Chapter V

FOLLICULAR DYNAMICS AND REPRODUCTIVE TECHNOLOGIES IN BUFFALO

Giuseppina Maria Terzano

Istituto Sperimentale per la Zootecnia
(Animal Production Research Institute)
Via Salaria 31, 00016 Monterotondo (Rome), Italy

The general characteristics of reproduction like seasonality, cyclicity and ovulation differ widely in mammals for the following reasons:

a) reproductive activity may take place during the whole year or at defined seasons, according to the species and their adaptation to environmental conditions; thus, photoperiod plays a determinant role in seasonal breeders such as rodents, carnivores and ruminants (sheep, goats, buffaloes, deer, etc.,). An extreme situation is observed in foxes with only one ovulation per year, occurring in January or February;

b) mammals may be distinguished according to the absence or presence of spontaneous ovulations: in the first group of mammals (rabbits, hares, cats, mink, camels, Llama), the ovulation is induced by mating and cyclicity is not obvious; in the second group, ovulation occurs spontaneously in each cycle, separating the follicular phase from the luteal phase;

c) the length of cycles is quite different among species: small rodents have short cycles of four or five days, farm animals and primates have longer cycles (sheep: 17 days; cow, goat, buffalo, horse and pig: 21 days; primates: 28 days), and dogs are characterized by long cycles of six to seven months, including a two month luteal phase (Concannon, 1993);

d) ovulation rates differ widely among species and breeds within a given species: in sheep for example, Merinos d'Arles or Ile-de-France breeds have only one ovulation per cycle, whereas average rates of two to four ovulations per cycle are observed in prolific breeds like Romanov or Finn (Land et al., 1973). An extreme situation is observed in some insectivores such as Elephantulus, with ovulation rates as high as 120 (Dryden, 1969).

Despite these differences, it is now established that common mechanisms regulate the ovarian function (Monniaux et al., 1997).

1. Oogenesis and the beginning of follicular growth

Oogenesis begins in the early life of the females and results in the constitution of a pool of primordial follicles. At the time of sexual differentiation, the foetal female gonad is constituted of oogonia and somatic cells from the mesonephros. Oogonia develop from primordial germ cells that have migrated into the ovary early in embryogenesis. Schematically, oogenesis involves three phases: a prolific phase (the oogonia divide actively), a meiotic phase (primary oocytes are formed), and an intense germ cells degeneration phase. The oocytes that survive to degeneration are arrested at the diplotene stage of the first meiotic division and are surrounded by a single layer of granulosa cells, constituting structures called primordial follicles. The three phases of oogenesis clearly overlap, but important differences of chronology are observed among the species studied. The meiotic phase occurs during the prenatal life in most mammals: in the rabbit oocytes formation begins two days after birth, in the pig oocytes formation also begins early during foetal life but oogenesis is completed only during the first weeks after birth, in sheep and cattle the oogonia and oocytes are formed during the first half of foetal life and in water buffaloes the formation of primordial follicles is finished completely before birth (at

127.84 ± 11.55 days, when crown-rump length (CRL) is = 22.84 ± 4.74 cm), whereas in woman the oogenesis finishes at birth (Figure 1).

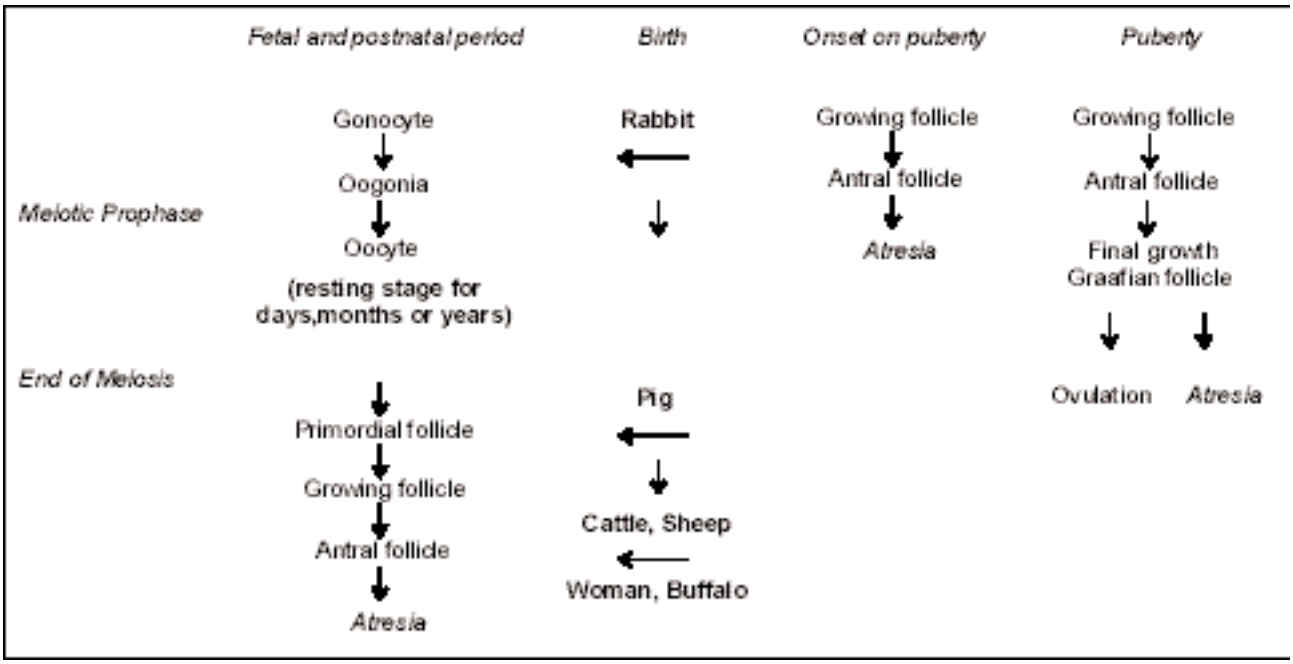

Figure 1. Gametogenesis in female mammals from fetal life to active sexual life

At the end of oogenesis, the ovary encloses millions of primordial follicles within a framework of interstitial tissue and is lined with ovarian epithelium erroneously called germinal epithelium: as oogonia completely disappear, the oocytes formed during the foetal and neonatal period are the only source of oocytes available during the entire sexual life. As soon as the primordial follicle reserve is constituted, it rapidly decreases by atresia and from the end of the period of oogenesis, some primordial follicles continuously begin growth, but up to puberty all disappear owing to atresia: for example a cow foetus that has 2 700 000 oocytes at day 110 of gestation has only 70 000 oocytes at birth; with continual follicular growth and maturation throughout her life, an old cow may have only 2 500 potential ova. In the human foetal ovaries the number of germ cells decreases from 7 000 000 to 1 000 000 between six months of gestation and birth.
It is now established that a number of oocytes, probably only one percent of the total, reach maturity and is released through ovulation.

Follicles are in a constant state of growth and maturation. A histological section of the cortex of a reproductively active female reveal these maturation stages. The primary follicle stage is followed by a proliferation of granulosa cells surrounding the potential ovum giving rise to a secondary follicle. Later in the development, an antrum will form by fluid collecting between the granulosa cells and separating them. At this stage the follicle is classified as a tertiary follicle, also called a Graafian follicle.

The ovary performs two major functions: a) the cyclic production of fertilizable ova and b) the production of a balanced ratio of steroid hormones that maintain the development of the genital tract, facilitate the migration of the early embryo and secure its successful implantation and development in the uterus. The follicle is the ovarian compartment that enables the ovary to fulfill its dual function of gametogenesis and steroidogenesis.

Macroscopically the features of the buffalo ovary (Figure 2) differ widely from those of cattle; the ovary of the buffalo was earlier described as roundish in shape, about 2.5 cm long and weighing 3.9 g (Lutkuke and Rao, 1962) whereas the latter is oval in shape, about 3.7 cm long and weighing 8.6 g (Sisson, 1953).

Physiologically, the buffalo ovary shows scarce reproductive potentiality because it contains less follicles (primary and antral) than those found in cattle and it seems that buffalo oocytes rapidly undergo atresic degeneration. The corpus luteum of the buffalo is deeply embedded in the ovary and is greyish in colour while in cattle it often juts out on the surface of the ovary and is yellowish. The size varies among buffaloes and active ovaries are larger than inactive ones.

Figure 2. Buffalo ovary

2. Advanced technology applied in buffalo

In buffalo important advances in artificial breeding and in the control of reproduction have been made over the past few years. The earliest development in reproduction technology was the use of artificial insemination (AI) which, through the widespread dissemination of genes carried by high quality males, has been considered, up until now, the most effective method for breeding.

Progress has also been made, but with less impact, with the development of oestrus synchronization, superovulation and the transfer of embryos derived "in vivo". Increased interest in the "in vitro" embryo-production (IVEP) technologies, for faster propagation of superior germoplasm, has led to the development of the transvaginal ultrasound-guided follicular puncture (Ovum pick-up or OPU): this latter technique, combined with the IVEP technology, has great potential for improving the genetic development of this species through the maternal lineage.

The recent application of ultrasonographic techniques in the study of buffalo follicles is elucidating the patterns of follicular growth, development and regression that can lead to the improvement of fertility in the female buffalo.

3. Follicular dynamics in buffalo

More recently, the development of ultrasonic probes used intrarectally to observe females' ovaries has clearly confirmed that ovarian follicular development during the oestrus cycle occurs in a wave-like pattern in cattle, thus providing the bases for improving fertility, synchronizing oestrus with more precision and enhancing superovulatory responses.

A good understanding of the processes involved in the growth and differentiation of vesicular follicles destined for ovulation is also essential in order to optimize buffalo reproduction. Diagnostic ultrasonography for the assessment of ovarian structures is a reliable and accurate method for identifying and measuring follicles, especially important since manual palpation through the rectum in buffalo is not completely accurate.

The studies and advancements that have led to our current understanding regarding patterns of follicular development, are listed below in chronological order:

1960 The two-waves concept for follicular growth during the bovine oestrus cycle was propounded. (Rajakoski,1960).

1972 and 1981 The lifespan and fate of individual follicles during the oestrus cycle of heifers

was directly examined. (Dufour et al., 1972; Matton et al., 1981).

1982-1983 Growth and differentiation of estrogen-active and estrogen-inactive follicles during the oestrus cycle were distinguished. (Ireland and Roche, 1982, 1983a,b).

1984 Ultrasound to monitor sizes of follicles during the oestrus cycle of heifers was used. (Pierson and Ginther, 1984).

1987 The concept of dominant follicles, as observed in primates, is applied to cattle and the three-wave hypothesis for development of dominant follicles during the oestrus cycle was proposed. (Ireland and Roche,1987).

1988 Ultrasound analysis and ovarian maps to track growth and atresia of individual follicles throughout the oestrus cycle of heifers were used. (Fortune et al., 1988, Savio et al., 1988, Sirois and Fortune, 1988).

1990-present The autocrine and paracrine role of intrafollicular factors in the regulation of follicular growth, differentiation and function is studied (Ireland et al., 2000).

1992 The radioimmunoassay (RIA) method for determining the transient peak in basal serum concentrations of FSH before each follicular wave was used. (Adams et al., 1992).

1993 The decreased episodic pattern of secretion of LH associated with termination of a follicular wave was studied. (Savio et al., 1993, Stock et al., 1993).

3a. Follicular population

In buffaloes, the follicular system has not been studied as much as in cattle. Singh et al. (1984) delineated the pattern of development and atresia of large follicles (8 mm) on the surface of the ovaries of buffalo heifers. In 65 percent of the postpubertal heifers they found larger follicles at mid-cycle than one to three days before oestrus concluding that these findings complied with the theory of Rajakoski (1960): the follicles at mid cycle become atretic and a new growth wave of follicles begins about mid-cycle and gives rise to the follicle ovulating after oestrus. The buffalo species is characterized by a reduced follicle reservoir compared to that of cattle: the number of primordial follicles has been reported to be approximately 12 000 - 19 000 in riverine buffalo heifers (Samad and Nasseri, 1979).

Furthermore, through ovarian histological evaluations, Danell (1987), studying the follicular system of cycling and non-cycling Surti buffalo heifers, reported 12 636 primordial follicles in cyclic buffalo heifers, (less than the 150 000 primordial follicles reported in cattle, Erickson, 1966) and 10 132 primordial follicles in the non-cycling animals, with a range of 1 222 - 40 327 in an ovary pair. He observed more atresia in buffalo follicles (66.7 percent) than in bovine follicles (50 percent) and detected the same pattern of follicular dynamics in buffalo as observed by Rajakoski (1960) in cattle. Le Van Ty et al., (1989) in a study of swamp buffalo reported a number of antral follicles equal to only 20 percent of those observed in cattle under similar conditions (47.5 *vs* 233.0) and the observed number of non atretic follicles (> 1.7 mm) was between one and five (average 2.9) for buffalo and 17 and 32 (average 22.1) for cattle.

The total number of surface follicles per ovary in abattoir buffalo ovaries at random stages of reproduction has been reported to range from 5.14 (2.5, 1.2, 0.82 and 0.62 follicles of 0-4, 5-8, 9-12 and > 12 mm diameter, respectively); Kumar et al., (1997) to 6.06 (5.30, 0.54 and 0.17 follicles of 0.4, 5-8 and 8 mm diameter, respectively); Madan et al., (1996).

In swamp buffaloes, Smith (1990) reported the number of ovarian follicles of various sizes including also those atretic at different age groups (Table 1).

Table 1. Number of primordial, growing, secondary, tertiary and atretic follicles in buffaloes at various ages (mean ± sd).

Follicle class	Age groups		
	2 years	**7-8 years**	**12-14 years**
Primordial	47.189 ± 39.23	5.996 ± 2.52	3.673 ± 1.97
Growing	4.233 ± 3.5	18.0 ± 13.95	17.0 ± 17.91
Secondary	324 ± 3.23	14.0 ± 11.4	8.0 ± 8.7
Tertiary	62.7 ± 37.78	9.0 ± 6.98	6.67 ± 5.7
Atretic	126.5 ± 22.5	139 ± 50.2	138 ± 37.2

The ovaries obtained from two-year old Swamp buffaloes showed a relatively high rate of transformation from the primordial developing to tertiary follicles; in seven to eight year old and 12 to 14 year old buffaloes this transformation was not noticeable.

The number of secondary follicles in the pubertal buffaloes was low, indicating a slower transitional rate of the growing follicles to secondary follicular stage (7.65 percent); a decline in the number of follicles, from growing (4.233 *vs* 18.0 *vs* 17.0) to tertiary stage (62.7 *vs* 9.0 *vs* 6.67) was observed with age. The rate at which the primordial follicles are stimulated to develop to pre-antral and, subsequently, to antral stage is, in part, dependent upon the size of the pool of primordial follicles (Krarup et al., 1969). A nearly ten-fold lower population of primordial follicles could be, in part, the major factor contributing to the lower number of antral follicles in buffalo compared with that in cattle. The transformation of primordial follicles through the growing stage to the tertiary stage appears to be very inefficient: this can also be seen in the high level of atretic follicles that in buffalo is higher than that reported in cattle (Settergren, 1964). In fact, in the earliest report on follicular atresia in buffalo, Danell (1987) and Ocampo et al., (1994) found a high level of follicles to be atretic (66.0 percent and 82.0 percent, respectively). Molar ratios of progesterone (P4) and oestradiol (E2) have been used to clarify cattle ovarian follicles into atretic and non atretic categories (Grimes et al., 1987). In recent studies, (Palta et al., 1998a, 1998b) classified buffalo ovarian follicles into oestrogen active (E2/P4 molar ratios > 100) and oestrogen inactive/atretic (E2/P4 molar ratios < 100) and observed 92 to 95 percent of follicles to be atretic in abattoir ovaries at random stages of reproduction. The percentage of atretic follicles was lower in large (74 percent) compared with medium (97 percent) and small (92 percent) follicles. In cattle, Danell (1987), using histological evaluation, reported a value of 50 percent; Grimes et al., (1987), using P4/E2 molar ratios, reported a value of 70 percent and Blondin et al., (1996), using flowcytometry in ovaries of cycling cattle reported a value of 16 to 38 percent.
The small number of follicles and the higher level of follicular atresia may explain in part the reported lower superovulatory response and embryo production in buffaloes compared to cattle (Table 2).

Table 2. Follicular and embryo-production efficiency in buffalo and cattle (Zicarelli, 1998).

	Buffalo	**Cow**
Primordial follicles (n)	20 000	100 000
Antral follicles (n)	47	233
Non atretic follicles (n)	39	207
Recovered oocytes (n)	10.6	14.7
Oocytes used for IVEP (%)	58	96
Cleavage rate (%)	30	86
Blastocysts/oocytes (%)	6.3	29.4
Blastocysts/head (n)	0.39	4.20

3b. Follicular dynamics

The use of ultrasound technology in animal reproduction has played an important role in the collection of data regarding ovarian follicular dynamics and related hormonal profiles in domestic animals (Fortune et al., 1991; Ginther et al., 1996; Pierson and Ginther, 1987) as well as other species (Figueiredo et al., 1997; Radcliffe et al., 2001) and has demonstrated that the follicular turnover during the oestrus cycle occurs in waves, each wave being characterized by the synchronous development of a group of follicles (Baruselli et al., 1997). Ovarian follicular growth in buffaloes is similar to that observed in cattle and is characterized by waves of follicular recruitment, growth and regression (Baruselli,1997; Baruselli et al., 1997); the same authors have shown that buffaloes typically show two follicular waves (63.3 percent) and three follicular waves (33.3 percent) during an oestrus cycle, with the first wave beginning around day 0 (day of ovulation). Cattle also commonly have three follicular waves (Sirois and Fortune, 1988; Savio et al., 1988) and two follicular waves (Ginther et al., 1989a, b) (Fig.1). Unlike cattle (Rhodes et al., 1995, Savio et al., 1988, Sirois and Fortune, 1988) buffalo do not display four wave cycles.

In each wave of follicular growth, one dominant follicle develops and suppresses the other follicles. Dominant follicles grow and reach maximum diameter in the middle of the oestrus cycle: when there are high levels of progesterone, there is no ovulation; regression starts allowing a new wave growth to occur. The dominant follicle that develops during the last wave of follicular growth in each oestrus cycle is the ovulatory follicle (Figure 3).

Based on ultrasound analysis, most animals have one *(First wave)* or two waves *(First wave, Second wave)* of follicular development during the luteal phase and a single wave of follicular development *(Ovulatory wave)* during the follicular phase. *Cohort* refers to a group of similar sized nearly synchronously growing follicles. *Emergence* marks the beginning of a wave and is the first day a 4 - 5 mm follicle is the largest in a new wave. The beginning of *selection* cannot be determined by ultrasonography, however, the end of selection occurs simultaneously with the onset of dominance. *Deviation* is when growth rates between the dominant and largest subordinate follicle begin to differ. *Dominance* occurs when the largest follicle in a wave is 1 to 2 mm larger than the next largest follicle and growth of all subordinate follicles ceases. *Subordinate follicles* are all nondominant follicles in a wave. Loss of dominance marks the end of a wave and is detected at emergence of the next wave. The *growing phase* for a follicle begins on the day of the oestrus cycle of its emergence and ends the day in which the diameter of the follicle ceases to increase. The *static phase* is from the day the follicle diameter ceases to increase (end of growing phase) until the day follicle diameter decreases minus one day. The *regressing phase* is the last day of the static phase until the follicle is no longer detectable, which is usually when it reaches four to five mm in diameter.

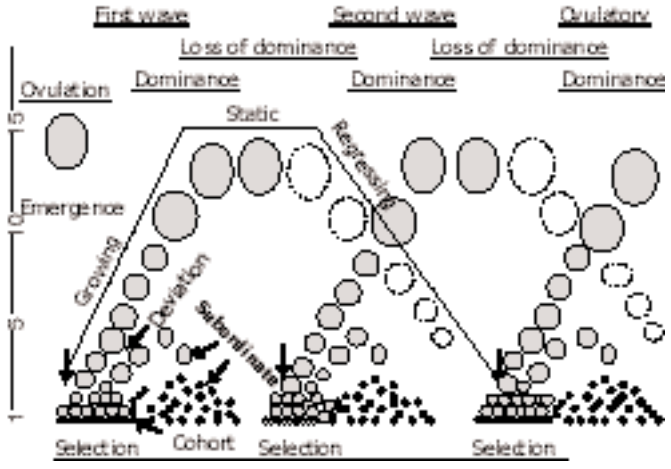

Figure 3. Description of the physiological terms associated with each wave of follicular dynamics during the oestrus cycle of animals. (Ireland J.J. et al., 2000).

3c. Follicular dynamics during an oestrus cycle

The dynamics of follicular turnover, including the length of the interovulatory interval (Knopf et al., 1989; Savio et al., 1990), emergence of waves, number of follicle 3 mm at emergence of waves (Knopf et al., 1989), persistence and maximum diameter of the dominant anovulatory and/or dominant ovulatory follicles (Table 3) (Fortune et al., 1988; Ginther et al., 1989a,b; Bo et al., 1995), have been expressed showing the similarity between buffalo and cattle.

