tenth century in Sicily, while at a later time, during the Swabian era, they presumably reached the Sele valley.

According to Zicarelli, the question as to whether it was the Lombards or the Arabs who introduced the species from which the modern-day water buffalo is derived has so far gone unanswered: incredibly, there is no documentary evidence irrefutably proving one hypothesis or the other. The first buffaloes reaching Europe after the Ice Age may be assumed to have arrived with the Huns – a people originating from Northern China, where the species was certainly raised starting 5000 BC; and through the Huns, other barbarian populations gained familiarity with them.

In any case, the first real testimony to the buffalo's presence in Italy can be found in certain documents discovered in the Farfa Abbey (in Lazio) between the twelfth and thirteenth centuries. For example, found among these documents was a decree by King Charles I of Anjou, ordering the return of a stolen work buffalo. Manuscripts from the San Lorenzo monastery in Capua have also

been discovered, relating to the offering to the Patron Saint – a piece of bread with mozza. Starting in 1300, there is testimony of a trade in buffalo meat and dairy products, usually sold at the markets in Naples and Salerno.

As the years went by, interest in the species grew. During Spanish rule, the practice of buffalo hunting spread (a tradition that remained until before the Second World War in the Caserta area, and until the 1950s in Venezuela, while still practised in some reservations in Brazil): in fact, "buffalo hunting" parties were organized, during which the court would visit the husbandry areas in the Volturno and Sele valleys (*Cristin, 1862*).

The Bourbons in particular gave much importance to this species, going so far as to set up a breeding farm on the Carditello estate, and even an experimental dairy. To this day, the Bourbons' hunting lodges, later used as dwellings by patrician families, can be found in the Sele valley. The existence in Bourbon times of production and trade in buffalo milk and dairy products, such as to result in the search for improvement techniques as well as in protection measures, shows the degree to which the Kingdom of the Two Sicilies was projected towards development.

The buffalo is to be credited with allowing degraded land and marginal areas to be used, thereby keeping them from being abandoned by people altogether. Indeed, with the barbarian invasions, vast territories were left to gradually become swampland – an abandonment that, especially during the war of the Vespers, coincided with the spread of malaria. One of the most well-known testimonies was written by *Goethe*, visiting the Paestum plain in 1786: "*In the morning we drove by rough and often muddy roads towards some beautifully shaped mountains. We crossed brooks and flooded places where we looked into the blood-red savage eyes of buffaloes. They looked like hippopotamuses. The country grew more and more flat and desolate, the houses rarer, the cultivation sparser*." This testimony shows that the only form of farming and animal husbandry in these areas was buffalo breeding, capable of transforming the marshland's forage resources into a product. In the same territories, other ruminants suffered high mortality rates, and were therefore unable to produce income (*Zicarelli and Campanile, 2001*).

The presence of the buffalo in certain areas of Southern Italy was linked to the characteristics of the terrain, to this species' particular adaptability to adverse climate conditions (like the hot and humid climate of marshy areas), and to its ability to feed on coarse foods and on the plants that grow in marshlands. With a physical prowess allowing them to unleash considerable driving power, buffaloes were also used to pull fishing nets ashore along the Sele, Volturno, and Garigliano rivers. They were in fact indispensable for cleaning the drainage channels in marshy areas called "*regi lagni*," and in riverbeds: this was a considerably important job since the cleaning of channels, by

permitting the outflow of mud and water during the rains, prevented the tragic consequences of flooding. Buffaloes were also used for draw loads through river fords, and for the periodic drainage of channels and canals, because by swimming in hordes when the waters were deep, or by treading on the bottom, they were able to envelope their limbs in aquatic plant life, thus uprooting it; in addition to this, by churning up the mud, they made it easier for the water to carry it away.

It is also to be borne in mind that the hide was highly valued and used for straps on carriages, footwear, and leather articles for soldiers, since it is far thicker and sturdier than cowhide. The horns were also highly sought-after, used for knife handles and for other luxury and ornamental items. In this regard, Maymone (1937) reports the following: *"This use of buffaloes was typical in the Pontine Marshes before the reclamation transformed the age-old great marsh into towns and a dense network of roads and small farms, where until a few years ago, 1930, about 200 buffaloes, subdivided into groups of about fifty head each, could drain a network of about 130 km of channels, some of which navigable."* The versatility and potential of this animal species, which was able to adapt splendidly to a variety of market needs, is therefore plain to see.

At the turn of the twentieth century, there about 20,000 head, a figure that declined to 12,000 after the Second World War (*Zicarelli, 2001b*). The transformation from an agricultural civilization, with a high rate of self-provision and self-consumption, to an industrial, consumer, no longer food-producing civilization, which took place during the years of the economic recovery and of the great exodus of rural populations towards stable jobs in the nascent industry, caused the buffalo to be considered at that time as an animal at future risk of extinction. The environmental conditions no longer existed to justify the economic rationality of buffalo raising. Policies were adopted to subsidize and finance intensive agriculture, low in hard-to-find labour, that could guarantee competitiveness on the market. Industrialized farming – a phenomenon that had already reached other European countries – became the objective. In Italy, in order to secure a minimum competitiveness for their products, some breeders introduced more productive breeds, to the detriment of quality, thereby destroying a husbandry heritage of inestimable value.

However, this phenomenon did not take place in the buffalo sector. In a few decades, the buffalo, from being an animal employed in order to utilize marginal territories, was transformed into an animal of highly specialized production; this transformation took place over a very short generational span in comparison with the development of other Italian husbandry practices. From 1947 to date, the buffalo population has seen exponential growth, reaching the more than 400,000 head discussed earlier.

It can then be stated with certainty that this is a species with high productive potential, that is also endowed with great versatility and diversification. But while on the one hand our breeders have been far-sighted in continuing to raise the buffalo, on the other they have been short-sighted in concentrating only on the albeit highly profitable sector of milk production. Promoting meat can be seen as a sound alternative to milk production for cheese-making alone. By using buffalo bulls and heifers no longer suitable for reproduction and replacement, meat production creates an activity capable of guaranteeing additional income that is by no means insignificant in volume.

1.3. Population and distribution in Italy

In terms of husbandry, ISTAT estimated, at 01 June 2017, a cattle and buffalo stock virtually unchanged from the same date in 2016, noting an insignificant decline of barely 3,000 head: the balance between the decline in cattle (-18,000 head, equal to -0.3%) almost entirely offset by the 15,000-head increase in buffalo (+3.8%). Among cattle, there was a greater decline for dairy cows (-31,000 head, equal to -1.9%), offset by the increase in buffalo cows (+28,000 head, equal to +7.5%). The sample survey was performed on about 9,000 farms in December, and about 6,000 in June. The sample was clearly random, and its breadth was determined in such a way as to guarantee estimates that were accurate regionally (December survey) and nationally (June) for all the considered species. The survey is performed every six months.
In Italy, the heads are distributed mainly in the regions of Campania (74%), Lazio (17%), and Puglia (2%), and particularly in the province of Foggia. The remaining population is widespread both in Central and above all in Northern Italy: in fact, starting in 1980, buffalo raising also spread to the northern provinces where some breeders, due to the problem of milk quotas, found it advantageous to convert the cattle-raising structure into this activity.

In the Campania region in particular, husbandry is practised by upwards of 1,200 farms (about 57% of the total of farms present in Italy), situated above all in the provinces of Salerno and Caserta, with small settlements in the provinces of Naples, Avellino, and Benevento as well.

In these areas, farms have achieved a level of specialization, so that the average farm size is decidedly larger than that normally found in dairy cattle farms (214 vs. about 100 heads per farm for buffalo and dairy cattle species, respectively; *BDN, Anagrafe Zootecnica Nazionale, 2020*).

The extraordinary capacity for adaptation of the species was one of the main reasons for its spread: as discussed earlier, the buffalo is in fact to be credited with having enabled the use of degraded territories, marginal areas, and swampland zones. Today, however, the main activity of buffalo raising is represented by milk production to be processed into cheese, as the use of the buffalo as a work animal is now entirely a thing of the past– and this is also in light of the progress in farm mechanization.

All this could take place thanks to the farsightedness of buffalo breeders, who continued to believe in this animal's potential and did not undertake the raising of other species, thus bucking the expectations of many analysts in the past who raised fears of a drastic reduction as a consequence of the reclamation of the marshlands.

1.4. Mozzarella cheese and Protected Denomination of Origin (PDO)

The reasons for the considerable development of the species are to be found in the promotion of the end product of buffalo milk processing: the *mozzarella di bufala Campana* PDO (*Cerrato, 1999*). This pasta filata cheese, made a typical designation in 1979, has since 1996 enjoyed the status of Protected Designation of Origin (PDO) – the prestigious European mark institutionally recognizing the organoleptic and commercial characteristics of this cheese, derived prevalently from the environmental conditions and from the traditional processing methods existing in the specific production area. Mozzarella is obtained from buffalo milk with a yield of about 25% (*Zicarelli 1992b; Cerrato, 1999*), much higher than cow's milk, which is generally between 12% and 14% (*Zicarelli, 1992b*). Thanks to this product, buffalo milk – which in terms of yield, as already discussed, is worth about 1.8 times more than cow's milk (24 ÷ 13 = 1.8) – is marketed by the producer at a price, over the course of the year, about 2.6 – 3 times higher than cow's milk.

As already described, mozzarella is a pasta filata cheese, and therefore, in a production process involving a scalding of the curd with hot (90°C) water, the pathogenic germs normally eliminated by the pasteurization process are destroyed: this is essential for the purpose of obtaining a sanitarily faultless product. However, it is necessary for the milk to have a low bacterial load to begin with, since an excessive presence of germs would result in problems for the curd and for the end product's organoleptic characteristics. The pasteurization of the milk, which could be the solution for using a milk with an excessively high microbial load, results in a product with organoleptic characteristics decidedly inferior to what can be obtained with raw milk. The reasons for this are to be sought in the fermentation of the lactobacilli present in raw milk, which are altered by heat treatment. The characteristics that give it its typical nature set the fat content (dry matter) at a minimum of 50%, a maximum humidity of 65%, and a provenance exclusively from fresh, whole buffalo milk (within 60 hours of milking), produced only in certain areas of the national territory (so called "DPO Zones").

It must also be borne in mind that this sector suffers from a recurring market crisis during the autumn and winter since those periods see a greater availability of milk linked to the characteristics of the species to be discussed below, and a lower demand for mozzarella. The calving calendar must therefore be modified in order to use only fresh milk for the production process. In the past (and, unfortunately, in some recent cases), the milk was frozen and reused in the spring and summer, in correspondence with the increased demand: the result was a poor-quality product that did not meet with the consumers' favour (*Cerrato, 1999; Zicarelli, 1992b*).

In Italy at present, the area of provenance of the milk, and of the production and processing of *Mozzarella di Bufala Campana*, comprises the following administrative territory:

- ✓ The Region of Campania, which includes the entire territory of the Province of Caserta and the Province of Salerno. It also comprises several municipalities in the Province of Naples and the Province of Benevento;
- ✓ Region of Lazio, which includes several municipalities in the Province of Frosinone, the Province of Latina, and the Province of Rome;
- ✓ Region of Puglia, which includes the Province of Foggia and the entire territory of the municipalities of Manfredonia, Lesina, and Poggio Imperiale, and part of the territory of the neighbouring municipalities;
- ✓ Region of Molise, which includes, in the Province of Isernia, the sole municipality of Venafro.

The Consortium for the Protection of the Mozzarella di Bufala Campana, born in 1981, is the only organization recognized by the Ministry of Agriculture, Food and Forestry for the protection, supervision, enhancement and promotion of this extraordinary cheese from Central and Southern Italy, appreciated all over the world. The Consortium's purpose is to protect the production and trade of Mozzarella di Bufala Campana, to protect the designation in Italy and abroad, to foster constant improvement of the means of production of Mozzarella di Bufala Campana and to consequently improve the quality of its production, to constantly monitor production and trade, and in particular to monitor the correct use of its designation of origin.

1.5. Critical points of the supply chain

Regional husbandry production, including both the cattle and buffalo sectors, represents approximately 4% of the country's GDP in quantity and value of national production. Dairy companies are quite heterogeneous in their size composition and strategic organizations. As a general rule, in the buffalo sector, the organizational characteristics, sturdiness of the production apparatus, and capacity for production enhancement are more substantial and larger in size, with productions that have for several years been integrated with investment aimed at innovation and at the spread of mechanization. There is a greater homogeneity and a larger concentration in specific territorial settings, with medium or medium/large-sized farms and more evident and consolidated processes of vertical integration among the players in the supply chain, as shown moreover by widespread membership in the Consortium for the Protection of the Mozzarella di Bufala Campana. However, there are still considerable limits in production, processing, and distribution.

As regards husbandry, it is still necessary to aim towards optimizing and streamlining feeding and selection techniques – and above all techniques that guarantee improved health and hygiene. In some areas, in spite of an on average large farm size and the operators' good professional skills, problems related to health and the environment can still be found; in fact, processing is still highly seasonal, while product standardization is low (inconsistent over time and among different production units).

In particular, from the standpoint of health, mention ought to be made of the resurgence of certain infectious diseases, such as brucellosis and tuberculosis, that undermine the equilibrium of the entire productive sector and might lead to consumers' disaffection with a product no longer deemed healthy. From this standpoint, it is essential to apply all the biosafety measures provided for in Ministerial Decree no. DM 429/2016, that unfortunately are currently implemented only by a small number of farms.

From an operational perspective, the greatest critical areas in buffalo husbandry relate to the concentration of calving in the autumn. This translates into milk production outstripping market demand during the autumn/winter period, when mozzarella demand declines, and a short supply during the summer when mozzarella consumption is more sustained. The greater presence of buffalo milk on the market during the winter places cheese producers in a situation of contractual weakness, forcing milk prices downwards. Contributing negatively to this situation are the intrinsic

characteristics of buffalo milk which, unlike cow milk, is not for human consumption but only for cheese-making, thus reducing the breeders' market power.

The discontinuous availability of buffalo milk, associated with an extreme fragmentation of the processing structures, also determines the end product's extreme variability, which in turn conditions commercial penetration into markets other than the regional one. However, many farms implement the OBMS techniques, in order to encounter market demand *(Zicarelli, 1994)*, with selective reproduction and genetics programmes in order to improve the buffaloes' lactation level. In these areas, the husbandry sector has played an irreplaceable role that goes much further that a mere economic and social function, contributing towards giving greater sustainability to the development of the territories of reference.

Added to this is the fact that mozzarella is at any rate a fresh product, with a limited shelf life due precisely to its respect for the traditional production characteristics, like processing raw milk. Therefore, as regards the specific sectors of processing and marketing, the main requirements appear to be precisely those of reducing product perishability, with the aim of increasing its shelf life: in addition to seeking new technological solutions downstream, the production of a milk flawless from the sanitary standpoint, and with a low bacterial load, would clearly contribute significantly to this process.

Another problem of particular importance for the sector is the Region of Campania's decision of 05 December 2017, enlarging to about 23% of the regional territory the areas vulnerable to nitrates, where, according to the Directive (EU) 91/676, the restriction of 170 kg of nitrogen (or 2 livestock units – LSU) per hectare/year, resulting from the spreading of livestock effluents, cannot be exceeded. This measure, initially blocked by an appeal brought by trade-union organizations and whose entry into force was forecast for March 2020, was then subject to several debates in the regional setting, culminating in regional government directive no. 152 of 17 April 2019, that mainly establishes an extraordinary programme for plant adjustment and environmental improvement in support of the buffalo sector in Campania, having as its prime objective the construction of tree – pre-siting criteria for provisioning plants and a consequent standardization of values.

Lastly, it bears emphasizing that buffalo raising operations are in reality single-income enterprises since, as already discussed, the meat market has never been developed. This results in a particular fragility of the system, since a crisis in the milk market, like the one that took place not many years ago, creates profound difficulties for buffalo entrepreneurs, with inevitable repercussions on the sector's health. The search for alternative solutions, such as the expansion and diversification of the

production range, representing important preconditions for developing and conquering new markets, are therefore hoped for.

With regard to these problems, it is necessary to emphasize both the proximity between the production and processing locations – which allows the milk to be processed in real time – and an established tradition in processing technique, with a large availability of specialized labour, thus making high-quality products available. Certainly contributing to the sector's good health is the buffalo milk traceability system, put in place in 2014 by Ministerial Decree no. 9406 of 09 September 2014, establishing the procedures for implementing the provisions pursuant to art. 4 of Legislative Decree no. 91 of 24 June 2014. Managed by SIAN and by Istituto Zooprofilattico Sperimentale del Mezzogiorno, the system collects the daily quantities of milk produced in total by lactating buffaloes on the farm, and brought for processing. In their turn, the buffalo milk processors are required to announce the quantities of milk and of semi-finished goods, also in frozen form, purchased for the making of transformed products, as well as the indication of those who brought them, and the produced quantities of Mozzarella di Bufala Campana DPO, of buffalo milk mozzarella, and of other processed products. The system has made it possible to discard those who were playing loose with the origins, and has benefitted the consumer who now finds a product on average better than that from some years ago. The addition of transparency to the sector provided by the milk traceability system has brought benefits to the whole sector – first and foremost to the breeder, who has become the "owner" of the produced milk, and to the cheese producers as well.

Equally important is the evolution of demand for typical products and those bearing a designation; in the case of Campania, these products are an important element of production, both for the number of specific features present in the Region, and for the ever-increasing economic weight of Mozzarella di Bufala. We may state that in spite of the critical areas that have been discussed, the sector's outlook is more than favourable.

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Chapter 2

CHAPTER 2

2.1. Optimization of puberty

At birth, the male buffalo calf weighs about 38-39 kg, and the female about 35-36 kg. This figure is fundamental, because what is to be emphasized here is that weaning is done precisely in relation to weight and not age; weaning, in fact, will be possible only when the calf has reached a weight of approximately 85 kg, which will allow it to ingest a quantity of dry matter functional to a daily weigh gain of 800-900g/day.

Over the years, numerous attempts have been made to lower weaning age, which is quite variable also in relation to farm management, the workforce, and available structures. In this case as well, we may note a difference from bovine calves because in the buffalo calf, weaning comes later: since the ingestion capacity is lower, the live weight must be greater in order to be able to guarantee the consumption of 1.2 kg of dry matter at the time of weaning – an amount indispensable for assuring harmonious growth. We may glean from this that nutritional management is essential right from the start for these animals, and that an error might result in delayed growth with negative repercussions on the productive and reproductive career, delaying both puberty and the complete expression of genetic potential.

Raising the buffalo heifer is a delicate phase for its future productive and reproductive career. In fact, it is during this period that the heifer develops the physical structure and metabolic functions that, along with longevity, will contribute to making it a "good" producer and reproducer.

In the breed referred to as the Italian Mediterranean buffalo, delayed puberty has been observed when insufficient energy levels are adopted in the previous months. Animals with daily weight gains (DWG) > 500 grams initiate puberty about 50 days earlier than those with lower DWG; this takes place if high energy levels do not alternate with low energy levels. On the other hand, a positive effect is recorded when low nutritional levels are followed by high ones.

Longevity, in addition to being an individual characteristic, is influenced by the level of management. The degree of productive inefficiency that a breeder can tolerate is in fact rather subjective. The raising of young cattle to be destined for replacement is in fact an activity that, if analyzed over the short term, represents exclusively a cost for the breeder, and not a revenue. The

breeder often tends not to follow this activity with particular attention and, above all, not to limit its costs, without assessing the long-term repercussions on future performance.

The need to reduce costs is certainly to be acknowledged, but the animal's growth ought not to be penalized to the point of delaying its entry into production. In fact, cutting costs in this phase improperly considered "unproductive," and thus delaying the start of the productive phase, is economically disadvantageous. The age at first calving determines the duration of the unproductive period, which has a negative impact on farm income: the longer this period is, the higher the cost will be that the breeder will have to incur in order to bring the animal to the start of its productive career.

Buffalo heifers initiate puberty at around 16-18 months, and males after 2-2,5 years of age. The recommended age for the first fertilization is 18-22 months, at a live weight of 380-400, so that the first calving takes place at 28-32 months. Using more extreme nutritional regimes, puberty can be reached earlier, with the age at first calving at about 24 months. A dual benefit is obtained in this way: on the one hand, the animal's unproductive period is reduced, with the economic return coming earlier for the breeder, and in the second place the species' genetic improvement is accelerated, since genetic progress is inversely proportional to the interval between one generation and the next. The earlier the animals initiate production, the shorter that interval, and the quicker the herd's progress, will be.

2.2. The reproductive seasonality

As discussed above, the buffalo is by tendency a photoperiod-negative seasonally polyoestrous species. Reproductive seasonality is a strategy adopted by various species in order to birth their offspring in the most favourable environmental conditions, and when there is a greater availability of food. In Italy, calving season is concentrated in the autumn and winter (*Zicarelli, 1997*). Most likely, this behaviour has ancestral motivations: the cradle of the buffalo species is the Indus Valley, the northern equatorial area situated between India and Pakistan (*Zicarelli, 1995*). In these areas, the best environmental conditions and the greatest availability of forage occur after the rainy season, which coincides with the period from July to December-January. Of course, the individuals born in said conditions, being those that survive and therefore prevail in numbers in the population, passed their particular reproductive characteristics on to subsequent generations. When the buffalo was exported to other countries, it conserved this particular feature, even in conditions unfavourable to the species' survival (as in Italy, in fact). Seasonality is a trait manifested in adult buffalo cows and less so in heifers. This might be due to the fact that the light stimuli perceived by the central nervous system during gestation and the first days of life can condition sensitivity to a long or short day.

Everything discussed thus far is regulated by an endocrine mechanism: the light source, depending on the intensity and duration, will stimulate the hypothalamic-pituitary-gonadal axis; this system involves the retina, the suprachiasmatic nucleus (SCN), the superior cervical sympathetic ganglion (SCSG), and, lastly, the pituitary.

Studies on the subject have shown that the SCN is an internal biological clock that regulates the endogenous circadian rhythm. The stimuli processed there are transmitted through the SCSG to the pituitary which functions as a transducer, converting nerve information derived from the alternation of the light/dark cycle. In the pineal gland, the message from the nerves stimulates the secretion rhythm of melatonin which, through complex mechanisms, regulates hypothalamic-pituitary activity, and therefore the function of the gonads.

Light regulates the secretion of melatonin in two ways: by stimulating a circadian pacemaker located in the suprachiasmatic nucleus (SCN), which in its turn controls the activity of the pineal gland, and by inhibiting melatonin production. The duration of melatonin secretion is therefore an endocrine index of the length of night and day (*Lincoln et al., 2005*). Some studies have shown that in the buffalo, too, melatonin is the endocrine signal of light/dark alternation (*Parmeggiani and Di Palo, 1994*). During the night-time hours, high plasma concentrations of this hormone are found, as they are in winter and autumn in comparison with spring and summer.

In OBSM-farms (see below), which is to say those where births are concentrated in the first seven months of the year, there is a trend opposite to what takes place in seasonal farms. A completely different circadian rhythm of melatonin is found among buffalo cows that calve spontaneously in the springtime and those that calve in the autumn (Figure 2).