In an earlier study Baruselli et al., (1997) described the follicular dynamics of an oestrus cycle in buffalo (Table 3).

Table 3. Characteristics of follicular turnover during an oestrus cycle in buffaloes having two or three-wave patterns (Baruselli et al.,1997).

	N° waves	
	2	**3**
Percent of Buffaloes	66.33	33.33
Length of interovulatory intervals (d)	22.27±0.89	24.50±1.88
Emergence of first wave (d)	1.16±0.50	1.10±0.32
Emergence of second wave (d)	10.83±1.09	9.30±1.25
Emergence of third wave (d)		16.80±1.22
N° of follicles 3 mm at emergence of wave		
First wave	7.72±4.64	7.50±2.75
Second wave	6.66±3.12	6.50±2.36
Third wave		5.11±1.37
Persistence of first dominant follicle (d)	20.67±1.18	17.9±3.47
Maximum diameter (cm)	1.51±0.24	1.33±0.18
Persistence of second dominant follicle (d)		13.30±2.96
Maximum diameter (cm)		1.11±0.21
Persistence of ovulatory follicle (d)	11.44±0.92	7.70±1.91
Maximum diameter (cm)	1.55±0.16	1.34±0.13
Growth rate of ovulatory follicle (mm/d)	0.131±0.01	0.172±0.02
Length of oestrus cycle (d)	21.84±1.01	24.00±2.24
Length of luteal phase (d)	10.40±2.11	12.66±2.91

In the above studies the growth and static phases of the dominant anovulatory follicles and the growth phase of the ovulatory follicle were also described (Table 4). Specifically, the beginning of a wave (also called emergence) is defined as the first day of the oestrus cycle when a growing follicle four to five mm in diameter in a new wave is detected by ultrasound. According to this definition, in an oestrus cycle with two waves, the first wave begins at approximately day one (day 0 = day of ovulation) and the second wave begins at approximately day 11, both in buffalo and cattle; for the first and second waves, the maximum size of each dominant follicle is 15 mm, reached on days 9 and 22 of the oestrus cycle. In a cycle with three waves, the waves emerge, on average, at days 1, 9 and 16 both in buffalo and in cattle (Baruselli et al., 1997; Manik et al., 1998a,b; Savio et al., 1988; Sirois and Fortune 1988; Ginther et al., 1989; Fortune et al., 1991). There were no differences between second- and third-wave cycles with regard to the day of emergence of the first wave; the second wave appeared earlier in the oestrus cycle with three waves than with two waves.

Two- and three-wave cycles were significantly different with regard to: the persistence of the

first dominant follicle (20.7 d *vs* 17.9 d), the length of the growing phase (7.39 d *vs* 5.50 d) and the static phase (6.88 d *vs* 5.30 d), the last day of the growing phase (8.55 d *vs* 6.60 d), the beginning of the regressing phase (15.4 d *vs* 11.9 d), the maximum diameter of both the first dominant follicle (1.51 cm *vs* 1.33 cm) and the ovulatory follicle (1.55 cm *vs* 1.34 cm); no difference was found with regard to the length of the regressing phases (6.40 *vs* 7.10). Two- and three-wave cycles were also significantly different with regard to the maximum diameter of both the first dominant follicles (1.51 cm *vs* 1.33 cm) and the ovulatory follicle (1.55 cm *vs* 1.34 cm). Terzano et al., (2001) also reported the diameter of ovulatory follicles ranging between 1.32 cm and 1.50 cm.

However, in two-wave cycles, no differences were observed between the maximum diameters of first dominant follicle and ovulatory follicle (1.51 cm *vs* 1.55 cm) and in three-wave cycles, the maximum diameter of the first dominant follicle was significantly larger than that of the second dominant follicle (1.33 cm *vs* 1.11 cm).

No correlation was observed between the diameter of ovulatory follicle (beginning of oestrus cycle), first dominant follicle (diestrus) and second ovulatory follicle (end of cycle).

Two- and three-wave cycles were significantly different with regard to the average length of intervals to oestrus and ovulation and the average length of luteal phase. The mean progesterone level during the luteal phase was lower in two-wave than in three-wave cycles.

Similar follicular waves can also be observed in prepubertal buffalo heifers (Baruselli et al., 1997a; Presicce et al., 2003).

Table 4. Characteristics of dominant follicles (DF) for each wave during two-and three-wave cycles in buffalo (Baruselli et al., 1997)

	N° waves	
	2	**3**
First dominant anovulatory follicle		
Growing phase		
Beginning day	1.16±0.50	1.10±0.32
End day	8.55±2.33	6.60±1.42
length (d)	7.39±1.55	5.50±1.22
growth rate (cm/d)	0.172±0.01	0.187±0.02
Static phase		
maximum diameter (cm)	1.51±0.24	1.33±0.18
beginning day	15.43±2.33	11.90±1.68
end day	21.83±1.20	19.00±3.12
length (d)	6.40±0.85	7.10±0.92
growth rate (cm/d)	0.148±0.02	0.113±0.02
Second dominant anovulatory follicle		
Growing phase		
Beginning day		9.30±1.25
End day		14.50±2.01
length (d)		5.20±0.61
growth rate (cm/d)		0.174±0.02
Static phase		
maximum diameter (cm)		1.11±0.21
beginning day		18.21±1.43
end day		22.60±3.06
length (d)		4.39±0.89
growth rate (cm/d)		0.136±0.02
Ovulatory follicle		
Growing phase		
Beginning day	10.83±1.09	16.80±1.22
End day	22.27±0.89	24.50±2.01
length (d)	11.44±0.91	7.70±0.55
growth rate (cm/d)	0.131±0.01	0.172±0.02

In all buffaloes studied, there was little variation between the numbers of follicles for the different waves. This finding agrees with the high repeatability observed in the number of follicles per wave in cattle (Boni et al.,1993) and suggests that the number of follicles recruited depends on the individual animal. No information was available on the heredity of this characteristic. However, the selection of animals based on the number of follicles per wave is encouraging because of the positive correlation found between the number of small follicles at the beginning of superovulatory treatment and superovulatory response (Romero et al., 1991).

To summarize, follicular dynamics in buffalo is similar to that observed in cattle; there may be marked individual variation in follicular dynamics among buffaloes, with as few as one to as many as three waves of follicular growth occurring within an oestrus cycle: the two waves pattern appears to be the most common; the number of follicular growth waves during an oestrus cycle is linked to the length of luteal phase and of the oestrus cycle.

The elucidation of patterns of follicular development in the buffalo ovary could provide new experimental models for studying the regulation of follicular development and dominance, and could generate information that could help to explain variability in response to oestrus synchronization and superovulatory protocols. Moreover it could also provide new ideas for future improvement of fertility in female buffalo.

3d. Endocrine basis of the wave pattern

Ovarian follicular dynamics in the buffalo species have been studied by several authors although these studies include little information on the hormonal aspects related to wave emergence and follicle development. However, because there are striking similarities between cattle and buffalo in terms of follicular dynamics, the basic endocrine mechanism leading to the occurrence of the wave pattern, could, presumably, be similar in the two species. It is now established also in buffalo that a transient rise in serum concentrations of FSH begins each follicular wave (Presicce et al., 2003), and a decreased episodic secretion of LH is associated with loss of dominance and with the end of a nonovulatory follicular wave.

Today it is clear that several intrafollicular growth factors identified in the follicular fluid of individual follicles, are also involved and some factors have been identified (Table 5). In vitro studies have shown that these growth factors could have endocrine, autocrine or paracrine actions that modify gonadotropin stimulated follicular growth and differentiation.

Table 5. Growth factors and animal reproduction

Acronym	Term
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
PDGF	Plateled-derived growth factor
IGF	Insulin-like growth factor (including IGF-binding protein)
TGF-β	Transforming growth factor-β (including inhibin and activin)
HGF	Haematopoietic growth factor (cytokines)

Their exact role in folliculogenesis is not yet clear but increasing evidence suggests that growth factors modulate follicle growth, acting in a paracrine or endocrine way and regulating proliferation, differentiation and survival of follicular cells (Monniaux et al., 1997).
Changes in insulin-like growth factor-1 (IGF-1) and IGF-binding proteins during follicular development have been reported by various authors in the rat (Zhou et al., 1991), pig (Hammond et al., 1993), human (El-Roeiy et al., 1993), sheep (Gordon I., 1999) and cattle (Kruip A.M., 1997). Changes in the profile of IGF binding proteins have suggested than these proteins may be an important regulator of IGF-1 action on cell proliferation and steroidogenesis within the ovary.

Other authors have reported evidence of paracrine and autocrine effects of epidermal growth factor (EGF) and fibroblast growth factor (FGF) on follicle and luteal function (Kaipia A. and Hsueh J.A., 1997).

4. Multiple ovulation and embryo-transfer (MOET) in buffalo - Limits and perspectives

In buffalo, multiple ovulation and embryo-transfer (MOET) technology is of relatively recent origin. The pioneering work of Drost et al., 1983 in the USA, resulted in the birth of the first buffalo calf through non surgical transfer. This work aroused considerable interest in buffalo-rearing countries leading to subsequent reports on this species (Table 6).

Table 6. Review of superovulation and embryo (E) collection in buffalo (Misra, 1997)

Country	Breed	Treat-ment	Donor super-ovulated	Donor flushed	Total CL (mean)	Total E (mean)	Viable E (mean)	Authors
USA	River	FSH	1	1	2	1	1	Drost et al., 1983
Malaysia	Swamp	PMSG PMSG GnRH	5 5		(5.2) (3.2)	0 0	0 0	Sharifuddin and Jainudeen, 1984
USA	River	FSH	26	19		18	1	Drost et al., 1985
Bulgaria	River	FSH PMSG	19 54			35 ↓		Vlahov et al., 1985
Bulgaria	Murrah & MurrahX	FSH PMSG	19 19		(4.3) (1.9)	24 11		Karaivanov, 1986
Bulgaria	Murrah & MurrahX	FSH PMSG		45 26		66 28		Karaivanov et al., 1987
Bulgaria	Murrah & Medit.	FSH	34	24	(4.0)	(1.2)	(1.7)	Drost et al., 1988
Bulgaria	Murrah & Medit.	FSH PMSG	126 75	123 66	(4.0) (3.4)	146 (1.2) 54 (0.7)	132 38	Alexiev et al., 1988
Thailand	Swamp FSH	PMSG	7 5	7 5	26 15	10 6		Chantaraprateep et al., 1988
Japan	Murrah & Swamp	PMSG	4	3	9	2	0	Ocampo et al., 1988
India	Surti	FSH	1	1	3	1	1	Misra et al., 1988
India	River	FSH	24	24	72	35	24	Yadav et al., 1988b
India	Murrah & Surti	FSH PMSG		145		165		Kurup, 1988
India	Murrah	FSH PMSG	12 12	12 11	57 47	22 8		Deshpande et al., 1988
India	Murrah	FSH PMSG	14	9	(4)	20	19	Sing et al., 1988c
India	Murrah	FSH PMSG	15 7	15 7	42 22	8 3	5 1	Madan et al., 1988
Pakistan	Nili-Ravi	FSH	12	7	(3.7)	(0.5)		Rail et al., 1988b

Country	Breed	Treat-ment	Donor super-ovulated	Donor flushed	Total CL (mean)	Total E (mean)	Viable E (mean)	Authors
Malaysia	Swamp	FSH PMSG	17 32		(3.6) (4.3)	1 1		Sharifuddin and Jainudeen, 1984
Bulgaria	Murrah	PMSG FSH	20 5	20 5	112 44	31 10		Karaivanov et al., 1990
India	Murrah	FSH	73	69	(3.7)	(2.7)	(1.5)	Misra et al., 1990
Italy	Medit	PMSG	10	6	27	16		Schallenberger et el., 1990
India	Murrah	FSH	14	14	31	11	5	Singla and Madan, 1990
India	Murrah	FSH	16	16	(6.8)	(4.4)	(3.1)	Misra et al., 1991
India	Murrah	FSH	22	22	82	52	30	Ambrose et el., 1991
Philippines	Murrah	FSH PMSG	7 4	7 4	720 6	14 0	4 0	Cruz et al., 1991
India	Murrah	FSH PMSG	115 10	70 10	249 31	60 0	33 0	Jain et al., 1992
Vietnam	Swamp	FSH	15	15	90			Uoc et el., 1992
Pakistan	Nili-Ravi	FSH	20	20	75	35	22	Ullah et al., 1992
India	Murrah	FSH PMSG	814	718		1452	804	NDDB, 1992
India	Murrah	FSH PMSG	217	175		353	174	NDRI, 1992
India	Murrah	FSH PMSG	35	29		45	28	NII, 1992
India	Murrah	FSH PMSG	556	497		1302	712	Misra et al., 1994
India	Murrah	FSH PMSG	41 158	41 154	(8.2) (6.3)	(5.1) (3.5)	(3.4) (2.2)	Rao, 1994
Italy	Medit	FSH	51		(5.6)	(1.8)	(1.4)	Zicarelli et al., 1994
China	Swamp	FSH	6	6	46	26	22	Wang et al., 1994
India	Murrah	FSH FSH	8 8		(3.38) (2.25)			Beg et al., 1997
India	Surti	FSH	13	29	(2.48)	(0.76)		Sarvaiya et al., 1997

Superovulation associated with embryo-recovery and embryo-transfer to synchronized recipient females is considered an effective means of increasing the contribution of high quality females to the gene pool of the population.The succesful application of multiple ovulation and embryo-transfer technology largely depends on superovulation for which the essential factor is the treatment with exogenous gonadotrophins. Superovulation, in fact, requires the stimulation of a significantly increased number of pre-ovulatory follicles by the administration of gonadotrophins simulating the effect of FSH. In cattle superovulatory response is much higher than in buffalo and ovulation rates of the order of 15 are frequently recorded: in general 60 to 70 percent of recovered embryos are suitable for transfer: in comparison with cattle embryo transfer (ET), the use of this technology in the buffalo is much more limited. Worldwide MOET technology in cattle has developed on a large scale and about 715 000 bovine transferable embryos, yielding an average of six transferable embryos (5.5 to 7.3), are collected from close to 120 000 donors; almost 50 percent were transferred as fresh embryos and 50 percent were transferred as frozen embryos (Table 7).

Table 7. Number of bovine in vivo-derived embryos transferred (AETE 2000)

Continents	Flushes	Transferable Embryos	Number of transferred embryos		
			Fresh	Frozen	Total
Africa	1 765	10 005	3 766	1 949	5 715
N.America	51 224	299 180	98 391	99 495	197 886
S.America	12 719	92 400	58 423	34 929	93 352
Asia	11 519	74 811	11 684	38 487	50 171
Europe	26 429	145 305	54 286	75 494	129 780
Oceania	15 508	92 655	29 182	14 626	43 808
Total	**119 164**	**714 356**	**255 732**	**264 980**	**520 712**

The procedure commonly used in buffalo for ovarian superstimulation was quite similar to that employed in cattle; however, MOET programmes in buffalo typically resulted in the recovery of small numbers of embryos (one to three) from donor females.

For many years the superovulatory effect of PMSG and FSH have been used to increase ovulation rates in buffaloes and have been applied in conjunction with progestagen and/or prostaglandin F2α treatments to regulate the oestrus cycle. Although the number of corpora lutea was often similar in FSH- and PMSG treated buffaloes, the recovery of embryos after flushing often favoured FSH (Table 6).

An endocrinological evaluation of superovulation by 3 000 IU of PMSG in buffaloes, attempted by Shallenberger et al., (1990) showed that PMSG treatment rapidly induced LH surges of low magnitude, causing unovulated follicles to become endocrinologically active; they further suggested that high oestrogen levels during the early luteal period may activate subclinical uterine infections, which may affect embryonic development.

Anti-serum to neutralize PMSG has resulted in a decreased number of large follicles with variable effects on the number of transferable embryos (Manik et al., 1999).

Palta et al., (1996a) examined the effect of 2 500 IU of PMSG on peripheral inhibin levels recording a sustained elevation in plasma inhibin which they speculated may result in the suppression of endogenous FSH secretion.

The effect of hCG and GnRH, given at oestrus, on the ovulation rate and embryo production in PMSG-treated buffaloes was reported by Ismail et al., (1993). They found that the embryo

recovery percentage was higher after hCG treatment (25 percent *vs* 9 percent in controls) but GnRH proved to be ineffective.

The response of Mediterranean buffaloes induced to superovulate with 2 500 IU of PMSG or 1 050 IU of human menopausal gonadotrophin (HMG) was reported by Alvarez et al., (1994). They recorded an average of 2.3 and 3.0 corpora lutea for PMSG and HMG, respectively. Progesterone levels in the donor animals at the start of superovulatory treatment were found to be extremely variable and this was considered to be a factor contributing to the poor ovarian response. The injection of a single i.m. injection of FSH in the post-scapular region has been reported as effective as the multiple dose regimen (Kasiraj et al., 1992) or to produce a lower superovulatory response compared to a multiple injection regimen (Misra, 1997).

Terzano et al. (2004a) evaluated the relationship of plasma inhibin A (analysed in duplicates by a human sandwich type of immunoassay) to ovarian follicular development in prepuberal Mediterranean Italian buffaloes subjected to two different ovarian stimulation protocols. The data suggested that the medium/large follicles are the most important source of hormone production and that serum inhibin A determined during FSH treatment may provide a useful marker in the control of ovarian hyperstimulation.

Despite these efforts, the variability in the ovulatory response and the low yield of transferable embryos have always been the most important factor affecting the economical use of embryo-transfer technology in this species. It was initially assumed that the low embryo recovery rate in buffalo was related to a poor follicular response to exogenous gonadotrophins. However, in recent studies undertaken by ultrasound evaluation, 9 to 14 ovulatory size follicles were consistently observed in buffaloes stimulated with FSH (Baruselli et al., 1999). This was associated, on average, with ovulation rates of 62.8 percent, a value similar to that found in cattle (Desaulniers et al., 1995; Shaw et al., 1995; Stock et al., 1996). In the same study, the number of ovulations presented a high correlation (0.86;$P < 0.01$) with the number of corpora lutea found on the day of embryo collection, but only one to three ova/embryos were recovered (average recovery rate/CL = 30 percent). In cattle, on the contrary, Shaw et al., (1995) reported a recovery rate proportional to the number of ovulations. In a subsequent study, evidence was obtained for a relatively low rate of transfer of oocytes to the oviduct in buffaloes (Baruselli et al., 2000). It was concluded that the recovery of a low number of embryos in MOET programmes was not necessarily a result of poor superstimulatory responses; rather, it would appear that the failure of oocytes to enter the fallopian tubes and/or impaired transport of ova/embryos in the reproductive tract are major contributing factors to low embryo recovery. This latter condition has implications for direct oocytes aspiration from follicles and the linking of this approach with in vitro fertilization. A negative correlation ($r = 0.31$;$P<0.07$) between the number of large (>0.8 mm) follicles present on the day of embryo collection and the number of embryos recovered was observed: follicles not ovulating in response to the endogenous LH surge continued to secrete large amounts of estradiol, adversely affecting the functionality of the infundibulum and passage of ova into the oviducts. It seems that the failure of some follicles to ovulate depends on incomplete follicular maturation and therefore on lack of sufficient LH receptors at the time of the preovulatory surge release of LH. A GnRH agonist-LH protocol, developed in cattle (D'Occhio et al., 1997, 1998, 1999) was used in buffaloes to verify whether it consistently induced ovulations and increased embryo recovery. In females treated with a GnRH agonist the endogenous pre-ovulatory surge release of LH is blocked and ovulation is induced by injection of exogenous LH. It would appear that the GnRH agonist-LH protocol provides full control on ovulations, including fixed-timed artificial insemination after follicular superstimulation and a reduced number of inseminations. Zicarelli et al., (2000) failed to find significant differences in ovarian follicular response in buffaloes treated with GnRH agonist LH protocol and in those treated with a conventional MOET protocol. Carvalho et al., (2002) reported the GnRH agonist LH protocol to be efficient in the control of follicular dynamics and in the time of ovulation in superovulated buffaloes but a relatively low embryo recovery rate remains a fundamental problem in buffaloes.

Several authors have attributed the poor superovulatory response of buffaloes to inherent endocrine patterns as well as to the characteristics of the follicular population and ovarian folliculogenesis. Recent interest and research activity in ovarian function have contributed greatly to our understanding of the ovary, particularly with respect to follicular dynamics and its control. Based on recent findings regarding endogenous mechanisms controlling follicular wave emergence, follicle selection and dominance, new ideas for artificial manipulation of ovarian function are being investigated. Up to now the most important trials on follicular dynamics were performed on bovines, using ultrasound examination of the ovaries. Attention has been given in recent investigations on buffalo to superstimulatory responsiveness with specific regard to the status of follicular wave development. An increase in the number of ovulations has been reported when superstimulatory treatments were initiated in the absence of a dominant follicle or when the dominant follicle was in a regressing or plateau phase (Taneja et al., 1995). The concept regarding the need to mobilize the small follicles to the stage considered to be responsive to superovulatory treatment has been the basis for trials on hormone pretreatment prior to main superovulatory regimen. Although in cattle some reports have shown that superovulatory response was improved by administering FSH at the start of the donor's oestrus cycle, in buffalo some studies failed to find evidence of any useful effect of such FSH priming (FSH on day three and four) (Joshi et al., 1992; Aggarwal et al., 1995). The folliculogenesis studies have shown a great variation regarding the day the second wave starts, showing the difficulty in standardizing the superovulatory schemes in the middle of the oestrus cycle (Barros et al., 1993; Beg et al., 1997). In monitoring follicular growth by ultrasound, authors reported a greater superstimulatory response when treatment started before (day one) rather than after (day five) manifest selection of the dominant follicle (Adams et al., 1992, 1994; Nasser et al., 1993). In a direct comparison of the superstimulatory response of first follicular wave versus the second one, the results revealed no differences in the number of ovulations induced or the number of ova/embryos recovered in heifers in which superstimulatory treatments were started on day of emergence of wave one or wave two. In cattle, several reports have confirmed that a superovulatory response could be elicited when begun at the time of wave emergence, near the expected time of the pre-wave FSH surge. Superstimulation of the first follicular wave after ovulation (wave one), rather than of the subsequent waves, was chosen because the day of ovulation (day 0) could be used as a convenient and consistent point of reference for the emergence of wave one. However this procedure is difficult to perform under field conditions. A way to perform this would be the synchronization of follicular waves by hormonal or mechanical methods and to perform superovulatory treatment at the onset of the second wave, as proposed for bovines (Bo et al., 1995, 1996). In buffalo a low individual variation was found for the number of follicles recruited for different waves of the same oestrus cycle. These results concur with the high repeatability found in the engagement of follicles in each wave of follicular growth in bovines (Boni et al., 1997b). This suggests that the number of follicles engaged depends on individual characteristics. Although no information exists on the heredity of this characteristic, the selection of female buffalo based on the number of follicles per wave is encouraging due to the positive correlations found between the number of small follicles at the beginning of superovulatory treatment and superovulatory response in cattle (Romero et al., 1991). This selection becomes more important in buffalo, showing a smaller number of follicles (Danell,1987; Le Van Ty et al., 1989).

In these first ventures, it is clear nowadays that the application of cattle ET technology to buffalo has met with limited success and much remains to be done in developing procedures specifically for this species.

5. Ovum Pick-up and in vitro embryo production

In buffalo the low efficiency of superovulation (SO) and embryo-transfer (ET) programmes had led to an increased interest in the in vitro embryo production (IVEP) technologies for faster propagation of superior germoplasm. One of the relatively recent breakthroughs in the practical world of animal reproduction is the combined application of the existing in vitro

fertilization technology and transvaginal ultrasound-guided follicular puncture (Ovum pick-up or OPU) to improve the genetic progress of this species through the maternal lineage. Within this framework, transvaginal oocyte recovery by puncture and aspiration of antral follicles has become a routine procedure in most laboratories where in vitro embryo production is part of the services offered to breeders.
Worldwide a considerable number of bovine oocytes have been collected (the number of approximately 160 000 is an underestimate) (Table 8). Japan, in particular, leads all other countries with around 8 000 in vitro-produced embryos. Europe, notably Italy and the Netherlands, is also actively involved in this in vitro production and transfer of embryos. Several hundred nuclear-transferred embryos have reportedly been transferred by Korean teams for experimental purposes.

Table 8. The number of bovine in vitro-produced embryos transferred (AETE 2000)

Continents	**Transferable embryos collected**	**Number of transferred embryos**		
		Fresh	**Frozen**	**Total**
Africa	421	31	17	48
N.America	1 384 (*)	2 182	117	2 299
S.America	92 (*)	27	42	69
Asia	136 751	4 089	6 114	10 203
Europe	24 146	6 074	7 314	13 388
Oceania	n.d	895 (*)	50	945
Total	**166 794**	**13 298**	**13 654**	**26 952**
(*) Only one country from this region has reported data				

The OPU technique is a non invasive and repeatable procedure for recovering immature oocytes from individual known donors. The possibility of collecting large numbers of meiotically competent oocytes, suitable for in vitro embryo production (IVEP), renders the OPU*IVEP technique competitive to SO for embryo production. Furthermore, the Ovum pick-up (OPU) technique can be performed in non cyclic females, in pregnant cows, in subjects with patent oviducts or genital tract infections, in animals not responsive to hormonal stimulation. It can also be employed as a means of obtaining embryos from clinically infertile but valuable animals. In buffalo the number of transferable embryos/donor/session is lower with OPU + IVEP *vs* MOET, but it is significantly higher over longer periods of time because the MOET programmes cannot be repeated before 100 days (Table 9).

Table 9. Embryo production efficiency in vivo (ET), in vitro (IVEP) and by OPU+IVEP in buffalo (Zicarelli, 1998).

	ET	**IVEP**	**OPU+IVEP**
Total embryos/session	1.8	0.4-0.8	0.17-0.37
Transfer embryos/session	1.7	0.3-0.6	0.15-0.33
Embryo production in 100 d	3.6	0.4-0.8	5.1-11.1
Transferable embryos in 100 d	3.4	0.3-0.6	4.5-9.9

The use of OPU+IVEP in the field could represent a valid approach to speed up genetic improvement by decreasing the generation interval. It has been estimated that a selection scheme based on this technique, applied in a closed nucleus of farms, will decrease the generation interval from 6.28 to 3.25 years and the genetic increase will be about 30 to 25 percent compared to progeny testing (Zicarelli 2003).
OPU has been successfully applied in the buffalo species since 1994 (Boni et al.) and subsequent studies dealing with this technique have been reported by several authors (Galli et al., 1998; Di Palo et al., 2001) showing a low yield of good quality oocytes per ovary, compared

to cattle (on average 2.4 *vs* 10.0, respectively) (Gordon, 1994; Gasparrini et al., 2000).

Boni et al., (1997b) recorded a high individual variability and a low repeatability of the follicular recruitment; the latter probably because the number of follicles recruited varied cyclically and follicular wave was observed. Although OPU resets the follicular population, a cyclic pattern is still observed, perhaps because of the autocrine mechanism. Nevertheless, it might be possible to predict the ability of animals to recruit follicles on the basis of the first four transvaginal ultrasound-guided follicular puncture sessions, as observed in cattle. In fact, the number of total and small follicles recruited within the first four puncture sessions were significantly correlated with the total production (r=0.72 and 0.83, respectively). The importance of this finding is highlighted by the correlation existing between the number of follicles and COCs (r=0.61), Grade A and B COCs (r=0.42) and blastocyst production (r=0.24). The possibility of undertaking a selection of the donors for OPU and embryo production programmes may further improve genetic progress. A limitation to this technique may be the functional exhaustion of the follicular pool (Zicarelli et al., 2003) after six months of OPU. In this trial a productive phase (first six months characterized by high blastocyst production) and an unproductive phase (lasting three months characterized by a low number of follicles and no embryo production) were observed. This finding highlights the need to further investigate this aspect and evaluate whether a resting period is required to better exploit the donor's potentials.

5a. In vitro maturation

Most attempts at producing buffalo embryos in vitro have been based on the methods employed in cattle (Gordon, 1994) and the majority of experimental work in this species utilized ovaries from slaughtered animals as a source of oocytes. Research in buffalo IVF technology has been mainly reported from the developing countries of Asia where the greatest number of buffaloes are found (of the 152 million buffaloes in the world, 96.6 percent are found in Asia), providing not only milk but also meat and consequently a high ovary availability from which to collect oocytes. In Italy, on the contrary, the estimated population of buffaloes is 250 000 *vs* 8.5 million cattle and the culling rate is also lower (12 percent *vs* 25 percent). In addition to this, buffaloes are bred mostly for milk production and are usually slaughtered at the end of their productive life span. Furthermore, the yield of good oocytes per ovary is low compared with cattle (2.4 *vs* 10, respectively) (Kumar et al., 1997; Gordon, 1994). Although the multi step process of IVEP has been successfully used for producing morulae/blastocyst (Madan et al., 1994a; Boni et al., 1999; Gasparrini et al., 2000; Caracciolo di Brienza et al., 2001) and pregnancy in buffalo (Madan et al., 1994b; Suzuki et al., 1992; Chauhan et al., 1997a) the efficiency, in terms of transferable embryos (TE) and development to term, has been very low (Madan et al., 1996). The IVEP technology involves several sequential steps, from the recovery of oocytes to the in vitro maturation (IVM) of the selected oocytes, in vitro fertilization (IVF) and in vitro culture (IVC) of zygotes up to the morula or blastocyst stage but so far many crucial questions still remain to be satisfactorily resolved.

A deeper knowledge of buffalo oocyte/embryo physiology, metabolism and culture requirements is necessary to optimize the efficiency of innovative reproductive strategies in this species.

The ultrastructure of buffalo oocytes during IVM is dealt with in a report by Boni et al., (1991). An ultrastructural study was carried out to assess whether oocyte maturation was accomplished also at a cytoplasmic level in a system that was previously shown to successfully support nuclear maturation (Boni et al., 1992); studies with confocal microscopy have shown that the highest proportion of MII oocytes occurs at a shorter time in buffalo compared to cattle (19 hours *vs* 24 hours, respectively) (Neglia et al., 2001).

During maturation, considered an important step for further development, the oocytes undergo a series of modifications necessary to acquire developmental competence. Therefore the development of a suitable IVM system is critical.

Authors are increasingly using defined rather than undefined media in evaluating the role of various factors in maturation rate and so several complex media (TCM-199 and Ham's F-10) (Totey et al., 1992, 1996; Ocampo et al., 1996), different sources of serum (foetal calf serum-FCS and buffaloes oestrus serum BES) (Totey et al., 1993; Chauhan et al., 1998; Samad et al., 1998) and hormones (Follicle stimulating hormone-FSH, Luteinizing hormone-LH and 17 estradiol) (Totey et al., 1992, 1993) have been evaluated. The role of granulosa cells in the maturation process has also been demonstrated (Bacci et al., 1991). Subsequent studies have been performed to evaluate the role of growth factors in oocyte maturation and post-fertilization development. In this regard the buffalo follicular fluid (BUFF), used as a supplement of IVM media and in replacement of hormones and serum additives, has yielded high maturation, fertilization and blastocysts rates (Chauhan et al., 1997). According to Palta et al., (1996, 1998) the beneficial effect of BUFF during IVM is related to the presence, in this supplement, of gonadotriphins, estradiol, progesterone and several growth factors. The latter play an important role in oocyte maturation and post-fertilization development, acting as a local modulator of gonadotrophin action on mammalian oocytes. IGF-1, IGF-2 and insulin enhance oocyte maturation in buffalo oocytes, as well as fertilization and development to the blastocyst stage (Pawshe et al., 1998), acting in synergy with FSH as autocrine and paracrine modulators of granulosa cells and therefore promoting mitosis, steroidogenesis and protein synthesis. EGF improves cumulus expansion, nuclear maturation and cleavage rate of cumulus-enclosed buffalo oocytes without affecting the post-fertilization embryonic development (Chauhan et al., 1999).

In buffalo Boni et al., (1992) have found that the oocytes and early embryos show extreme sensitivity to oxidative damage, due to their high lipid content. It is known that glutathione (GSH) plays a critical role in protecting mammalian cells from oxidative stress; the latter is thought to be a major factor affecting in vitro mammalian embryo development; GSH content increases during in vivo maturation in the ovary and this reservoir protects the oocytes in the later stages of development (Perreault et al., 1998). Based on these observations, De Matos et al., (1997, 2000) showed that cysteamine, a low molecular weight tiol compound, added to the maturation media, improves bovine and ovine embryo development and quality increasing GSH synthesis. Gasparrini et al., (2000), by supplementing the IVM medium with 50 M of cysteamine, obtained an increased proportion of tight morula and blastocyst-stage buffalo embryos (22.6 percent *vs* 14.9 percent) and, more interestingly, embryo quality was also improved. However, no beneficial effect was recorded on maturation and cleavage rates. The authors speculated that cysteamine-induced GSH synthesis may significantly enhance buffalo embryo development either by protecting the embryos from oxidative stress or by affecting the delicate process of cytoplasmic maturation, that in buffalo may be impaired by the fact that oocytes are often surrounded by only a few layers of cumulus cells. Eppig (1996) suggested that GSH production is critical for the acquisition of development competence of oocytes at a cytoplasmic level and de Matos et al., (1995, 1997) proposed the measurement of GSH at the end of IVM as a reliable indicator of cytoplasmic maturation.

5b. In vitro fertilization

Relative to cattle, buffalo sperm appears to have poor fertilizing capacity and low viability if the semen is frozen with liquid nitrogen. In fact, despite a similar maturation rate (87 percent *vs* 94 percent) a significantly lower cleavage rate (65 percent *vs* 84 percent) is observed in buffalo *vs* cattle (Gasparrini, 2003). In contrast with previous reports (Totey et al., 1992; 1993); Chuangsoongneon et Kamonpatana, 1991; Bacci et al., 1991), Wilding et al., (2003) reported non significant differences between frozen and fresh buffalo semen in penetration and cleavage rate (69.4 percent *vs* 79.6 percent and 60.3 percent *vs* 70.5 percent, respectively). In the same trial the mitochondrial activity of buffalo semen was also assessed showing it to be only slightly lower in cryopreserved *vs* fresh semen. These results suggest that other factors may contribute to the low efficiency rate in buffalo IVF.
As with cattle and other farm animals, considerable variability exists among buffalo bulls in the fertilizing capacity of sperm (Totey et al., 1993b): an accurate screening of the sperm of

several bulls is required in order to identify a suitable semen for IVF programmes.
Sperm needs to undergo capacitation to acquire fertilizing ability; this process can be induced in vitro either by pre-incubation with heparin (Chauhan et al., 1997; Boni et al., 1999) or by adding heparin to the IVF medium (Totey et al., 1996; Gasparrini et al., 2000). In a previous report Totey et al., (1993) showed heparin was able to capacitate buffalo sperm in a dose-dependent manner. High sperm motility is required to accomplish fertilization, although when frozen-thawed sperm is employed, this can be carried out by the swim-up method (Boni et al., 1994a,b; Chauhan et al., 1997a; Nandi et al., 1998) or by Percoll density gradient (Totey et al., 1993b; Boni et al., 1999; Gasparrini et al., 2000). Different motility-inducing substances are used during IVF such as caffeine (Bacci et al., 1991; Madan et al., 1994b; Chauhan et al., 1997a), theophilline (Jainudeen et al., 1993) or a mixture of penicellamine, hypotaurine and epinephrine (Totey et al., 1993b; Madan et al., 1994b), all enhancing the sperm motility and fertilization rate. Basic media such as Tyrode's modified medium (TALP) (Totey et al., 1996; Gasparrini et al., 2000) or Brackett and Oliphant (BO) (Madan et al., 1994a, b; Chauhan et al., 1997a; Nandi et al., 1998) have been found suitable for IVF in buffalo. In this regard, several studies have suggested that BO medium supported higher fertilization and cleavage rates than TALP medium (Totey et al., 1992; Madan et al., 1994a; Ocampo et al., 1996) with average fertilization and cleavage rates of 30 percent to 78 percent and 28 percent to 69 percent respectively. As described above, oocyte maturation in vitro, at least at nuclear level, occurs earlier than in cattle (Neglia et al., 2001), but results are not improved by anticipating the IVF; moreover a linear decrease in efficiency is observed starting from 27 hours of maturation (Gasparrini, 2003).

The efficiency of IVF is also affected by the sperm concentration and, consequently, by the length of sperm-oocyte incubation. Increasing sperm concentration from one through five to 10*106/ml increase polispermy from 24 percent to 43 percent and 64 percent, respectively (Ocampo, 1996), which can be reduced by shortening the co-incubation time. Authors suggested the use of a concentration of 2*106/ml, which yields a high fertilizing rate, avoiding the occurrence of polispermy (Totey et al., 1993b). The positive effect of cumulus cells at the time of IVF has been observed also in buffalo, similar to cattle (Zhang et al., 1995).

5c. In vitro culture

The development of a suitable system for supporting in vitro embryonic development is the most critical step to increase the buffalo IVEP efficiency. Although buffalo embryos have been successfully cultured in ligated rabbit (Chantarapatreep et al., 1989c) and sheep (Galli et al., 1998) oviducts, the use of intermediate hosts is unsuitable for large-scale embryo production. Following the development of co-culture systems in sheep (Gandolfi and Moore, 1987), several authors (Chuansoongneon et al., 1991; Jaunuden et al., 1993) developed a buffalo oviductal epithelial cell co-culture system, with or without a cumulus cell monolayer (Madan et al., 1994b), supporting embryonic development up to the blastocyst stage, but with very low efficiency (8.10 percent). In cattle, established cell lines in a pathogen free-form, such as Buffalo Rat Liver (BRL) cells (Reed et al., 1996) and Vero cells (Lay et al., 1992) have been successfully used for culturing buffalo embryos in vitro (Boni et al., 1994 a,b; 1999), avoiding any risk of transmitting infectious diseases by way of oviductal cells from slaughterhouse material. The use of chemically defined cell-free medium termed Synthetic Oviductal Fluid (SOF) or Potassium Simplex Optimized Medium (KSOM), earlier used in cattle (Tervit et al., 1992), has become necessary to acquire a better understanding of metabolic pathways and biochemical requirements of buffalo embryos in vitro which, in turn, would allow the formulation of an optimal species specific culture system.

A higher blastocyst rate and improved quality was obtained when embryos were cultured in SOF medium compared with the co-culture system with BRL cells (13.5 percent *vs* 7.0 percent, respectively) (Boni et al., 1999). Recently, Caracciolo di Brienza et al., (2001) have reported a higher blastocyst rate, evaluated on the total COCs, in SOF (22.6 percent) and in KSOM (23.8 percent) culture systems.

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Chapter VI

REPRODUCTIVE APPLICATION OF ULTRASOUND IN BUFFALO

Giuseppina Maria Terzano

Istituto Sperimentale per la Zootecnia
(Animal Production Research Institute)
Via Salaria 31, 00016 Monterotondo (Rome), Italy

The use of ultrasound as a diagnostic technique to evaluate reproduction has enhanced our understanding of the ovarian and uterine processes during the oestrus cycle and pregnancy and our ability to manipulate these processes in order to improve reproductive performance and increase genetic improvement of farm animals. Its use has also provided a "window" to examine the environment of the foetus in order to better understand the interaction between the foetus and its mother and to accurately predict foetal sex. The advent of ultrasound has changed the static glimpses that were achieved with palpation, laparoscopy or post-mortem examination into real-time images.

The earlier methods such as those based on the Doppler principle have now been superseded by real-time B-mode ultrasound and equipped with a linear-array 5 or 7.5 MHz intra-rectal probe. The method is non-invasive and interactive and a principal reason for the increased routine use of ultrasound in farm animals has been the development of inexpensive, portable equipment. Several companies now offer excellent ultrasound units for diagnostic examination of large or small animals (i.e Aloka, Universal Medical System, Classic Medical Supply Inc., E.I. Medical).

Today ultrasound is used for the following examinations:

- Ovarian status determination
- Onset of puberty determination
- Follicular monitoring for diagnosis or pharmacological treatments
- Ovulatory follicles and ovulation time determination
- Ovulation time or anovulatory condition determination
- Corpus luteum monitoring
- Stage of the oestrus cycle determination
- Luteal persistence and anovulatory conditions differentiation
- Establishment of optimal time for artificial insemination
- Oocytes recovery through ultrasound Ovum Pick-up
- Recipients testing for MOET programmes
- Early diagnosis of pregnancy
- Embryo growth characterization
- Foetal viability and age determination
- Foetal number and gender determination
- Post-partum uterine involution determination
- Embryonic death rate (by lack of heartbeat) determination

In this chapter some useful applications will be reported regarding the use of ultrasound for monitoring reproduction in buffalo.