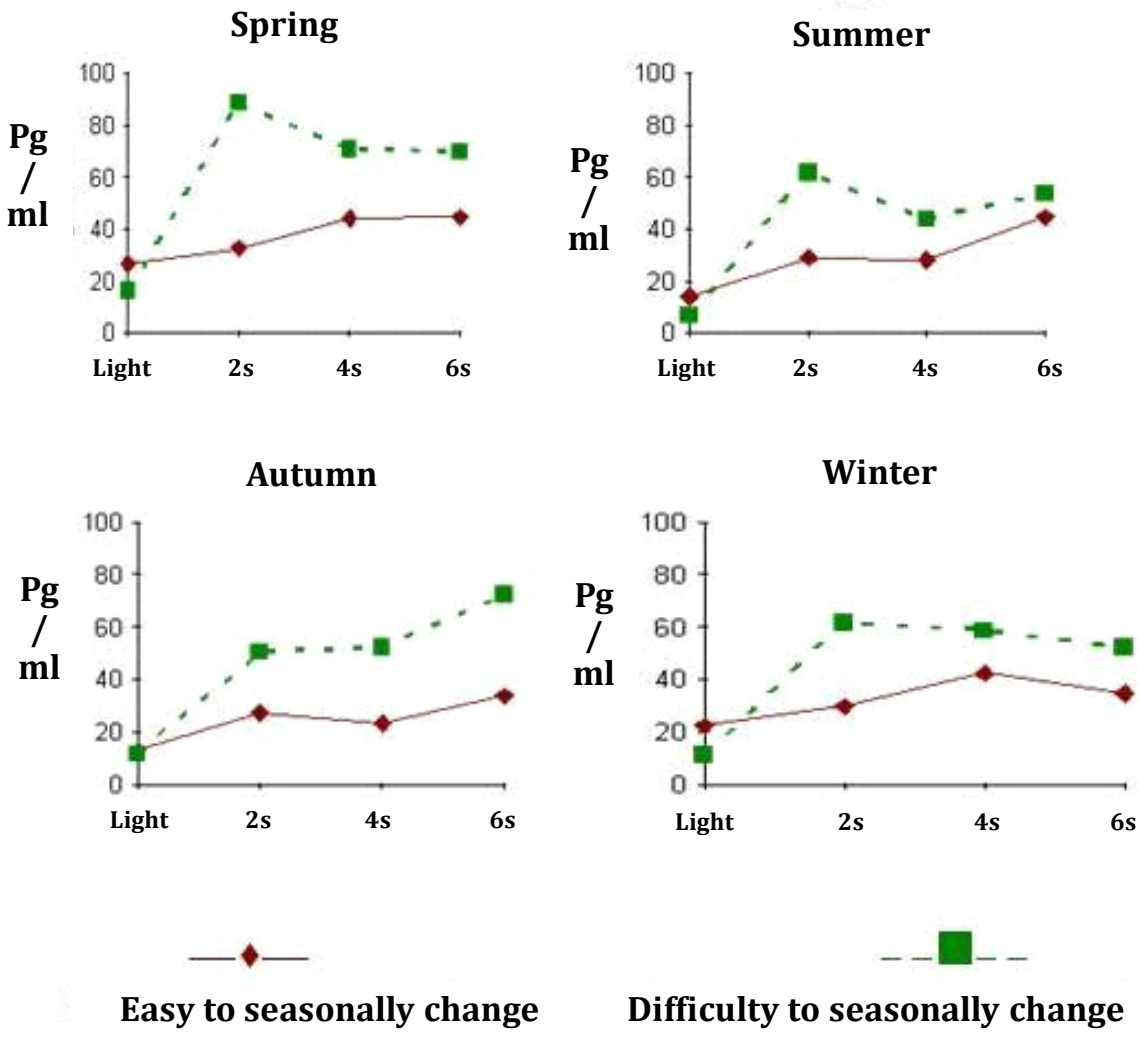

Figure 2 Melatonin levels in OBSM female buffaloes 2 hours before sunset and 2, 4, and 6 hours after sunset, measured in the four seasons.

In particular, two hours from sunset, the individuals most sensitive to the photoperiod, and therefore more seasonal, show, during the winter season but above all in the spring, a rise in melatonin levels greater than that recorded in buffalos that calve spontaneously in the spring, and that are thus less sensitive to the photoperiod (*Zicarelli, 1997*). The levels measured two hours after sunset, excluding those measured in the autumnal period favourable to the species' reproductive activity, show a repeatability (*Di Palo et al., 1993*), assessed on plasma levels recorded in the other 3

seasons, equal to 0.733. There are therefore good possibilities that this trait, since it is repeatable, is also heritable: should the heritability be high, this might be inserted into the genetic selection programmes (*Zicarelli, 1993*).

The phenomenon of seasonality also relates to the male: just as females outside the reproductive season encounter the phenomenon of anoestrus, males present a reduced libido as well, also accompanied by a lower quality of ejaculate. To get around this problem, farms generally increase the male/female ratio to 1 to 15 or 1 to 20, or they introduce young individuals that are less sensitive to the photoperiod and have a higher libido than adults.

2.3. The Out of Breeding Season Mating (OBSM) Technique

According to that described above, it is unequivocally clear that:

- ✓ the buffalo cow, albeit showing reproductive activity during any period of the year, accentuates it during the months when the hours of daytime light decrease or, although increasing (January north of the equator, July south of the equator), hours of darkness still prevail over the course of the day;
- ✓ where the light/dark ratio is constant over the course of the year (the equator), although there is no tendency for seasonality, calving noticeably grows in frequency at a rate varying from zone to zone and from year to year; it appears, in practice, that the activation of the reproductive cycle necessitates particular environmental conditions or unanimous or group sexual manifestations.

The reproductive seasonality of the species is a physiological characteristic that has considerable economic implications. In Italy, as discussed, the economic motivation for buffalo breeding is the production of mozzarella, obtained exclusively with buffalo milk. This production requires a continuous availability of milk or, better, a greater quantity of milk during the spring/summer period, when consumption increases and there is at the same time a noticeable decline in the calving schedule at the farm. It is therefore clear that the demand for milk to meet market needs contrasts with the concentration of the species' calving (Figure 3). Years ago, the lack of milk during the spring/summer period was made up for using milk frozen during the winter, but the end product had qualitative and organoleptic characteristics decidedly inferior to what was obtained from fresh milk.

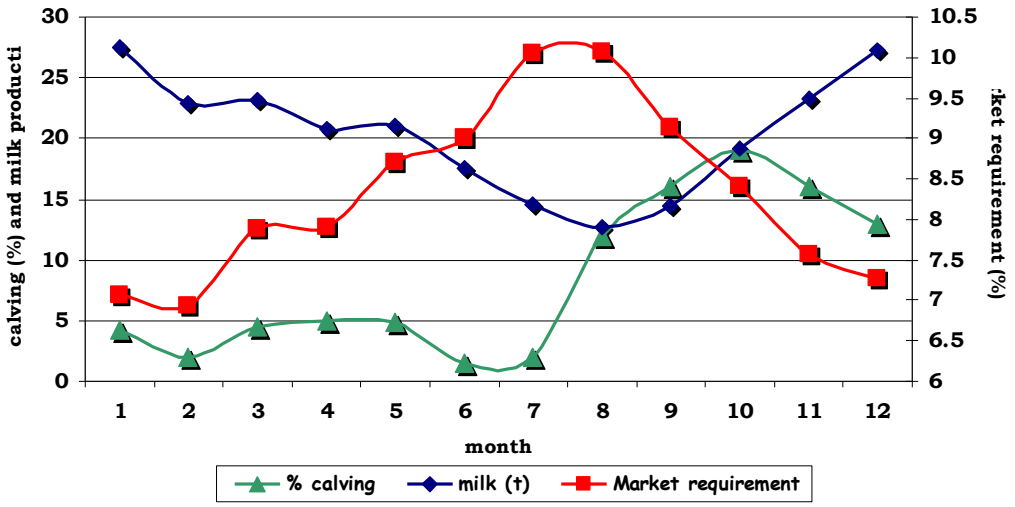

Figure 3. Ratio between percentage of calvings, milk production, and market demand for the production of mozzarella di bufala Campana DPO over the course of the year, in conditions of natural mating.

The strategy first used to freeze the milk during the winter in order to make up for the shortcomings during the spring, with the consequent decline in the qualitative and organoleptic characteristics, gave rise to a mediocre product and to a considerable downturn in the market for Campanian buffalo mozzarella. Moreover, starting in 1996 with the approval of the Regulation governing the production of mozzarella di bufala Campana DPO, freezing milk was forbidden, and it thus became necessary to seek other techniques to invert the calving schedule. This gave rise to the need to modify the calving schedule by intervening in the time of mating, through out of breeding season mating (OBSM) technique. The technique consists of interrupting sexual promiscuity during the period of maximum reproductive activity, and leaving the bull in the herd during the period between March and late September, in such a way as to obtain calving between late January and early August (*Zicarelli, 1997*).

Doubtlessly, the application of this technique results in reduced fertility, as it keeps the animals from mating during periods when the species is most fertile (which is to say when hours of daylight diminish) and induces them to mate when the hours of daylight increase. Precisely for this reason, two different techniques were developed: the gradual and the drastic OBSM techniques. The choice of one or the other is closely dependent upon a variety of factors:

- ✓ **Degree of herd seasonality**. This indicates the percentage of animals that calve during the autumn-winter period: if the degree of seasonality exceeds 70%, there is a preference for employing a "soft" technique to be implemented over the course of three years, while, if seasonality is 40-50% the applied technique will be drastic. A gradual OBSM over 3 years, which is generally implemented at farms where the OBSM has never been done, calls for removing the bulls from the herd during the first year between late October and late January, during the second year between mid-October and mid-February, and during the third year between early October and early March, in order to dilute the loss of fertility that should take place in 1 out of 3 years.
- ✓ **Availability of pubescent heifers**. As discussed earlier, the youngest animals are less affected by the photoperiod, and can therefore be more easily subjected to the OBSM technique. A good availability of heifers (not a very difficult event), given the considerable longevity of the species which allows the replacement rate to be reduced, makes it possible to partially make up for the loss of fertility that takes place in pluriparous buffaloes for the application of the technique. In any event, it bears emphasizing that heifers can be placed in production during any period of the year, provided they have reached a weight that oscillates between 350-400 kg of live product;
- ✓ **Commercial conditions of the area.** Market demand is the main factor to take into consideration prior to applying the OBSM technique. For example, a dairy located in a large city will paradoxically have less demand for mozzarella (and therefore milk) during the month of August, and more in September for the return from holiday.

The reduction in fertility, which can vary from 10 to 35%, is due to the two main reproductive problems found in the buffalo species in Italy:

- ✓ *embryonic mortality*, which is to say interruption of gestation in very early stages;
- ✓ *anoestrus*, encountered in the springtime months especially by elderly pluriparous buffaloes (*Zicarelli, 1993*).

Some studies have underscored how the technique determines an increase of the calving interval – which depends on the herd's reproductive efficiency, and grows greater the more the problems there are concerning the buffaloes' reproductive sphere. Farms that have applied the OBSM for a number of years (more than thirty), eliminating the animals more subject to the photoperiod, show a lower calving interval than farms that have applied them for less time.

2.4. Physiology of oestrus

Buffalo cows have an oestrus cycle averaging 22-23 days, with variations ranging from 17 to 33 days (*Zicarelli, 1992*; *Di Palo et al., 2001; Neglia et al., 2007*). The duration of heat varies from a few hours to 2-3 days (*Zicarelli, 1992*), and the moment of ovulation is also unpredictable: on average, the heat/ovulation interval averages about 50 hours, while the end-of-heat/ovulation interval is about 20 hours, with a coefficient of variation virtually double the former, and conditioned also by the season of the year, with a longer duration during the cold periods (January-March). Short-duration heats, and behavioural manifestations starting and ending during the night-time hours, are often found with high incidence. Ovulation is an event that follows the sharp spike in LH blood levels that in the buffalo cow takes place more or less at the start of heat, while ovulation takes place at a distance from the peak that ranges between 26 and 33 hours, although in about 30% of the animals, the phenomenon of double ovulation has been described. In the buffalo cow, as in the bovine cow, the oestrus cycle is marked by a series of follicular waves, and differs both among species and within the same species. During the oestrus cycle, in most buffalo cows (about 63%), 2 follicular waves are observed, divided by an interval of about 11 days, for both natural (*Baruselli et al., 1997*) and synchronized (*Neglia et al., 2007*) oestrus.

The buffalo's reproductive physiology is marked by a reduced number of cycles that succeed one another after calving, after which, if conception does not take place, anoestrus occurs, which accentuates the tendency towards seasonality (*Campanile et al., 2009*). The behavioural manifestations typical of oestrus in buffaloes are far less evident than in cows: the percentage of buffaloes in heat that have homosexual manifestations is extremely low, in general less than 30% (*Ohashi, 1994*; *Baruselli, 2001*), and the animals often tend to let themselves be mounted by bulls even when there are no heats. Symptoms like swollen vulva, vaginal discharge, and increased urination are not highly significant for detecting oestrus. The most effective method is using a vasectomized bull with a heat mount detector and/or visual detection twice a day, to be done preferably during the early hours of the morning and evening.

2.5. Anoestrus

Anoestrus refers to the condition of absence of oestrus in the animal.

In this conditions, the ovaries are moderately inactive and there are neither ovulations nor corpora lutea. There can be multiple causes for this phenomenon:

- physiological events like pregnancy, lactation, the presence of offspring, nutrition, environmental and seasonal conditions;
- pathological events like persistent corpus luteum, corpus luteum cysts, ovarian hypoplasia (*Spelta and Corbella, 1999*).

Therefore, there are various types of anoestrus that we shall proceed to analyze below.

Gestational anoestrus: Gestational anoestrus is due to high concentrations of progesterone produced by the corpus luteum and/or by the placenta, that act at the hypothalamic level with a negative feedback mechanism, inhibiting the production of GnRH while FSH and LH gonadotropin are at basal levels, for which there is no ovulation. Oestrus behaviour may be present during gestation in 3-5% of the cases in both bovines and ovines. Similar cases have also been found in buffaloes (*Di Palo et al., 2001*): approximately 1-2% of heats found during the use of pedometers were detected precisely during gestation, in correspondence with very high levels of progesterone (> 1.5 ng/ml).

After calving, progesterone levels decline and remain low for several days, to permit uterus involution. The average interval for completing uterus involution in the buffalo is between 17 and 52 days, although ovarian follicular activity can resume earlier, at around 15-30 days. In particular, the pituitary gland's reactivity to exogenous GnRH takes place by the 20th day in buffalo milk cows (*Palta and Madan, 1995*). According to some studies, the first postpartum heat in buffalo takes place between 44 and 87 days after calving (*El-Wishy, 2007*), although some evidence exists of this condition already taking place around the 17th day.

Lactation anoestrus: in nearly all species, ovarian activity is interrupted during lactation, to prevent another pregnancy from occurring. The calf's nursing results in inhibiting the secretion of gonadotropin by the pituitary with a negative feedback mechanism for the release of GnRH, which determine the release of basal levels of LH. The various factors responsible for this phenomenon also include, for example, the viewing and the olfactory/auditory recognition of offspring.

Nutrition anoestrus: This is due mainly to ancestral factors according to which buffalo cows, being prey, would not leave their offspring unattended, which would risk their young becoming prey; but anatomical/functional factors also contribute, in which the postpartum period sees a reduced ingestion of dry matter, since the body, and the digestive glands in particular, need to adjust to the new condition. In the immediate postpartum period, the animal is in negative energy balance (NEB) due to the greater energy expenditure caused by milk production and the decreased physiological ingestion of dry matter. There is therefore a reduction in blood glucose, an increase in GH and a decline in T4 (temporary hyperthyroidism), an alteration in the insulin/glucagon ratio tilting in favour of the latter, a different break down of glucose between SNC and teat, and an activation of neoglucogenesis. All this is translated into use of the body's stores, and into the animal's growing thinner. The animal has priorities in energy use and, in particular, exploits energy to maintain basal metabolism, circulation, neuronal activity, and thermoregulation. In the event of an energy deficit (negative energy balance – NEB), it can reduce milk production, growth, and locomotion, and may suspend reproductive activity.

Seasonal anoestrus: Seasonal anoestrus is a natural phenomenon that guarantees the birth of offspring during the period most favourable in terms both of weather conditions and of abundant food, providing assurance of survival in nature. The season influences various aspects of reproduction, such as the quality of oocytes, the characteristics of the oviduct fluid, and the concentrations of progesterone (*Campanile and Neglia, 2007*). Generally, the dosage of progesterone in the milk, as an element assessable for the purposes of a pregnancy diagnosis, presents an average reliability of 80%, with variations during the autumn and spring periods of 93.5% and 64.3% respectively (*Zicarelli et al., 1987*). The lower reliability recorded during the spring might be explained with the greater number of oestrus cycles and the increased incidence of embryonic mortality that takes place during this period. This condition is found above all in primiparous heifers (more accentuated NEB) and in pluriparous cows more than 10 years of age. In a study conducted by Professor Zicarelli, the phenomenon of seasonal anoestrus was found to be more present in primiparous heifers (about 80%) than in pluriparous cows (circa 44%) calving between late winter and early spring. Studies relating to this phenomenon were also carried out at a later time (*Borghese et al., 1993; Zicarelli, 1994*).

Seasonal anoestrus causes enormous economic damage to the breeder, since it results in a lengthened calving interval, slowed genetic progress, production of milk during periods when there is less market demand, and increased spending for the therapeutic treatments needed to induce oestrus. To maintain a calving interval of 13-14 months (390-420 days) in buffalo cows,

fertilization must be achieved in the first 85-115 days following calving (*El-Wishy, 2006*). It may be calculated that acyclic buffaloes during the first 70 days post partum extend the calving interval by approximately 28 days for primiparous heifers, and by 62 days for pluriparous cows (*Zicarelli et al., 1985*). In fact, only 31.9% of buffaloes that are acyclic during the first 70 days after calving manage to conceive within 120 days; 68.1% of the animals conceive more than 200 days after calving (*Di Palo et al., 1993*). This has allowed temporary anoestrus to be differentiated from deep anoestrus. The former is seen in animals that conceive within 150 days, and that generally calve in early spring: these animals show a tonic uterus, and smooth ovaries with small/average follicles that are unable to ovulate. Deep anoestrus, on the other hand, is seen in animals that calve in the winter and that conceive after 150 days. In these animals, the uterus is not tonic, the ovaries are small and hard, and no follicle is clinically noted.

Based on the time of calving, the condition of seasonal anoestrus changes: in it, the animals that calve in the spring encounter (progressively) a reduction in hours of daylight, and the animal therefore enters into the ideal season for reproductive purposes. Otherwise, the animals that calve during the winter encounter a period of growing light, and if they are not impregnated within 70/90 days after calving, they thus enter into a deep anoestrus.

The incidence of anoestrus and the activity of the corpus luteum are also influenced by the bull's presence within the herd (*Campanile et al., 1992*). This phenomenon was confirmed by subsequent studies done on buffalo cows raised, during the transition period, with vasectomised bulls (*Zicarelli et al., 1997*). These animals showed a greater incidence of spontaneous oestrus (from 69% to 92%) and a greater pregnancy rate following both natural (42.5 vs 18.9%) and artificial (51.1 vs 33.3%) service, than those where the bull was not present (*Zicarelli et al., 1997*). A reduced incidence of animals going into anoestrus can be obtained through the selection of animals less sensitive to the photoperiod, proper nutritional management, proper postpartum management, and the use of hormonal treatments. Good postpartum management along with the administration of oxytocin and $PGF_{2\alpha}$, allows us to have greater uterine contractility and, consequently, a rapid resumption of the uterus involution process. Of course, it is indispensable to maintain hygienic conditions on the level of the uterine cavity that can be obtained through infusions of disinfectants and, where applicable, broad-spectrum antibiotics.

To reduce seasonal anoestrus, a variety of hormonal treatments, based on both progesterone and on GnRH, have also been used. Progesterone-based treatments involve the use of intravaginal devices, subcutaneous devices, or intramuscular injections for six days. These treatments are able to remove anoestrus in up to 100% of the animals (*Borghese et al., 1993; Neglia et al., 2003*). Treatment with

the intravaginal release device has been used in acyclic buffaloes subject to artificial insemination (*Neglia et al., 2003*), bringing to 100% the animals showing a resumption of ovarian activity, and a 50% pregnancy rate. Treatments with subcutaneous devices or subcutaneous injections, on the other hand, have guaranteed a treatment-conception interval ranging between 27 and 42 days. Progesterone-based treatments (PRID®, CIDR®, CRESTAR®, parenterally-administered progesterone), on their own or in combination with gonadotropin, have proved effective in stimulating ovarian activity during the hot season. PRID® in association with PMSG has yielded a 65.3% pregnancy rate, against 54.5% for PRID alone®. The interval between the removal of the PRID® device and LH peak is about 61.0 ± 12.05 hours (against 46.9 ± 21.53 hours in winter controls), so that a double insemination is generally recommended for increasing the conception rate. An alternative consists of fixed-time insemination through the use of prostaglandin PGF2α or similar compounds. GnRH may be administered in association with the protocols to reduce the variation of LH peak times and to improve the synchronization of ovulation. The administration of gonadotropin to stimulate the resumption of cyclic activity has been shown to be a failure, due probably to the brief half-life and the follicular cohorts' variable sensitivity to gonadotropin.

2.6. Embryonic mortality

Embryonic development in the buffalo is quicker than in bovines, and this justifies the earlier formation and functionality of the corpus luteum in the former than in the latter. It in fact appears that the relationship between initial development of the zygote, functionality of the corpus luteum, preparation of the uterus, and the mother's recognition of the pregnancy is linked to the time factor in buffaloes more closely than in bovines. The embryo implantation phase would appear to be a critical period for determining the outcome of reproduction in the buffalo (*Campanile et al., 2010*). Interruption of pregnancy is correlated with delayed resumption of the cyclic activity, and in fact involves only 5% of cyclic animals, against 16% of buffaloes that are acyclic 70 days after calving.

Embryonic mortality is the second cause of hypofertility in buffaloes, especially in animals subjected to the OBSM technique. It is differentiated into early embryonic mortality (EEM), which tales place before 21 days after insemination, and late embryonic mortality (LEM), which takes place between 21 and 45 days after insemination (*Campanile et al., 2016*). Both negatively influence the herd's reproductive efficiency. When the embryo reaches the uterus (about 5 days after fertilization), it must be able to send the mother the signal preventing luteal regression, maintaining a suitable level of plasma P_4 to enable the production of all the uterine secretions necessary for embryonic implantation and development. The pregnancy is maintained thanks both to the embryo's ability to send a signal of recognition, and to the mother's ability to receive this signal and to maintain a uterine environment adequate for maintaining the pregnancy. Towards this end, the product of conception produces a protein, Interferon-tau (*Roberts et al., 1992*), capable of preventing luteal regression by means of 2 mechanisms:

- By preventing the development of oxytocin receptors in the endometrium (*Robinson et al., 1999*);
- By activating a prostaglandin antagonist (*Thatchter et al., 1995*).

The overall results of a recent study suggest that cortisol acts as a modulator and/or mediator of IFN-Tau actions in the bovine uterus (*Majewskaet al., 2012*). The main cause for EEM lies in the embryo's inability to be recognized by the mother, thus preventing luteolysis. Studies on buffaloes (*Campanile et al., 2010a*) have demonstrated the relationship between EEM and delayed secretion of P_4. This phenomenon is in fact reduced through the use of pharmacological protocols that increase the levels of P_4 in circulation (*Campanile and Neglia, 2007; Campanile et al., 2008b; Campanile et al., 2016*). Treatment with P_4, hCG, and GnRH on day 5 after insemination increases

the concentration of P_4 in circulation, and reduces early embryonic mortality. The same treatment can be applied from day 20 to day 25 after insemination, to reduce late and foetal embryonic mortality (*Campanile et al., 2016*).

The fundamental role of progesterone is to allow the embryo to implant. A decline in P_4 on the tenth day after insemination can reduce the embryo's vitality and cause problems in the pre-implantation phase (*Russo et al., 2010*). P_4 has a higher value on day 10 after insemination in buffaloes pregnant on day 45 after insemination. (*Campanile et al., 2005*): in fact, the period between 5 and 10 days after insemination is important for the growth of the corpus luteum and crucial for establishing a pregnancy (*Vecchio et al., 2012*).

Maintaining gestation is strongly correlated with the vascularisation of the corpus luteum and of the pre-ovulatory follicle (*Russo et al., 2010; Neglia et al., 2012*). Proving this, it is observed that ultrasound examinations on day 25 demonstrate that poorly developed embryos suffer more from LEM (*Neglia et al., 2012*).

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Chapter 3

Estrus synchronization and artificial insemination

CHAPTER 3

3.1. Reasons for estrus synchronization

A method for avoiding the problem of detecting heat might be that of oestrus synchronization, which would make it possible to set a fixed time for IS without having to observe heat, thereby making herd management simpler. It would in this way be possible to:

- ✓ concentrate calving, and thus concentrate maximum milk production during the months of greatest market demand – the summer months;
- ✓ create homogeneous groups of animals;
- ✓ restore proper ovarian activity in animals with post partum and seasonal anoestrus;
- ✓ reduce to a minimum the number of treatments per/conception.

Oestrus synchronization relies on the use of hormonal treatments that induce heat, and therefore ovulation. However, this biotechnology has thus far yielded decidedly variable results, comprised between 25 and 60% of pregnancies (*Zicarelli, 1997c; Baruselli et al., 1999; Baruselli, 2001; Neglia et al., 2003; Neglia et al., 2020*). Whatever protocol is used, synchronization appears to work especially if the buffalo cows are cyclic. This depends on the season, the climate, the stage of lactation, and the presence/absence of the bull. The best results are obtained in the autumn, a period when the duration of oestrus is shorter, and the interval between heat and ovulation is more similar to that in bovines.