1. Monitoring Ovarian Structures

Before the ultrasound, evaluation of ovarian follicles was limited to palpation, laparoscopy or visual examination of excised ovaries. With the advent of ultrasound, however, non-invasive, repeated monitoring of follicular and luteal development became possible (Figure 1).
Resolution and clarity of ovarian images depend on the quality of the ultrasound equipment

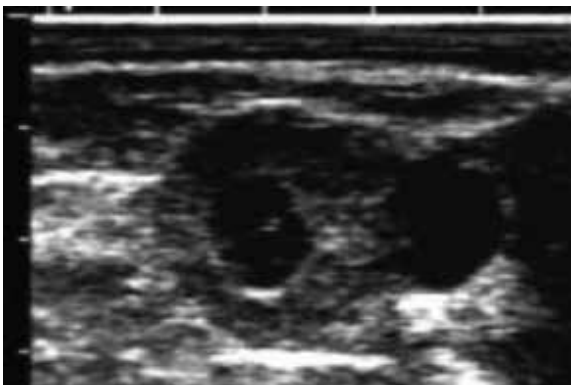


Figure 1. Ultrasound image of the buffalo ovary
with a corpus luteum (on the left) and follicle (on the right).

and the experience of the operator (Sirois and Fortune, 1988). However, ultrasound is a more sensitive method than palpation via the rectum for detecting and measuring ovarian follicles, especially those within the ovarian stroma (Pieterse M.C.et al., 1990). In heifers correlation coefficients between ultrasound measurements and counts obtained by slicing ovaries after slaughter ranged from 0.80 to 0.92 for follicles detected in various size categories and was 0.97 for diameter of the largest follicle (Pierson and Ginther, 1987).

Up to now the most important trials on follicular studies have been performed on bovines, using ultrasound examination of ovaries.

In buffalo, ultrasound monitoring of ovarian function has also been used to determine that follicular development occurs in two or three waves throughout the oestrus cycle.

Ovarian follicular growth in buffaloes is similar to that observed in cattle and is characterized by waves of follicular recruitment, growth and regression (Baruselli, 1997a; Baruselli et al., 1997b). By ultrasound the same authors have shown that buffaloes typically show two follicular waves (63.3 percent) and three follicular waves (33.3 percent) during an oestrus cycle, with the first wave beginning around day 0 (day of ovulation). Also cattle commonly have three follicular waves (Sirois and Fortune,1988; Savio et al.,1988) and two follicular waves (Ginther et al., 1989 a,b, Karaivanov, 1986) (Fig.1). Unlike in cattle (Rhodes et al., 1995, Savio et al., 1988, Sirois and Fortune, 1988), ultrasound monitoring proved that buffalo do not show four wave cycles.

Following each wave of follicular growth, one dominant follicle develops and suppresses the other follicles. Dominant follicles grow and reach maximum diameter in the middle of the oestrus cycle. When there are high levels of progesterone, there is no ovulation; regression starts allowing a new wave growth to occur. The dominant follicle that develops during the last wave of follicular growth in each oestrus cycle is the ovulatory follicle (Fig.2).

The echotexture characteristics of the dominant follicle may be correlated with the functional and endocrine status of the follicle. In cows, after the dominant follicle reaches its peak diameter, referred to as the static phase, granulosa cells are sloughed into the antrum and this debris increases the echogenic heterogeneity of the antral fluid. The changes in follicular echotexture measured by computer-assisted echotexture analysis coincided with the ovulatory potential of the follicle and steroid content of the follicular fluid (Singh et al., 1998; Tom et al., 1998). At present, however, there is no method to determine the physiological status of a large follicle without serial examinations and retrospective analysis. Future use of computer assisted image analysis may improve the diagnostic potential of ultrasound to determine the health of a large follicle in a single examination: in buffalo this will be of significant importance in detecting the health of ovulatory follicles after the application of oestrus synchronization protocols for fixed time artificial insemination. In addition, based on recent findings regarding

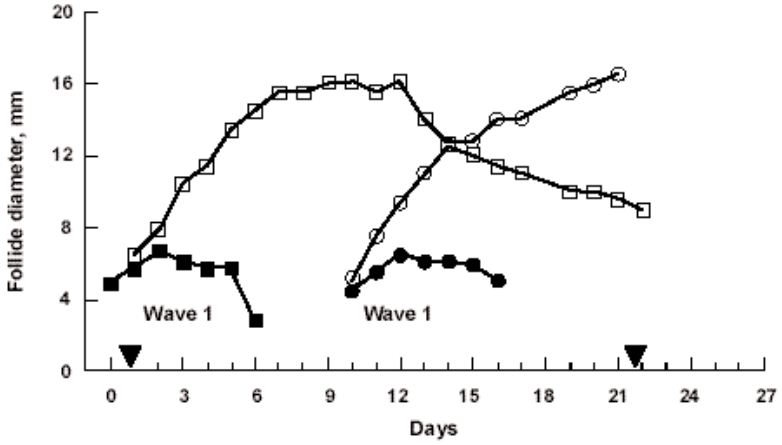


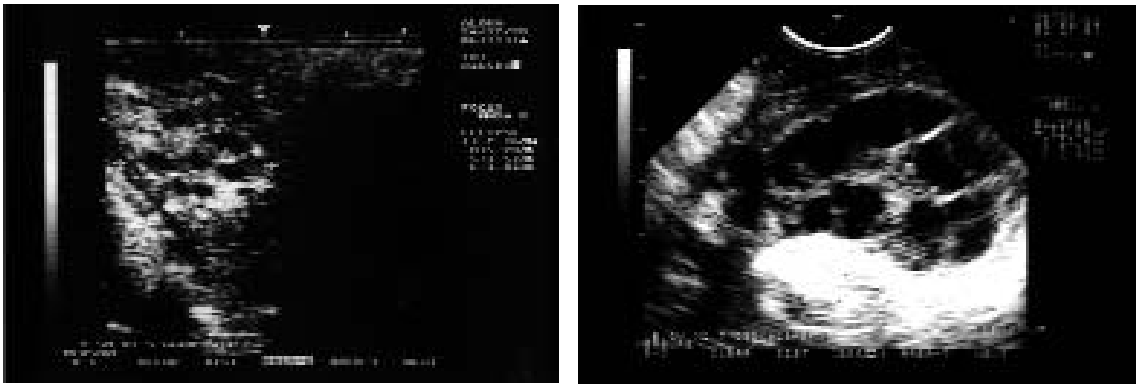
Figure 2. Buffalo oestrus cycle characterized by two follicular waves

endogenous mechanisms controlling follicular wave emergence, follicle selection and dominance, new ideas for artificial manipulation of ovarian function are being investigated.

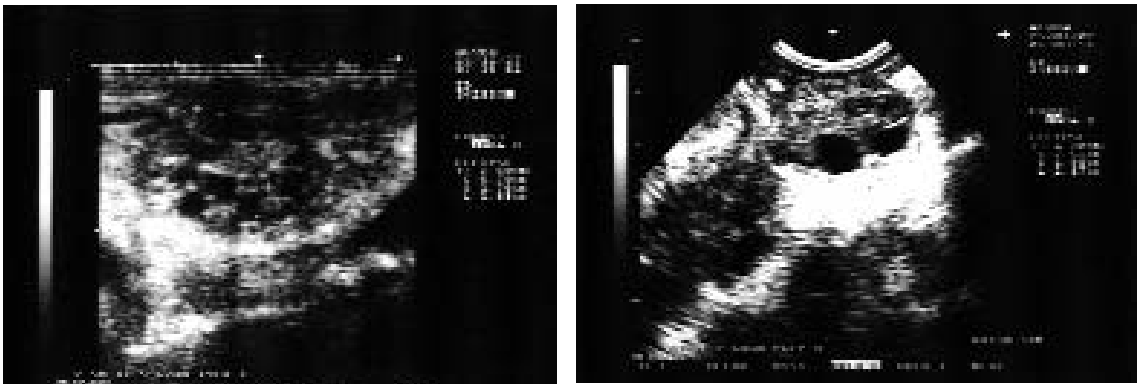
Recent ultrasound investigations in buffalo have paid particular attention to superstimulatory responsiveness with specific regard to the status of follicular wave development. An increase in the number of ovulations has been reported when superstimulatory treatments were started in the absence of a dominant follicle or when the dominant follicle was in a regressing or plateau phase (Taneja et al., 1995).The ultrasound folliculogenesis studies have moreover revealed a great variation on the day the second wave starts, demonstrating the difficulty in standardizing the superovulatory schemes in the middle of the oestrus cycle (Barros et al., 1993; Beg et al., 1997). When monitoring follicular growth by ultrasound, authors reported a greater superstimulatory response when treatment was started before (day 1) rather than after (day 5) manifest selection of the dominant follicle (Adams et al., 1992, Nasser et al., 1993). In a direct comparison of the superstimulatory response of the first follicular wave vs the second one, the results revealed no differences in the number of ovulations induced or the number of ova/embryos recovered in heifers in which superstimulatory treatments were started on the day of emergence of wave 1 or wave 2. In cattle several reports have confirmed that a superovulatory response could be elicited when begun at the time of wave emergence, near the expected time of the pre-wave FSH surge. Superstimulation of the first follicular wave after ovulation (wave 1), rather than of the subsequent waves, was chosen because the day of ovulation (day 0) could be used as a convenient and consistent point of reference for the emergence of wave 1. However this procedure is difficult to perform under field conditions. A way to perform this would be to synchronize the follicular waves by hormonal or mechanical methods and to perform superovulatory treatment at the onset of the second wave, as proposed for bovines (Bo et al., 1995, 1996). By using ultrasound in buffalo a low individual variation was found for the number of follicles recruited for different waves of the same oestrus cycle.

Ovulation is detected by ultrasound as the acute disappearance of a large follicle (≥10 mm) that was present at a previous examination. As in buffalo the corpus luteum (CL) is deeply embedded in the ovary, its ultrasonic detection may be more sensitive than detection by palpation, this being dependent on the experience of the individual performing rectal palpation (McDougall et al., 1999). Detection of a CL with ultrasound is based on the differences in echogenicity between the stroma and the luteal tissue. In buffalo a mature developing CL was recognizable within the first one to three days from ovulation by an increasingly distinct border separating it from the remaining ovarian stroma together with a darker grey granular echotexture (Senatore et al., 2002). The ability to discern CL from the stroma depends on the skill of the ultrasound technician. Occasionally it can be difficult to differentiate the CL from the stroma due to the size of the CL and the area of the ovary occupied by the CL. Usually the stroma can be differentiated from the CL by the presence of small follicles dispersed throughout

the stroma (Terzano, unpublished data, ISZ). Ultrasound machines with expanded gray scale capabilities enhance the ability to differentiate ovarian structures due to subtle differences in echogenicity.



Ovary before FSH-4d (a) Ovary after FSH 4d (b)



Ovary before PMSG+FSH (c) Ovary after PMSG+FSH (d)

Figure 3. Representative ultrasonographic images of ovaries of buffalo heifers before (a and c) and after (b and d) treatment with 4-d FSH and FSH + PMSG.

Ultrasonography may provide a good method of evaluating the follicular development after synchronization with different hormonal protocols for artificial insemination (Terzano et al., 2001; Barile et al., 2004) and after different superstimulatory treatments (Terzano et al., 2004a,b) (Figure 3) and of evaluating corpora lutea in embryo transfer programmes. In fact, embryo-transfer practitioners often reject recipients presented for transfer based on the absence of palpable luteal tissue or the presence of a small, irregular, fluid-filled or soft CL; ultrasound may provide a better method of evaluating CL's in embryo-transfer recipients (Beal W.E., unpublished).

2. Ultrasound evaluation of the uterus

The ultrasound appearance of the buffalo uterus, as in cattle, is dependent on the stage of the oestrus cycle. Variation in the appearance of the uterus involves changes in endometrial thickness, vascularity and the presence of intraluminal fluid. During oestrus the endometrium is very echogenic, the endometrial/miometrial border is evident and throughout the uterine lumen it is possible to see small fluid accumulation. The echogenicity and puffed up appearance of the uterine endometrium decreases by three or four days after ovulation. The uterine horns are extended during and immediately after oestrus (Bonafos et al., 1995).

Real-time, B-mode ultrasound has been reported to detect pregnancy in cattle as early as 9 (Boyd et al., 1988) or 12 days into gestation (Pierson and Ginther, 1984). The potential advantages of using ultrasound for pregnancy diagnosis are that the presence of an embryo can be detected earlier than by palpation per rectum and that direct physical manipulation of the gravid reproductive tract is unnecessary with ultrasound. The latter fact should reduce the risk

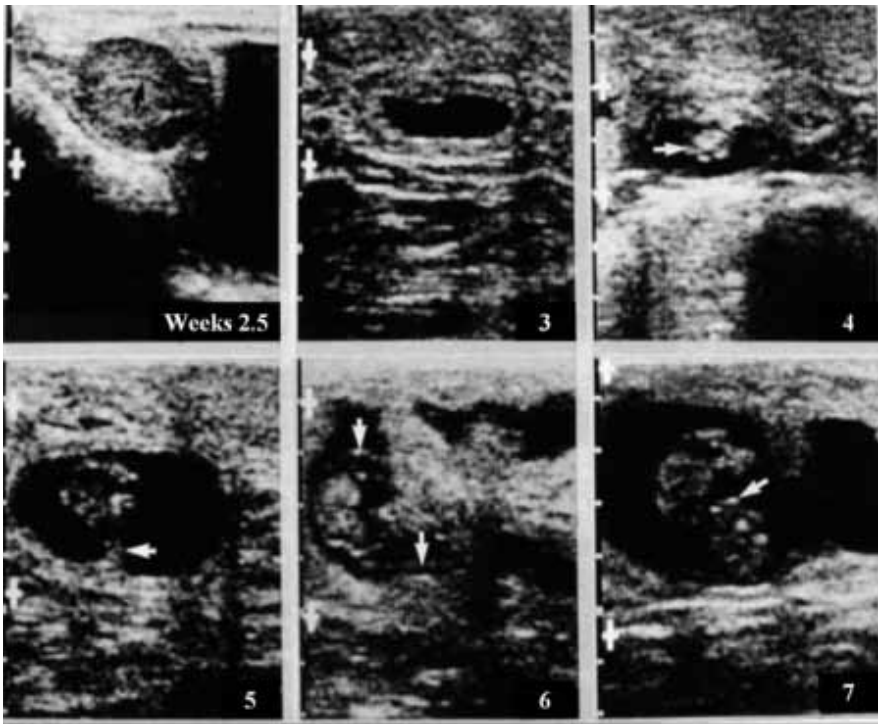


Figure 4. Ultrasound images of buffalo embryos and foetuses.

of inducing embryonic mortality. The use of ultrasound rather than palpation per rectum may also improve the consistency of early (< 40 days) pregnancy diagnosis by reducing the variation in accuracy among practitioners.

In buffalo, pregnancy was determined as early as 20 days with ventral view of the foetus (Presicce et al., 2001).

The embryo is defined as a distinct echogenic structure within the nonechogenic, fluid-filled vesicle. Presence and vitality of the embryo can be confirmed by the detection of a heartbeat at as early as three weeks of gestation: the embryo initially appears as a short, straight echoic line (three weeks), later becomes C shaped (four weeks) and by 4.5 weeks of gestation assumes an L shape (Figure 4).

3. Determination of foetal age

Various ultrasound methods for estimating animal foetal growth have been described in the literature (Kahn W., 1991; Noia et al., 2002). These techniques are based on serial measurements of specific somatic parameters in the foetus: measurements of crown rump length, head diameter and trunk diameter are actually the easiest predictive measurements to estimate gestational age (Figure 5).

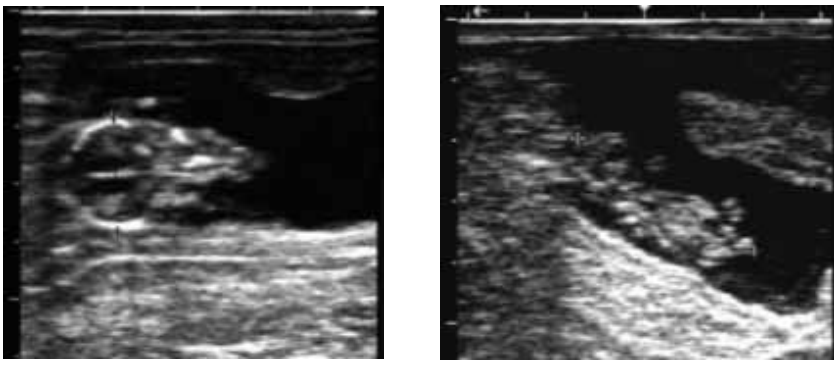


Figure5. Determination of buffalo foetal head (left panel) and crown rump length (right panel) by ultrasound.

Crown rump length (distance from the tail head to the greater curvature of the skull) is easily measured in embryos of foetuses presented in frontal or sagittal view. Head and trunk diameter measurements (recorded at their maximal diameters) require a cross-section or frontal presentation. Experience has revealed that crown rump length is best for estimating ages of embryos less than 50 days and head or trunk diameters are more easily obtained for foetuses over 50 days old.

The regressions and correlation coefficients between the development of the ovine (Noia et al., 2002) and bovine (Kahn W., 1991) foetus and age of gestation has been obtained for several different features. The use of these measurements in formulas to estimate age results in the least variation between the estimated and actual ages.

4. Foetal gender determination

The genital tubercle is embryonic tissue that gives rise to the clitoris in the female and to the glans penis in the male. It originates between the rear legs of the foetus and migrates just caudal to the umbilicus in the case of the male and ventral to the anus in the female. After day 50 of gestation, male and female foetuses can be differentiated by the relative location of the genital tubercle (presumptive penis or clitoris) and development of genital swellings into a scrotum in the male foetus. Diagnosis of sex should be made by visualization of either male or female sex organs and should be nearly 100 percent accurate. Determinations made on the basis of absence or inability to identify the organs either ventral to the tailhead or caudal to the umbilicus may result in lower accuracy.
Ultrasound imaging of 28 buffalo foetuses on day 50 to 65 (period considered critical for foetal gender determination) has been performed every day (Presicce et al., 2001).The position of the genital tubercle was considered to be diagnostically relevant for both males (n=16) and females (n=12) by day 57, with confirmation of the sex to occur by day 59. The hyperechogenic image of the buffalo genital tubercle did not show any appreciable differences from the bovine genital tubercle. A good flat ventral view of the foetus at day 57 was essential for gender determination and at this stage a good view was always reached within two minutes of ultrasound scanning for each animal. Echographic confirmation of gender was performed from day 65 to 67 and 100 percent efficiency was verified.

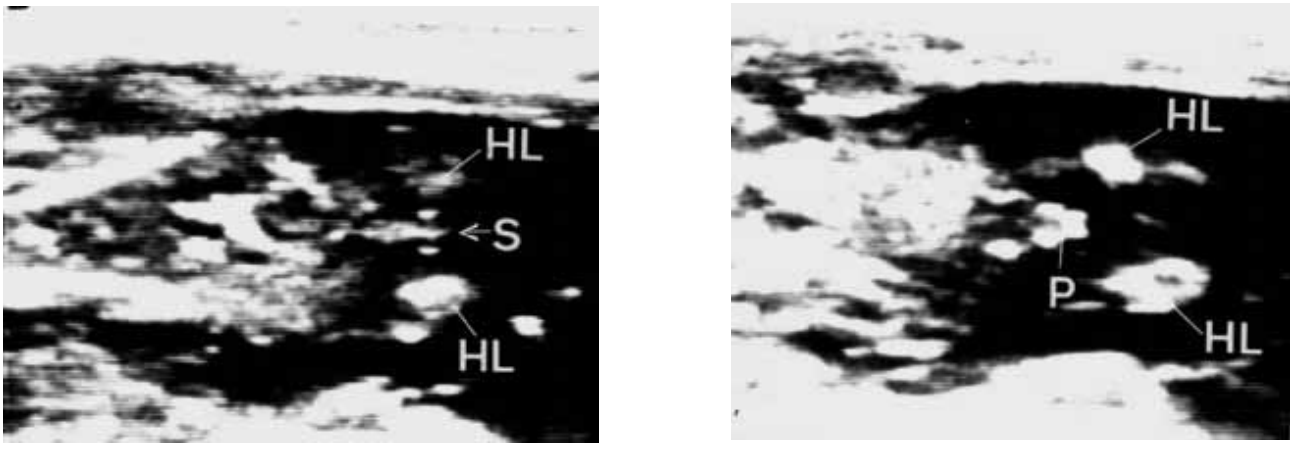


Figure 6. Ultrasound images of a male foetus (frontal view).
Right panel shows hind limbs (HL) and penis (P); left panel shows scrotum (S).

The ultrasound transducer must be manipulated within the rectum to provide a frontal, cross-sectional or sagittal image of the ventrum of the foetus. The umbilicus and tail serve as excellent landmarks when determining the location of the genital tubercle or the presence or absence of the scrotum.

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Chapter VII

NUTRITIONAL REQUIREMENTS IN BUFFALO COWS AND HEIFERS

Stefano Terramoccia, Settimio Bartocci and Antonio Borghese

Istituto Sperimentale per la Zootecnia
(Animal Production Research Institute)
Via Salaria 31, 00016 Monterotondo (Rome), Italy

In order to provide the appropriate feeding when considering the varying physiological phases of animals, the evaluation of the nutritional requirements becomes a determining factor. There are two different periods in the life of the buffalo cow: the lactating period and the dry period. The dry phase is defined by the lapse of time between the end of lactation, the parturition and the onset of the next lactation, which in buffalo lasts for approximately 270 days. In this survey the nutritional requirements of heifers and the buffalo herd, lactating and non lactating will be analysed.

The evaluation of the nutritional requirements of the dry buffalo herd

As mentioned above the dry phase is the period which elapses between one lactation and another, in buffalo this dry phase lasts approximately four months, the gestation period is longer than in bovines, and considered as an unproductive phase by some breeders. Since the dry period in buffaloes is longer than that of dairy cows, possible rationing errors, even though slight, could have negative repercussions with significant consequences both for the subsequent lactation and for the wellbeing of the animal itself (Zicarelli, 2000). Proto (1993) carried out the first research to evaluate the nutritional requirements in buffaloes providing indicative values for rations of non-lactating buffaloes (Table 1). In the dry period the buffalo herd must procure its own maintenance requirements in addition to the gestation demands since the needs of the foetus increase compared to the previous months and additional food supplements are essential. Proto (1993) considered the nutritional requirements applied to dairy cows adequate also for non-lactating buffaloes and suggested an energy-protein level of 0.65 Milk FU/kg DM and 10.5 percent of crude protein. Similar data was reported by Bertoni et al. (1994), who recommended the following energy-protein trend for diets of the non-lactating buffalo herd: 0.63-0.65 Milk FU/kg DM and 10-11 percent of crude-protein (Table 2), confirming Proto's study. During the dry phase Bertoni et al. (1994) recommended that the amounts of protein in the rations should be higher than 10 percent because, with a lower quantity the rumen activity could be compromised. By providing these indications, for the non lactating buffalo diet, the issue arose (Bertoni et al., 1994) whether the high recycle of urea could allow for at least a 10 percent reduction of dry matter in the crude protein content of maintenance rations; during the dry phase the requirements are almost identical to maintenance. Di Lella (2000) provided the first response; an *ad libitum* diet for the non-lactating buffalo herd should be able to provide an energy content not inferior to 0.65 Milk FU/kg DM and above all a protein concentration lower than 10 percent, with a suggested value of 9 percent. During the dry phase the animals should be fed with fresh forage or hay of good nutritional value and it is advisable to provide 15 percent DM with a concentrate, therefore re-establishing the reserves of liposoluble vitamins, oligominerals and, by means of hydrosoluble vitamins, to normalize the rumen fermentation and hepatic functions. The diet that characterizes this phase has a low rumen fermentation rate, which conditions the production of volatile fatty acids and favours the proliferation of cellulolytic bacteria. While still in this phase a decrease of the absorbent process with a drop in the rumen papillae activity is noticed. The nutritional requirements increase following parturition and the diets show differing characteristics with a great increase of non structural carbohydrates (NSC) and in protein content. The sudden changes in the diet are not supported by variations within the rumen such as an increase of the amilolytic

population and development of the rumen papillae, which occur at a slower rate. Therefore it is important for the dry buffalo to be fed the same diet as when lactating; this new breeding technique could start at least three weeks before the presumed parturition by forming a group "near partum". This group should be administered with a suitable feed with an energy content of at least 0.90 Milk FU/kg DM, a reduction of structural carbohydrates in the diet and an increase of NSC. In addition to the energy content, the diet for buffaloes "near partum" must guarantee the appropriate amount of nitrogen compounds: according to Di Lella (2000) the optimal protein requirement in the last phase of gestation should be 13 percent.

In 1999 the Technical-scientific Committee of the consortium for the protection of Campania Buffalo Mozzarella Cheese was established. The Committee drafted standard guidelines for the regulation of both hygiene and nutrition in buffalo herds related to the Campania Buffalo Mozzarella Cheese DOP, published in 2002. In the cited work all the indications concerning the nutrition of buffaloes are presented. Furthermore the Technical-scientific Committee suggested, where possible, to subdivide the animals according to a breeding technique in which the Body Condition Score (BCS) is evaluated, the aim being, by means of the correct diet, to achieve the ideal weight within the ninth month of gestation. Table 3 reports the nutritional requirements for the non-lactating buffalo herd. The average energy level of the last three months is approximately the same as that previously suggested by various authors (Proto, 1993; Bertoni et al., 1994; Di Lella, 2000), while the optimal level for crude protein is indicated at 800 g/d, with a protein level of approximately 7.5 percent. Particular attention is given to mineral content, especially when comparing the calcium:phosphorus ratio. In fact from the ninth month the Ca:P ratio must be 1:1.1 to avoid the possibility of vaginal and/or uterine prolapse (Zicarelli et al., 1982). In the diet high Ca:P ratios cause an alteration of the normal Ca:Mg ratio in the blood, as a result the excitability of the uterine-vaginal muscle fibre undergoes alteration, causing atonicity of the organ leading to prolapse (Campanile et al., 1989). An excess of calcium during the dry phase can cause a minor parathyroid activity with consequent low values of calcemia at calving. Integration by means of hyper phosphorus salts tends to draw the ratio of the macro-elements closer together stimulating the parathyroid activity (Campanile et al., 1995). The mineral supplement, which is calculated bearing in mind the calcium, phosphorus and magnesium content in the diet, can be added to the concentrate or given separately. To provide a well-balanced supplement it is essential to assay the mineral composition of the foodstuff administered to the animals. Due to this the Technical-scientific Committee considers it unwise to administer poliphita hays, alphapha hay and Italian ryegrass hay, during the dry phase which accounts for 4.0-7.0 g/kg DM of calcium and 2.5-4.8 g/kg DM of phosphorus. From this point of view oat hay, wheat straw and maize silage (in quantities not higher than 5.0-7.0 kg/head/d) appear to be more suited being poor in minerals. The ample variations of these elements in the diet do not influence the final content.

The nutritional requirements of the non-lactating buffalo herd have also been evaluated by Bartocci et al. (2002). Twenty farms were assessed in the Lazio region (central Italy) and were subdivided according to the daily milk yield: high yield (> 9 kg), intermediate yield (8-9 kg) and low yield (< 8 kg). The diets for the non-lactating buffalo herd were characterized by an average energy content of 0.64 Milk FU/kg DM, no significant differences emerged between the farm categories. The protein content demonstrated an average of 7.5 percent, with a higher statistically significant difference between the low yield farms and the other two categories (6.3 vs 8.0, 8.1 percent; $P<0.05$). Bartocci et al., (2002) evaluated the nutritional requirements of the dry buffalo herd only on the farms with high and intermediate milk yields (Table 4). Such a low protein content (7.9 percent) could be justified by the nitrogen metabolism in buffaloes that differs from cattle (Abdullah et al., 1990; Kennedy et al., 1990). Moreover, studies carried out by Puppo et al., (2002) indicate a greater protein digestibility in buffaloes compared to cattle in diets with a high content of structural carbohydrates. This all leads to the conclusion that buffaloes have a greater capacity to utilize protein sources at least from those diets adapted for the dry phase, therefore giving the breeder the opportunity to formulate diets with a low protein content.
It is clear that great progress has been made with regard to the understanding of the

nutritional requirements of non-lactating buffaloes. Not so many years ago Proto (1993) and Bertoni et al., (1994) asserted that the non-lactating buffalo could be fed in the same way as the dairy cow, during the same physiological period. However, results from subsequent studies (Technical scientific Committee, 2002; Bartocci et al., 2002) demonstrate that the energy level in the non lactating buffalo herd can fluctuate from 0.60 to 0.65 Milk FU/kg DM, while the protein level can drop to 7.5 percent DM in the diet. In our opinion this protein level, which may appear low when compared to cattle, requires further research. In addition particular attention should be given to the Ca:P ratio which should be 1:1.1 from the ninth month.

The evaluation of nutritional requirements in the lactating buffalo herd.

The standard lactation phase in buffaloes is 270 days, the milk yield increases after calving and reaches a peak between the fourth and the sixth week. Besides quantity variations buffalo milk is also subject to a variation in the chemical composition during lactation, this phenomenon is much more evident in this species compared to cattle. This implicates greater attention when observing the lactation curve, bearing in mind the chemical variations when calculating the production requirements. According to Proto (1993), particular attention should be given to the milk fat percentage variation which ranges from 6.0 to 12.0 percent and influences the energy requirements. In the same way the protein level which varies between 3.5 to 5.5 percent influences the protein requirements. Table 5 records the protein and energy requirements for the production of 1 kg of buffalo milk relative to the fat and protein content (Proto, 1993). In addition to the energy and protein requirements, the mineral demands should be considered, with particular attention to the calcium, phosphorus and magnesium contents. The production requirements of these three elements, according to the same author, can be considered the same as for bovines bringing the levels to 6.7 g calcium, 2.2 g phosphorus and 0.9 g magnesium, per kg of milk yield. Other criterium suggested by Proto (1993) was to transform buffalo milk into milk standardized to 4.0 percent fat and 3.1 percent protein, by using Di Palo's equation (1992):

kg of standard milk = kg of milk produced * (((g fat - 40)+(g protein - 31))* 0.01155+1.0)

Once the conversion of buffalo milk was carried out Proto considered that the energy requirements of bovines were suitable when calculating buffalo requirements: 0.44 Milk UF/kg of milk normalized to 4 percent fat, and subsequently applying the production requirements for milk determined by the Institut National de la Recherche Agronomique, France (INRA, 1988).

Bertoni et al., (1994) proposed that one breeding technique could be to divide the lactating animals into two groups: one with a yield higher than 8-9 kg and the other with a lower yield. In the former group the suggested ration has an energy density of 0.80-0.85 Milk FU/kg DM and 13.5-14.5 percent CP; in the latter group the density drops to 0.76-0.80 Milk FU/kg DM and 12.5-13.5 percent CP (Table 6). The same authors recommended a diet containing mainly forage since the buffalo utilizes this much better than concentrates; furthermore, in order to avoid digestive problems, in the rumen or intestine, the crude lipid and starch + sugar content must not exceed respectively 4.0-4.5 percent DM and 16.0 17.0 percent DM (Bertoni et al., 1994).

Zicarelli (1999) likewise paid particular attention to buffalo diets during the lactation phase. When employing the equation of Di Palo (1992) and comparing buffalo milk to that of dairy cows, with the same energy produced per 1 kg of milk (Table 7), the fact emerges that buffalo milk is characterized by a lower protein and phosphorus value compared with that produced by dairy cows. According to the same author, analogous to variations of dairy cow milk, in the first 50 days circa of lactation buffaloes register a dry matter intake lower than their requirements, which leads to an inevitable weight loss. As a consequence the milk yield tends to decrease since the animals have the ability to accumulate reserves as a precautionary measure for periods of scarce forage availability, thus aiding their wellbeing while not favouring the galactopoiesis. Possible excesses of energy intake in buffaloes do not cause the "fat cow syndrome" typical in bovines, but modify the chemical composition of the milk, especially the lipid content. As the milk yield gradually augments during lactation, the requirements increase

according to the quantity of the milk yield: on average it can be considered that with a rise of 1 kg milk the requirements increase to 0.76 Milk FU, while the intake of dry matter rises to 0.475 kg. After 150 days from parturition the buffaloes tend to ingest more than their requirements, therefore accumulating excessive reserves. In order to prevent excessive weight gain in this phase the energy density should be lowered, the NDF increased and the starch reduced (not higher than 18 percent DM). A greater adipose reserve is most common in animals that exceed 270 days of lactation due to fertility reasons, or in animals with low yields. With the remaining animals this phenomenon appears less evident since the previous condition is easily re-established in the dry phase. As previously mentioned the buffalo milk protein quota, compared to energy produced is lower than that of dairy cows. One of the characteristics of the buffalo lies in the protein degradability in the rumen which is greater than that in cows (Terramoccia et al., 2000); furthermore the permanence time of foodstuff in the buffalo rumen is greater in comparison to cattle, while there is an inverse tendency in the intestinal tract (Bartocci et al. 1997). This characteristic favours the by pass proteins employed to a lesser degree than in cattle, therefore avoiding fertility or mastitis problems in the event of excessive protein. Zicarelli (1999) suggests a protein ration of 2.47 g CP for every gram of protein in the milk (similar values to those of dairy cows). At the onset of lactation as the intake is lower, it is advisable to increase the protein quota by 10 percent, bearing in mind that the requirements are not adequate if a diet containing less than 13.5 percent CP is used (Campanile et al., 1995). Each kg of buffalo milk contains 1.8-2.0 g calcium and 1.1-1.2 g phosphorus; as far as the maintenance requirements are concerned values provided by INRA (1988) for dairy cows apply. Zicarelli (1999) calculated that for milk production, the calcium requirements reach the value of 5.2-5.8 g/kg milk circa and the phosphorus requirements are 2.1-2.3 g/kg milk. Table 8 reports the conversion factors which consent the technician to calculate the milk yield normalized to 8.30 percent fat and 4.73 percent protein and subsequently to calculate the requirements and formulate the ration.

Another research which provides indications for the nutritional requirements of the lactating buffalo herd (Table 9) is that elaborated by the Technical-scientific Committee of the consortium for the protection of Campania Buffalo Mozzarella Cheese (2002). This work combines the experience gained in the various research centres (University of Naples - two faculties and the Animal Production Research Institute, Rome) that have studied this species to a greater extend. According to the authors the intake of dry matter depends on: the weight, the production level and the physiological phase of the animal, also on the forage: concentrate ratio and lastly on the quality of the feeds used to formulate the ration. The requirements reported in Table 9 have been evaluated considering 20 percent primiparous incidence within the lactating group. Moreover the possibility of weight gain recovery was considered which in buffaloes occurs between 100 and 170 days after calving, this period corresponds to the passage from the catabolism to the anabolism phase of the lactation curve. The considered Milk FU were calculated by evaluating the energy necessary to assure the milk production of the herd. As regards protein content the Technical-scientific Committee has decided to quote the values obtained by the research centres which are part of the working group. These values differ from the theoretic requirements because they not only consider the production of protein in the milk, the growth development of the primiparous and weight recovery of the animals, but also what endocrine - metabolic effects the feed proteins have on the buffalo milk yield. For example, the percentage of crude protein suggested by the Technical-scientific Committee for a group of buffaloes that produce 12 kg/d of normalized milk is 15.9 percent, compared to requirements calculated at 13.2 percent. Slight excesses of protein in the buffalo diet do not determine those negative effects that are usually detected in the dairy cow. Studies on lactation buffaloes demonstrate that protein concentrations greater than those arising from the calculation regarding only the requirements, show a rise of azotemia but also result in an increase of glycemia and a reduction of insulinemia. This particular metabolic condition guarantees a greater availability of glucose for the udder due to the synthesis of lactose, which in turn favours the galactopoiesis due to the osmotic effect. When formulating the rations for the lactating buffalo herd it must be considered that elevated levels of structural carbohydrates limit the ingestion capacity and that greater concentrations of highly fermentable starches and

sugar can lead to an excessive weight gain which results in a shorter lactation curve. The calcium and phosphorus contribution is correlated to the productive requirements of the herd; so in this case the Ca:P ratio must be 2:1, so that the quantity of these two minerals is in proportion to the amount of milk produced (Technical-scientific Committee, 2002).

Table 10 reports the indicative requirements of the lactating buffalo herd elaborated by Bartocci et al. (2002). These data were obtained by evaluating the amount of dry matter intake, the chemical composition, the nutritional value and the milk yield for an entire lactation phase of 258 buffaloes, on 20 buffalo farms. In order to estimate indicative requirements of lactating buffaloes regression equations were calculated ($P<0.01$) between the normalized milk quantity (8.30 percent fat, 4.73 percent protein) and the average daily net energy consumed, protein, structural and non-structural carbohydrates of the diets administered *ad libitum* on 20 monitored farms:

Milk FU/d = 7.16 + 0.66*kg of milk ($R^2 = 0.80$)
CP (g/d) = 314.72 + 187.35*kg of milk ($R^2 = 0.87$)
NDF (g/d) = 8864.30 - 198.92*kg of milk ($R^2 = 0.76$)
NSC (g/d) = 4762.92 + 150.36*kg of milk ($R^2 = 0.81$)

The data for net energy ingestion of proteins and structural and non-structural carbohydrates, resulting from the previous equations are considered an estimate of the nutritional requirements of the lactating buffalo herd, corresponding to a normalized milk yield varying from 7 to 12 kg/d. When dividing the above-mentioned daily requirements, calculated by means of the previous equations, per ingestion of dry matter, the concentrations of nutritional principles of the diet are obtained which are necessary to satisfy maintenance requirements and the milk yield (Table 10). The data refers to a buffalo herd with 20 percent circa primiparous, the average weight for the multiparous of 650 kg and for primiparous of 570 kg. The maintenance requirements were evaluated by employing the INRA method (1988) for dairy cows, as specific data are not available. In order to obtain a normalized milk yield of 10 kg/d, an average live weight increase of 18.8 kg was estimated, which takes into account a weight reduction in the first forty days and a consequent gain between 100 and 170 days of lactation. From the daily weight gain for primiparous, estimated at 300 g/d, it was possible to calculate the energy and crude protein needed to produce 1 kg of normalized milk in 0.72 Milk FU and 145 g. When confronting the data of the nutritional requirements (7-12 kg/d) reported in Tables 9 and 10 the following considerations emerge: the daily ingestion of dry matter to produce 7 kg of milk is 16.0 kg according to Bartocci et al., (2002) while the Technical-scientific Committee (2002) estimated an intake of 14.7 kg DM. This difference, 1.3 kg DM, with the increase of the milk yield almost tends to disappear, 0.65 kg DM per 10 kg of milk; the same intake of dry matter (17 kg) with 12 kg of milk produced. Therefore, for the highest milk yield, the difference between the two studies when evaluating the dry matter intake is minimal; conflicting values were reported for the lower milk yields. However the intake capacity of the buffalo species needs to be considered as it has yet to be defined due to the differing results obtained from the various research centres. The total energy intake required to produce 7-10-12 kg of milk according to Bartocci et al., (2002) is 11.84, 13.74, 15.13 Milk FU/d vs 12.05, 14.16, 15.64 Milk FU/d of the Technical-scientific Committee (2002); consequently there is substantial agreement between the two studies concerning the total energy to administer in order to obtain the same milk yield. The crude protein amounts calculated to produce 7-10-12 kg of milk/d are almost equal, and precisely 1 626, 2 188 and 2 565 g/d of crude protein for Bartocci et al. (2002); 1 617, 1 996 and 2 240 g/d of crude protein for the Technical-scientific Committee (unpublished data). The higher values of crude protein (2 102, 2 463, 2 705 g/d) reported in Table 9 refer to the recommended (not calculated) values which also take into account the endocrine metabolic effect of the proteins. When comparing the fibre contribution no substantial differences emerge in the values of NDF obtained by the two works. The evaluation of the non structural carbohydrate (NSC) contribution appears rather interesting; Bartocci et al., (2002) report higher values compared to those of the Technical-scientific Committee (2002) because in the latter work the protein level increased and also the fat content in the diet was considered.

In conclusion the optimal protein level is the one recommended by the work of the Technical-scientific Committee (2002). As this is the latest work which takes into account not only the calculated protein, parameters used by Bartocci et al. (2002), but also the endocrine metabolic effects that an addition of protein has on milk yield. The two studies agree on the total energy required for the various productions; the dry matter intake needs to be specified for the medium-low milk yields (7-9 kg of milk/d) of the buffalo herd.

The evaluation of nutritional requirements of buffalo heifers

In many countries the requirements of heifers are not a problem; the heifers stay on pasture, often on very poor pasture, or they are fed with straw or with bad hay. But this is not the correct and economic approach; in fact, as already stated in Chapter IV, the age of puberty and therefore the reproduction efficiency of the herd is affected by many factors: both genetic (breed, sire, etc.) and environmental factors (i.e. season, climate, management, feeding, etc.). The age of puberty is particularly influenced by the diet energy level that enhances growth and sexual maturity.

Therefore in some countries, such as Italy, farmers prefer to give the correct diet to heifers satisfying the necessary requirements, in order to obtain high daily gains, to anticipate sexual maturity, to realize early puberty, early conception and early calving, and thereby reducing the unproductive period in the herd.

In this connection, a series of experiments was performed at the Animal Production Research Institute in Rome in order to determine what daily gain is the optimum and with which feeding stuffs it is possible to realize such gains, and thereby ascertain, the most efficient system for the feeding and management of buffalo heifers in economic terms and in reproduction efficiency.

Experiment 1: Different farms
This first trial (Borghese et al.,1993; Esposito et al., 1993) was carried out at the Tormancina farm (TM), 18 km north east of Rome (42° latitude North) and in three other farms (D-J-S) situated in the Campania Region of southern Italy (40.5 41° latitude North). The heifers of TM farm were housed in open feed-lots and fed unifeed ad libitum (maize silage 55 percent, alfalfa hay 17 percent, wheat straw 12 percent, beet pulp 9 percent, soya bean meal 1 percent, brewer grain 6 percent, 0.76 Milk FU/kg DM), while in D-J-S farms the animals were fed unifeed (maize silage, hay, straw, concentrates) in restricted diets: 4.21 3.73 3.83 Milk FU/day between 400 and 500 days of age and 5.10 4.42 5.25 Milk FU/day between 500 and 650 days of age, respectively, on the three farms.