To date, several different synchronization protocols are proposed for buffaloes. In particular, the hormones used to control the animals' reproductive cycle may be distinguished in three large categories:

1) **Hormones that act upon the emergence of the follicular wave**: such as the gonadotropin releasing hormone (GnRH), lutenizing hormone (LH), human chorionic gonadotropin (hCG), estradiol + progesterone ($E_2 + P_4$).
2) **Hormones that act upon luteal regression**: such as prostaglandin ($PGF_{2\alpha}$), estradiol (E_2) and progesterone (P_4).
3) **Hormones that allow ovulation to be controlled**: such as gonadotropin releasing hormone (GnRH), lutenizing hormone (LH), human chorionic gonadotropin (hCG), estradiol (E_2).

Those most used in the field will be discussed below.

3.1.1. Double prostaglandins treatment

Several analogues of prostaglandin (cloprostenol, luprostiol, dinoprost, etc) are actually used in buffaloes. These molecules' effectiveness is attributed to their luteolytic action on the corpus luteum, which triggers a rapid diminution of progesterone within 24 hours (*Bachalus et al., 1980*) and, consequently, to ovulation, which is recorded within 60 – 72 hours. A rapid decline in luteal blood flow would appear to be one of the main luteolytic actions of the prostaglandins and their analogues. This diminished luteal blood flow occurs during both normal and $PGF_{2\alpha}$-induced luteolysis (*Knickerbocker et al., 1988; Azmi et al., 1982*). Generally, at least in bovines, luteal regression is initiated by an exogenous injection of prostaglandins released after the 7^{th} day in the normal oestrus cycle (*Schallenberger et al., 1984*). This is due to the increased intraluteal production of vasoactive substances like endothelin-1 (ET-1) (*Ohtani et al., 1998*) and angiotensin II (Ang II) (*Hayashi et al., 2001*), both of which play an important role in the luteolytic cascade (*Miyamoto et al., 1997; Meidan et al., 1999*). To the contrary, the role of prostaglandins during the first days in the oestrus cycle, when there is no active corpus luteum present in the ovary, is still unclear, although some studies have demonstrated a higher pregnancy rate in buffalo cows treated with cloprostenol at the moment of AI (*Neglia et al., 2008*). The protocols, which are based on the use of prostaglandins, call for two administrations, 9-11 days apart. In fact, as specified above, the prostaglandins are able to induce luteolysis only after day 5 of the cycle and within day 17-19 (depending on the length of the cycle). Therefore, a single administration would be able to induce oestrus only in animals showing an active corpus luteum (dioestrus phase). On the other hand, in the animals in oestrus, metaoestrus (0-3 days of the cycle) or proestrus (18-22 days of the cycle), and that therefore have no active corpus luteum and do not respond to the first administration of prostaglandins, a response to the second administration is supposed, since they should be in dioestrus after 9-11 days. The main limits of this protocol are represented by the period of the year when it is used, and the difficulty of predicting ovulation. In reality, due to seasonality (*Zicarelli, 1997*; *Campanile et al., 2009*), this protocol is recommended particularly during the reproductive season, when a high incidence of cyclic animals is recorded. In this case, a second treatment with prostaglandin induces oestrus and ovulation in more than 80% of buffalo cows, with a 50% pregnancy rate (*Dhaliwal et al., 1988*; *Brito et al., 2002*), regardless of the mode of administration of the prostaglandin (*Dhaliwal et al., 1987*). To the contrary, it is difficult to record a pregnancy rate exceeding 25-30% outside of the reproduction period (*Chohan et al., 1995*), even though a high number of buffaloes show signs of oestrus (*Sahasrabudhe& Pandit, 1997*). Moreover, it bears

emphasizing that the incidence of heats characterized by a subsequent good luteal phase after treatment with $PGF_{2\alpha}$ is also influenced by the bull's presence (*Zicarelli et al., 1997*). It has been observed that following the administration of prostaglandin, there is a strong variation in heat duration, oestral behaviour (36 to 96 hours) and ovulation (from 60 to 100 hours) (*Baruselli, 1994*; *Porto-Filho et al., 1999*). This is particularly evident if the cows are treated before or after day 10 of the cycle, thus confirming that the success of the administration of prostaglandin depends on the presence of functional corpora lutea in a specific phase of the oestrus cycle (*Porto-Filho et al., 1999*). Lastly, it is noteworthy that this has also been used to perform a preliminary screening (*Pre-synch Protocol*) of head that can be subsequently synchronized, both in bovine (*Moreira et al., 2001*) and buffalo (*Oropeza et al., 2010*) species. This leads to a higher pregnancy rate both for the choice of cyclic animals and for a better response to other, subsequent synchronization treatments.

3.1.2. Progestin-based treatments

Progesterone or progestinics can be used via various modes of administration: intravaginal, by ear implant, or injectable (in the countries where this is allowed). These protocols are particularly utilized in buffaloes out of the breeding season, because it has been widely demonstrated that progesterone is able to act on the hypothalamus-pituitary-ovarian axix, with a consequent resumption of ovarian cyclicity in anoestrus animals (Rhodes et al., 2003; Neglia et al., 2003**;** Baruselli, 2001). Several hypotheses have been performed to explain the effect of progesterone or progestinics treatment on hormone concentration and follicular development (Rhodes et al., 2003). In fact, as progesterone concentrations reach levels similar to those recorded in nature for luteal activity during treatment, there is an increase in LH pulse-frequency, associated with increased estradiol synthesis (Rhodes et al., 2002). Therefore, sensitivity of the hypothalamus to the negative feedback effects of estradiol is reduced, the follicular growth is stimulated and the largest ovarian follicle matures and responds to exogenous estradiol or a gonadotrophin (Rhodes et al., 2003). For this reason, treatment with progesterone is often associated with estradiol (benzoate or valerate) and/or Pregnant Mare Serum Gonadotrophin (PMSG).

Another hypothesis is based on studies done on rat pituitary cells, demonstrating that progesterone blocks GnRH growth at the hypothalamic level and at the same time acts at the level of pituitary beta cells, thus determining the synthesis of gonadotropins, which, however, cannot be released (*Park et al., 1996*). Since progesterone in vivo decreases the frequency of GnRH pulses secreted into the hypothalamic–hypophyseal portal circulation (Karsch et al. 1987) and GnRH is also an important regulator of the number of GnRH receptors, it has been hypothesized that progesterone could decrease pituitary responsiveness to GnRH by reducing the frequency of GnRH pulses, which in turn would lead to reduced synthesis of GnRH receptors (Nett et al., 2002). It is likely that exogenous progesterone supplementation may increase LH storage in pituitary gland and augment GnRH induced LH release (Nett et al. 2002). This is supported by the evidence that an increase in pituitary content of gonadotropins following treatment with progesterone is observed in ovariectomized and hypothalamic-pituitary-disconnected ewes (Di Gregorio & Nett, 1995) probably for a double effect, increased protein synthesis and lack of secretion of LH and FSH, due to removal of GnRH.

Usually treatment with progesterone in buffalo is performed by using intravaginal device (containing 1.5 to 1.9 g of natural progesterone) for 10-12 days, and blood concentrations of 4-5 ng/ml are reached (Neglia et al., 2003). Auricular implants, containing norgestomet (17a-acetoxi-

11b-metil-19-norpreg-4-en-3,30-diona), a progesterone analogous, that is more potent than natural progesterone, are also utilized to synchronize estrus cycle and ovulation. Since norgestomet is more potent than natural progesterone, lower doses are required. During the treatment (day 6 or day 7 after insertion), or when the device is withdrawn, it is recommended to perform a prostaglandin administration, in order to remove corpora lutea on the ovaries. The day of prostaglandin and PMSG administration does not seem to affect pregnancy rate after AI in adult buffaloes, that is reported between 30 and 50% (Neglia et al., 2003; Barile et al., 2007). This treatment is also utilized to synchronize estrus cycle in buffalo heifers (Barile et al., 2001). In fact, it is known that the age at puberty in buffalo species ranges from 16–22 to 36–40 months in different Countries (Borghese et al., 1994) and the delay in puberty, and the consequent delay in conception, is one of the problems that causes the low reproductive efficiency of this species. Although many factors may affect age at puberty, such as breed, season climate, nutrition and growth rate, it has been demonstrated that mimicking the hormonal changes occurring around puberty can induce sexual maturity in heifers. Treatments based on progesterone releasing intravaginal device, are able to anticipate the puberty, when applied in animals at peripubertal age (Barile et al., 2001). Therefore, progesterone plus PMSG treatment of heifers may have a strong economic impact on buffalo production because a greater proportion of heifers could be bred earlier than with other synchronization protocols.

3.1.3. Ovsynch-TAI Program

Due to the difficulty of detecting oestrus in buffalo cows, the most commonly used oestrus synchronization protocols are based on monitoring follicular development and ovulation. These techniques permit fixed times for artificial insemination (AI), thus avoiding the observation of oestrus and facilitating the herd's reproductive management and the use of these biotechnologies in the field. In fact, the administration of GnRH makes it possible to have peaks of LH, FSH, and estradiol during any phase of the oestrus cycle, which will promote the ovulation of a dominant follicle or the luteinization and/or atresia of the pre-dominant follicles. Consequently, a new follicular wave emerges two or three days later (*Pursley et al., 1995*).

The response to the LH peak is influenced by the dose of GnRH and by the diameter of the dominant follicle. In fact, 100 mg of natural GnRH (gonadorelin) can induce ovulation in 100% of treated buffalo cows, against the 33% obtained with 50 mg (*Rastergania et al., 2004*).

In any event, the administration of GnRH in the buffalo induces ovulation in 60-86% of treated animals (*Baruselli, 2001; De Araujo et al., 2002; Neglia et al., 2003b; Neglia et al., 2016*) and the interval between the administration of GnRH and ovulation is 33±8.3 hours (*De Rensis & Lopez-Gatius, 2007*).

Different synchronization protocols have been conceived to monitor follicular development and ovulation, by extrapolating information on studied species, like bovines and sheep. All these methods are based on the ovulation of the dominant follicle and the regression of the corpus luteum using prostaglandin. The protocol far more used is the Ovsynch-TAI program, developed for cattle (*Pursley et al, 1995*) and successfully applied to buffaloes as well (*Baruselli et al., 1999; De Araujo et al., 2002; Neglia et al., 2003b; Neglia et al., 2016*), which consists of an injection of GnRH on day 0, an injection of prostaglandin on day 7, and an additional injection of GnRH on day 9, with fixed-time artificial insemination performed on day 10 (about 72 hours after the administration of prostaglandina and 16-18 hours after the last administration of GnRH).

It has been shown that a percentage equal to 78-90% of treated buffalo cows respond to synchronization treatment, with a conception rate varying between 33 and 60% (*Baruselli, 2001; Neglia et al., 2003; Paul &Prakash, 2005*). These pregnancy rates tend to diminish if the treatment is applied during the transition period (which coincides with the passage from reducing to increasing hours of daylight), due to the high incidence of embryonic mortality (20-40%)

(*Campanile & Neglia, 2007*), and during the non-reproductive season, due to the high incidence of buffalos in anoestrus, that are unable to respond to treatment (*Baruselli, 2001*).

Recent studies have shown that synchronization, and, as a consequence, the pregnancy rate can, with this treatment, be increased by the response to the first administration of GnRH (*Neglia et al., 2016*). In fact, animals that present ovulation after the first administration of GnRH show ovulation and the start of the new follicular wave that makes it possible to obtain, at the moment of AI, a young follicle, without atresia, inside of which there is a non-degenerated oocyte capable of being fertilized. One of the factors conditioning the response to the first GnRH is the presence of a functional corpus luteum, which permits follicular growth under the influence of high progesterone rates, thereby improving oocyte maturation.

The *Ovsynch-TAI* treatment has been successfully used in the reproductive management of the buffalo herd, thereby permitting a considerable reduction of the calving interval. Recent studies (*Rossi et al., 2014*) have in fact demonstrated that this protocol can be used throughout the year, by subjecting the buffalo cows to a precise and careful management that starts as early as during the postpartum phase. Immediately after calving, all the animals receive a double dose of prostaglandin, which is useful for improving the lochia flow, and as early as the 37^{th} day after calving, the first dose of GnRH in the *Ovsynch* program is administered. On day 43, the animals are sonagrammed to assess the functionality of the corpus luteum, using echo-colour Doppler: all the buffalo cows that do not have a functional corpus luteum are discarded and resynchronized, while the others are administered prostaglandin. At the time of AI, an additional ultrasound is performed, with the purpose of assessing the presence of a follicle of a size exceeding 2 cm: in this case as well, the buffaloes that do not present this characteristic are discarded and resynchronized, while the remaining ones are inseminated. On day 20 after insemination, all the animals receive a dose of GnRH, with a dual purpose: in pregnant animals, an increase in serum levels of P_4 can be determined, due to the formation of an accessory corpus luteum, while, for non-pregnant animals, it is the first GnRH of the *Ovsynch-TAI* protocol. On day 27 after AI, the pregnancy diagnosis is made by ultrasound: in this case as well, the non-pregnant animals with a functional corpus luteum receive an injection of prostaglandin, two days after an additional administration of GnRH, and on day 30 a second IS. This protocol may be repeated up to 7 times, although it has been shown that with the first three inseminations, more than 90% of the animals can be impregnated (*Rossi et al., 2014*), thereby obtaining a considerable reduction of the calving interval (just over 400 days, as against a calving interval of approximately 460 days obtainable by natural service). In this regard, it is also to be borne in mind that recent studies have demonstrated the possibility of making a very

early “non-pregnancy” diagnosis as early as day 8 after AI, by assessing the characteristics of the corpus luteum between the 5th and the 10th day after AI (*Neglia et al., 2014*). This method, clearly to be considered still experimental, has good reliability and specificity, and might permit additional improvements in the efficiency of buffalo AI in the near future.

A treatment similar to the *Ovsynch-TAI* program is the *Co-Synch* (*Stevenson, 2011*), in which a second injection of GnRH is performed simultaneously with the AI (72 hours after the administration of the prostaglandins). There are no experiences in buffalo with this treatment, although in recent years the standard *Ovsynch-TAI* protocol has been associated with an additional administration of GnRH on day 10 (day of AI) and an injection of prostaglandin, in order to better synchronize ovulation and increase the levels of progesterone of the corpus luteum that will be formed (*Neglia et al., 2008*).

3.1.4. Ovsynch-TAI Program + Progesterone

This treatment is an improvement of the *Ovsynch-TAI* protocol (*De Rensis et al., 2005*). In this case, at the moment of the first GnRH, an intravaginal, progesterone releasing device is inserted and maintained in place for 7 days. At the time of removal, the prostaglandins are administered, and two days later the GnRH, with fixed-time insemination on day 10 (in this case as well, about 72 hours after the prostaglandin and 16-18 hours after the GnRH). The protocol is particularly effective on animals in seasonal anoestrus, permitting the resumption of cyclic activity and a good response to the prostaglandins. The chief limit of this protocol is the cost which, albeit justified by the considerably increased conception rate (about 50% - obtainable also with *Ovsynch-TAI* alone – during the period of highest fertility, but the only one capable of increasing the rate outside this period as well), remains particularly high.

3.2. Artificial Insemination (AI) in buffalo

What has been described thus far provides an idea of how complicated reproductive management in buffalo is, and how many factors are to be taken into consideration to optimize it. This is even more difficult when intending to apply certain reproductive biotechnologies such as artificial insemination (AI). Its limited use in the buffalo is due to many factors, including the low number of tested bulls, the difficulty of identifying oestrus, and its not always encouraging efficiency. Due to its difficult use in the buffalo, it was in the past used nearly exclusively for progeny testing, although in recent years its employment has considerably increased following the application of innovative methods capable of guaranteeing a considerable improvement in the species' reproductive performance.

The essential point for the proper and fruitful use of AI is correctly identifying the favourable moment for insemination, in order to guarantee the encounter between the spermatozoa and the female gamete in the fallopian tube. Insemination done at too great an interval after ovulation may contribute towards lowering the conception rate, due to the limited vitality of the spermatozoa, and above all of the egg cell (*Brackett et al., 1980*; *Campanile et al., 1988*). Identifying the optimal moment for insemination is rather labour intensive: it would in practice be necessary to repeatedly explore the female genital apparatus until the follicle is near dehiscence. However, quite often, even this is insufficient since, although there is the clinical sensation of the follicle about to burst, this same follicle can reach dehiscence in a period ranging between 12 and 72 hours or can even fail to burst at all, and enter into luteinization.

It must therefore be specified that the farms engaged in using artificial insemination must be organized in such a way as to reduce to a minimum the problems discussed above, committing themselves to a management of the buffalo herd that permits the correct detection of heat, or other practices like synchronization. In particular, it is important:

- ✓ To see that puberty is reached as quickly as possible.
- ✓ To regularly check – with veterinary assistance and, where applicable, the aid of ultrasound – the physiological, reproductive, and pathological state of the genital apparatus, as well as the oestrus phase in each animal.

- ✓ To apply, where necessary, pedometers, and to constantly record the data on the animals' movements.
- ✓ To properly use and stock the frozen semen to be used in artificial insemination, in order to guarantee good spermatozoa motility and concentration.
- ✓ To properly apply the oestrus synchronization protocols developed for buffalo.
- ✓ To see to proper application of artificial insemination both by the veterinary concern and by animal raising operatives.

3.3. AI technique

The AI technique performed for the buffalo is similar to that described for bovines. Before the semen is thawed, a careful sonographic gynaecological checkup should be performed to assess follicular diameter (which should be more than 1 cm), indicative of the peak state of oestrus, and therefore the right moment for artificial insemination (C*ampanile et al., 2009; Rossi et al., 2014*).

One of the great advantages of this technique is the non-invasiveness of the procedure, and its ability to be performed several times on the same animal, with no negative effects. Normally, the buffalo's reproductive apparatus is checked through endorectal palpation and the use of linear ultrasound sensors; during the procedure, the operator removes the faecal material from the rectum, introduces the sensor, and scans the uterine horns and the ovaries.

The insemination device used in the buffalo is the same used for bovines. One of the chief steps for success of AI is the management of the semen which, once frozen in liquid nitrogen at a temperature of -196°C, can be conserved for an undetermined time. The critical temperature is about -80°C; semen exposed to temperatures exceeding this (even for a brief period of time) and then returned to the storage tank can be irreversibly damaged. The amount of damage depends on how much time the semen is exposed to high temperatures. The recommendation for a proper thawing of semen straws involves heating in hot water at 32-35°C for at least 40 seconds, after which the straw is carefully dried and inserted into the insemination gun. To prevent the transmission of disease and contamination, protection sheaths must be used, as well as sanitary smocks, where possible.

Before performing the AI, the vulva region must be cleaned with a single-use material to keep the inside of the reproductive tract from being contaminated and potentially infected. The last step, which is essential to the procedure, is depositing the semen. The semen must be deposited in the body of the uterus, after passing through the cervix. The body of the uterus in the buffalo is shorter than in bovines, and for this reason the operation must be done with caution. The right site for depositing the semen may recognized from the change in the consistency of the uterine tissue: from hard and compact in the region of the cervix and the cervix itself, to soft and spongy in the body of the uterus. However, the use of ultrasound can allow the structure of the endometrium and myometrium (physiologically thickened during the oestrus phase) to be assessed, and the possible presence of the preovulatory follicle on the right or left ovary to be detected: in this way, the semen might be positioned near the horn where the presence of the follicle was detected.

However, this technique would not be wholly advisable in buffalo cows; several studies done using sexed semen have in fact recorded a lower conception rate in animals inseminated in the horn, compared with those inseminated in the body of the uterus (*Campanile et al., 2011*). The authors attributed this worsening to the enormous variability in terms of the animals' ovulation (double ovulations, etc.), that do not allow the horn where the follicle's ovulation will take place to be fully predicted.

3.4. References

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Chapter 4

The color doppler technique

CHAPTER 4

4.1. Doppler ultrasonography in animal breeding

The advent of B-mode ultrasonography in bovine reproduction in the 1980s promoted tremendous advances in research and clinical practice, because it allowed non-invasive visualization of the internal reproductive organs. Although organ morphology can be evaluated using this technique, it cannot, however, provide information about the organ function, such as vascular perfusion. For the past 10 years, color Doppler sonography is being increasingly used for blood flow studies. Bollwein et al. (2000, 2002a) used this imaging method to demonstrate changes that occurred in uterine circulation during the estrous cycle and pregnancy in cows. Doppler sonography has also been used to investigate blood flow to the ovaries (Acosta et al. 2002, 2003, 2005; Miyamoto et al. 2005).

4.2. Principles and Techniques of Color Doppler Sonography

Diagnostic ultrasound originates from crystals that have piezoelectric properties and are activated by an electric charge. As a result, the crystals expand and contract, thus emitting sound waves (Dudwiesus et al. 1993). These ultrasound waves penetrate the body and are reflected by the various tissue surfaces depending on their acoustic impedance. When ultrasound waves hit static surfaces, the frequency of the reflected echoes corresponds to that of the waves emitted. In contrast, when the waves strike moving structures, such as red blood cells, the frequency of the reflected waves changes from that of the emitted waves, resulting in a Doppler shift. This shift is positive, i.e. the frequency of the reflected waves is higher than that of the emitted waves when the red blood cells move towards the transducer. When the blood cells move away from the transducer, the frequency of the reflected waves is lower than that of the emitted waves, and then the Doppler shift becomes negative. The Doppler shift Δf can be calculated using the following formula:

$$\Delta f = 2 * f0 * v * \cos\alpha / c;$$

where:

- ✓ *f0* is the frequency of the emitted ultrasound waves;
- ✓ *v* is the velocity of the reflector relative to the transducer;
- ✓ α is the angle between the ultrasound beam and the direction of movement of the reflector;
- ✓ *c* is the speed at which the ultrasound waves are dispersed within a tissue.

In ultrasound machines with color Doppler capacity, the Doppler shifts are color-coded on the screen. Positive shifts (blood flow towards the transducer) are usually indicated in red and negative shifts (blood flow away from the transducer) in blue. The brightness of the color pixels indicates the amplitude of the frequency shift: the higher the frequency shift, the brighter the pixels.

The two types of Doppler systems currently in use are continuous-wave Doppler and pulsed-wave Doppler. Continuous-wave Doppler incorporates two separate piezoelectric crystals: one crystal continuously emits waves and the other continuously receives the reflected waves. Continuous-wave Doppler is able to measure very high blood flow velocities. A disadvantage is that there is no

depth selection and that all frequency shifts and thus, all blood flow in all the vessels within the range of the ultrasound beam is imaged (Marsal 1993). With the pulsed-wave Doppler system, on the other hand, one can select the depth at which the Doppler shift is received, and the blood flow in specific vessels can be evaluated selectively. In this system, the same piezoelectric crystals alternately emit and receive the ultrasound waves. The Doppler wave only consists of single segments because of the alternating pattern of emission and reception of the waves. To reconstruct the wave form accurately from the individual impulses, the pulse rate must be twice that of the Doppler frequency, which limits the maximum detectable speed (Dickey 1997).

4.3. Evaluation of Color Doppler Sonographic Findings

In order to describe the color Doppler sonography of blood flow in bovine reproduction, qualitative, semi-quantitative and quantitative methods are differentiated (Dickey 1997). Blood flow can be evaluated qualitatively analyzing by subjectively the number and brightness of the color pixels or the pitch and volume of the sound produced by the Doppler shift (Marsal 1993). Describing the shape of the Doppler wave is another qualitative method of assessment; various classification schemes have been developed for this purpose (Dickey et al. 1994; Delorme and Zuna 1995; Tekay et al. 1996) and are based mainly on the presence and continuity of diastolic blood flow. In addition, changes in the direction of blood flow are recorded during a cardiac cycle. However, this classification of the Doppler waves does not allow for differentiation of subtle changes in ovarian and uterine blood flow during the estrous cycle (Dudwiesus 1995).

Blood flow is typically evaluated semi-quantitatively using the so-called Doppler indices. These indices measure the downstream impedance to blood flow and are only indirect estimates of the flow volume. They are most useful for estimating the blood flow resistance in vessels distal to the point of examination. As the values of the indices increase, so does the blood flow resistance and vice versa (Dickey 1997). The Doppler indices are relative quantities obtained from the maximum systolic (S), minimum (Min), end-diastolic (D) or mean frequency shift (Mean) during one cardiac cycle (fig. 3).

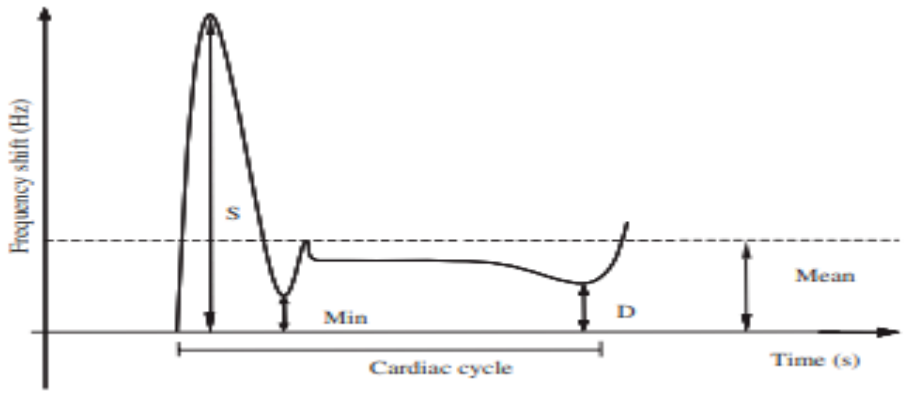

*Fig.3. Schematic representation of a Doppler wave with the maximum systolic (S), minimum (Min), end-diastolic (D) and mean (Mean) frequency shift during one cardiac cycle (*K. Herzog and H. Bollwein, 2007).

This means that knowledge of the angle α, which is required for determination of blood flow velocity, is not required (Maulik 1997). The resistance index (RI) is calculated using the formula:

$$RI= (S-D)/S; (fig3).$$

However, differentiation of blood flow with an end-diastolic flow that goes to 0 is not possible using this index because, by definition, it assumes the maximum value of 1 (Chaoui et al. 1989; Dickey 1997). The pulsatility index (PI) is used to analyze this type of blood flow, whereby two indices can be differentiated: the peak to peak pulsatility index (PPI) and the PI. The PPI is used in tissues with a high vascular resistance, in which there is backflow of blood during diastole. The PPI measures the total distance from the top to the bottom of the systolic peak, and divides this by the mean velocity over the cardiac cycle. It is expressed as:

$$PPI= S/velocity\ M.$$

Where:

- ✓ *S* is the peak systolic velocity;
- ✓ *Velocity M* is the time-averaged maximum velocity (TAMV) over the cardiac cycle.

The PI, also known as the Mean PI, is expressed as:

$$PI= (S - D)/velocityM$$

Where:

- ✓ *S* is the peak systolic velocity;
- ✓ *D* is the end diastolic velocity;
- ✓ *velocityM* is the TAMV over the cardiac cycle (Gosling et al. 1971).

Velocity is calculated from the average of three or four cardiac cycles. Because it does not go to 1.0 if early or end-diastolic flow goes to 0, the PI is often used for vessels where flow is absent during the entire diastole or parts thereof (Dickey 1997). For quantitative evaluation of blood flow, the blood flow volume (BFV) is determined using the formula:

$$BFV = v * A$$

Where:

- ✓ *v* is the blood flow velocity;
- ✓ *A* is the surface area of the vessel in cross-section (Dudwiesus 1995).

The blood flow velocity *v* is obtained from the Doppler shift formula:

$$v = \Delta f^* c/2^* f0 \ ^*\cos\alpha;$$

and the cross-sectional area of the vessel is calculated from its diameter, which is measured using B-mode ultrasonography. However, the latter is difficult to measure because the pulsation of the blood flow during the cardiac cycle results in changes in the arterial lumen (Loch 1995). Relative errors that occur in the calculation of the BFV have a lesser impact on vessels with large diameters (Dudwiesus 1995). Another important source of error in the calculation of the BFV is the determination of the blood flow velocity. For this, the angleα between the Doppler beam and the direction of blood flow must be set manually by the examiner. Because the BFV is, among other factors, a function of $\cos\alpha$, a small angle is associated with a much smaller error in the calculation of the blood flow velocity than a larger angle. The aim, therefore, was to maintain as small an angleα as possible between the Doppler beam and the blood vessel.

4.4. Luteal blood flow

Transrectal color Doppler sonography is being used in recent years to evaluate ovarian perfusion, especially luteal and follicular blood flow (Acosta et al. 2002, 2003, 2005; Miyamoto et al. 2005, 2006). Baumgartner investigated the cycle-associated changes in the luteal blood flow using transrectal color Doppler sonography (Baumgartner 1998). The results of these studies indicated that the morphological changes of the corpus luteum (such as size) are not as pronounced as the physiological changes of the corpus luteum, such as the luteal blood flow and the peripheral progesterone concentration in cows.

The study of luteal blood flow reflected luteal function better than luteal size specifically during luteal regression in cows. Relating to the whole estrous cycle, correlation between LBF and P_4 is higher than the correlations between the cross-sectional area of the CL (luteal size [LS]) and P_4 (Herzog, 2010). It has also been observed that angiogenesis is positively correlated with blood P4 concentrations during CL development, and the apoptosis of endothelial cells is involved in both functional and structural luteolysis (Fraser and Wulff, 2003). The close association between LBF and P_4 is explainable because steroid precursors are provided to the CL via blood supply and the release of P_4 into the circulation is also dependent on adequate luteal blood flow (Janson, 1981).

Regarding important features of luteal blood flow, a study demonstrated that the blood flow area (BFA) measured on Day 7, and paired BFA and TAMV recorded on Day 14 could represent reliable predictors of pregnancy in cows (Kanazawa et al., 2016). Indeed, the most important application for the assessment of CL blood flow is the early detection of non-pregnant females (Neglia et al., 2015; Samir and Kandiel, 2019; Siqueira et al., 2019). The latter approach has the potential to improve buffalo reproductive efficiency by allowing earlier resynchronization of open females and, consequently, a reduction in the interval between services.

Several studies have been performed applying the color Doppler technique in buffalo. Pregnancy in buffaloes was associated with greater blood flow to the CL on Days 10, 20, and 25 after AI (Vecchio et al., 2012; Russo et al., 2012). There is evidence to suggest that greater activity of the CL in buffaloes that establish a pregnancy is related to increased angiogenesis (Galvão et al., 2012). In cattle, vascularization of the CL and blood flow is closely linked with P4 synthesis and release (Stormshak et al., 1963; Kobayashi et al., 2001). Similar relationships between vascular density and P_4 synthesis were observed in buffaloes at the mid- and late-luteal phases (Asahara et al., 1995; Zimmermann et al., 2001). In a more recent study in buffaloes, the expression of vascular

endothelial growth factor (VEGF) by the CL varied during the estrous cycle and was related to circulating concentrations of P4 (Papa et al., 2007). This was similar to findings in cattle (Schams et al., 2004). The normal role for VEGF in the developing CL is to act as a mitogen at endothelial cells to induce vascular permeability and stimulate angiogenesis (Fraser et al., 2001; Young et al., 2000).

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Chapter 5

The metabolomics: a new approach

CHAPTER 5

5.1. Introduction to metabolomics

Metabolomics is the study of the alterations of the metabolic functions of biological systems using techniques applied to the characterization of fluids and of tissue samples. *Harvard Magazine* has summed it up as "*the simplest and more reflective of the health status. It incorporates environmental influences, for example, from exercise and diet*."

Metabolomics can be used both to describe existing pathologies and to identify pre-pathological states that can be linked to risk factors for the occurrence of disease, or states that may be gleaned from variables dependent upon alterations of genetic or environmental origin that contribute towards increasing or decreasing the possibility of occurrence of disease, and knowledge of which can help implement prevention plans. Moreover, the employment of metabolomic methods can help provide an overall vision of the problem, which is lacking if the individual biological processes are considered as isolated events. In fact, the biological processes can be represented through networks that connect organs and functionalities of living systems with very complicated mechanisms.

We are currently witnessing transitional phase from purely “reductionist” approaches (analyzing complex phenomena through their separation in to simple, individual aspects) to approaches more oriented towards a "holistic" vision.

This vision in fact falls within the perimeter of what are referred to as "omics," which is to say those sciences, like genomics and proteomics, that study everything that involves relationships between groups of similar molecules. The spread of "omic" disciplines was made possible thanks mainly to the development of sophisticated techniques of high-throughput investigation capable of generating enormous quantities of data linked to different hierarchical levels of biological complexity (DNA, proteins, metabolites), thus helping revolutionize the approach to the study of living beings.

In a hypothetical "pyramid" of life (Figure 4), all the basic information of living systems is contained in the genetic code, and the study of gene expression allows alterations in the code to be detected and placed in relation with pathologies.

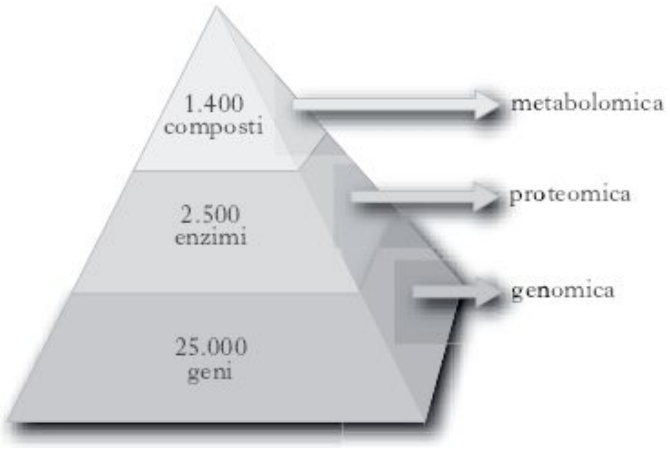

Figure 1. The pyramid of life.

Compounds metabolomics

Enzymes proteomics

Genes genomics

One level higher, the study of proteomics adds an important piece to the characterization of the effects of the alterations of the genetic code on the functions of the individual biological systems. At the top of the pyramid, the expression of metabolites is an indicator on the mode of evolution of the biological process being studied. However, while gene expression is a process within the systems, proteomic and metabolic expressions also related to outside factors, like nutrition, the influence of environmental factors, and so on. Moreover, metabolic and proteomic expressions depend upon interactions among the various biological processes, and therefore allow the adaptation and reaction processes that biological systems implement in the presence of pathologies to be studied as well. The models constructed with metabolomics are able to connect the various levels (genomics, proteomics, metabolomics) that characterize biosystems, and to represent the interactions and dependencies that generate their complexity, thus helping to identify specific profiles of pathologies. In the current state, high-resolution mass spectrometry and the combination of liquid chromatography and mass spectrometry allow infinitesimal quantities of chemical substances to be detected, and are some of the most accurate and precise examinations available for monitoring and detecting the specific causes (nutritional, behavioural, and metabolic) of health problems.

Currently, the bionutritional properties of products of animal origin and of the interactions between the animal genotype, environment, and nutrition, has prompted many researchers to face the study of animal productions with a holistic approach, integrating several disciplines with one another. As new knowledge and sophisticated methods of analysis in the fields of genomics, transcriptomics, proteomics, lipidomics, and metabolomics are gradually acquired, zootechnical research is grappling in a global fashion with the productive activities of animals, thereby allowing, among other things, genes associated with Mendelian or quantitative traits, and chromosome regions containing one or more genes that influence a multifactorial nature, to be identified.

Nowadays, the most current challenges of scientific research include studying the interactions between genes and the qualitative characteristics of animal productions, which is essential for understanding the mutual interactions between nutrients, metabolism, and gene and biochemical expressions. Interesting applications relate to analyzing the protein components of products like meat, milk, and dairy products using two-dimensional gel electrophoresis and mass spectrometry, and lipidic components and volatile fractions using chromatography techniques associated with mass spectrometry. The environmental conditions, along with age and genetics, characterize the lipidic and enzyme components (enzymes for the synthesis and storage of intramuscular fat and of bioactive molecules)

.

5.2. Sampling and preparation

The purpose of metabolomics is to identify the metabolites present in biological fluids (amino acids, organic acids, carbohydrates, lipids). These are present in different concentrations, and are not always found in all biological fluids and tissues. Once the decision is made as to what biological matrix to be worked on, the sample is prepared, and techniques are used to quantify the metabolites that are present. During the experiment, it is recommended that control samples of uniform quality be included (QC), whose estimated variations may be employed to retrospectively assess an experiment's statistical power (*De Vos et al., 2007*). Among other things, an experimental design appropriate for metabolomic investigation includes analytical replications, positive and negative controls, and verifications suitable for assessing the quality of the data.

Experimental reproducibility is influenced by the collection and management of the sample. Recently, some studies have stressed that manipulation and conservation influence the composition of the metabolites (*Zivkovik et al., 2009*). In proteomics, there are guidelines (*Rai et al., 2005*) that can be easily applied to metabolomics. According to these guidelines, the sample must be kept at -80°C or in liquid nitrogen to prevent the metabolites from decaying. Moreover, the collection of biological fluids requires considering such factors as the type of syringe, the vacuum system / "vacutainer" for the collection of blood, the conservation container, the anticoagulant, temperature, and speed and duration of centrifugation.

On the other hand, to collect and prepare solid samples, several methods are available, including freeze-drying, pulverization / homogenization, tissues cell lysis (for example, milling of liquid N_2, manual or electric homogenization and ultrasonic cell lysis). Other factors influencing the quality of the samples include the methods for freezing the samples, the method for cleaning the samples, time, duration of tissue collection, etc.

Another sensitive phase in sample processing is extraction, both for the chemical diversity in the metabolome and for the dynamic nature of the metabolite turnover. Metabolite extraction methods include liquid-liquid extraction (LLE), solid-liquid extraction (SLE), solid phase extraction (SPE), microwave-assisted extraction, accelerated extraction of the solvent, protein precipitation, etc.; of these, LLE, SLE, and SPE are the three most common ones.

In LLE, the choice of solvents is made taking account of the chemical properties of the metabolites, and should also consider their compatibility with the analytical tools. In LES, sample preparation must be optimized to prevent the decay or modification of the metabolites caused by extraction

conditions or enzyme activity in the samples. SPE, on the other hand, allows the sample to be extracted and interfering substances, like salts for example, to be removed.

5.3. Liquid chromatography and mass spectrometry (LC/MS)

Metabolomic data are acquired using specific analysis techniques. To process our data, LC/MS, which we will briefly describe, was used. LC/MS is a technique that employs liquid chromatography and mass spectrometry. A mass spectrometer has three components:

- An ion source
- A mass analyzer
- A detector

The ion source converts the sample's molecules into ions, and the analyzer conveys them to an electromagnetic field before they are quantified by the detector. Several options are available, including electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI), atmospheric-pressure photoionization (APPI), and fast atom bombardment (FAB).

Due to the metabolites' different chemical properties, it is often necessary to analyze the biological sample in both modes of ionization – + ve (positive) and −ve (negative) – in a scanning range of m/z 50–1000 to maximize metabolic coverage. ESI is by far the method of choice in metabolomic studes based on LC-MS, because it's capacity for "gentle ionization" yields a large number of ions through the exchange of charge in the solution, and often forms intact molecular ions that aid initial identification. APCI and APPI also generally induce little or no in-source fragmentation and are considered relatively tolerant of high swab concentrations.

Mass analyzers may be classified as: quadruple (e.g. Agilent 6100 Single Quadruple; Thermo MSQ plus;), ion trap (IT, e.g. Thermo LTQ; Bruker Daltonics amaZon ion trap; Agilent 6300 ion trap), time-of-flight (TOF, e.g. Bruker Daltonics MicrOTOF; AB Sciex Triple TOF; Agilent Accurate-Mass TOF), Orbitrap (Thermo Scientific), and Fourier transform ion cyclotron resonance (FTICR, e.g. BrukerApex FTICR; Thermo Scientific FT Ultra). Tandem mass spectrometers combine two or more analyzers. Modern high-resolution mass spectrometers (HRMS), like FTICR, Orbitrap, and TOF, can provide accurate measurements of the mass to facilitate the identification of metabolites and also provide a precise quantification of the metabolites.

Most metabolomic studies use a method of separation prior to the mass spectrometry analysis. High-performance liquid chromatography (HPLC), a versatile separation method, permits the separation of compounds with a broad interval of polarity through isocratic elution (the solvent composition in water remains constant during separation) or a gradient elution (the solvent

composition in water registers changes during separation). Acetonitrile, methanol, and tetrahydrofuran (THF) are the organic solvents most commonly used. Isocratic elution is preferred for simple samples (that is to say less than 10 components). Gradient elution provides an overall quicker analysis, closer peaks, and a resolution similar to isocratic elution (*Schellinger et al.,2006*).

A good chromatographic separation improves the detection sensitivity of MS, and also determines an improved quality of MS data due to the reduced background noise. Therefore, more efficient separation approaches are needed in order to reduce the complexity of the sample and to improve the chromatographic resolutions of the superimposed metabolites. One of these approaches is multidimensional liquid chromatography (MDLC) which permits the combination of two or more independent separation phases in order to increase peak capacity and improve the separation of metabolites in complex samples.

Beyond biological variability, which must always be taken account of when studies on several subjects are performed, the data obtained using LC/MS present a significant variability due to analytic reasons (preparation of the samples, conditions of the instrument at the moment of use, and operating environment). For this reason, assessing the samples a number of times during the experiment is recommended (*De Vos, 2007*). The raw data, once obtained using LC/MS, will be subjected to pre-processing, consisting of several phases, through which the data will be converted into peaks so as to be easily interpreted. Statistical analysis will then be tasked with detecting those peaks whose levels of intensity are significantly altered among distinct biological groups. The specific choice of statistical methods often depends on the study's particular design, while some methods can be applied to various types of studies.

5.4. Metabolite identification

About 2,000 metabolites are estimated to exist in the human body, while the total number of metabolites in nature might equal 1,000,000. In the current state, metabolite identification in untargeted metabolomic analysis is obtained through mass search followed by a manual verification. The m/z value of a molecular ion is searched in the database (*Wishart et al., 2007*). Metabolite identification is complicated since even an accuracy of less than 1 ppt is not sufficient for unequivocal identification; given the presence of very similar molecular weights, metabolite identification in mass cannot discriminate isomers that have the same elemental composition but different structures; lastly, we have a database coverage that is limited. Generally under 30% of the ions detected in a typical metabolomic experiment based on LC-MS can be univocally identified through mass search, thus leaving most of the ions unidentified or with multiple putative identifications.

5.5. Clinical applications

The use of metabolomics in the clinic is destined for success: identifying patterns of metabolites or individual metabolites allows us to understand a pathology's aetiology and to follow its progress over time.

From the clinical standpoint, we have three potential applications:

- ✓ Characterization of pathological phenotypes: identifying the metabolites that discriminate different groups of subjects among themselves enables the metabolomic description of a particular phenotype of a disease. In this case, the identified metabolites may be considered potential biomarkers. And in fact, many studies are carried out with the purpose of highlighting changes borne by the metabolites prior to the appearance of symptoms.
- ✓ Determination of predictive profiles of pathology.
- ✓ Pharmacometabolomics: identifying markers before drug treatment that are able to predict the effectiveness or toxicity of a treatment in a given subject.

5.6. Metabolomics in humans

Metabolomic analysis can be performed on biological materials sampled non-invasively, as in the case of milk and urine, and with little invasiveness as in the case of blood, and allows excellent results to be obtained even on minimum quantities of sample. A growing number of studies is taking an interest in application with the metabolomic approach.

In paediatrics, some studies have focused on how certain physiological variants, age, and nutrition can influence children's metabolic profiles. In fact a study on preterm infants was done, and different metabolic patterns could be distinguished depending on gestational age (*Atzori et al., 2011*). Another study assessed the effect of diet on two groups of children fed differently: one with meat protein and the other with milk protein, by simply comparing these subjects' urinary profiles (*Bertram et al., 2007*).

A recent study made it possible to identify early biomarkers of sepsis. These biomarkers allow a healthy subject to be discriminated from one with sepsis, thanks to the presence of metabolites produced following the increased demand for energy, and in the presence of a state of inflammation (*Mickiewinez et al., 2013*). Starting from urine samples, it was possible to use metabolomic analysis to identify the biomarkers in order to employ them to improve the diagnosis, prognosis, and treatment of jaundice in subjects suffering from liver disease. The considered study identified 44 metabolites, thanks to which it was possible to discriminate sick subjects from healthy ones. Moreover, in patients with JS, the metabolism of alanine, aspartate, and glutamate, and the synthesis and decay of ketone bodies, are altered. This study shows how metabolomics can be employed as a tool for diagnostic purposes, can provide new information on physiopathogenetic processes, and enables the monitoring of the metabolic alterations taking place during pathological processes (*Wang et al., 2012*).

5.7. Metabolomics in veterinary sciences

While in humans metabolomics is widely employed to identify many pathologies, in the veterinary field its use is still not very widespread. The following are some studies done using metabolomics.

One study used it a tool for determining the post-mortem interval (PMI) in stillborn calves. The study compared the metabolic profiles of calves with different times of death and those of calves born alive. Plasma and urine from 21 calves born healthy and 75 stillborn calves (5 hours postpartum or a gestation exceeding 260 days). The metabolomic analysis shows that 26 metabolites in the plasma and 29 in the urine changed significantly, and six metabolites increased simultaneously in plasma and urine: acetate, sn-glycero-3-phosphocholine (GPC), leucine, valine, creatine, and alanine. These metabolites can be used as potential biomarkers for PMI (*Jawor et al., 2019*).

Chronic hepatopathies present a diagnostic challenge, with different diseases being associated with similar clinical and laboratory findings. Among other things, characterization of dogs with chronic hepatopathies requires costly and invasive diagnostic procedures such as acquiring a liver biopsy specimen. One study has aimed to identify patterns of metabolites able to discriminate dogs with chronic hepatopathies from those with vascular anomalies like portosystemic shunts. Serum samples were collected from 12 healthy dogs, 10 dogs with congenital portosystemic shunts, and 6 dogs with chronic hepatitis. Quantitative differences were found with regard to the metabolites present (50 metabolites were different among groups) and increased aromatic amino acids and xylitol was found in dogs with congenital portosystemic shunts (*Lawrence et al., 2019*).

Given the greater incidence of overweight dogs and the poor knowledge of the metabolic processes correlated with this event, a study was performed with the purpose of investigating the postprandial response of metabolites and in particular the variations present between overweight and lean dogs. A low concentration of carnitine was found in overweight dogs compared with lean ones. This might indicate an insufficiency of carnitine correlated with altered adiposity and lipid metabolism in overweight dogs (*Soder et al., 2019*).

Studies were conducted to investigate the effects of physical exercise and aging on metabolic profile (*Deda et al., 2017*).

Metabolomics also allows the metabolic alterations derived from toxic food rich in mycotoxins, or secondary metabolites of funguses, to be identified. The study in question showed that the alterations in bovines depend on concentrations of ZEN and STC. Contamination of feed with several mycotoxins can alter the systemic metabolic processes, including the metabolites associated with the generation of ATP, amino acids, conjugated glycin, organic acids, and purine bases. The results that were obtained indicate that a two-week treatment period is not sufficient to improve the levels of urinary metabolites, suggesting that chronic contamination with mycotoxins can have harmful long-term effects on the systemic metabolism of bovines (*Toda et al, 2017*).

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Chapter 6

Effect of live body weight and method of synchronization on ovulation, pregnancy rate and embryo and fetal loss in buffalo heifers

CHAPTER 6 –

EFFECT OF LIVE BODY WEIGHT AND METHOD OF SYNCHRONIZATION ON OVULATION, PREGNANCY RATE AND EMBRYO AND FETAL LOSS IN BUFFALO HEIFERS*

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6.1. Introduction

In buffalo species the optimization of breeding techniques in Italy allowed to reduce the age at first calving from about 45 months to less than 30 months in 30 years (*Bhatti et al., 2007*). The onset of puberty and, consequently, the age at first calving, is deeply affected by several factors, such as management, together with genotype and climate (*Campanile et al., 2009*). However, one of the main aspects influencing the reaching of puberty is the live body weight (LBW; *Campanile et al., 2009*). An anticipation of puberty has been observed in both Egyptian (*El Nouty et al., 1971*) and Mediterranean buffaloes (*Campanile et al., 2001*) that showed a daily weight gain after weaning of 0.45 and 0.55 kg, respectively. A reduction of the generation interval can be obtained anticipating the age at first calving and by the utilization of artificial insemination (AI). Recently, several synchronization protocols have been developed in buffalo, doubling the efficiency of AI (25%–50% pregnancy rate) over a period of approximately two decades (*Zicarelli et al., 1997*; *Neglia et al., 2015*). However, the majority of studies has been primarily performed in adult buffaloes (*Neglia et al., 2016; Monteiro et al., 2018*), while few trials were carried out in heifers (*Carvalho et al., 2017; Neglia et al., 2018*). In cyclic buffalo heifers with normal Corpus Luteum (CL) function, the response to estrus synchronization by Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; *Zicarelli et al. 1997*) or Ovsynch-Timed Artificial Insemination (O-TAI) program (*Neglia et al. 2018*), is comparable to the response observed in cattle. However, at present, no information are available on the relationships between the body weight and reproductive performance in buffalo heifers treated by different synchronization protocols. Therefore, the aims of this study were: i) to assess the influence of LBW

on reproductive performance in buffalo heifers and ii) to evaluate if the synchronization by double $PGF_{2\alpha}$ or O-TAI program may affect pregnancy rate in buffalo heifers.