Experiment 2: Low and high feeding levels
The heifers were housed in open feed-lots, subdivided in two groups, and fed two different diets according to standard requirements in order to obtain 450 g (low level group) or 650 g (high level group) daily gains respectively (Terzano et al., 1993; Borghese et al., 1994). The forage/concentrate ratios were: 4.42:1 - 2.46:1 respectively in the low level and in the high level groups; the diet components were: hay (81.4-70.7 percent respectively), soya bean meal (10.1-8.7 percent respectively), maize meal (8.5-20.6 percent respectively).

Experiment 3: Intensive feeding versus grazing feeding
The heifers were housed in feed-lots and randomly assigned to intensive feeding or to the pasture system: in the intensive system they received maize silage ad libitum (DM 33 percent, crude protein 8 percent, crude fibre 21 percent, 0.85 Milk FU/kg DM), plus hay and protein-mineral-vitamin supplement; the natural pasture botanical composition was: 50 percent grass, 40 percent legume and 10 percent other species (DM 20-70 percent, crude protein 14 percent, crude fibre 30 percent, 0.50-0.85 Milk FU/kg DM) (Terzano et al., 1996). The trial was repeated for two consecutive years taking into account that the pasture could be subjected to variability due to the different seasons, so the feeding systems are reported as maize silage 1 - pasture 1

for the first year and maize silage 2 - pasture 2 for the following year. Experiments 2 and 3 were carried out at the same farm (Tormancina).
Protein requirements (100-150 g PDI/100 kg live weight) were satisfied in all the trials except in the grazing one during the dry season.

Experiment 4: Maize silage and unifeed versus grazing feeding
The trial was carried out on 27 Mediterranean buffalo heifers, housed in feed-lots, treated against helminthes and randomly assigned to three groups at the average initial age of 8.5-9.0 months (Borghese et al., 1997).
1. Maize silage - nine heifers were fed maize silage ad libitum (DM 33 percent, crude protein 8 percent, crude fibre 21 percent, 0.85 Milk FU/kg DM) plus hay (about 20 percent on fed maize silage) and protein-mineral-vitamin supplement.
2. Pasture - eight heifers were fed natural pasture (50 percent graminaceae, 40 percent leguminosae and 10 percent other species, DM 20-70 percent, crude protein 10-21 percent, crude fibre 18-35 percent, 0.50-0.85 Milk FU/kg DM).
3. Unifeed - ten heifers were fed unifeed (DM 43.7 percent, crude protein 15.3 percent, crude fibre 22.4 percent, 0.84 Milk FU/kg DM).

During each trial the animals were weighed monthly in order to evaluate their growth rate; starting from about the thirteenth month of age they were tested every ten days by rectal palpation in order to determine the presence of follicle and corpus luteum and to assess the development of ovaries, cervix and uterine horns. At the same time blood samples were collected and plasma progesterone (P4) was assayed by RIA. Heifers were considered to have achieved puberty and cyclic ovarian activity when plasma P4 levels exceeded 1.5 ng/ml for two consecutive samples with a low value interval. After two cycles, as confirmed by rectal palpation, the heifers were mated.

Results of the four trials

The puberty age of all the reported trials (Table 11) shows a large variability depending on several factors. The pre-weaning and weaning systems which had influenced the daily gain obtained before the trials started could be important in promoting growth and achieving puberty. In fact, considering a mean of 40 kg body weight at birth, the animals that had shown a higher daily gain before the trial reached puberty in a shorter time.

Most of the heifers required a body weight of 380-420 kg to achieve puberty. In this case the feeding level plays a pivotal role in order to promote weight gain, and body and sexual growth. In experiment 1 on the TM farm, where the heifers received 4.5-5.5 Milk FU/d, all the 30 animals achieved cyclicity before 20 months at a body weight of 421 kg (679 g/d), while on J farm, where the heifers received 3.7-4.4 Milk FU/d, the lowest daily gain (472 g/d) was registered and only seven animals (24 percent) achieved puberty before two years. On D farm (4.2-5.1 Milk FU/d), where a daily gain of 525 g was recorded, 28 heifers out of 30 became cyclic and 25 conceived. Contrary to the other farms, where a constant daily gain was recorded for the whole trial period, on S farm 300 g daily gains were recorded up until 500 days with 3.8 Milk FU/d, after 500 days a high compensative increase (740 g/d) was achieved with 5.2 Milk FU/d and all 30 heifers became cyclic and conceived, even if at a higher age (658 d) and at a lower weight (358 kg) than on the TM farm. On this farm the best feeding efficiency (7.36 Milk FU/kg gain) was executed. This trial showed how a proper feeding level may anticipate the onset of puberty and affect the incidence of pregnancies.

In experiment 2 (Table 11) significantly higher daily gains and more favourable ages and weights at puberty were achieved with a high feeding level (5.6 Milk FU/day) than with the low level (4.4 Milk FU/day). The feed efficiency in the low and high level diets was about the same. These results confirmed the feeding level effect on growth and on body and sexual development and on the onset of puberty, as noted by other authors in Swamp heifers in Malaysia (Dollah et al., 1989), in Nili-Ravi in Pakistan (Chaudhary et al., 1983; Asghar et al., 1983) and in Murrah

in India (Kaur and Arora, 1989). Most of the animals had cyclic ovarian activity when the first P4 >1.5 ng/ml appeared. Two buffalo heifers showed ovarian disorders; one persistent corpus luteum and one luteinic cyst. In this trial, as in experiment 1 at the same farm (TM), the start of cyclic ovarian activity was influenced by decreasing photoperiod with the highest concentration in the autumn. Nine animals, born between December and May, achieved puberty from the following October to February at about 22 months of age (614 d in the high level group, 686 d in the low level group), while 15 heifers, born after May were not able to achieve puberty within the favourable season of the following year and delayed ovarian activity until the next autumn, at an average age of 27 months (796 d in the high level group, 825 d in the low level group). Therefore it was also confirmed in this study that the age at puberty is affected by the season of birth.

In Experiment 3 during the course of the first year (maize silage 1 - pasture 1) significantly higher (+42 percent) gains were obtained with the intensive system (693 g/d) than with the grazing one (488 g/d). In the second year (maize silage 2 - pasture 2) the differences between the intensive and grazing groups were notably reduced: 679 (+6.6 percent) versus 637 g/d, certainly due to the better conditions of the pasture and the climate of the second year, which permitted constant daily gains similar to that obtained with ad libitum feeding. On the contrary the poor pasture of a very hot summer (the first year) halted the heifers' growth, determining even a diminution of their body weight which, however, was followed by a prompt recovery of growth in the autumn. In both trials, the puberty age was about the same in the intensive and pasture groups (Table 11), due to the balancing growths realized by the heifers on pasture, that were able to attain the same body and sexual development during the autumn, the season which normally promotes the onset of cyclic ovarian activity. Very early puberty was realized by the maize silage group (16 months, 23 days before the pasture group) at 402 kg body weight (22 kg more than the grazing one, in the first year), while in the following trial, puberty age was delayed until 20 months with the maize silage and until 19 months in the grazing one, achieving body weights comparable to those of the previous year. Feed efficiency was also about the same and more convenient in comparison to that of the previous trials characterized by more intensive feeding systems. The grazing system was the most convenient in economic terms. All the animals had cyclic ovarian activity, as detected by rectal palpation, when the first progesterone >1.5 ng/ml appeared and so the animals conceived at a very early age; less than 20 months (first year) and at about 22 months in the second year without variations between groups.

In Experiment 4, seven heifers from the maize silage group (77.8 percent), seven of the pasture (87.5 percent), and all ten animals of the unifeed group achieved puberty within two years of age (Table 12). Therefore data are reported on 24 animals. All the animals showed cyclic ovarian activity, as detected by rectal palpation and by progesterone assay, when the first P4>1.5 ng/ml appeared, without following anoestrous period. No persistent corpus luteum nor luteinic cyst were found.

The heifers in this trial achieved puberty between July and October, due to the favourable effect of decreasing photoperiod on cyclic ovarian activity by melatonine intermediate action (Borghese et al., 1995). Since these heifers were born in the winter (December-March), they showed a longer anoestrous period than heifers born in the spring-summer (May-August), which had been utilized in other trials; the latter also achieved puberty in the autumn (October-December) at a very early age (15-18 months), since these animals had been born near the autumn, while the heifers of this trial, born in the winter, achieved puberty at 18-20 months. Therefore, as in previous trials, the age at puberty is confirmed to be affected by the season of birth.

How the feeding system affected body weight during the trial (Fig.1). The unifeed group showed higher body weight particularly between 498-550 days of age ($P<0.05$). During this period the animals obtained the maximum average daily gain (Fig. 1), that was more than 1.0 kg/d, but this group demonstrated another period (366-466 days) with a 600-800 g/daily gain which is

similar to the values attained by the other feeding groups. The maize silage group was more uniform in the daily gain during the whole trial (600-800 g/d) and consequently for body weight trend. Heifers on pasture showed a minimum daily gain at 366 days (600 g/d) during winter when the pasture was poor, but later they realized balancing growths of more than 1.0 kg/d (Fig. 1) at 426 days during the spring when the pasture was rich. At the end of the trial all the groups demonstrated the lowest daily gain since body maturity was achieved at about 20 months of age and 420 kg of weight. The highest average daily gain obtained with unifeed (824 g, Table 12) significantly affected ($P<0.05$) the age of puberty, which was 17.7 months in comparison with 19 on pasture and 20 months with maize silage. The heifers on pasture achieved puberty with the lowest body weight (386 kg, Table 12), about 38 kg less than the other groups, one month later than the unifeed groups and one month prior to the maize silage group.
The heifers on pasture achieved these reproductive performances with the lowest cost in terms of feeding stuffs and management.

Six heifers on pasture (85.7 percent) conceived at 668.5 days of age, about 100 days after the onset of puberty, 47 days after being bull exposed. One heifer did not conceive within the two months with the bull. Seven heifers fed maize silage (100 percent) conceived at 697 days of age, about 61 days after the onset of puberty, 56 days after being bull exposed. No heifer with unifeed conceived in the same period, though they were bull exposed at 582 days of age for two months, but it was due to the bull's fertility. Therefore the pasture system promoted the best performances in buffalo heifers, due to the economy of feeding and management, with favourable daily gains and an early age at puberty and at conception.

The conclusion of these experiments is that the best results are obtained by using unifeed which guarantees the integration of different feeding stuffs, this means the optimum of crude protein (12 16 percent) and crude fibre (20-24 percent) concentration, good mineral and vitamin content, good energetic concentrations (0.76-0.84 Milk FU/kg DM), convenient daily gains (680-800 g), the best feed efficiency (5.8-7.0 Milk FU/kg daily gain), early puberty (530-600 days) at a correct body weight (400-420 kg) and early conception before two years.

These results are valid for the Mediterranean Italian Breed, but probably they could be extended with some variations to all River breeds.

The requirements average in heifers, commonly used to prepare diets on Italian farms, is reported in Table 13.

Table 1. Indicative characteristics of requirements of the dry buffalo herd, average live weight = 600 kg (Proto, 1993).

Dry matter (kg)	10.5
NE_L (Milk FU/kg DM)	0.65
CP (% DM)	10.5
Dig. Prot (% DM)	7.0
CF (% DM)	30.0
NDF (% DM)	60.0
Starch + Sugars (% DM)	9.0

Table 2. Indicative characteristics of requirements of the dry buffalo herd (Bertoni et al., 1994).

Dry matter (kg)	10-12
NE_L (Milk FU/kg DM)	0.63-0.65
CP (% DM)	10-11
NDF (% DM)	52-58
Starch + Sugars (% DM)	8-10

Table 3. Nutrient requirements during gestation of the dry buffalo herd (multiparous: 600 kg; primiparous: 500 kg) in relation to the gestation months (Technical-scientific Committee, 2002)

Months of pregnancy		**Milk FU/d**	**CP (g/d)**	**Ca (g/d)**	**P (g/d)**
8	multiparous	5-7	700	40	35
	primiparous	6-7	830	40	35
9	multiparous	6-7	700	40	35
	primiparous	6-7.5	830	40	45
10	multiparous	6-7	800	40	45
	primiparous	7-8	900	40	45

Table 4. Indicative characteristics of requirements of the dry buffalo herd (Bartocci et al., 2002).

Dry matter (kg)	10.61
NE_L (Milk FU/kg DM)	0.63
CP (% DM)	7.90
NDF (% DM)	49.10
NSC (% DM)	33.10

Table 5. Energy and protein requirements for the production of 1 kg of buffalo milk relative to the fat and protein content (Proto, 1993)

Energy equirements (Milk FU/kg of milk)													
Milk fat	6.5	7.0	7.5	8.0	8.3	8.5	9.0	9.5	10.0	10.5	11.0	11.5	12.0
NE_L	0.61	0.64	0.67	0.70	0.72	0.73	0.76	0.79	0.82	0.85	0.87	0.90	0.93
Protein requirements (g/kg of milk)													
Milk protein (%)			3.5	3.7	3.9	4.1	4.3	4.5	4.7	4.9	5.1	5.3	5.5
CP			99	105	111	116	122	128	134	139	145	151	157

Table 6. Indicative characteristics of rations for the lactating buffalo herd (Bertoni et al., 1994).

Milk yield	**DM (kg)**	**NE_L (Milk FU /kg DM)**	**CP (% DM)**	**NDF (% DM)**	**Starch + Sugars (% DM)**
>8-9 kg/d	15.5-16.5	0.80-0.85	13.5-14.5	42.0-46.0	14.0-16.0
<8-9 kg/d	14.5-15.5	0.76-0.80	12.5-13.5	46.0-50.0	12.0-14.0

Table 7. Energy and quality of cattle and buffalo milk and indicative requirements (Zicarelli, 1999).

	Cattle milk 4% (FCM)	**Buffalo milk**	**Buffalo milk (same energy as cattle milk 4% FCM)**
Energy and quality			
Energy (kcal/kg)	740	1258	740
Milk protein (g/kg)	31	45	26.47
Milk fat (g/kg)	40	87	51.18
Ca (g/kg)	1.2	2.0	1.18
P (g/kg)	0.9	1.2	0.71
kcal/g protein	23.9	28	28
Requirements/kg of milk			
Crude protein (g)	85	123	73
NE_L (Milk FU)	0.44	0.74	0.44
Ca (g)	3.5	5.80	3.43
P (g)	1.7	2.3	1.33

Table 8. Conversion factors to calculate the milk yield normalized 8.30 percent fat and 4.73 percent protein (Technical-scientific Committee, 2002).

	Fat (%)											
	6.0	**6.5**	**7.0**	**7.5**	**8.0**	**8.3**	**8.5**	**9.0**	**9.5**	**10.0**	**10.5**	**11.0**
Protein (%)												
3.8	0.779	0.813	0.847	0.881	0.915		0.943	0.984	1.019	1.053	1.087	1.121
4.0	0.792	0.827	0.860	0.845	0.929		0.964	0.998	1.032	1.066	1.101	1.135
4.2	0.806	0.840	0.874	0.909	0.943		0.977	1.012	1.045	1.080	1.114	1.149
4.4	0.820	0.853	0.888	0.923	0.957		0.991	1.025	1.060	1.094	1.128	1.162
4.6	0.833	0.868	0.902	0.936	0.971		1.005	1.039	1.073	1.108	1.142	1.176
4.73												
4.8	0.847	0.881	0.916	0.950	0.984		1.019	1.052	1.087	1.121	1.156	1.190
5.0	0.861	0.895	0.929	0.964	1.000		1.032	1.066	1.101	1.135	1.170	1.204
5.2	0.875	0.909	0.943	0.977	1.012		1.046	1.080	1.114	1.149	1.184	1.217
5.4	0.888	0.923	0.957	0.991	1.025		1.060	1.094	1.128	1.163	1.197	1.231
5.6	0.902	0.936	0.971	1.005	1.039		1.070	1.180	1.142	1.177	1.210	1.245

Table 9. Indicative characteristics of rations for the lactating buffalo herd (average live weight= 650 kg; normalized milk: fat=8.30 percent and protein=4.73 percent) (Technical-scientific Committee, 2002).

	Production of normalized buffalo milk (kg/d)							
	<6	6	7	8	9	10	11	12
Advised intake (kg DM/d)	13.3	14.2	14.7	15.1	15.6	16.1	16.5	17.0
NE_L (Milk FU/d)	0.75	0.79	0.82	0.84	0.86	0.88	0.90	0.92
CP (%DM)	13.0	13.9	14.3	14.6	15.0	15.3	15.6	15.9
NDF (%DM)	52.0	47.0	46.0	44.0	43.0	42.0	40.0	39.0
NSC (%DM)	25.0	27.0	28.0	29.0	30.0	30.0	31.0	32.0

Table 10. Indicative characteristics of rations for the lactating buffalo herd (average live weight= 650 kg; normalized milk: fat=8.30 percent and protein=4.73 percent) (Bartocci et al., 2002).

	Production of normalized buffalo milk (kg/d)					
	7	8	9	10	11	12
Advised intake (kg DM/d)	16.00	16.25	16.50	16.75	17.00	17.00
NE_L (Milk FU/d)	0.74	0.76	0.79	0.82	0.85	0.89
CP (%DM)	10.16	11.16	12.13	13.06	13.97	15.08
NDF (%DM)	46.70	44.76	42.87	41.05	39.27	38.10
NSC (%DM)	36.35	36.71	37.07	37.41	37.75	38.63

Table 11. Performances of buffalo heifers during different trials until puberty (Borghese et al., 1996).

Exper.	Groups	N	Initial age (days)	Initial weight (Kg)	gain/d before trial (g)	Puberty age (days	N	Puberty weight (days)	gain/d (g)	DM/d (Kg)	Milk FU/d	Milk FU/d Kg DM	Milk FU/Kg gain
1	TM	30	371	274^{a}	631^{A}	598	30	421^{a}	679^{a}	6.5	5.00	0.76	7.36
	D	30	383	267^{a}	593^{A}	612	28	392^{ab}	525^{b}	601	4.65	0.76	8.86
	J	29	385	235^{b}	506^{B}	624	7	385^{ab}	472^{b}	5.5	4.09	0.75	8.66
	S	30	372	204^{c}	441^{C}	658	30	358^{b}	538^{b}	6.6	4.61	0.70	8.57
2	Low level	12	237	143	435	767	12	388	465^{b}	6.4	4.42	0.69	9.50
	High level	12	241	141	419	736	12	410	562^{a}	7.3	5.56	0.76	9.89
3	Maize silage 1	6	319	280	752	490	6	402	693^{a}	5.1	4.34	0.85	6.09
	Pasture 1	4	333	285	736	513	4	380	488^{b}	5.8	3.92	0.67	7.41
	Maize silage 2	9	132	107	508	602	9	426	679	5.8	4.93	0.85	7.26
	pasture 2	8	138	109	523	569	8	387	637	6.5	4.65	0.70	7.30

Different letters in the same column of the same trial mean significant differences between groups (A, B, C: <0.01; a, b, c: P<0.05)

Table 12. Weight and age at puberty, feeding efficiency in buffalo heifers (Borghese et al., 1997).