6.2. Materials & Methods

6.2.1. - Animals

The investigation was carried out in accordance with EU Directive 2010/63/EU and the Animal Ethics Committee of the University of Naples, Federico II. The study was carried out on 146 Italian Mediterranean buffalo heifers, previously chosen (see below), with a mean age of 25.3±13.4 months and a mean LBW of 424±47 kg, between February and March 2017. To ensure the best welfare conditions, buffalo heifers were maintained in open yards that allowed 10 m2 for animal and received a total daily mixed ration consisting in 8 kg of dry matter, 0.85 UFL/kg dry matter, 13% crude protein, 18% starch and 44% NDF. Twenty and ten days before the start of the trial, the animals underwent ultrasound examination with a portable Sonoace Pico (Medison®, Seoul, South Korea) equipped with a 10 MHz linear transducer for trans rectal examination. Only heifers in good health, without any abnormalities of the genital tract and with the presence of a CL in at least one examination were included in the study. Furthermore, in correspondence with the second ultrasound examination, all the animals were weighed. Only the animals with a LBW > 270 kg (n=146) were chosen. This because puberty is related to the age at first estrus, (at about 16.5–19.0 months) and to the LBW (at around 340–360 kg) (*Borghese et al., 1994*).

6.2.2. Estrus synchronization treatments, AI and pregnancy assessment

The selected heifers were divided into two homogeneous groups, according to LBW and age. Heifers in OVS (n = 72) underwent synchronization of ovulation by O-TAI Program (*Neglia et al., 2016*). Heifers in PGF (n = 74) underwent synchronization by a double $PGF_{2\alpha}$ analogue (Dinoprost, 25 mg; Dinolytic®, Zoetis, Rome, Italy) injection on day 0 and 12, at a random day of the cycle. AI were performed by the same operator and each buffalo was inseminated twice: at 16 and 40 hours after the second injection of GnRH in OVS and at 60 and 84 hours after the last $PGF_{2\alpha}$ in PGF (*Neglia et al., 2008*). Because of the low intensity of estrous behavior in buffaloes (*Ohashi 1994*), the heifers were palpated *per rectum* to assess the estrous status (tonic uterus in presence or absence of vaginal mucus discharge) and underwent ultrasound examination to record preovulatory follicle. The ovulation rate was assessed by ultrasound in each group, 24 and 48 hours after the first insemination. Frozen/thawed semen of two bulls of proven fertility was utilized in the AI program in both groups. Finally, the body condition score was assessed on the day of AI, by using a 1 to 9 scale (*Wagner et al., 1988*).

Twenty-five days after AI, buffaloes underwent trans rectal ultrasonography to assess embryonic development and the heartbeat. Pregnancy diagnosis was confirmed on day 45 and 90 after AI: heifers pregnant on day 25 but not on day 45 were considered to have undergone late embryonic mortality. Similarly, buffaloes pregnant on day 45 but not on day 90 were considered to have undergone fetal mortality.

6.2.3. Statistical analysis

Differences in ovulation rate after 24 and 48 hours after the 1st AI and pregnancy between treatments were assessed by Chi-square test. Differences in age, LBW, BCS, pregnancy rate, LEM and FM between treatments were assessed by ANOVA. Two separate logistic regression models were estimated to assess the ovulation rate at different time within each treatment with respect to the LBW. Pregnancy outcome was evaluated by a logistic regression second order model using LBW and treatment as independent variables. All statistics were performed using the IBM SPSS Statistics for Windows, version 20.0" program. (*IBM Corp. Launched, 2011*).

6.3. Results

Eleven animals did not respond to the synchronization treatments and were excluded from the trial. A total of 92.5% synchronization rate was recorded, without any difference between the two synchronization treatments (93.1 vs. 91.9% in OVS and PGF, respectively). LBW was ($P<0.05$) higher in animals that were inseminated, compared to those that did not respond to the synchronization treatment (450.0±3.2 vs. 423.2±9.6 kg in inseminated and not inseminated heifers, respectively), whereas a similar age was recorded (796.5±9.0 vs. 789.7±40.7 days in inseminated and not inseminated heifers, respectively). No differences were recorded in preovulatory follicle dimensions between OVS and PGF, neither on the day of TAI (1.27±0.02 vs. 1.32±0.03 cm, in OVS and PGF, respectively) nor at 24 h (1.49±0.06 vs. 1.50±0.04 cm, in OVS and B, respectively). Ovulated buffaloes showed a significantly ($P<0.05$) higher LBW compared to not-ovulated counterparts (Table 1) regardless of the treatment, and, in particular, in the PGF Group and a similar total ovulation rate was recorded between OVS and PGF.

Table 1. Effect of live body weight on synchronization, ovulation and pregnancy rate in buffalo heifers synchronized with Ovsynch-TAI Program (OVS) and double prostaglandin (PGF).

	OVS		PGF		Statistical Significance
Synchronization rate	Yes	No	Yes	No	
%					
(n)	(67)	(5)	(68)	(6)	
LBW	457.1±4.4	434.0±21.2	449.4.7[a]	414.2±2.2[b]	P = 0.04
Ovulation rate	Yes	No	Yes	No	
%	60	7	59	9	
(n)	(89.6)	(10.4)	(86.8)	(13.2)	
LBW	450.8±4.5	454.0±19.2	454.4±5.0[a]	413.2±6.7[b]	P = 0.03
Pregnancy Rate (90 d)	Yes	No	Yes	No	
%	47.8	52.2	57.4	42.6	
(n)	(32)	(35)	(39)	(29)	
LBW	456.3±5.7	446.3±6.6	458.0±6.1	436.8±6.9	

However, within each treatment, only ovulated heifers of PGF showed a significantly (P<0.01) higher LBW and age compared to those not ovulated, whereas no differences were recorded in animals synchronized by O-TAI Program (Table 1).

Neither the LBW nor the age affected ovulation rate at 24 and 48 hours after insemination (data not shown). However, the ovulation rate recorded in OVS at 24 h tended to be higher (P = 0.06) compared to that recorded in PGF (86.7 vs. 72.9% in OVS and PGF, respectively).

No differences were recorded on both embryonic and fetal mortality in buffaloes treated by Ovsynch-Tai Program or Double prostaglandin treatment (Table 2).

Table 2. Effect of live body weight on embryo and fetal loss in buffalo heifers synchronized with Ovsynch-TAI Program (OVS) and double prostaglandin (PGF).

Embryo Loss %	Yes	No	Yes	No
	15.0	85.0	7.1	92.9
(n)	(6)	(34)	(3)	(39)
LBW	427.8±12.5	455.1±5.5	453.3±6.9	458.0±6.1
Fetal Loss %	Yes	No	Yes	No
	5.9	94.1	0.0	100.0
(n)	(2)	(32)	(0)	(39)
LBW	434.5±13.5	456.3±5.7		458.0±6.1
Total embryo and fetal loss %	Yes	No	Yes	No
	20.9	79.1	7.1	92.9
(n)	(8)	(32)	(3)	(39)
LBW	429.5±9.6	456.3±5.7	453.3±6.9	458.0±6.1

The multiple logistic regression analysis showed that ovulation rate tended to be influenced by LBW, independently of the treatment (odds ratio = 1,030; P = 0.08). If only heifers in PGF were considered, ovulation rate was significantly affected by LBW (odds ratio = 1,032; P < 0.05), while no effects were recorded in OVS. Similarly, ovulation rate at 24 h was significantly influenced by LBW only in PGF (odds ratio = 1,033; P < 0.01), but no effects were observed in OVS. No influence of LBW was observed for the ovulation rate at 48 h. Data on pregnancy rate are reported in Table 1. If only ovulated animals were considered, LBW tended to be higher (P = 0.08) in pregnant heifers compared to not pregnant counterparts (457.2±4.2 vs. 445.7±5.3, in pregnant and not pregnant heifers, respectively), whereas a similar age was recorded. No differences were found between OVS and PGF (Table 1) in terms of pregnancy rate and a similar live body weight and age were recorded between pregnant and not pregnant heifers within each treatment. However, a significantly higher pregnancy rate was recorded in heifers ovulated after 48 h in PGF (12/16; 75%) vs OVS (3/8; 37.5%). The regression logistic analysis for pregnancy, did not assess any influence of LBW or age on pregnancy. Finally, no differences were recorded for BCS in both pregnant and not

pregnant heifers in both groups (7.2±0.2 vs. 7.3±0.2, in pregnant and not pregnant heifers of OVS, respectively and 7.1±0.2 vs. 7.2±0.2, in pregnant and not pregnant heifers of PGF, respectively).

6.4. Discussion

The onset of puberty in farm species is a multifactor event that heavily influences productivity, especially in dairy breeds. Genetics, farm management, nutrition, climate and, in buffalo, also photoperiod all contribute to the high variability in the timing of its occurrence, conditioning the age at first calving and, hence, the beginning of the productive life. Anticipating the age at first calving and reducing the generational interval favour an early evaluation of the subjects and of the bulls used in progeny tests. From an economic point of view, even if the milk yield in the first lactation is lower in younger heifers, the total net income from birth to the end of the first lactation is significantly higher at a lower calving age. Thus, it is clear that an optimal management in this category of subjects influences the age at first calving and allows to anticipate the start of their productive life, reducing the generational interval and favoring the genetic improvement of the herd.

This study aimed to assess the influence of LBW on reproductive efficiency in buffalo heifers. Accordingly, reproductive development in heifers and the attainment of puberty are determined primarily by nutrition from the time of weaning (*Campanile et al., 2001*). Some studies performed in buffalo (*Campanile et al., 2001, 2009*), demonstrated that post-weaning weight gain may influence reproductive performance in heifers and the LBW on the day of insemination further affects reproduction, particularly when different protocols of synchronization are applied. In our study, LBW significantly influenced either the response to the synchronization treatment and the ovulation rate. In Mediterranean Italian buffaloes, different studies showed an age at first oestrus, stated as the first blood P_4 rise at a value over 1 ng/ml, at about 16.5–19.0 months of age and at around 340–360 kg of body weight (*Borghese et al., 1994*). Cyclic ovarian activity, with a P_4 value > 1 ng/ml at about a ten-day interval was achieved at 20.7 months of age and at a body weight of 380-390 kg (*Borghese et al., 1994*). On these bases, in our study all the heifers with LBW lower than 370 kg were excluded from the trial. It is worth pointing out that the influence of LBW was only observed in PGF. Synchronization by double prostaglandin needs a regular cyclic activity of the animals, as demonstrated by the evidence that anoestrus or prepubertal subjects are not responsive (*Stevenson and Pursley, 1994*). After the onset of puberty, LH decreased at a level similar to that of 4 months before puberty and a positive correlation between LH and body weight was found during the prepubertal period (*Haldar and Prakash, 2006*). On the contrary, the LBW did not affect the ovulation rate in animals synchronized by O-TAI Program. The latter is largely applied in both buffalo (*Neglia et al., 2016; Sharma et al., 2017*), and bovine species (*Pursley et al.,*

1995; Carvalho et al., 2015), because of its feasibility and practical and economic advantages. The ovulation that occurs following exogenous GnRH administration is due to the presence of a follicle with ovulatory capability. In cattle, a follicle requires on average 7 to 10 days to go through the stages of emergency, deviance and dominance and then reaches the preovulatory stage or encounters atresia (*Ginther et al., 1989*). In buffalo the deviation phase, and hence the acquisition of LH receptors, is reached at a mean diameter of 0.75 cm (*Baruselli et al., 2001*). The ovulation rate recorded at 24 h tended to be higher in heifers of OVS compared to those of PGF. It is known that the last GnRH of the O-TAI treatment aims to induce an endogenous LH surge that is responsible for the synchronization of ovulation (*Pursley et al., 1995*). On the contrary, treatment by double prostaglandin is based on CL regression, a consequent decrease of circulating progesterone that in turn causes an increase of GnRH and LH pulse frequency, that can act on the preovulatory follicle. Therefore, in this case the ovulation is not induced and it is dependent from the maturation and the oestradiol levels of the preovulatory follicle (*Kastelic and Ginther, 1991*). In a previous trial carried out in adult buffaloes synchronized by O-TAI Program, it was demonstrated that the LH surge occurs about 1 hour after GnRH injection (*Campanile et al. 2008*). On the other hand, with the double $PGF_{2\alpha}$ protocol LH surge occurs naturally and GnRH peak and hence the ovulation may be slightly delayed. In fact, in heifers ovulated at 48 h a significantly higher pregnancy rate was recorded compared to Ovsynch-treated buffaloes. In any case, no differences were observed between the two synchronization protocols in terms of pregnancy rate, suggesting that both protocols can be used in buffalo heifers. The treatment by double prostaglandin resulted in a 10% more pregnancy rate, despite of the low number of ovulated buffaloes at 24 h and the requirement of two AI. An improvement in this protocol may be obtained by administrating a GnRH two days later the last prostaglandin in order to synchronize the ovulation rate at 24 h. On the contrary, the O-TAI Program is characterized by a short duration and by the possibility of reducing the number of inseminations allowing economic advantages. In any case, a key point is the selection of the heifers, that would reach a reasonable weight and ovarian cyclicity.

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Chapter 7

Corpus Luteum Color Doppler Ultrasound and Pregnancy Outcome in Buffalo During the Transitional Period

CHAPTER 7 –

CORPUS LUTEUM COLOR DOPPLER ULTRASOUND AND PREGNANCY OUTCOME IN BUFFALO DURING THE TRANSITIONAL PERIOD*

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7.1. Introduction

The growing demand for meat and milk worldwide has caused a steady increase in the buffalo population in the last few years in both developed (Campanile et al., 2010b) and developing countries (2,3). Buffaloes show high adaptability to different climates and conditions, and can be considered to be an important source of animal-derived protein for human consumption (1). Although breeding is often managed on small farms, representing the basal rural economy in Asia, intensive breeding systems are performed in other countries such as Italy (4). However, its productive efficiency is strongly related to the reproductive pattern, which is influenced by the photoperiod. Indeed, this species is considered to be a short-day breeder, meaning that it increases its reproductive activity when daylight hours decrease (4). In Italy, these reproductive characteristics are opposite of the market requirement for milk production, suggesting that the application of the out-of-breeding season mating technique is responsible for a decline in reproductive efficiency, particularly during some periods of the year (4). Embryonic mortality represents one of the main factors that are involved in pregnancy failure in both buffalo (5,6) and bovine (7) species.

The advent of color Doppler ultrasonography promoted tremendous advances in research and clinical practice in animal reproduction, because it allowed non-invasive visualization of the

vascularization in reproductive organs. In particular, the corpus luteum blood flow reflects luteal function better than luteal size in ruminants. Since buffaloes are a seasonal species, it is important to evaluate corpus luteum functionality also during the non-breeding season, through blood flow examination and early pregnancy diagnosis. Several studies demonstrated that early development of the corpus luteum (CL) together with high progesterone (P4) levels are involved in adequate embryonic growth and reduction of embryonic mortality (8,9). In particular, a study performed during the breeding season showed that successfully pregnant buffaloes had larger CL development, higher P4 concentrations, and increased blood flow to the CL, measured as the time average medium velocity (TAMV), compared to nonpregnant counterparts (10). The latter experiment was performed during the breeding season from Day 5 to Day 10 post-timed artificial insemination (TAI). Furthermore, a low pregnancy rate was associated with delayed vascularization of the CL after Day 5. However, it cannot be ruled out that CL development occurs differently during the transitional period from mid-winter to spring when the females show a decrease in reproductive activity in response to increasing day length. The incidence of embryonic mortality during this period increases to 40% on some farms (11,12). Therefore, the aims of this study were to: (i) evaluate CL development during the transition to the seasonal nadir in reproductive activity (transitional period from decreasing to increasing daylight length) through blood flow examination; (ii) determine whether CL blood flow parameters or progesterone levels can be used to estimate the likelihood of pregnancy by using the receiver operating characteristics (ROC) analysis, even if very early during CL development.

7.2. Materials & Methods

7.2.1. - Animals

The Ethical Animal Care and Use Committee of the Federico II University of Naples approved the experimental design. The trial was performed in February 2018 on 29 pluriparous Italian Mediterranean buffaloes that were 120.2 _ 18.3 days in milk and bred on a commercial farm in the South of Italy between 40.5_ N and 41.5_ N parallel. The animals were maintained in open yards that allowed 15 m2 per animal and 80 cm manger space.

The buffaloes were fed a total mixed ration consisting of 55% forage and 45% concentrate, containing 0.91 milk forage units/kg of dry matter and 15% crude protein.

To assess the cyclic status, all the animals underwent two ultrasound examinations, spaced 12 days apart (MyLab 30Gold, Esaote, Genova, Italy) using a 7.5 MHz transrectal linear probe, before the start of the synchronization protocol. Only animals with a functional CL in at least one examination were included in the study.

7.2.2. Synchronization of ovulation and Timed Artificial Insemination

Buffaloes were synchronized using the Ovsynch TAI program, which was developed for cattle (13), and it is widely used in buffalo (14). Briefly, 20 µg of Gonadotropin-releasing hormone (GnRH) analogue (Buserelin acetate, 12 µg; Receptal®, Intervet, Milan, Italy) was administered on Day 0 followed by a synthetic analogue of prostaglandin F2α (Cloprostenol®, 0.250 mg/mL MSD, Milan, Italy) on Day 7 and an additional GnRH analogue administration on Day 9, with TAI on Day 10. All the animals underwent ultrasound examination on Day 10 of the synchronization protocol. Only those with a follicle that was larger than 1 cm and with a tonic uterus, with or without mucus vaginal discharge, were inseminated by the same experienced operator 20 hours after the second GnRH injection. The dimensions of the preovulatory follicle were recorded on the day of TAI, and the follicle area was calculated according to the following equation:

$$FL\ area = \left(\frac{a}{2}\right) * \left(\frac{a}{2}\right) * \pi$$

Where:

FL = Follicle
a = major axis of the follicle
b = minor axis of the follicle
π = 3.14

Furthermore, ultrasound examination were made also one day post AI to be sure that all the animals underwent ovulation.

7.2.3. Corpus Luteum and Blood Flow Evaluation

CL ultrasonography examinations were performed daily from Day 5 to Day 10 post-TAI using a portable ultrasound unit (MyLab 30Gold, Esaote, Genova, Italy) that was equipped with a 7.5 MHz linear transducer, designed for transrectal examination in large animals (LV 513). To obtain a better definition of the CL, once the ovary was visualized, the image was adjusted and then frozen to measure both the long and short axes. The CL area was calculated as follows:

$$CL\ area = \left(\frac{a}{2}\right) * \left(\frac{a}{2}\right) * \pi$$

Where:

CL = Corpus luteum
a = major axis of the corpus luteum
b = minor axis of the corpus luteum

To assess blood flow characteristics, the color Doppler mode was activated in order to evaluate the corpus luteum blood flow and, when the CL was clearly visualized, the sample gate was placed at the level of the primary branch of the ramus tubarius of the ovarian artery, just before hugging the corpus luteum. At this level, the artery was reasonably accessible and sufficiently large (2 to 3 mm in diameter) in order to measure flow rates accurately and in particular the flow indexes (TAMV, Resistivity Index (PI) and Pulsatility Index (PI); Figure 1). Color gain setting, velocity setting, and a color-flow filter setting were standardized during all the procedures and all the analyses were carried out by the same technician. In particular, the pulse repetition frequency was set at 0.7 kHz, the lowest possible that did not result in aliasing artifact, the power Doppler frequency was set to 1.5 kHz with a gain of 47, and the pulsed wave Doppler was set at 8 kHz, gain 67. Real-time B-mode/Color Doppler images of the continuous scans of the CL were recorded and then analyzed retrospectively. B-mode and color-flow mode short video clips (7 s duration) were stored in the internal memory of the ultrasound machine. Later, as described by Siqueira et al. (15), the CL area and the area of the cavity (where present) were assessed using the machine's internal calipers. To justify fluid-filled cavities, CLS was assessed by subtracting the area of the cavity from the entire CL area

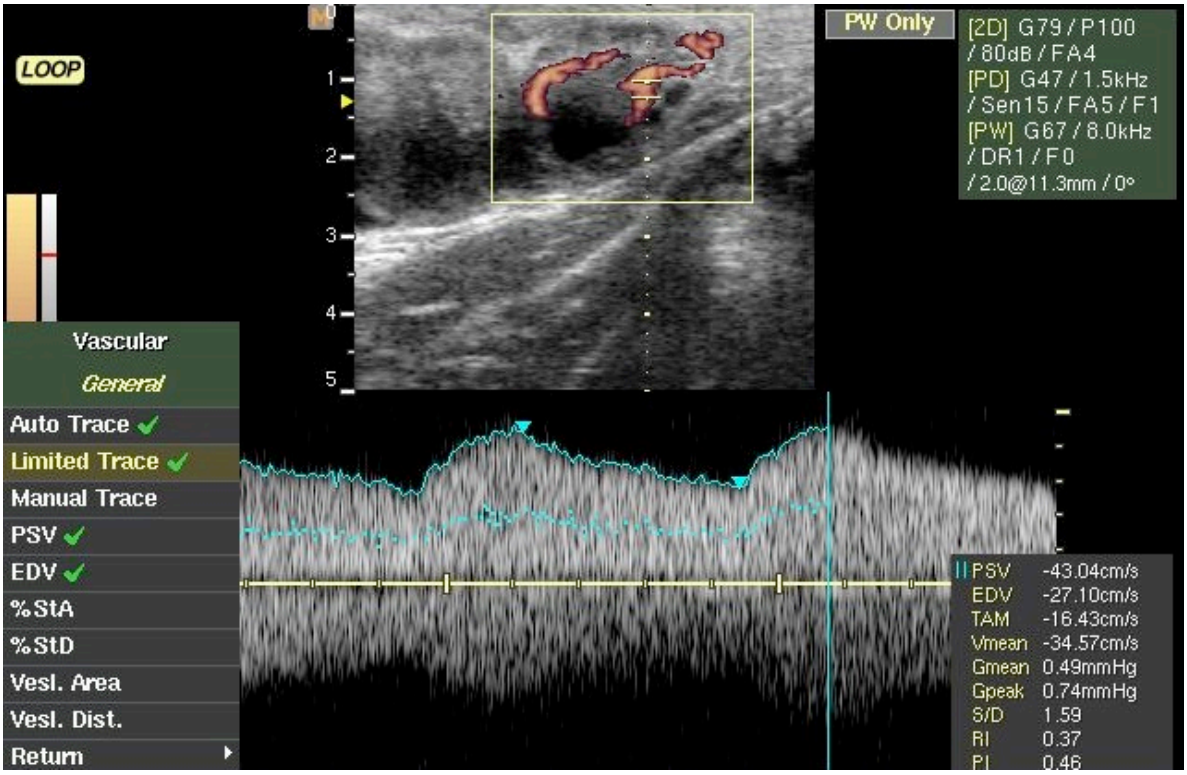

Figure 1. Corpus luteum blood flow assessment in buffaloes with the aid of eco-color Doppler mode.

7.2.4. Progesterone (P_4) Assay

Blood P_4 levels were assessed using a radioimmunoassay (RIA) according to (16) to determine CL functionality. All the analyses were performed in the Laboratory of Veterinary Physiology of the University of Udine. Blood samples were collected from the jugular vein into heparinized tubes on the same days as ultrasound examination (from Day 5 to Day 10). The samples were centrifuged at 800 x g for 15 min and the plasma was stored at -20 °C until it was required for the P_4 assay, which was performed at the same time for all samples.

The minimum detectable amount of P4 was 2.2 _ 0.07 pg and the intra-assay and inter-assay coefficients of variation were 6.4% and 12.1%, respectively. Values of 1.2 ng/mL were considered to indicate the presence of a functional CL, according to previous studies (10).

7.2.5. Pregnancy diagnosis

Pregnancy diagnosis was performed on Day 27 post-TAI using the same ultrasound machine as described above. Pregnancy status was confirmed on Day 45 and 70 post-TAI. Animals that were pregnant on Day 27 but not pregnant on Day 45 were considered to have encountered late embryonic mortality (LEM). Similarly, those that were pregnant on Day 45 and not pregnant on Day 70 were considered to have undergone fetal mortality.

7.2.6. Statistical analysis

A repeated measure ANOVA was performed to evaluate the differences between pregnant and nonpregnant animals for P_4 levels, CL area, TAMV, PI, and RI values that were recorded from Day 5 to Day 10 (17). Because of the low number of animals, the latter analysis was confirmed by another repeated measure ANOVA using a nonparametric approach [18,19]. Chi-square analysis was performed to evaluate differences in the pregnancy rate between buffaloes that showed an early or delayed increase in P_4. A linear regression model was applied to evaluate the effects of TAMV, RI, PI, and CL area on P_4 blood levels, either on each day or throughout the experimental period. Moreover, a multiple logistic regression assay was performed to calculate the odds ratios for pregnancy using the TAMV, RI, PI, and CL area on P_4 blood levels as dependent variables (17). All data were expressed as the mean ± standard error of the mean (SEM). Additionally, receiver operating characteristics (ROC) analyses were performed, focusing on CL characteristics (CL area, RI, PI, TAMV, and P_4 concentrations) on Day 5 to Day 10 post-TAI to identify the optimal cutoff value for predicting pregnancy. The latter was determined from the data point that minimized the distance and reported the relative sensitivity and specificity percentages and area under the curve (AUC) value.

7.3. Results

7.3.1. – Pregnancy rate

Total pregnancy rate on Day 27 was 57.7% (15/26), and it was reduced to 50.0% on Day 45 after TAI. This means that the incidence of embryonic mortality was 13.3% (2/15). For the subsequent analysis, buffaloes that underwent LEM were included in the non-pregnant group. No fetal mortality was recorded. No differences were recorded between follicular area in both groups (data not shown). Average CL area was similar from Day 5 to Day 10 in pregnant animals compared to their non-pregnant counterparts (Table 1), although a ($P<0.05$) difference was recorded on Day 10. Pregnant buffaloes also showed higher ($P<0.01$) P_4 concentration for the Day 5 to Day 10 period compared to non-pregnant buffaloes, although significant differences were recorded from Day 7 on. The P_4 levels that were higher than 1.2 ng/mL on Day 5 post-TAI were recorded in 42.3% of buffaloes (11/26) and 81.8% (9/11) of them were pregnant. In 69.2% (9/13) of the pregnant buffaloes, P_4 concentrations that were higher than 1.2 ng/mL were recorded, whereas 30.8% (4/13) showed lower P_4 concentrations on Day 5 post-TAI. No effect of the day on pregnancy and no interactions were found with the repeated measures ANOVA.

Table 1. Corpus luteum (CL) area and circulating concentrations of progesterone (P_4) from Days 5 to 10 in buffaloes that were subsequently diagnosed as pregnant (P) or non-pregnant (NP).

	CL area *(cm^2)*		***P_4*** *(ng/mL)*	
Day	P (n=13)	NP (n=13)	P (n=13)	NP (n=13)
5	1.80±0.1	1.72±0.2	1.24±0.1	1.11±0.1
6	1.86±0.2	1.84±0.2	1.45±0.1	1.26±0.2
7	2.32±0.2	2.10±0.2	$1.70±0.1^{a}$	$1.32±0.1^{b}$
8	2.49±0.2	2.14±0.1	$1.91±0.1^{A}$	$1.25±0.1^{B}$
9	2.70±0.2	2.25±0.2	$2.09±0.1^{A}$	$1.32±0.2^{B}$
10	$2.66±0.1^{a}$	$2.23±0.1^{b}$	$2.28±0.1^{A}$	$1.36±0.1^{B}$
Day 5 to Day 10	2.31±0.1	2.05±0.1	$1.78±0.1^{A}$	$1.27±0.1^{B}$

Values are expressed as mean ± standard error. a,b, A,B For each endpoint, values with different superscripts within the same row differ. a,b: $P < 0.05$; A,B: $P < 0.01$.

7.3.2. – Corpus luteum characteristics

No differences were recorded for the RI (0.41±0.0 vs. 0.41±0.0, in pregnant and non-pregnant buffaloes, respectively) and the PI (0.56±0.0 vs. 0.55±0.0, in pregnant and non-pregnant buffaloes, respectively) values, either on single days or throughout the experimental period. However, the mean TAMV value from Day 5 to Day 10 was (P<0.01) higher in pregnant compared to non-pregnant buffaloes (14.05±0.45 vs. 10.39±0.57 cm/s), and significant differences were present from Day 6 (Figure 2).

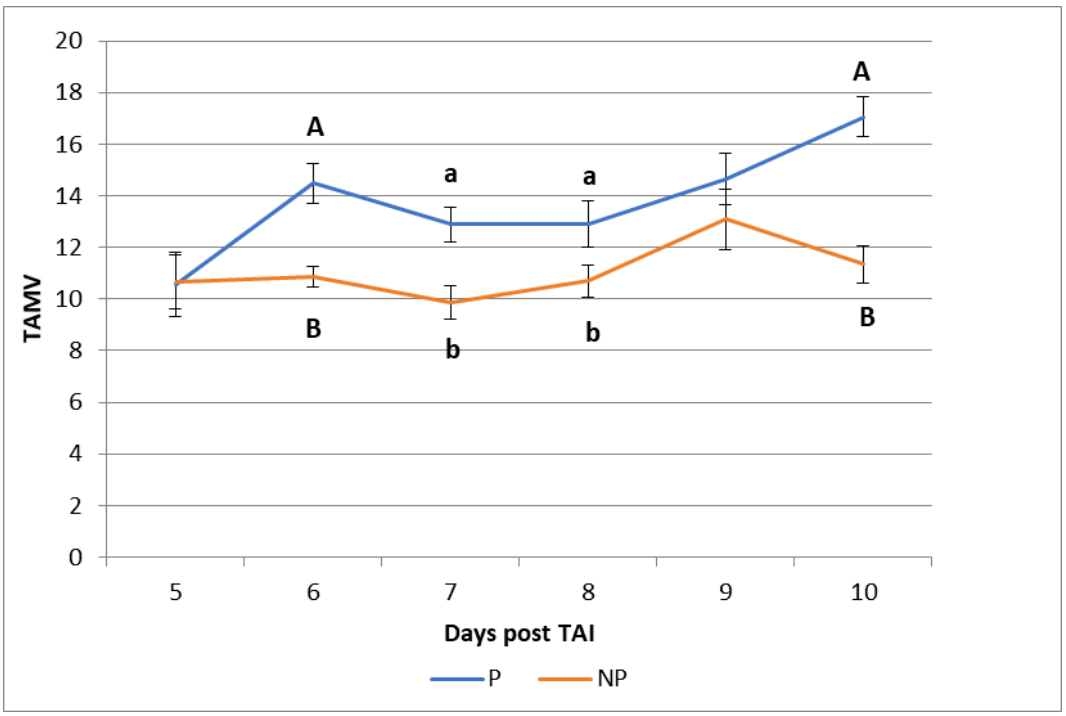

Figure 2. Time average medium velocity (TAMV) values recorded in pregnant (P) and non-pregnant (NP) buffaloes from Day 5 to Day 10 post-timed artificial insemination (TAI). [A, B] Values with different superscripts are significantly different; P<0.01; [a, b] Values with different superscripts are significantly different; P<0.05.

The characteristics of the CL blood flow that were evaluated by the eco-color Doppler technique showed a delayed vascularization in six buffaloes (19.2%). Blood flow features (TAMV, RI, PI) on Day 5 post-TAI were not recorded in these animals. Among these animals, only one buffalo

(17.0%) was diagnosed as pregnant, whereas a higher pregnancy rate (60.0%; 12/20; $P<0.05$) was recorded in buffaloes that showed a competent blood flow on Day 5 post-TAI.

7.3.3. – Regression analysis

No differences were recorded for the RI (0.41±0.0 vs. 0.41±0.0, in pregnant and non-pregnant buffaloes, respectively) and the PI (0.56±0.0 vs. 0.55±0.0, in pregnant and

The multiple linear regression analysis, considering the average of all parameters between Day 5 and Day 10, showed a significant relationship ($R^2 = 0.394$; $P<0.05$) between P_4 levels and TAMV. The CL area tended ($P = 0.096$) to influence P_4 levels, according to the following equation:

$$P_4 = 0.287 + (0.263 \times \text{CL Area}) + (0.081 \times \text{TAMV}) \quad (1)$$

The same analysis was performed each day. In this case, the influence of TAMV and the CL area on P_4 levels was recorded only on Day 8 ($R^2 = 0.303$; $P<0.05$), according to the following equation:

$$P_4 = 0.117 + (0.334 \times \text{CL Area}) + (0.082 \times \text{TAMV}) \quad (2)$$

The multiple regression analysis in pregnancy that was performed using the average values from the period Day 5 to Day 10 of P_4, CL area, TAMV, RI, and PI showed that pregnancy outcome was significantly influenced only by TAMV (odds ratio = 3.808; $P<0.01$). If the multiple regression analysis was performed considering the same parameters on each day, a significant influence of TAMV was recorded also on Day 6 (odds ratio = 2.581), Day 7 (odds ratio = 1.899), and especially on Day 8 (odds ratio = 10.325).

7.3.4. – Predicting pregnancy

The ROC analyses indicated that both TAMV and P_4 were useful to predict pregnancy compared to other endpoints starting on Day 6 (Table 2 and 3). Based on the distance from the ideal point, our analysis defined the best TAMV cutoff value as 11.88 cm/s for pregnancy prediction on Day 6 (sensitivity 76.9% and specificity 76.9%; P <0.01). The P_4 cutoff value was 1.14 ng/mL for the pregnancy prediction on Day 6 (sensitivity 84.6% and specificity 61.5%; P <0.05). However, Day 10 after TAI showed the best results for TAMV and P_4 in terms of sensitivity and specificity. The TAMV cutoff value was set at 13.19 cm/s, with an increase in sensitivity (92.3%) and specificity (84.6%; P < 0.01), while the P_4 cutoff value was set at 1.69 ng/mL, with an increase in sensitivity (92.3%) and specificity (92.3%; P < 0.01). For the other parameters, it was possible to set a cutoff value (2.38 cm2) for the CL area only beginning on Day 10, with a sensitivity of 76.9% and a specificity of 53.8% (P <0.05). It was not possible to set a cutoff value for RI and PI.

Table 2. Summary of the ROC analyses of the Time Average Medium Velocity (TAMV) from Day 6 to Day 10 post TAI in buffaloes during the transitional period.

Day	Items	Cutoff value	AUC value	Sensitivity (%)	Specificity (%)	P value
6	TAMV	11.88 cm/s	0.90	76.9	76.9	P<0.01
7	TAMV	10.53 cm/s	0.87	84.6	69.2	P<0.01
8	TAMV	11.00 cm/s	0.82	84.6	61.5	P<0.01
9	TAMV	12.13 cm/s	0.75	69.2	69.2	P<0.05
10	TAMV	13.19 cm/s	0.94	92.3	84.6	P<0.01

ROC, receiver operating characteristics; AUC, area under the curve.

Table 3. Summary of the ROC analyses of the Progesterone (P_4) levels from Day 6 to Day 10 post TAI in buffaloes during the transitional period.

Day	Items	Cutoff value	AUC value	Sensitivity (%)	Specificity (%)	P value
6	P_4	1.15 ng/mL	0.73	84.6	61.5	P<0.05
7	P_4	1.34 ng/mL	0.73	84.6	61.5	P<0.05
8	P_4	1.47 ng/mL	0.89	92.3	76.9	P<0.01
9	P_4	1.60 ng/mL	0.88	92.3	69.2	P<0.01
10	P_4	1.69 ng/mL	0.95	92.3	92.3	P<0.01

ROC, receiver operating characteristics; AUC, area under the curve.

7.4. Discussion

This study analyzes the detailed CL development from day 5 to day 10 post-TAI in buffalo species during the transitional period to the seasonal nadir in reproductive activity. Although some studies of this type were performed (10), CL growth and characteristics were only investigated during the breeding season. Thus, we based our experimental design on that paper, and we performed this study to confirm the results obtained by Neglia et al. (10) during the buffalo non-breeding season. The CL is known to play a pivotal role in pregnancy establishment in several mammalian species, because of its capability to secrete P_4 (20). The sensitivity to decreasing daylight hours deeply affects the reproductive efficiency of the buffalo, as demonstrated by the reduced CL functionality in some periods of the year (21,22). This is responsible for the decline in reproductive efficiency and the increase in embryonic mortality (6).

The CL functionality can be assessed using several tools. The CL ecotexture that was assessed by ultrasound was one of the first approaches that was used to evaluate the CL (23,24), and the main limit of this technique was its subjective determination, which does not allow large-scale utilization. However, two reliable indicators of CL functionality are P_4 blood levels and/or the CL blood flow (25). In our study higher P_4 levels were recorded in pregnant buffaloes during the Day 7 to Day 10 period, which can be considered to be a critical window for embryonic growth and development. These results are in agreement with previous trials that were performed during the breeding season (10, 25), in which the same trend was observed. High P_4 levels, and particularly an early increase in P_4 levels during the first days after mating, is responsible for larger embryonic development, high interferon-tau levels, and, consequently, reduced embryonic mortality, which represents one of the main causes of reproductive failure in buffalo (6, 9, 26). This is also demonstrated by the evidence that animals that ovulated after treatment with a GnRH agonist on Day 5 after TAI showed an early increase in the P_4 concentration and a high pregnancy rate (11). In our trial, significantly higher P_4 levels between pregnant and non-pregnant buffaloes were recorded starting from Day 7 and were definitely higher from Day 8. This interesting aspect further confirms the hypothesis that this period is critical for embryonic development, as previously observed (10). Furthermore, more than 80% of buffaloes that showed P_4 levels that were higher than 1.2 ng/mL on Day 5 post-TAI were diagnosed as pregnant on Day 45, which strongly supports the evidence that an early increase in P_4 levels is fundamental for pregnancy outcome. It is still unclear if the CL dimensions may be considered to be a reliable indicator for P_4 production and/or the pregnancy rate.

Although CL area tended to be larger in pregnant buffaloes starting from Day 8 after TAI, a significant difference was only observed on Day 10 and similar values were recorded on average during the Day-5 to Day-10 period. The influence of CL dimensions on pregnancy rate is controversial. While in some studies, pregnant buffaloes showed a higher CL area compared to non-pregnant subjects (10, 27), no differences were observed in other studies (28,29). In a recent trial (30), a difference in CL dimensions between pregnant and non-pregnant buffaloes was observed only 14 days after insemination, while no differences were recorded during the Day-5 to Day-10 period. Therefore, further investigations are needed to clarify this aspect. Conversely, according to other authors, CL blood flow can be considered to be a consistent indicator of luteal functionality rather than its dimensions (31). The results obtained in our trial further confirm this interesting aspect because TAMV values significantly affect the likelihood of pregnancy, as observed in previous studies (10). After ovulation, the developing CL needs blood substrates for P_4 production and several studies support a strong correlation between blood flow and P_4 levels, either in cattle (32) or in buffalo (10). However, a significant increase of blood flow has been correlated with pregnancy in cattle only 15 days after insemination (33), although some recent studies performed in Holstein cows highlight a difference between pregnant and non-pregnant cattle from Day 7 post estrus (28). In the present trial, TAMV values recorded on Day 8 post-TAI seem to significantly influence the likelihood of pregnancy in the buffalo species. This was supported by a recent study in which a significantly higher luteal blood flow and TAMV values were observed on Day 7 post-estrus in successfully pregnant Holstein cows, which were used as recipients for ET and treated with GnRH on Day 5, compared to non-pregnant counterparts (28). However, according to these authors, the simultaneous evaluation of TAMV and the blood flow area may increase the accuracy of pregnancy prediction in cattle. In our trial, the blood flow area was not measured, but a significantly influence of TAMV values was observed on Day 8, rather than Day 7. However, a delayed evaluation of blood flow on Day 8 cannot be ruled out, which may also increase the chances of pregnancy prediction in cattle. If these interesting findings are confirmed, this parameter may be used for an early pregnancy diagnosis either in cattle or in buffalo.

To determine the reliability of our results and to use them in a practical way, we used the ROC curve (34) to set a good cutoff value to predict pregnancy. Our results confirmed that both TAMV and P_4 blood levels at Day 6 could already provide some important information, but more reliable results in terms of sensitivity and specificity could be recorded directly on Day 10. Kanazawa et al [29] made a similar experiment in cattle and reported that, already at Day 7 it was possible to set up a cutoff value for both TAMV and P_4. Considering these findings, both TAMV and P_4 measurements seem to be better indexes of luteal function compared to the CL area. Moreover, the

use of color Doppler ultrasonography could avoid stressful conditions for an animal that could not undergo blood sampling. We are aware that, going very early during the CL development, we had cut off values with low sensitivity and specificity. In our study, the highest values for sensibility and specificity among the days evaluated (5 to 10) are observed on day 10. Actually, day 10 may not be the best day to perform a non-pregnancy diagnose based on CL vascularization, in the perspective of a practical use. However, postponing this specific time window will delay the resynchronization of the open females, and allow breeders to save money. Moreover, there will always be false positives, such as animals that, due to asynchronized ovulations, fail to respond to the TAI or that undergo embryo mortality. We are aware that further studies and a higher number of animals are necessary to confirm our results.

7.5. Conclusions

In conclusion, this study demonstrated that proper CL functionality and development from Day 5 to Day 10 post-TAI is crucial for pregnancy maintenance. The evaluation of CL blood flow using an eco-color Doppler technique is a noninvasive procedure that can be practically used to obtain useful information on CL functionality. Finally, the evaluation of the TAMV in the CL from Day 8 after TAI can be used to estimate likelihood of pregnancy, and thus to estimate the potential benefits of using an early resynchronization strategy to increase the number of pregnancies in buffaloes.

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Chapter 8

Milk Metabolomics Reveals Potential Biomarkers for Early Prediction of Pregnancy in Buffaloes Having Undergone Artificial Insemination

CHAPTER 8 –

MILK METABOLOMICS REVEALS POTENTIAL BIOMARKERS FOR EARLY PREDICTION OF PREGNANCY IN BUFFALOES HAVING UNDERGONE ARTIFICIAL INSEMINATION *

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8.1. Introduction

Buffalo (*Bubalus bubalis*) breeding has achieved remarkable production standards in the last twenty years (1). The competitiveness of breeding is further implemented by the use of reproductive biotechnologies, enabling selective objectives to be reached more quickly (2). Among these, artificial insemination (AI) has been largely applied (3–5), reaching considerable results, comparable or higher than those obtained for cattle (6). In fact, recently, the difficulties that lead to low fertility in buffaloes after AI have been largely overcome (6) through a deeper understanding of the physiological mechanisms that regulate the ovarian cycle (7), the factors that influence embryonic mortality (8,9), and the development of new protocols for the synchronization of estrus (6) and ovulation (10). The latter two aspects are fundamental in buffaloes, since, in this specie, the estrus behavior is less evident and difficult to detect naturally, compared to cattle (11). All these features allowed to gain a pregnancy rate higher than 50%.

The combined effect of consecutive AI treatments and the utilization of ultrasound is a reliable tool for reducing the days open, and consequently the intercalving period, increasing the reproductive efficiency of the herd in both pluriparous buffaloes (12) and heifers (13). In fact, the possibility of utilizing some resynchronization protocols in non-pregnant buffaloes allows to reduce the interval

between an unsuccessful AI and the subsequent insemination on the same animal (14). However, one of the main limitations of these schedules is the time required for pregnancy detection, that can be hardly performed before 25 days post-mating (15). Today, the ability to determine the pregnancy status of cows as soon as possible after artificial insemination (AI) has become the most important thing to obtain in an ideal farm. Several efforts have been made to discover biomarkers of early pregnancy but, as of today, without any particular result. Most of the studies were carried out on non-invasive and cheap biological fluids, such as milk. Several attempts have been carried out to identify biomarkers of early pregnancy in cattle (16–18) but without satisfactory results. One of the first attempts that was developed to achieve an early pregnancy diagnosis was the detection of blood progesterone (19). The latter is an indirect method for pregnancy and particularly non-pregnancy diagnosis in many livestock species including cattle, buffaloes, sheep, and goats (19–21). In buffalo, the detection of plasma progesterone allows the diagnosis of non-pregnant animals after 21 days post AI (20), but it is reliable only if AI or breeding dates are known/recorded and has low accuracy due to the variable length of the estrus cycle. Some studies were carried out on pregnancy-associated glycoproteins (PAG), a large family of proteins that are produced by the placenta of ruminants (22,23). Although the diagnosis through PAG assay is particularly reliable, it cannot be performed before 28 days of pregnancy (24). In cattle, the potential of miRNA biomarkers for early pregnancy detection serving as a 'biomarker signature' in milk has been recently analyzed but without success for a diagnostic use (25).

The characterization of the metabolome can represent a new promising approach for identifying pregnant subjects. Metabolomics is a field of "omics" sciences very useful for the characterization of large numbers of small molecules within a biological sample using high-throughput methodologies (26,27). Several techniques, such as liquid chromatography tandem-mass spectrometry (LC–MS), nuclear magnetic resonance (NMR) spectroscopy and gas chromatography-mass spectrometry (GC-MS), have been developed and used for metabolite detection purposes (28–30). The levels of these metabolites reflect the metabolic and health status of the subjects and may be used for discriminating among different phenotypes (31). Evidence indicate that the presence or absence of specific combinations of milk metabolites is strongly correlated with its properties and the physiological status of animals (32). Furthermore, some studies performed over the last 30 years demonstrated the fundamental importance of either metabolic hormones (such as GH, IGF1, insulin, leptin, and thyroxine) and metabolic factors (glucose, fatty acids) in the reproductive field (33).

Therefore, the aim of this study was to investigate the milk metabolomic profile for the identification of novel potential biomarkers to be used in early prediction of pregnancy in buffaloes that have undergone AI.

8.2. Materials & Methods

8.2.1. - Farm and animals

The trial was carried out on 20 animals (51.85 ± 0.72 days in milk and 2.95 ± 0.20 parity), selected in a large group of 31 buffaloes (125.90 ± 15.54 days in milk) during the transition period from decreasing to increasing day light length. The animals were bred in a commercial farm located in the South of Italy between 39.0 °N and 41.5 °N. Lactating buffaloes were maintained in open yards in 12 m^2/head and 80 cm manger. Animals were fed a total mixed ration consisting of maize silage, oat hay, corn meal and soybean meal and characterized by 0.91 Milk Forage Units (MFU), 15% crude protein on dry matter, and 20% starch, with a forage concentrate ratio of 60:40. Buffaloes were milked twice daily, and milk yield was recorded in each milking by the software in the milking machine (Afifarm, TDM, Nutriservice, Brescia, Italy). The reproductive management of the herd was carried out by using a re-synch protocol, as described in another study (10). At the beginning of the study, all buffaloes underwent clinical examination 12 days apart by clinical examination and ultrasound monitoring to evaluate the presence of a corpus luteum (CL) in at least one examination. Only buffaloes with a functional CL assessed by eco-color Doppler technique (34) and without any gross abnormality of the genital tract were included in the study.

8.2.2. - Synchronization Treatment and AI

The buffaloes underwent synchronization of ovulation and timed artificial insemination by the Ovsynch-TAI protocol (5). Briefly, it consists in a GnRH analogous administration on Day 0 (buserelin acetate, 12 mg; Receptal, Intervet, Milan, Italy), a prostaglandin F2α on Day 7 ($PGF_{2\alpha}$ analogue, luprostiol, 15 mg; Prosolvin,Intervet, Milan, Italy), and a further GnRH agonist administration on Day 9. Timed AI was performed on Day 10, at 60 and 16 hours from the $PGF_{2\alpha}$ and the last GnRH, respectively. Because of the low intensity of estrus behavior in buffaloes, animals that showed a tonic uterus and a follicle higher than 1 cm, with or without mucus vaginal discharge, were considered to be in estrus and inseminated. Artificial inseminations were performed by the same technician using frozen/thawed semen of one bull of proven fertility.

Furthermore, on the day of AI each buffalo underwent an ultrasound examination with a portable machine (MyLab 30Gold, Esaote, Italy) equipped with a trans-rectal 7.5 MHz linear probe: preovulatory follicle (FL) dimensions were measured, and FL area was calculated according to the following equation:

$$FL\ area = \left(\frac{a}{2}\right) * \left(\frac{a}{2}\right) * \pi$$

Where:

FL = Follicle
a = major axis of the follicle
b = minor axis of the follicle
π = 3.14

8.2.3. - Milk Sampling and Ultrasound Examination

Milk was sampled at the start of the synchronization treatment (Day -10; D10), on the day of AI (Day 0; D0), 7 days after AI (+7; D7) and 18 days after AI (+18; D18). After collection milk was stored at −80 °C until analyses, that were performed after pregnancy assessment. Pregnancy diagnosis was performed on day 27 post-TAI using the same ultrasound machine described above and was confirmed on day 45 and 70 post-TAI. Animals that were pregnant on day 27 but not pregnant on day 45 were considered to have undergone late embryonic mortality (LEM), whereas those pregnant on day 45 but not on day 70 were considered to have experienced fetal mortality (FM). As described above, after pregnancy diagnosis assessment, 20 pluriparous buffaloes (between second and third parity) that have undergone the first AI (10 pregnant and 10 non-pregnant) were selected. The differences between pregnant and non-pregnant subjects were retrospectively analyzed by a metabolomic approach.