	Maize silage	Pasture	Unifeed	RMSE
Initial age (d)	267	260	258	28.236
Initial weight (kg)	195.8	164.7	197.2	37.919
Weight at puberty (kg)	425.7	386.6	423.1	38.130
Age at puberty (d)	603^{a}	569^{ab}	532^{b}	39.361
Daily gain (g)	684	718	824	0.088
DM (kg/d)	5.8	6.5	5.7	
Milk FU/d	4.93	4.65	4.80	
Feed efficiency Milk FU/kg	7.21	6.48	5.83	

Different letters mean significant differences for $P<0.05$

Table 13. Average requirements in Italian heifers

	WEIGHT (kg)			
	100-200	200-300	300-400	400-500
Dry Matter kg	3.5-4.5	4.5-7	7.9-9	9.5-11
Crude protein % DM	15-16	15-16	15-16	13
Neutral Det. Fibre % DM	35	35	38	40
Mcal/kg DM	1,4	1,4	1,4	1,3
Calcium % DM	0,6	0,6	0,48	0,45
Phosphorus % DM	0,4	0,4	0,32	0,3
vit A UI/kg DM	300	300	3 400	3 200
vit D UI/kg DM	1 100	1 100	1 300	1 200
vit E UI/kg DM	31	31	34	32

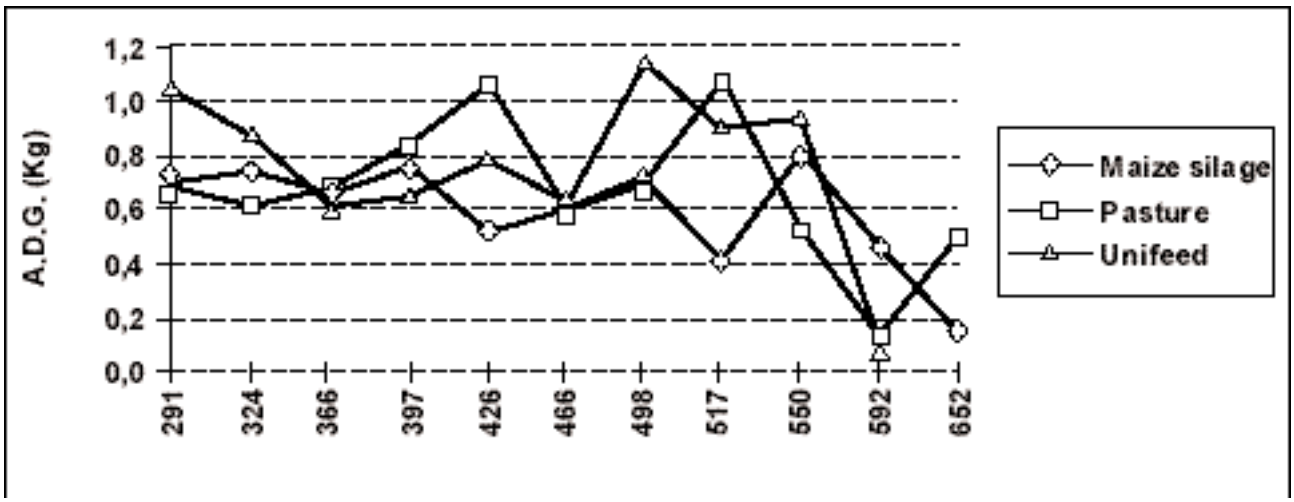

Figure 1. Average daily gain trend in buffalo heifers (Borghese et al., 1997)

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Chapter VIII

NEW ACQUISITIONS ON THE DIGESTIVE PHYSIOLOGY OF THE MEDITERRANEAN BUFFALO

Settimio Bartocci, Stefano Terramoccia and Simonetta Puppo

Istituto Sperimentale per la Zootecnia
(Animal Production Research Institute)
Via Salaria 31, 00016 Monterotondo (Rome), Italy

The high demand for buffalo mozzarella cheese and the problem of milk quotas in cattle breeding has led to an increased diffusion of the buffalo species, with about 230 000 head bred in Italy in 2001, according to recent estimates reported by the National Association of Buffalo Breeders. This present situation is a radical transformation of the original circumstances dating back to 1950, when the buffalo population reached its minimum historical number of 5 000 head (Lucifero, 1998). At that time the nutritional requirements of dry and lactating buffaloes were almost completely unknown. In recent years studies have been published by researchers from various institutions (Proto, 1993; Bertoni et al., 1994; Zicarelli, 1999; Bartocci et al., 2002) and by the Scientific Committee of the Consortium for the Protection of Campania buffalo mozzarella cheese (2002) which give estimates of the nutritive requirements and suggested suitable diets for buffaloes in their different physiological conditions.

In order to ascertain an exact definition of such requirements it is also necessary to know the digestive physiology of the species and different research centres have carried out studies in this field (Masoero et al., 1994; Infascelli et al., 1995; Di Francia et al., 2000). In addition the Istituto Sperimentale per la Zootecnia has made its own contribution with a research activity covering the following aspects: the passage rate of fluids and solids in the gastro-intestinal tract, the rumen degradability of feeds and the rumen microflora content in relation to its in vivo digestibility. As a result of this research activity four studies have been published in such scientific reviews as Animal Science (Amici et al., 1997; Puppo et al., 2002) and Livestock Production Science (Bartocci et al., 1997; Terramoccia et al., 2000), in order to contribute to the knowledge regarding the digestive physiology in buffaloes.

The research activity was carried out using four Mediterranean buffaloes (*Bubalus bubalis* L.) bulls and four Holstein Friesian bulls, two year of age; the average live weight was 417.1 and 509.2 kg respectively. Eight weeks prior to the research all the animals were fitted with a rumen silicon cannula. The animals involved in this trial were supervised in compliance with the Italian laws and regulations regarding experimental animals. Adequate procedures to minimize pain and discomfort were adopted during the operative and post operative periods. Four isoproteic diets (about 14 percent of crude proteins, CP) were administered differing in the following way with regard to forage:concentrate ratio: Diet 1= 87.5:12.5; Diet 2= 75:25; Diet 3= 62.5:37.5; Diet 4= 50:50. The forage of the diets were alfalfa hay and maize silage mixed in a ratio of 65 to 35 on a dry matter (DM) basis. The concentrate contained in a decreasing amount: barley, maize, extracted soya bean meal and a vitamin-mineral supplement. The components of the diets were accurately mixed together. Each animal was fed each of the four diets, over four consecutive periods, according to a Latin square design.

The administration of the feed was at maintenance level twice daily, at 08:00 and 16:00 hours, with an amount equal to 50 g of dry matter/kg of metabolic weight. Each period, lasting 21 days, was divided into a first stage of feeding adaptation of 14 days and a second one of seven days, which was used for the whole collection of faeces for the determination of in vivo digestibility (ASPA, 1982). Samples of feeds and faeces were analysed for: DM, CP, crude fibre (CF), ether extract (EE), ash, neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL). The analyses were carried out according to AOAC methods (1984) and

according to Goering and Van Soest (1970). The chemical composition of feeds and diets used during the research is reported in Table 1.

Buffaloes and cattle, after the period of diet adaptation, received directly in the rumen, through the rumen cannula, 300 g of hay mordanted with $Na_2Cr_2O_7$ (Udén et al., 1980) and 50 g of Co-EDTA diluted in 300 ml of distilled water, in order to determine the passage rate of the solids and fluids; the markers were administered the morning before the first meal. 28 samples of faeces were taken from each animal, directly from the rectum, from 0 to 152 hours after the administration of markers; in order to determine the amount of chrome and cobalt. The samples were analysed by atomic absorption spectroscopy (Williams et al. 1962). In order to describe the digesta movements in the gastrointestinal tract the individual faecal concentration curves were evaluated with three gamma (2, 3, 4) age-dependent one-compartment models (Pond et al. 1988), one gamma age dependent/age-independent two-compartment model (Matis, 1972; Pond et al. 1988) and one multicompartment model (Dhanoa et al. 1985). The coefficients in the various models have the following meaning: k_1 and k_2 represent the constant outflow rate per hour; $1/k_1$ and $1/k_2$ are constants which are associated with the passage rate in the reticulo-rumen and in the caecum-proximal colon; (passage time) is the time delay in hours from the administration of the marker to its first appearance in the faeces. The total retention time (TTR) in the whole gastro-intestinal tract is calculated as follows: $TTR = 1/k_1 + 1/k_2 + \tau$ in the multicompartment model (age independent); $TTR=n/l + \tau$ in one compartment models (age dependent) where n corresponds to the distribution gamma order and l to the passage rate per hour ($k_1=l*0.59535$ for Gamma2, $k_1=l*0.47454$ for Gamma3, $k_1=1*0.40857$ for Gamma 4); $TTR = n/l + 1/k_2 + \tau$ in the two mixed compartment age dependent and independent models.

After the period of diet adaptation, ground maize silage, concentrate and hay, were separately introduced into the rumen by nylon bags (3 g of DM for each sample). The bags were removed from the rumen after 2, 4, 8, 24, 48 and 72 hours (and 120 hours only for hay); the value of degradability at 0 time was obtained by immersing the bags in the rumen fluid for three minutes. In order to determine the feed degradability at different times of incubation, the content of CP and of protein-free dry matter (PFDM) of each bag was calculated. The CP rumen degradability was obtained by $((CP_1-CP_2)/CP_1)*100$, while the PFDM degradability (degradability of structural and non structural carbohydrates) was obtained, at the different times, by $(((DM_1-CP_1)-(DM_2-CP_2))/(DM_1-CP_1))*100$, where DM_1 and CP_1 are the amounts of dry matter and crude protein of the feed before the incubation, while DM_2 and CP_2 after incubation. The data of CP and PFDM rumen degradability at different times of incubation were processed by the procedure SAS/NLIN (SAS, 1993), with the exponential model of Ørskov and McDonald (1979): $dg(t)=a + b * (1 - exp(-c*t))$, where dg(t) is the rumen degradability at time t, a is the rapidly soluble fraction at zero time, b the potentially degradable fraction, c the degradation rate constant of fraction b and t the incubation time. By using the three previously calculated parameters (a, b, c), and the reticulo-rumen passage rate constant of solids (k_1) we obtained the effective rumen degradability of CP and PFDM by the following equation: $a + (b * c/ (c + k_1))$; the single values of k_1 for species and for diets, used in the above equation, were experimentally calculated and are reported in Table 3.

In order to determine the total viable counts of rumen bacteria, samples of the whole rumen content were withdrawn, after the period of diet adaptation, at 08:00 hours before the morning meal, for three consecutive days. They were immediately gassed with CO_2 and treated with a blender-homogenizer to detach bacterial cells from the feed particles. The anaerobic technique used was that of Hungate (1950) modified by Bryant (1972), which provides an anaerobic glove-box (atmosphere: 95 percent CO_2 and 5 percent H_2). The incubation lasted five days at 39°C and the total viable counts were determined according to Harrigan and McCance's procedure (1976).

During the research the weight of the buffaloes did not undergo relevant changes, thus

demonstrating that the amount of daily dry matter administered (50 g/kg of metabolic weight) is a suitable amount for the maintenance level in buffaloes as already fixed for cattle.
From the two by two comparison of the residual deviations of the five models taken into consideration, the multi compartment model proved to be the best for the study of faeces elimination kinetics of the marker (Cr) of solids both in cattle and the buffaloes, while for the study of the faeces elimination kinetics of the marker (Co-EDTA) of fluids the best model proved to be, in both species, the Gamma4.

The reticulo-rumen passage rate in the first compartment (k_1) of the marker of the solid particles (Table 2), evaluated by the multicompartment model, is significantly higher in cattle compared to buffalo (2.99 *vs* 2.46 percent h_{-1}, $P<0.05$), so the slower passage rate of the marker of solid particles causes a longer average retention time ($1/k_1$) in the buffalo rumen than that of cattle (40.65 *vs* 33.44 h, $P<0.05$). Ponappa et al. (1971) confirmed a longer residence time of feeds in the buffalo rumen; Colucci et al. (1990) found an average rumen retention time in cattle equal to 32.25 h, similar to ours. The difference in the average rumen retention time of the marker of solid particles in the two species shows a different rumen digestion of feeds. The passage rate (k_2) of the marker of the solid particles in the caecum-proximal colon (the second compartment), which can be considered the digestive tract following the abomasum, in which the blend of digested feed takes place, shows values significantly higher in buffaloes than in cattle (11.37 *vs* 10.02 percent h_{-1}, $P<0.05$). The mean retention time ($1/k_2$) of this tract is equal to 8.79 *vs* 9.98 h, with a difference of only 1.2 h and even if it is significant, this datum marginally influences the difference between the two species with regard to the total retention time in the whole gastro-intestinal tract. The time between the administration of the marker of solids and its first appearance in the faeces (passage time, τ) resulted to be significantly lower in buffalo than in cattle (6.98 *vs* 19.06 h, $P<0.05$); Colucci et al. (1990) found similar results in cattle. The total retention time in the whole gastrointestinal tract ($1/k_1+1/k_2+\tau$) is significantly longer in cattle than in buffalo (64.55 *vs* 57.73 h, $P<0.05$) and such difference is specifically caused by the passage time (τ). Buffalo have a longer average residence time of the marker of solid particles in the rumen compared to cattle and a shorter residence time in the post-rumen tract, consequently it can be deduced that there is a different residence time of feeds and so a different behaviour of the two species with regard to their digestive physiology.

The reticulo-rumen passage rate (k_1) of fluids marker, evaluated with the Gamma4 model (Table 2) does not show any difference between the two species: 6.98 percent h_{-1} in the buffalo and 6.57 percent h_{-1} in the cattle; with a retention time ($1/k_1$) of 14.33 h for buffalo and of 15.22 h for cattle. As regards this coefficient, Hume and Sakaguchi (1991) did not find any difference between the two species, while Kennedy (1990) found that the fluids passage rate in buffalo is higher than in cattle; in our opinion, this difference could come from the different models used for evaluating this coefficient. There is no significant difference between the species, also with regard to the time between the administration of the fluids marker and its first appearance in the faeces (τ), which is equal to 4.76 h in buffalo and 6.19 h in cattle; however the total retention time ($n/l + \tau$) is significantly different favouring the cattle (31.59 *vs* 28.93 h, $P<0.05$). Another important data is represented by the rumen volume which was calculated by the determination of the marker concentration (Co-EDTA) in samples of rumen fluid withdrawn at different times, using an exponential model to describe the disappearance of the marker. The volume of the buffalo rumen resulted significantly greater than that of cattle (65.80 *vs* 59.10 l, $P<0.05$); the outflow rate of rumen fluid also resulted to be significantly higher in buffalo (4.34 *vs* 3.77 l/h, $P<0.05$).

The values of reticulo-rumen passage rate (k_1) of the solid particles used for the calculation of the effective CP and PFDM degradability are reported in Table 3. The effective CP rumen degradability of the feeds used, is reported in Table 4. Significant differences in favour of buffalo were observed in the degradability of the concentrate (64.8 *vs* 58.8 percent, $P<0.05$); in cattle, a value close to ours was obtained by Murphy and Kennelly (1987). When changing the

diet, we noticed a higher degree of variability in the CP degradability of concentrate in cattle (standard deviation:±6.2 for cattle and ±1.1 for buffalo), showing a higher sensitiveness to the increase of concentrate. The effective CP degradability of hay is always higher in buffalo (62.7 *vs* 57.0 percent, $P<0.01$); values similar to ours were obtained for cattle by Erdman et al. (1987). If we consider the diets, with regard to the CP degradability of hay, buffalo showed a lower variability (standard deviation: ±2.5 for cattle and ±0.8 for buffalo). The CP rumen degradability of maize silage is significantly higher in buffalo than in cattle (68.6 *vs* 58.7 percent, $P<0.01$); with regard to the latter species, values comparable with ours were obtained by Miller (1981) and Susmel et al. (1990). Infascelli et al. (1995) obtained a higher CP rumen degradability in buffalo but in a comparison study with sheep. Table 5 reports the effective PFDM rumen degradability of the three feeds used by the two species. As regards concentrate and maize silage, we notice significant differences ($P<0.01$) in favour of buffalo (70.0 *vs* 64.1 percent and 64.8 *vs* 56.0 percent, respectively); in the case of hay no significant difference can be noted: 49.2 percent for buffalo and 48.2 percent for cattle, however also in this case the highest value is that of the buffalo.

If we globally consider the degradability response, we can deduce a different ability of the two species of degrading both CP and PFDM of feeds; therefore the percentage of carbohydrates and amino acids, available in the small intestine, is higher in cattle, while in buffalo the level of ammonia and energy available for rumen micro-organisms is higher.

The results regarding the total viable bacteria in buffalo and cattle are reported in Table 6; the significances are referred to the transformed data ($\log_{10}$), since they do not have a normal distribution. Buffaloes have a higher and significant number of rumen bacteria (11.88×10^{10} *vs* 1.61×10^{10} cells/g of dry rumen content, $P<0.01$). As the concentrate in the diet increases, we notice an increase in bacterial number in buffalo, with a significant difference between diet 1 and the other three diets (2.03×10^{10} *vs* 8.75×10^{10}, 11.66×10^{10} and 25.10×10^{10} cells/g of dry rumen content, $P<0.01$). These values are similar in buffalo and cattle only for diet 1; as regards the latter species, as the concentrate increases, we do not notice any significant difference in the total number of rumen bacteria. By comparing the four diets between species, the total viable bacteria of buffalo shows higher values compared to cattle; such difference is significant for diet 2 ($P<0.05$) and for diets 3 and 4 ($P<0.01$). The higher microbial synthesis in buffalo comes from a higher ammonia rumen level; in this study, even if the ammonia level was not measured, a higher CP rumen degradability by buffalo was observed: therefore we can affirm that the rumen ammonia level is different in the two species, and it is higher in buffalo, as it was also reported by Bittante et al. (1994), Sangwan et al. (1990) and Kennedy et al. (1992a). Bertoni et al. (1993), in a research in which cattle and buffalo were fed isoproteic diets with different energetic levels, found that the level of urea in blood is quite constant in buffalo; by contrast in cattle this value significantly decreased when the dietary energy increased. This trend is attributable to the decline of ammonia in the cattle rumen when the concentrate increases, presumably resulting from a limited ability to recycle blood urea to the rumen. Also the level of available energy in the rumen is higher in buffalo because of the higher degree of degradability of the protein-free dry matter.

Considering the apparent digestibility of the most significant parameters (Table 7), the digestibility of the organic matter results to be higher in cattle (69.6 *vs* 67.6 percent, $P<0.05$). Di Francia et al. (2000) found an average digestibility in buffalo, with regard to this parameter, equal to 68.5 percent, similar to the data reported by us. The better digestibility of the organic matter by cattle is caused by the longer residence time of ingesta in the post-ruminal tract. The average time of rumen retention of feeds in buffalo is significantly longer compared to cattle, while the total retention time in the whole gastro-intestinal tract is significantly longer in bovine (Table 2). By comparing the four diets between the two species the digestibility values of the organic matter are always higher for cattle, but significantly ($P<0.01$) only for diet 4, while within the species, there is a significant difference ($P<0.05$) between diet 1 and diet 4 either for buffalo (66.1 *vs* 68.8 percent) or for cattle (67.6 *vs* 71.4 percent). Pannu and Kaushal (1985), found that the Hariana cattle digest the organic matter better than the buffalo Murrah

with a diet 50:50 of forage: concentrate ratio. Settineri and Puppo (1998), by using eight different feeds, found that the in vitro digestibility values of the organic matter were significantly higher favouring cattle, for six of the feeds.

The crude protein digestibility data show that the Mediterranean buffalo and the Holstein Friesian cattle have the same utilization of proteins (67.1 and 66.7 percent respectively). By comparing the diets within each species, cattle show a significant difference between diet 1 and the other three (63.2 *vs* 66.0, 68.7, 68.8 percent, $P<0.05$). On the contrary, the CP digestibility in buffalo is almost constant for all diets. The CP digestibility values of diet 1 are significantly higher in buffaloes than in cattle (66.2 *vs* 63.2 percent $P<0.05$) and diet 2 also shows a CP digestibility, which even if not significant, higher in the buffalo species: therefore we can affirm that the best utilization of proteins can be found in buffaloes with diets with a high content of structural carbohydrates. This result is confirmed by Sangwan et al. (1987), who, with a forage:concentrate ratio equal to 77:23, found higher CP digestibility in buffalo (76.5 *vs* 70.3 percent, $P<0.05$). Furthermore, Moran et al. (1979) feeding Swamp buffaloes with sorghum hay ad libitum, found that they had a better CP digestibility compared to Shorthorn cattle.

Cattle show a better aptitude to the NDF digestive utilization (54.8 *vs* 51.1 percent, $P<0.05$), because of the better digestibility of cellulose (62.1 *vs* 50.9 percent, $P<0.01$), while no differences were found with regard to the digestibility of hemicelluloses. Contradictory results can be found in literature: Kennedy et al. (1992b) found that NDF digestibility is higher in cattle, on the contrary Hussain and Cheeke (1996) found a better NDF digestibility in buffalo. By comparing the species for each diet, the NDF digestibility is significantly ($P<0.05$) higher for cattle only for diet 1, while the cellulose digestibility is significantly higher ($P<0.05$) with regard to diets; no differences can be noted with regard to the digestibility of hemicelluloses.

Fig.1 shows the digestibility trend of cellulose and hemicelluloses in the two species in relation to the increase of forage in the diet. The regression equations regarding cellulose and hemicelluloses are significant ($P<0.05$) for both species. The straight lines of cellulose tend to be parallel when the forage percentage is higher than 62.5 percent, so cattle digest cellulose in higher percentage and quite constantly as the forage increases. The values of hemicelluloses digestibility are similar and tend to be convergent when the contribution of forage is over 75 percent.

In buffaloes, the increase in rumen micro-organisms and the decrease in the structural carbohydrate digestibility show that the number of micro-organisms does not reflect the digestibility of NDF and cellulose. In a comparison test between buffalo and cattle, regarding the rumen degradability of the NDF, with a diet consisting of 75 percent of hay and 25 percent of concentrate, Settineri et al. (1994) found that the rumen degradability of only hay is similar in the two species, while that of hay + concentrate is higher in buffalo (69.1 *vs* 66.0 percent, $P<0.05$). Buffaloes, therefore, are more capable of degrading the most digestible structural carbohydrates in the rumen, consequently the higher degree of digestibility of NDF and cellulose in cattle, as already found with regard to the digestibility of the organic matter, is due to a longer residence time of the structural carbohydrates in the post-rumen tract. In spite of the higher ruminal microbial numbers in the buffalo for all diets, the cattle have higher, even if not always significant, digestibility coefficients for most nutrients except crude protein (diets 1 and 2) and hemicelluloses.