8.2.4. - Metabolomic analysis

The milk metabolites extraction was carried out mixing 100 µl from each sample with 300 µl of methanol (Sigma-Aldrich, city, state, USA) and vortexing for 30 s at room temperature. Mixtures were centrifuged at 12,000 × g for 15 min at 4 °C. Then, extracted supernatants were used. LC–MS analyses for detection of milk metabolites and for acetyl carnitine (3-Acetoxy-4-(trimethylammonio)butanoate) identification and quantification were done using an HPLC 1260 Infinity Series (Agilent Technologies, Santa Clara, CA, USA) coupled to a Q-TOF mass spectrometer model G6540B (Agilent Technologies) with a Dual Electrospray Ionization (ESI) source and equipped with a DAD system (Agilent Technologies). Ascentis® Express C18 column (2.7 µm, 50 mm x 3.0 mm i.d., Supelco©, Bellefonte, PA, USA) was used for separations. Flow-rate was set at 0.500 mL/min. The elution was done at constant temperature of 40 °C, using a linear gradient composed by A: 0.1% (v/v) formic acid (FA) in H_2O and B: 0.1% (v/v) FA in acetonitrile (ACN). The gradient was as follows: starting condition 5% B, ramping to 95% B in 12 min, lowering to 5% B in 1 min and equilibration at 5% B for 5 min. UV spectra were collected by DAD every 0.4 s from 190 to 750 nm with a resolution of 2 nm. Targeted MS and targeted MS/MS parameters were set with Agilent MassHunter Data Acquisition Software, rev. B.05.01. The instrument operated in positive mode, as [M + H] + ions; MS spectra were recorded in centroid mode, with an m/z 50–1700 mass range and with a speed of 3.3 spectra/s. Capillary voltage was set at 2000 V, fragmentor at 180 V, cone 1 (skimmer 1) at 45 V, Oct RFV at 750 V. Drying gas flow was set at 11L/min at a temperature of 350 °C, and the nebulizer was set at 45 psig. The injected sample volume was 5 µL.

In order to perform real-time lock mass correction, an Isocratic pump (1260 Infinity Series, Agilent Technologies) was used to infuse a standard solution consisting of two reference mass compounds: purine ($C_5H_4N_4$, m/z 121.050873, 10 µmol/L) and hexakis (1H,1H,3H-tetrafluoropentoxy)-phosphazene ($C_{18}H_{18}O_6N_3P_3F_{24}$, m/z 922.009798, 2 µmol/L). Flow rate was set at 0.06 mL/min, while the detection window and the minimum height were set at 1000 ppm and 10000 counts, respectively, for reference mass correction.

8.2.5. - Metabolite Identification and Quantification

Raw data were evaluated using Mass Hunter Qualitative Analysis Software, rev B.06.00 (Agilent Technologies), while compound identification was carried out using a freely available electronic database Milk Composition Database (MCDB) and an in-house database. N- acetyl carnitine was quantified by comparison with a standard compound purchased from Sigma-Aldrich.

8.2.6. - Statistical Analyses

Data on milk yield and follicle area between pregnant and non-pregnant buffaloes were analyzed by ANOVA. Statistical analysis of metabolomic data was carried out by using Mass Profile Professional, version 13.1.1 (Agilent Technologies). Specifically, a one-way analysis of variance, one-way ANOVA (p-value < 0.05), was performed and results obtained were subjected to principal components analysis (PCA) and hierarchical clustering.

8.3. Results

8.3.1. – Reproductive Activity

Total pregnancy rate detected 27 days after AI was 54.8% (17/31) and declined to 45.2% (14/31) on day 45, which means an incidence of late embryonic mortality (LEM) of 17.6% (3/17). No fetal mortality was recorded during this investigation. Further data are reported only for 20 selected buffaloes. No differences were found on milk yield between pregnant and non-pregnant buffaloes (Table 1).

Table 1. Average of milk yield (kg) in successfully pregnant (P) and non-pregnant (NP) buffaloes on Day 70 post artificial insemination (AI), measured at the beginning of the synchronization treatment (Day -10), at the artificial insemination (Day 0), and later at 7 (+7) and 18 days (+18).

Groups	**Time (Days)**			
	-10	0	+7	+18
P	8.78 ± 0.9	8.15 ± 0.9	9.02 ± 1.0	8.25 ± 0.8
NP	7.48 ± 0.8	6.88 ± 0.9	7.43 ± 0.8	7.49 ± 0.8

Data are expressed as means ± standard error

The size of the preovulatory follicle recorded on the day of Ovsynch-TAI protocol (TAI) was significantly ($p < 0.05$) lower in pregnant buffaloes than non-pregnant counterparts (1.21 ± 0.1 vs. 1.40 ± 0.1 cm, respectively).

8.3.2. – Milk Metabolic Profile

The milk metabolic profile differed between pregnant buffaloes and those non-pregnant. Distinct separation between these two groups was evident in principal components (PC1–PC2) of the variance in the LC–MS dataset. More in detail, PC1–PC2 values were accounting 76.79–99.49% and 68.49–82.68%, respectively, for each sample (Figure 1 A, B, C, D).

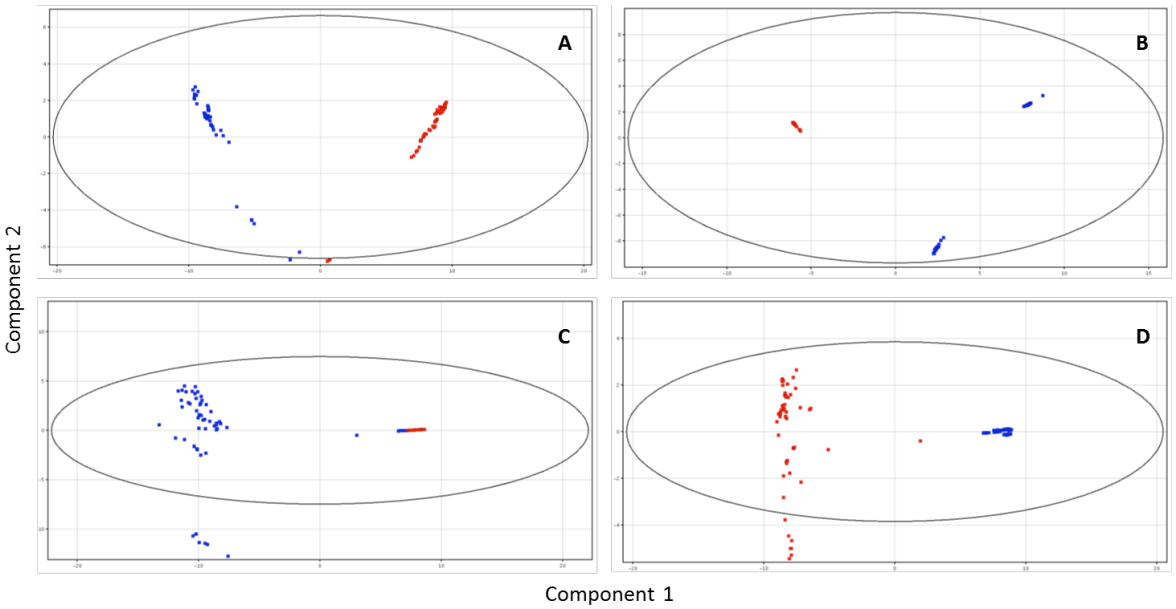

Figure 1. Principal components analysis (PCA) scores plots of the LC–MS data acquired for the four milk samplings from pregnant (in red) and non-pregnant (in blue) buffaloes. (A) (first sampling): PC1 occupies 69.37% and PC2 7.42% of total variance; (B) (second sampling): PC1 72.26% and PC2 27.23%; (C) (third sampling): PC1 61.45% and PC2 7.04%; and (D) (fourth sampling): PC1 79.85% and PC2 2.83%.

Moreover, hierarchical clustering analysis also showed a clear separation of pregnant and non-pregnant buffaloes. Based on the PCA loadings, a list of metabolites (Table S1–S4, reported in supplementary materials) whose changes in milk led to the clustering is presented in Figure 2 A, B, C, D). Clustering for the two groups of buffaloes highlighted the total variances within the PCA data. Differences between pregnant and non-pregnant buffaloes were clearly associated with differences in chemical composition (Figure 2).

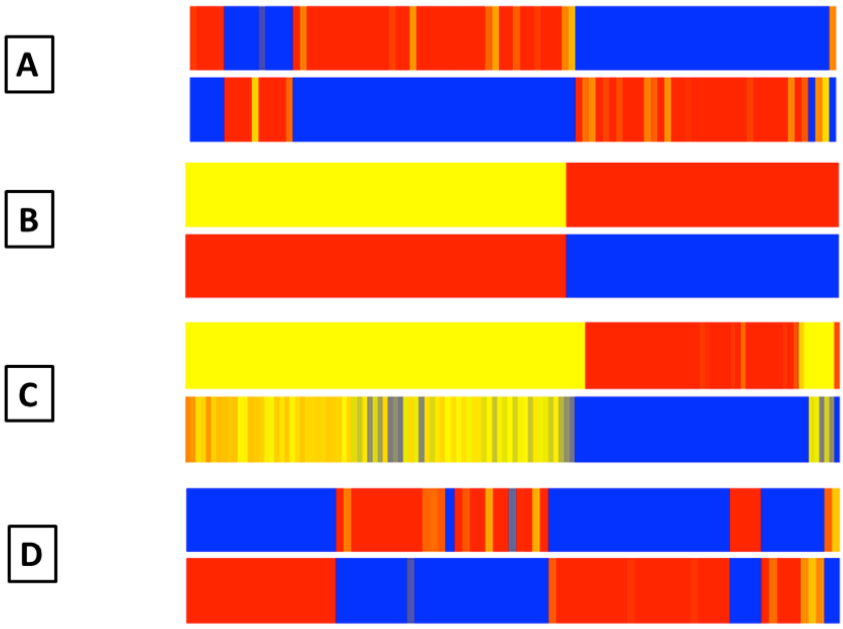

Figure 2. Hierarchical clustering of 80 ((A) first sampling), 67 ((B) second sampling), 103 ((C) third sampling), and 81 ((D) fourth sampling) differentially expressed compounds in pregnant (P) and non-pregnant (NP) buffaloes. The differently expressed compounds are reported in Table S1, S2, S3 ,S4.

Metabolomic analysis data revealed the presence of several metabolites differentially expressed (80, 67, 103, and 81 at D10, D0, D7, and D18, respectively; Table S1, S2, S3, S4) in the milk of pregnant and non-pregnant buffaloes. Among these, five metabolites were identified by comparison with an online database and a standard compound. The identified compounds were arginine-succinic acid hydrate, 5'-O-{[3-({4-[(3Aminopropyl)amino]butyl}amino)propyl]carbamoyl}-2'-deoxyadenosine, N-(1-Hydroxy-2-hexadecanyl) pentadecanamide, N-[2,3-Bis(dodecyloxy)propyl]-L-lysinamide), and acetyl carnitine (Table 2).

Table 2. Secondary metabolites identified in the milk samples. Identifications were confirmed by comparing results with known compounds present in a freely available electronic database Milk Composition Database (MCDB), an in-house database / standards and selecting matching, with a score ≥95%.

Metabolites	Sampling times			
	D10	D0	D7	D18
Acetyl carnitine (3-Acetoxy-4-(trimethylammonio)butanoate)	↓	↓	↓	↑
Arginine-succinic acid hydrate	↑	↑	↔	↔
5'-O-{[3-({4-[(3 Aminopropyl)amino]butyl}amino)propyl]carbamoyl}-2'-deoxyadenosine	↔	↓	↓	↔
N-(1-Hydroxy-2-hexadecanyl) pentadecanamide	↑	↑	↔	↔
N-[2,3-Bis(dodecyloxy)propyl]-L-lysinamide	↔	↔	↔	↓

↑Increased production of the metabolite in pregnant vs. non-pregnant. ↓Decreased production of the metabolite in pregnant vs. non-pregnant. ↔Unchanged production of the metabolite in pregnant vs. non-pregnant.

This metabolomic profile was dominated by the presence of acetyl carnitine. Targeted analysis of N-acetyl carnitine revealed its occurrence in milk samples from non-pregnant buffaloes starting from D10 up to D18, with a consistent decrease over the sampling time (Table 3). On the contrary, in milk from pregnant buffaloes, acetyl carnitine was observed only in the last sampling time (D18) (Table 3).

Table 3. N-acetyl carnitine (g/mL) quantification recorded in successfully pregnant (P) and non-pregnant (NP) buffaloes on Day 70 post AI, measured at the beginning of the synchronization treatment (Day -10), at the artificial insemination (Day 0), and later at 7 (+7) and 18 days (+18).

Group	N-acetyl carnitine (g/mL) Sampling times			
	D10	D0	D7	D18
P	N.D.	N.D.	N.D.	0.3 ± 0.5
NP	2.1 ± 1.1	3.3 ± 3.2	0.8 ± 0.5	0.2 ± 0.4

N.D. = Not detectable. Data are expressed as means ± ES.

8.4. Discussion

The improvement of reproductive efficiency is a key point to reduce the intercalving period and optimize the economic sustainability of the buffalo farm. This is particularly important in Italy, in order to guarantee milk availability during the period of greatest market demand for mozzarella cheese production (35). The application of re-synchronization protocols, together with eco Color-Doppler technique, allowed to gain an intercalving period of about 400 days (12). However, a reliable pregnancy diagnosis can be carried out at least 25 days post-AI by ultrasound, limiting the efficiency of the technique and increasing the number of days open. This can be achieved by both identifying real potential biomarkers of early pregnancy, such as those compounds that differ between pregnant and non-pregnant animals on D10 or D18 and recording different molecules before insemination. In fact, on D10 and D18 a pregnancy is effectively ongoing, whereas differences in milk metabolites on D10 and D0 may account for the incapacity of conception, i.e., for the development of the preovulatory follicle: therefore, they can be defined as biomarkers of potential conception.

The pregnancy rate recorded in this trial is comparable to that obtained in other studies, as well as the incidence of LEM (12,36). The latter is considered one of the main causes of fertility loss, in particular in some periods of the year (8,9). Both the seasonality of the species and the market requirements of milk in Italy, oblige to mate the animals out of the breeding season, increasing the incidence of LEM till 40% in some farms (37). On the contrary, an incidence of LEM higher than 10% is rarely recorded during the breeding season (38,39).

According to our results, the diameter of the preovulatory follicle may play a key role in the improvement of AI efficiency. In fact, a significantly smaller diameter has been recorded in pregnant buffaloes compared to non-pregnant counterparts. Several studies carried out in cattle demonstrated the deleterious effects of a prolonged follicle dominance on oocyte competence and embryo development (40). It is likely that, particularly in animals synchronized by the Ovsynch-TAI Program, the failure of ovulation following the first GnRH administration may be responsible for a persistent dominant follicle (41). This interesting aspect demonstrated in cattle (42) may be confirmed by a recent trial carried out on buffalo species (5), in which the follicular response to the first GnRH of an Ovsynch-TAI program on pregnancy outcome was evaluated. In this study buffaloes that ovulated after the administration of the first GnRH showed smaller area of the ovulatory follicle compared to those that did not ovulate. Interestingly, these animals had also a

significantly greater ovulation rate to the second GnRH (5). Further studies are needed to evaluate this aspect in buffalo.

The main aim of this study was to identify potential biomarkers for early pregnancy diagnosis in buffalo through a metabolomic approach. Over the last years, this technique was tested in several fields of veterinary science, using different biological fluids (43–45). In dairy animals the milk represents an ideal substrate, as the procedure for collection is easy, non-invasive and without any stress for the animals. Blood metabolites are concentrated in the milk by filtration, thus reflecting the metabolic status of the subjects. To our knowledge this is the first study that utilizes LC–MS technique for the identification of novel biomarker of early pregnancy in buffalo milk.

Several pathways involved in the milk biosynthesis were different between pregnant and non-pregnant buffaloes in this study. Milk metabolite profiling has been found to be successfully related to the onset of early pregnancy after AI treatment.

Among the compounds differently expressed, only acetyl carnitine was quantified. Interestingly, this compound was present in milk samples from non-pregnant buffaloes collected in each sampling time, while only on D18 was detected in pregnant animals. Likely, the source of acetyl carnitine in the milk is relocated or lost in favor of other tissues.

Metabolic analysis in early pregnancy is crucial for the identification of molecular pathways useful for the definition of new treatment strategies Among metabolites in milk from pregnant buffaloes, a positive modulation was observed for N-(1-Hydroxy-2-hexadecanyl) pentadecanamide (D10, D0), arginine- succinic acid hydrate (D10, D0), and acetyl carnitine (D18). Instead, unchanged or negatively modulated metabolites of nutritional value were 5'-O-{[3-({4-[(3 Aminopropyl)amino]butyl}amino)propyl]carbamoyl}-2'-deoxyadenosine, and N-[2,3-Bis(dodecyloxy)propyl]-L-lysinamide.

N-(1-Hydroxy-2-hexadecanyl) pentadecanamide is a derivative of pentadecanoic acid, a fatty acid with exogenous origin (primarily ruminant), which constitutes 1.05% of milk fat and 0.43% of ruminant meat fat. It belongs to the odd chain saturated fatty acids (OCS-FAs) family and is produced in relatively high levels by rumen microbial fermentation and microbial de-novo lipogenesis, which then transfers into the host animal (46). Moreover, the OCS-FAs produced by the animal rumen are then utilized by the mammary gland for the production of milk fat (47). Increased consumption of dairy products has been associated with an increase of OCS-FAs plasma levels. Indeed, pentadecanoic acid has been gaining interest within the scientific community as it

has been found to be important as low-cost internal standards in quantitative analysis, a blood biomarker for milk fat intake, and biomarker for coronary heart disease (48).

Acetylcarnitine from food source or as a supplement is known for its antioxidant activity, with particular regard to the neuroprotective action against central and peripheral nervous system injury (49–51).

Arginine- succinic acid hydrate is an arginine derivative. L-Arginine, is known to be particularly abundant in certain foods, such as meats and nuts, is the substrate for the enzyme nitric oxide synthase (NOS), involved in the production of nitric oxide (NO) (52,53). Recently, arginine-rich foods were shown to be inversely associated with endothelial dysfunction in hypercholesterolemia patients. In particular, evidence show that long-term L-arginine intake increases insulin sensitivity, improves glycemic indices, and reduces cardiovascular complications (54–56).

5'-O-{[3-({4-[(3Aminopropyl)amino]butyl}amino)propyl]carbamoyl}-2'-deoxyadenosine is an adenosine derivative. Adenosine, an essential metabolite distributed in several mammalian tissues (57,58), acts as a ubiquitous endogenous cell signaling and modulator agent since it directly affects a variety of synaptic processes and signaling pathways, thus playing an important role in the regulation of several neurotransmitters in the central nervous system (59). Moreover, adenosine produced in hypoxic, ischemic, or inflamed environments reduces tissue injury and promotes repair (60). Due to its extremely short half-life in human blood, prolonged release systems to improve its efficacy or food source capable of sustaining its intake are of great interest (61–64).

Finally, our metabolomic analysis showed unchanged levels of N-[2,3-Bis(dodecyloxy)propyl]-L-lysinamide from D10 up to D7. This metabolite is a derivative of lysine, an essential amino acid commonly assumed as dietary supplement, especially in countries where insufficient L-lysine is ingested from food. Indeed, supplemental L-lysine benefits include increase in the intestinal calcium absorption in osteoporosis, anxiety reduction, and muscle recovery in exercise or sarcopenia support (65–67).

8.5. Conclusions

On the whole, findings of this study reveal a peculiar metabolic profile of milk produced by pregnant and non-pregnant buffaloes with significant differences in their content. Beside the chemical and nutritional characterization of milk, these data suggest the effectiveness of the metabolomic analysis for the identification of novel potential biomarkers in early prediction of pregnancy in buffaloes after AI.

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Chapter 9

A Preliminary Study on Metabolome Profiles of Buffalo Milk and Corresponding Mozzarella Cheese: Safeguarding the Authenticity and Traceability of Protected Status Buffalo Dairy Products

CHAPTER 9 –

A PRELIMINARY STUDY ON METABOLOME PROFILES OF BUFFALO MILK AND CORRESPONDING MOZZARELLA CHEESE: SAFEGUARDING THE AUTHENTICITY AND TRACEABILITY OF PROTECTED STATUS BUFFALO DAIRY PRODUCTS*

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9.1. Introduction

The authenticity, integrity, and traceability of food products is very important for market protection in global food systems (1–4). This applies particularly to food that has a market niche and for which consumers pay a high price. The premium price of niche food makes it susceptible to substitution with falsely-labelled, non-authentic food (5). Examples are substitution in olive oil (6,7) and dairy products (8–10). Italian mozzarella cheese is made from the milk of Italian Mediterranean water buffalo (*Bubalus bubalis*) and is recognized globally for its exceptional eating qualities. The water buffalo undergo intense selection for efficiency of production and product quality, and today has an important commercial and cultural niche in several regions of Italy. Genuine Italian mozzarella is known as "Mozzarella di Bufala Campana." It has European Union protected designation of origin (PDO) and protected geographical indication (PGI) status. This identifies genuine mozzarella with four regions in Italy (Apulia, Campania, Lazio, Molise) (11,12). Attempts are regularly made to

place non-authentic mozzarella in premium markets as a substitute for buffalo mozzarella cheese (13–15). Consumers can be denied a genuine product and confidence in certified farmers is threatened. The potential for product substitution has led to interest in the development of screening technology that ensures the authenticity of buffalo mozzarella and traceability to geographical origin (14,16,17).

The characterization of the metabolome of food is a promising approach for establishing authenticity (18). The metabolome signature of biological material can be obtained using gas chromatography/mass spectrometry (GC-MS) (19,20), NMR spectroscopy (21,22), and high-resolution magic angle spinning (HRMAS) NMR spectroscopy (23). Some initial screening of buffalo mozzarella cheese has used NMR (16), HRMAS NMR (16), and GC-MS/LC-MS (14,15,17). To date, individual studies have looked at the metabolome of either buffalo milk or mozzarella. Substitution can occur at different stages of the supply chain and it is important that authenticity can be established from primary product (milk) through to secondary product (mozzarella). There are also limited studies in dairy on the impacts of breeding, diet, and geographical location on product quality (24). The present study sought to compare, for the first time, the metabolomes of both unprocessed milk and corresponding mozzarella cheese for buffalo in PDO and non-PDO regions in Italy. Differences in the milk and/or mozzarella metabolomes between buffalo in PDO and non-PDO regions have potential practical application in safeguarding the authenticity and traceability of protected status buffalo dairy products.

9.2. Materials & Methods

9.2.1. - Sample collection

The study utilized 20 commercial buffalo dairies. Eleven dairies were located in a protected designation of origin (PDO, Campania, Italy) region and nine dairies were in non-PDO regions in Italy. All dairies had a processing facility that produced mozzarella cheese exclusively from their own milk. Milk and mozzarella cheese quality was assessed the day of the sampling and the average results from PDO and non-PDO areas are reported in Tables 1 and 2. Pooled samples of raw milk (100 mL) and mozzarella cheese (100 g) were obtained from each dairy. The samples underwent similar processing and were obtained approximately 2 h after preparation. Mozzarella samples were immersed in mozzarella whey and stored at 25 °C until analysis.

9.2.2. - Metabolomics Analysis

Metabolite Extraction and Derivatization

Metabolite extraction, purification, and derivatization were carried out using the MetaboPrep GC kit according to the manufacturer's instructions (Theoreo srl, Montecorvino Pugliano (SA), Italy).

From each mozzarella sample 10 ± 1 mg was transferred to an Eppendorf microcentrifuge tube containing the extraction solution. The samples were then centrifuged at 800× *g* for 30 min, before putting the samples in an ultrasonic bath at 30 °C for 30 min. The samples were then centrifuged for 5 min at 10,000× *g* at 4 °C. From the supernatant, 200 µL was removed and transferred to an Eppendorf microcentrifuge tube containing a purification mixture, and then vortexed at 800× *g* for 30 sec. The sample was again centrifuged at 10,000× *g* (at 4 °C). Finally, 175 µL supernatant was transferred into a 2-mL glass autosampler vial and freeze-dried overnight.

To facilitate derivatization, "50 µL of pyridine/methoxamine (1/1 *v*:*v*) were added to the sample and centrifuged at 800× *g* (25 °C) for 90 min. Then 25 µL of the second derivatization mixture containing *N*,*O*-*bis*(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were added and vortexed at 800× *g* (25 °C) for 90 min. The solution was centrifuged for 5 min at 10,000 rpm× *g* (4 °C) before injecting into the GC-MS."

GC-MS Analysis

Samples (2 µL) of the derivatized solution were injected into the GC-MS system (GC-2010 Plus gas chromatograph coupled to a 2010 Plus single quadrupole mass spectrometer; Shimadzu Corp., Kyoto, Japan). Chromatographic separation was achieved with a 30 m 0.25 mm CP-Sil 8 CB fused silica capillary GC column with 1.00 µm film thickness (Agilent, J&W Scientific, Folsom, CA, USA), with helium as carrier gas. The initial oven temperature of 100 °C was maintained for 1 min and then raised by 6 °C/min to 320 °C with a further 2 min of holding time. The gas flow was set to obtain a constant linear velocity of 39 cm/s and the split flow was set at 1:5. The mass spectrometer was operated with electron impact ionization (70 eV) in full scan mode in the interval of 35–

600 *m*/*z* with a scan velocity of 3333 AMU/sec and a solvent cut time of 4.5 min. The complete GC program duration was 40 min.

9.2.3. - Metabolites Identification

Metabolite identification was performed according to Troisi et al. [44]. Briefly, untargeted metabolites were identified by comparing the mass spectrum of each peak with the NIST library collection (NIST, Gaithersburg, MD, USA). The linear index difference max tolerance was set to 10, while the minimum matching spectra library search was set to 85% (level 2 identification according to Metabolomics Standards Initiative [MSI]) [45]. Fifteen samples out of the over 200 signals per sample (7.5%) produced by gas chromatographic–mass spectrometry were not investigated further because they were not consistently found in other sets of samples (either too low in concentration or of poor spectral quality to be confirmed as metabolites).

9.2.4. - Statistical Analyses

Data regarding milk and mozzarella cheese quality are expressed as mean ± SE. Differences were assessed by Student's t-test, and $p < 0.05$ value was considered significant. The chromatographic data were tabulated with one sample per row and one variable (metabolite) per column. Data pre-treatment consisted of normalizing each metabolite peak area to that of the internal standard (2-iso-propyl malic acid) followed by generalized log transformation and data scaling by autoscaling (mean-centered and divided by standard deviation of each variable). Statistical analysis of data from three biological replicates for each farm was performed by ANOVA Bonferroni correction (p-value < 0.05) by applying GraphPad PRISM software. Only metabolites significantly different between the farms were considered for further analyses. Principal component analyses (PCA) and heatmap representations were conducted by the online tool ClustVis (http://biit.cs.ut.ee/clustvis/). Unit variance scaling was applied to rows (metal/metabolite values in each farm) and single value decomposition (SVD) with imputation was used to calculate principal components. The heatmaps were generated clustering columns (Farms) by correlation distance and McQuitty linkage. Samples were classified considering geographical area of origin (North/South) and also the presence or absence of PDO trademark (Yes = Y; No = N).

Moreover, partial least square discriminant analysis (PLS-DA) (46) was performed using the statistical software package R (Foundation for Statistical Computing, Vienna, Austria). Class separation was achieved by PLS-DA, which is a supervised method that uses multivariate regression techniques to extract, via linear combinations of original variables (X), the information that can predict class membership (Y). PLS regression was performed using the plsr function included in the R pls package (47). Classification and cross-validation were performed using the corresponding wrapper function included in the caret package (48). A permutation test was performed to assess the significance of class discrimination. In each permutation, a PLS-DA model was built between the data (X) and the permuted class labels (Y) using the optimal number of components determined by cross validation for the model based on the original class assignment. Variable importance in projection (VIP) scores were calculated for each metabolite. The VIP score is a weighted sum of squares of the PLS loadings, taking into account the amount of explained Y-variation in each dimension.

9.3. Results

No differences were observed in the quality of milk and mozzarella cheese from PDO and non-PDO regions (Tables 1 and 2). A total of 185 metabolites were detected consistently. In particular, 113 compounds were detected in milk, 102 in mozzarella cheese, and 30 compounds out of 185 were found in both matrices. The PLS-DA score plots clearly differentiated PDO and non-PDO milk and mozzarella samples (Figure 1(A1, B1)). The 15 highest scoring variable importance in projection VIP variables (VIP score > 1.5) identified by PLS-DA are shown in Figure 1(A2, B2).

Table 1. Average composition of milk samples from different farms located in protected destination of origin (PDO) and non-PDO regions in Italy. Data are expressed as means ± ES.

BUFFALO MILK					
Nutrition Facts for 100 g of Product (Reg. UE 1169/2011)	**NON-PDO REGION (n = 9)**		**PDO REGION (n = 11)**		***p* Value**
	U.M	**VALUE**	**U.M**	**VALUE**	
Energy net value	KJ/100 g	473 ± 8.4	KJ/100 g	479 ± 7.9	0.82
	Kcal/100 g	112 ± 3.5	Kcal/100 g	116 ± 3.7	0.77
Total protein	g/100 g	4.5 ± 0.7	g/100 g	4.6 ± 0.6	0.85
Total fat	g/100 g	8.4 ± 0.9	g/100 g	8.6 ± 0.8	0.69
Saturated fat	g/100 g	4.9 ± 0.5	g/100 g	4.9 ± 0.5	0.91
Total carbohydrates	g/100 g	5.2 ± 0.4	g/100 g	5.2 ± 0.5	0.82
Sugars	g/100 g	0.8 ± 0.1	g/100 g	0.9 ± 0.1	0.86
Salt	g/100 g	Nd	g/100 g	Nd	
Ashes	g/100 g	0.8 ± 0.0	g/100 g	0.8 ± 0.1	0.90

U.M. Unit measure.

Table 2. Average mozzarella cheese composition of samples from different farms located in protected designation of origin (PDO) and non-PDO regions in Italy. Data are expressed as means ± ES.

BUFFALO MOZZARELLA CHEESE					
Nutrition Facts for 100 g of Product (Reg. UE 1169/2011)	**NON-PDO REGION (n = 9)**		**PDO REGION (n = 11)**		***p* Value**
	U.M.	**VALUE**	**U.M.**	**VALUE**	
Energy net value	KJ/100 g	1129.9 ± 9.4	KJ/100 g	1131.1 ± 8.2	0.72
	Kcal/100 g	268.6 ± 4.0	Kcal/100 g	269.2 ± 3.2	0.84
Total protein	g/100 g	13.4 ± 1.0	g/100 g	13.5 ± 0.9	0.86
Total fat	g/100 g	23.3 ± 2.6	g/100 g	23.5 ± 2.9	0.73
Saturated fat	g/100 g	14.6 ± 0.4	g/100 g	14.6 ± 0.6	0.79
Total carbohydrates	g/100 g	0.8 ± 0.0	g/100 g	0.8 ± 0.0	0.91
Sugars	g/100 g	0.8 ± 0.1	g/100 g	0.8 ± 0.0	0.88
Salt	g/100 g	0.9 ± 0.1	g/100 g	0.9 ± 0.1	0.90
Ashes	g/100 g	1.4 ± 0.2	g/100 g	1.4 ± 0.2	0.93

U.M. Unit measure.

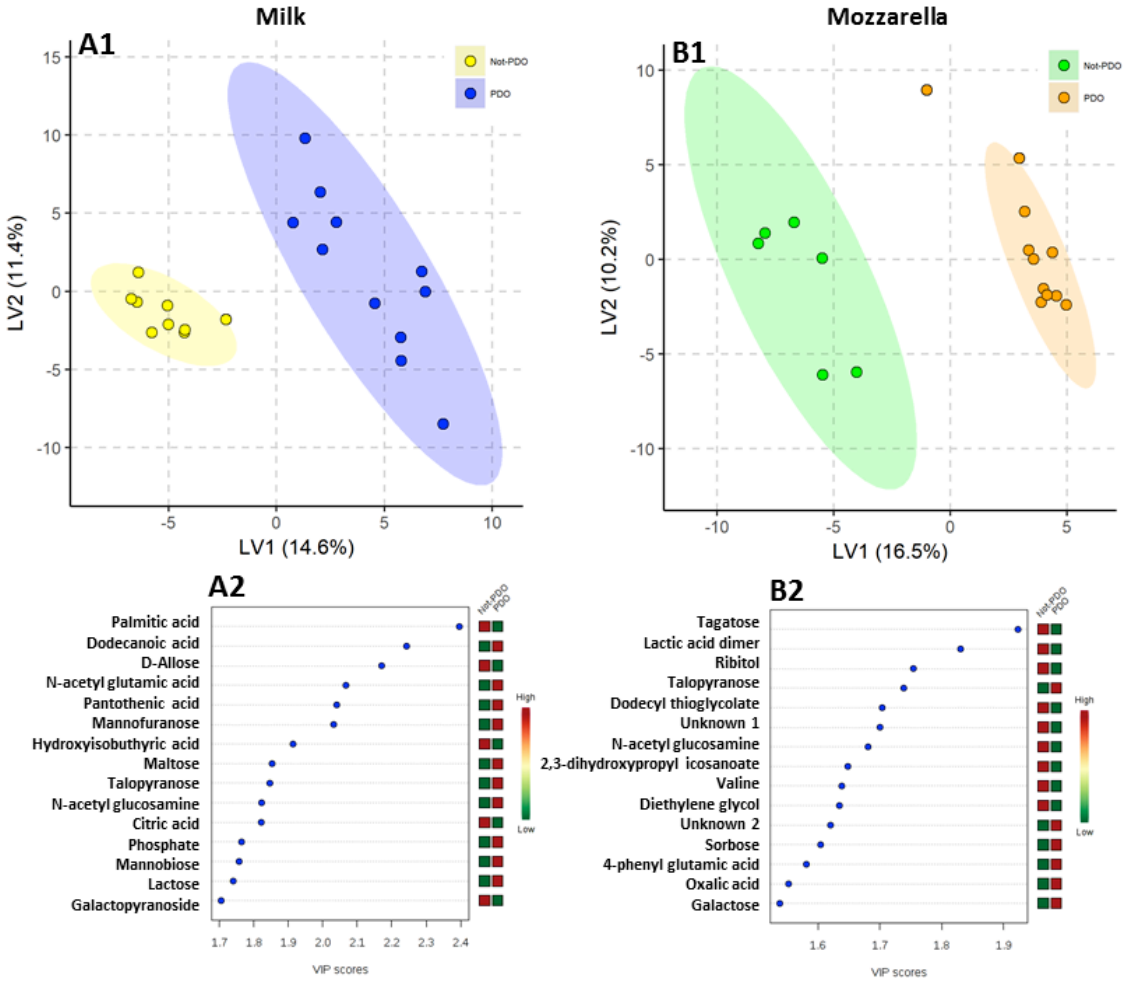

Figure 1. Partial least square discriminant analysis (PLS-DA) models to discriminate PDO and non-PDO buffalo milk (**A1**) and mozzarella (**B1**) samples. The explained variance of each component is shown in parentheses on the corresponding axis. (**A2**) and (**B2**) panels show the 15 top-scoring variable importance in projection (VIP) metabolites (VIP-score ≥ 1.5) for milk (A2) and mozzarella (B2) samples. The colored boxes on the right indicate the relative amount of the corresponding metabolite in each group under study.

Differences in metabolite concentrations between PDO and non-PDO raw milk samples allowed the metabolites to be separated into two classes: those with lower ($p < 0.05$) concentrations in PDO milk (galactopyranoside, hydroxybuthyric acid, allose, citric acid) and those with higher ($p < 0.05$) concentrations in PDO milk (talopyranose, pantothenic acid, mannobiose, maltose, phosphate, mannofuranose, dodecanoic acid, lactose, palmitic acid, n-acetyl glutamic acid, *n*-acetyl glucosamine) (Figure 2).

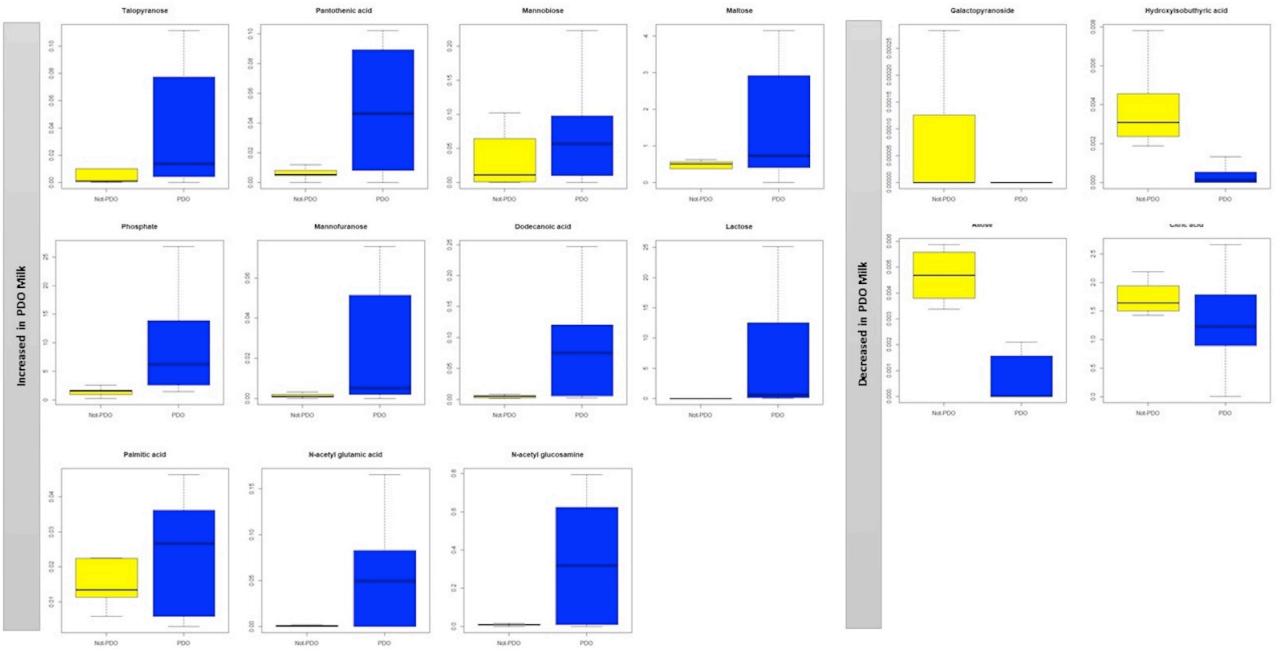

Figure 2. Box and Whisker plot of the VIP metabolites in buffalo milk samples. Boxes represent non-PDO (yellow) and PDO (blue) milk samples. The vertical axis reports the log of the gas chromatography mass spectrometry values of the normalized area of each metabolite.

Metabolites could also be separated into two classes for mozzarella cheese samples: those with higher ($p < 0.05$) concentrations in PDO mozzarella (talopyranose, 2, 3-dihydroxypropyl icosanoate, sorbose, 4-pnehyl glutamic acid, oxalic acid, galactose) and those with higher ($p < 0.05$)

concentrations in non-PDO mozzarella (tagatose, lactic acid dimer, ribitol, dodecyl thioglycolate, *n*-acetyl glucosamine, valine, diethylene glycol) (Figure 3).

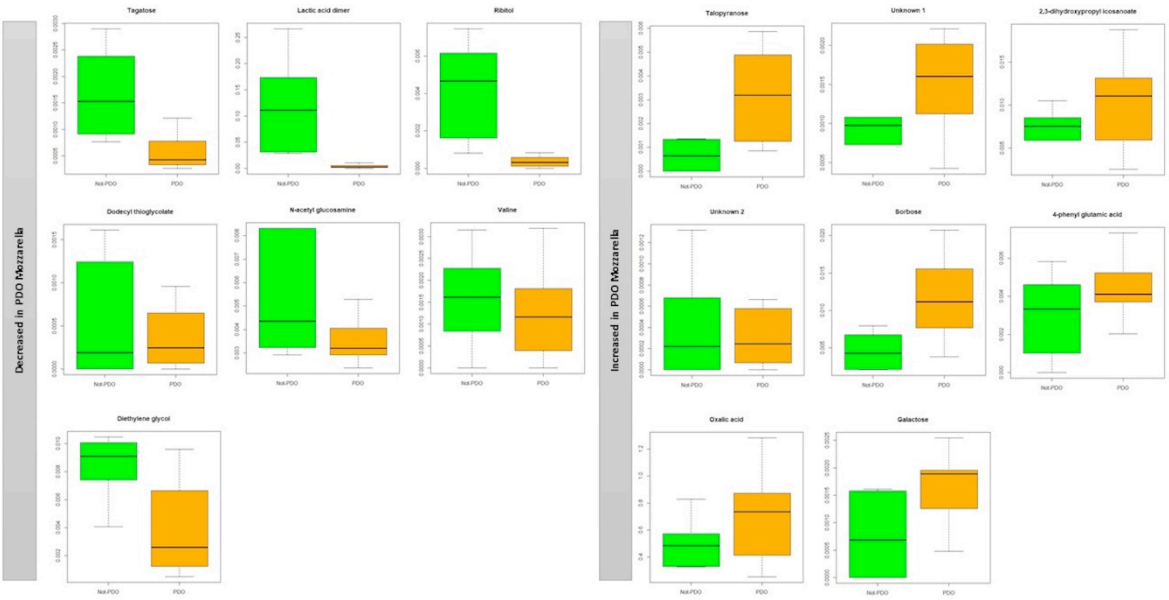

Figure 3. Box and Whisker plot of the VIP metabolites in buffalo mozzarella samples. Boxes represent non-PDO (green) and PDO (orange) mozzarella samples. The vertical axis reports the log of the gas chromatography mass spectrometry values of the normalized area of each metabolite.

9.4. Discussion

The present study sought to compare the metabolomes of unprocessed milk and corresponding mozzarella cheese for buffalo in PDO and non-PDO regions in Italy. It was found that the milk metabolome differed between buffalo in PDO and non-PDO regions. The mozzarella metabolome also differed between buffalo in PDO and non-PDO regions. This is the first time that some metabolites have been detected in both the metabolome of unprocessed milk and corresponding mozzarella cheese in buffalo. It is also the first time that differences have been found between buffalo in PDO and non-PDO regions in both milk and mozzarella metabolomes. The number of individual farms from PDO (n = 11) and non-PDO (n = 9) regions might be considered a relatively small sample size, although it was comparable to numbers in previous reports that looked at the metabolome [24–26]. Notwithstanding the sample size, the combination of GC-MS and mass spectral libraries (NIST library) proved to be a robust technology platform for determining the metabolomes of buffalo milk and mozzarella. This platform, together with a rigorous analysis of the data, has provided a sound foundation to inform further studies. In particular, a number of notable metabolites identified in unprocessed milk (n = 15) and mozzarella cheese (n = 13) could be used to validate the utility of the metabolome for safeguarding the protected status buffalo dairy products. Candidate metabolites that differentiated PDO from non-PDO milk included several carbohydrates (d-allose, mannofuranose, maltose, and talopyranose). Candidate metabolites that differentiated both milk and mozzarella between PDO and non-PDO origin were talopyranose and *N*-acetyl glucosamine. Talopyranose is the pyranose form of talose and an epimer of glucose. Talose was already reported to be a "differential" marker between bovine milk and goat milk (27). *N*-acetylglucosamine is a derivative amide of the monosaccharide glucose and a secondary amide between glucosamine and acetic acid. It is significant in several biological systems and a major component of the cell walls of most fungi (28). Talopyranose is of particular interest as amounts were substantially higher in both milk and mozzarella of PDO origin. A number of saccharides (tagatose, talopyranose, sorbose, galactose) differentiated mozzarella of DPO and non-DPO origin. Two metabolites in mozzarella cheese had markedly different concentrations between PDO and non-PDO samples. These metabolites could not be identified from public mass spectral libraries. However, they require further study as they may emerge as highly valuable in establishing the authenticity and integrity of protected status buffalo mozzarella cheese.

Irrespective of PDO or non-PDO origin, the milk aqueous fraction was rich in short-chain saturated carboxylic acids (carbonic acid, acetic acid, propanoic acid, butanoic acid, octanoic acid, decanoic

acid) and long-chain saturated carboxylic acids (lauric acid, palmitic acid, stearic acid). Free amino acids in milk included serine, threonine, and valine. Compared with milk, mozzarella cheese contained lower amounts of some short-chain saturated carboxylic acids (acetic, propanoic, nonanoic acid) and only two long-chain saturated carboxylic acids were found in mozzarella (palmitic acid, stearic acid). Mozzarella was, however, richer in free amino acids (serine, leucine, isoleucine, alanine, proline, valine, norvaline). Some of the metabolites found in mozzarella samples in the present study (lactic acid dimer, valine, oxalic acid, and galactose) were also identified in a study that looked at the metabolomic and microbiological differences of Italian mozzarella cheese produced with buffalo or cow milk (29).

Processing alters milk constituents and their concentrations. For example, milk monosaccharide levels change in response to heat treatment (30) and storage (31). The composition of the starter, which can be influenced by the environment, management and cheese-making technologies, also affects cheese characteristics by altering the milk quality. (32–34). In PDO mozzarella, there may have been a synergistic effect between different lactic acid bacteria and yeast species, which ferment residual galactose and lactose and thereby increase lactic acid production. The processed cheese industry uses citrate or phosphate salts to sequester Ca^{2+} from residual colloidal calcium phosphate. This solubilizes caseins which can then emulsify fat globules. The acidity of whey at drainage, and the rate of acid development, are important parameters that determine the mineral content, acidity, and quality of cheese (35). Milk of PDO origin had higher phosphate content and lower citric acid compared to non-PDO milk. Differences in milk salt composition most likely contributed to the differences in metabolites between PDO and non-PDO mozzarella cheese. Differences in packaging could also modify the metabolomic profile of mozzarella cheese. The higher diethylene glycol concentration in samples of non-PDO mozzarella compared to PDO was attributed to packaging (36).

Climatic conditions influence the rate of uptake of metabolites by plants (37,38). Also, lower temperatures and frequent rainfall can affect the drying process of forages; essentially, reducing sugars, mineral salts, and soluble nitrogenous substances, while increasing fermentation (39). Rainfall is usually less frequent in the PDO regions in the present study compared with the non-PDO regions, and the higher concentrations of some sugars (maltose, lactose, mannobiose, talopyranose) in milk of PDO origin may have been due to differences in climatic conditions. The climate and soils of a region directly and indirectly impact the biochemical and biophysical properties of food products. For example, the isotopic and elemental composition of milk is closely related to geographical origin and this carries over to the properties of cheese (39). To have PDO

certification, at least 70% of the dry matter of fodder, or 40% of the dry matter of the ration, must come from the PDO region. The use of local fodder helps to maintain the strict relationship between product and region for protected status of buffalo mozzarella cheese. There are close relationships between the environment, rumen microbiome, and animal metabolome. While the major families of microbes in the rumen are broadly similar across diverse landscapes (40), the relative populations of different microbes change according to local conditions of climatic, soil, feed, and management (40–42). The amount of crude protein, neutral-detergent fiber, and acid detergent lignin are higher in cultivars from the PDO region (43). It can be assumed that this would have influenced the rumen microbial populations and ruminal metabolome, which could have contributed, at least in part, to differences in the milk and mozzarella metabolomes between PDO and non-PDO regions.

In conclusion, a robust GC-MS and mass spectral library technology platform was used to identify for the first time the metabolome of unprocessed milk and corresponding mozzarella cheese in buffalo. Differences in both the milk and mozzarella metabolomes between buffaloes in PDO and non-PDO regions were also shown for the first time. A number of candidate metabolites in milk and mozzarella were identified that will be important in validation studies that aim to develop practical protocols to distinguish between PDO and non-PDO buffalo milk and mozzarella. Talopyranose was a particularly notable candidate metabolite as it differed substantially between PDO and non-PDO buffalo, for both milk and mozzarella. The development of quality assurance and certification protocols for milk and cheese will help to ensure the authenticity and traceability of primary (milk) and secondary (mozzarella) protected status buffalo dairy products. This is necessary to ensure that the investment in breeding, feeding, and management of buffalo in PDO regions is safeguarded.

9.5. References

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