In conclusion, for the two animal species considered in this research, the best fit for the marker associated with solid particles was obtained with the multicompartment model while the Gamma4 model was more appropriate for the fluid marker. Buffaloes retain particles in the rumen longer than cattle, although the retention time in the whole digestive tract was shorter because of their lower residence time in the gut. The mean retention time of fluids was shorter in buffalo than for cattle. The buffalo degrades a greater amount of both crude protein and protein-free dry matter than cattle. The total number of rumen bacteria is higher in buffalo and does not reflect the trend in organic matter digestibility which is always higher in cattle as it

is also for NDF and cellulose. The crude protein digestibility is similar in both species, but it is higher in buffalo fed diets with a higher content of structural carbohydrates.

Table 1. Dry matter (percent as fed) and chemical composition (percent DM) of feeds and of four experimental diets.

	DM	CP	CF	EE	NSC	Ash	NDF	ADF	ADL
Feeds									
Concentrate	90.20	14.42	8.02	2.40	49.15	8.70	25.33	10.87	3.14
Alfalfa hay	87.34	16.25	34.41	1.20	19.55	8.98	54.02	40.92	9.94
Maize silage	33.67	9.03	22.45	2.71	32.43	5.13	50.70	28.52	4.15
Diets (Forage:Conc.)									
1] (87.5 : 12.5)	71.00	13.81	27.38	1.81	26.88	7.77	49.73	33.37	7.32
2] (75.0 : 25.0)	73.94	13.89	24.67	1.89	30.42	7.90	45.90	30.15	6.71
3] (62.5 : 37.5)	76.20	13.98	21.89	1.96	33.57	7.98	42.51	26.87	6.11
4] (50.0 : 50.0)	79.37	14.07	18.92	2.06	36.70	8.17	39.00	23.73	5.53

DM=dry matter; CP = crude protein; CF = crude fibre; EE = ether extract; NSC = non-structural carbohydrates; NDF = neutral detergent fibre; ADF = acid detergent fibre; ADL = acid detergent lignin.

Table 2. Effect of animal species on excretion patterns of solid marker (Cr) and fluid marker (Co-EDTA) in faeces.

		Passage rate of solid particles (multicompartment model)		Passage rate of fluids (Gamma4 model)	
		Buffalo	**Cattle**	**Buffalo**	**Cattle**
1° compartment (reticulo-rumen)					
Passage rate	$k1$ (%h^{-1})	2.46^{b}	2.99^{a}	6.98	6.57
Constant outflow rate	$1/k_1$ (h)	40.65^{a}	33.44^{b}	14.33	15.22
2° compartment (caecum-proximal colon)					
Passage rate	(%h^{-1})	11.37^{a}	10.02^{b}	-	-
Constant outflow rate	$1/k_2$ (h)	8.79^{b}	9.98^{a}	-	-
Time delay between marker administration and its first appearance in the faeces	τ(h)	6.98^{b}	19.06^{a}	4.76	6.19
Total time of retention in the gastro-intestinal tract	TTR (h)	57.73^{b}	64.55^{a}	28.93^{b}	31.59^{a}

a, b: $P<0.05$

Table 3. Solid marker (Cr) and fluid marker (Co-EDTA) passage rate constant k1 (% h^{-1}) of the four diets in buffalo and cattle reticulo-rumen.

Diets	Buffalo	Cattle
	Cr marker	
1]	2.80	3.57
2]	2.42	2.82
3]	2.39	2.86
4]	2.24	2.67
Mean	2.46^{b}	2.99^{a}
	Co-EDTA marker	
1]	7.95	7.52
2]	7.44	6.37
3]	6.43	6.40
4]	6.12	6.01
Media	**6.98**	**6.58**

a , b : $P<0.05$

Table 4. Effective crude protein rumen degradability of the three feeds.

	Concentrate			Alfalfa hay			Maize silage	
Diets	**Buffalo**	**Cattle**		**Buffalo**	**Cattle**		**Buffalo**	**Cattle**
1]	63.3	51.8		61.5	54.5		67.8	56.3
2]	64.8	56.0		63.0	55.4		68.4	57.1
3]	64.9	61.7		63.1	58.3		68.5	59.7
4]	66.0	65.8		63.1	59.9		69.8	61.9
Mean	**64.8^{a}**	**58.8^{b}**		**62.7^{A}**	**57.0^{B}**		**68.6^{A}**	**58.7^{B}**

A, B: $P<0.01$ a, b: $P<0.05$

Table 5. Effective protein-free dry matter rumen degradability of the three feeds.

	Concentrate			Alfalfa hay			Maize silage	
Diets	**Buffalo**	**Cattle**		**Buffalo**	**Cattle**		**Buffalo**	**Cattle**
1]	69.1	61.5		48.0	47.1		67.8	53.2
2]	70.1	62.2		48.7	48.4		64.4	56.8
3]	70.3	66.0		49.0	48.6		65.3	57.0
4]	70.4	66.6		51.2	48.8		67.1	57.0
Mean	**70.0^{A}**	**64.1^{B}**		**49.2**	**48.2**		**64.8^{A}**	**56.0^{B}**

A, B: $P<0.01$ a, b: $P<0.05$

Table 6. Total viable bacteria (n x 1010 cells per g dry rumen content) in buffalo and cattle given four diets.

	Buffalo	**Cattle**
Mean	$11.88^{\#}$	$1.61^{\#}$
1]	2.03^{B}	1.72
2]	$^{a}8.75^{A}$	$^{b}2.52$
3]	$^{A}11.66^{A}$	$^{B}1.36$
4]	$^{A}25.10^{A}$	$^{B}0.82$

Means in the same column followed by different superscripts are significantly different (a,b,c: P<0.05; within each species).
Means in the same column preceded by different superscripts are significantly different (a,b: P<0.05; A,B: P<0.01; between species, for all diets and for each diet).

Table 7. Apparent digestibility coefficients (percentage) of organic matter (OM), crude protein (CP), neutral detergent fibre (NDF), cellulose and hemicelluloses in buffalo and cattle given four diets

	OM	CP	NDF	Cellulose	Hemicelluloses
Buffalo	$^{b}67.6$	67.1	$^{b}51.1$	$^{B}50.9$	66.6
Cattle	$^{a}69.6$	66.7	$^{a}54.8$	$^{A}62.1$	65.3
Buffalo					
1]	66.1^{b}	$^{a}66.2$	$^{b}53.1^{a}$	$^{B}54.6^{a}$	68.4
2]	67.0^{ab}	67.0	52.2^{ab}	$^{B}52.5^{a}$	66.5
3]	68.5^{ab}	67.8	50.7^{bc}	$_{B}50.5^{ab}$	65.9
4]	$^{B}68.8^{a}$	67.4	48.3^{c}	$^{B}46.0^{b}$	65.4
Cattle					
1]	67.6^{b}	$^{b}63.2^{b}$	$^{a}57.8^{a}$	$^{A}66.0^{a}$	67.6^{a}
2]	69.1^{ab}	66.0^{a}	55.5^{ab}	$^{A}63.0^{ab}$	66.6^{ab}
3]	70.2^{ab}	68.7^{a}	53.6^{b}	$^{A}60.5^{b}$	63.7^{ab}
4]	$^{A}71.4^{a}$	68.8^{a}	52.4^{b}	$^{A}58.8^{b}$	63.3^{b}

Means in the same row with same superscripts differ significantly (P<0.01)
Means in the same column followed by different superscripts are significantly different (A,B: P < 0.01; within each species).
Means in the same row preceded by different superscripts are significantly different (a, b: P < 0.05; A, B: P < 0.01; between species, for each diet).

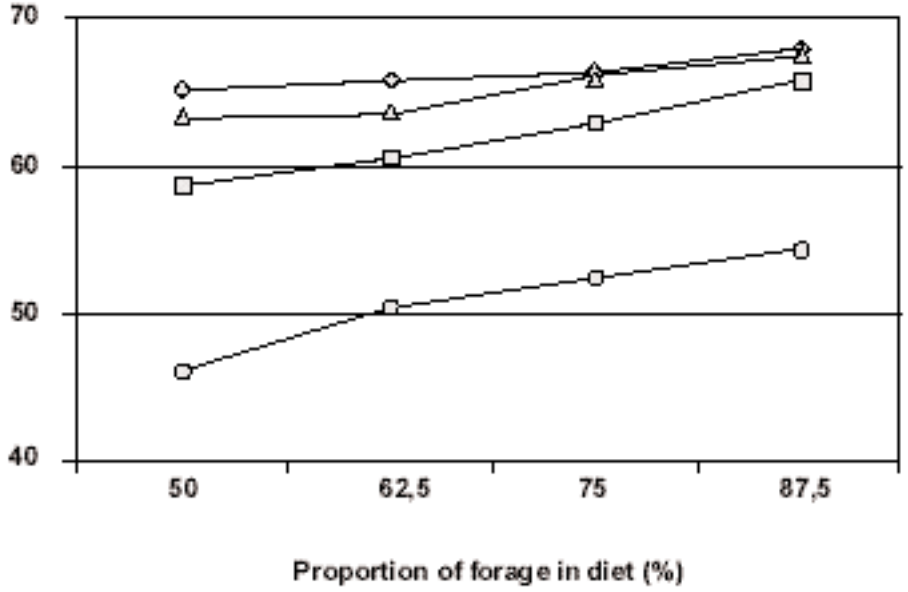

Figure 1. Cellulose (buffalo=□; cattle=□) and hemicellulose (buffalo◆; cattle=△) digestibility in the two species in relation to the increase of forage in the diets.

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Chapter IX

BUFFALO MILK QUALITY

Carmela Tripaldi

Istituto Sperimentale per la Zootecnia
(Animal Production Research Institute)
Via Salaria 31, 00016 Monterotondo (Rome), Italy

1. Introduction

In recent years, the buffalo population in Italy has increased from 200 000 head reared in 2001 (Zicarelli, 2001a) to the 265 000 head of today (ANASB, 2003). The principal motive for this trend, which began in the 1950s/1960s, was the potentiality for converting the buffalo farming system from extensive to intensive utilizing the structures and management systems in place for the dairy cow. Another reason was the high market demand for mozzarella cheese, a typical Italian cheese made from fresh buffalo milk, recognized as DOP and called "Mozzarella di Bufala Campana", when produced in traditional buffalo-rearing areas. Table 1 indicates the positive trend of milk yield and quality over the last years. The improvement in buffalo milk production is principally due to new feeding criteria, changed diets and rearing conditions and selective breeding (Di Palo, 2002).

Almost all buffalo milk is assigned to cheese making, mainly to mozzarella cheese, therefore it is important to produce milk which in turn will yield a good quality cheese in high quantities. A characteristic of buffalo milk is the very high fat content and the fat to protein ratio is about 2 : 1. Another characteristic is the high casein to protein ratio (81-84 percent) (Tripaldi et al., 1997; Tripaldi et al., 2003) compared to bovine milk (78 percent). Moreover the high calcium content of casein micelles results in a faster rennet coagulation, increased curd tension and a faster syneresis (Addeo et al., 1996) and its rennet ability is considered to be very good. Mozzarella from buffalo milk is richer in fat and presents sensorial characteristics very different from the more common bovine Mozzarella (Addeo et al., 1993). The present paper analyses the main factors influencing buffalo milk quality.

2. Feeding and milk quality

Buffalo feeding has been the object of numerous studies which have often been aimed at defining the appropriate requirements of this species. A lack of such defined requirements makes it more difficult to explain the results of research.

2.1. Energy content of the diet.

The results of two investigations on some commercial herds are shown in Table 2 including data relating to the whole lactation where diets with different energetic levels were administered, respectively, 0.78-0.82 vs 0.70-0.72 Milk FU/kg DM, (Bertoni et al., 1991) and 0.82 vs 0.77 and 0.73 Milk FU/kg DM (Bartocci et al., 2002). The dietary energy positively and significantly affected milk yield when 0.82, 0.77 and 0.73 Milk FU/kg DM diets were compared (Bartocci et al., 2002). Milk protein and fat content in both studies (Bertoni et al., 1991; Bartocci et al., 2002) were significantly higher consistent with the higher energetic levels of diets. Milk from buffaloes fed with rations with a higher energetic content also had better renneting ability and a higher estimated yield of Mozzarella cheese ($P < 0.001$) (Bartocci et al., 2002).

The results of the experimental trials carried out on multiparous buffalo cows at early-mid lactation (Verna et al., 1994; Tripaldi et al., 1997) were as follows: no significant differences in milk yield but a positive effect of high energy content (0.78 vs 0.68 and 0.83 vs 0.77 Milk FU/kg DM) on milk protein and fat. However the differences between high and low energy diets were

not significant.

In the same experimental trials when energy was increased there was a corresponding increase in casein content, even if not significant, and an improvement in the milk coagulation parameters, in particular clotting and curd firming time decreased while curd firmness increased; these are the characteristics of milk necessary for the production of good quality cheese (Tripaldi et al., 1997). The estimated yield of "Mozzarella cheese" was also higher for higher energy diets.

It has been largely demonstrated that the protein and casein content of cow's milk is positively affected by the energy level of diets (Remond, 1985). While, other authors when referring to bovine milk, indicate that total N, protein and casein content increased with energy supplies equal to or lower than INRA recommendations (Vertes et al., 1989; Macheboeuf et al., 1993). Whereas if the animals are fed energy supplies equal to or higher than INRA recommendations, the previous nitrogen fractions do not differ (Laurent et al., 1992).

In dairy cows it has been shown that fat content decreases when the energy level in the diet is higher (Journet and Chilliard, 1985). The higher fat content of buffalo milk when the diet has a higher energy level can be explained by a greater mobilization of lipidic reserves (Bertoni et al., 1991; Bertoni and Piccioli Cappelli, 1994). Masoero et al. (1994) found that, when the dietary energy was higher, rumen fermentations were oriented more towards butyric acid content and this acid favours mammary synthesis of fat. Zicarelli (2001b) asserted that if the dietary energy was in excess of the requirements, milk quality would be modified. Based on existing knowledge, the increase of fat in buffalo milk, due to an increase of energy level in the diet, is difficult to interpret. It is evident that the subject requires further investigation.

According to Tripaldi et al. (1997), the energy level of diets affected the composition of milk fat, as observed in the milk of dairy cows (Grummer, 1991). The short chain fatty acids increased when energy availability was higher, otherwise the long chain fatty acids prevailed when food energy was lower. The latter condition favoured fat mobilization and a higher long chain fatty acids content in the blood, than in the milk.

Numerous trials concerning the addition of fat to buffalo diets have been carried out and their effects on milk quality have been analysed. In Table 3 it can be noted that if the fat, calcium salts or crio-crystallized fatty acids, were added to diets in the first two months of lactation, milk yield and ECM milk increased, fat percentage was not significantly different and protein percentage was significantly lower only in one trial. When fat was administered after the first two months of lactation, the results of the two reported trials were contradictory. According to Di Palo et al. (1997) and Zicarelli (2001b) the increase of the dietary energy after the peak lactation phase had a positive effect only on milk fat percentage and not on milk yield. The possible explanation of these authors is the change from a phase characterized by energy deficit, where fat addition increased milk yield, to a phase where dry matter intake is regularized and that an increase in dietary energy only produces an increase of milk fat content. Polidori et al. (1997) observed a higher milk yield and a decrease in protein content in mid-late lactation, while milk fat content was not modified. According to these authors the lack of increase in body condition score and in milk fat content indicates that the administered fat was probably utilized to support the increased oxidation processes related to higher milk production.

The beneficial impact on milk yield of adding fat in the first two months of lactation is evident, the effect in the second part of lactation needs further investigation.

There was a significantly higher content of short and medium chain fatty acids in the milk fat of buffaloes fed on calcium soaps in the first two months of lactation (Cheli et al., 1991), but it has not yet been established what would be the effects on the sensory and texture characteristics of the cheese.

The feeding also affected milk acidity: the higher dietary energy caused an increase in milk protein content and a decrease of pH values (Bertoni and Piccioli Cappelli, 1994; Tripaldi et al., 1997). According to Zicarelli (2001b) in two farms where the dietary energy, but mainly protein content, was increased, milk titratable acidity increased respectively from 6,1 and 6,6 to 8,8, and 8,4 °SH percent, these latter values being normal in buffalo milk.

2.2. Protein content of the diet.

Table 4 records the results of an experimental trial where levels of 12 and 14 percent of dietary protein were compared in buffaloes at the beginning of lactation, yielding an average of 10 kg/day of milk. The only effect was an increase in NPN content corresponding to the higher protein content of the diets (Tripaldi et al., 1997).

Dietary protein of 12 percent compared with 9 percent in buffaloes 132 days in milk and with 10 kg/day of milk yield, increased milk yield, protein content and protein quantity (Campanile et al., 1998). In another trial using the same levels of dietary protein on buffaloes 164 days in milk, yielding 7 kg/day of milk, no effects on milk yield and quality were observed (Campanile et al., 1998).

According to Bertoni et al. (1993), buffaloes seem to be more adaptable than dairy cows to lower dietary protein. However, very low dietary protein, in theory not meeting buffalo requirements, can affect milk yield and quality. In fact in both the above-mentioned trials (Campanile et al., 1998) the higher level of dietary protein caused an increase in blood urea content and the stabilization of the milk freezing point (-0.54 vs -0.52°C), which with lower dietary protein proved to be above the contractual level.

If a dietary protein level of 17 and 19 percent was used on multiparous buffalo cows between 45 and 165 days in milk and yielding an average of 14 kg/day of milk, there were no differences in milk yield and characteristics (Sarubbi et al., 2000). Milk urea content was very high (47.7 and 51.8 mg/100 ml, respectively) when compared with the milk urea content in the above trials using 12 percent dietary protein (35.0 mg/100 ml) (Campanile et al., 1998).

In a survey lasting fifteen months covering nine buffalo herds fed 13.4 percent protein and yielding an average of 8.5 kg/day of milk, the average milk urea content observed was 40.8 mg/100 ml (Di Francia et al. 2003). These results confirm that buffalo milk urea content is higher than that of the dairy cow as already indicated (ASPA, 1999) and can be justified by higher amino acid catabolism and/or more efficient renal urea reabsorption.

3. Somatic cell count and milk quality

Somatic cell count is usually utilized as a sanitary control of milk and specifically as an indicator in the presence of sub-clinical mastitis. Inflammation of mammary epithelium, in addition to reducing milk yield, modifies milk composition, and this in turn affects cheesemaking properties, cheese yield and composition. Some studies have been carried out on cow milk (Politis and Ng-Kwai-Hang, 1988a; 1988b; 1988c; Auldist et al., 1996), but little is known about buffalo milk either with regard to the effects of somatic cell count on milk quality or on the physiological threshold of the somatic cell count (Esposito et al., 1997; Tripaldi et al., 2003).

Italian regulations regarding the hygienic and sanitary characteristics of buffalo raw milk only established a limit for the total bacterial count while no limit was set for the somatic cell count. European standards have established a limit of 400 000 somatic cells/ml for buffalo milk assigned to raw milk products, as is the case for cow's milk. The European directives have established the same limit both for cow and buffalo milk, and therefore it is likely that Italy will soon set a threshold also for buffalo milk.

According to Galiero et al. (1996), who studied 28 buffalo farms, 79 percent of herds produced

milk having less than 400 000 somatic cells/ml. The average value of somatic cell count observed in 37 farms of Italian buffalo from 1997 to 2000 was 191 808/ml (APA Latina, 2000). During a one-year survey on 20 farms, the average value of somatic cell count was 221 280/ml (Tripaldi et al., 2003). The somatic cell number observed in Surti, Murrah and Sri Lankan buffaloes varied from 50 000 to 375 000/ml with an average of 140 000/ml (Silva and Silva, 1994). The average value of somatic cell count revealed in 2 693 Murrah buffaloes' milk samples, obtained monthly from 1997 to 2000, was 63 610/ml. (Cerón Muñoz et al., 2002).

In Table 5 the average values of milk yield, pH, protein and casein content, casein to protein ratio and coagulating properties according to different somatic cell classes are reported. Milk yield decreased when the somatic cell number increased, contrarily, the milk pH increased. The protein and casein content and casein to protein ratio decreased when the somatic cell count increased. The coagulating properties deteriorated when the somatic cell count increased. According to Pasquini et al. (2003) the casein content of buffalo milk increases by about 10 percent when the somatic cell count decreases from 1 500 000 to 13 000/ml.

In another trial (Di Bernardini, 2004) the somatic cell classes were <200 000, 200 000-1 000 000 and >1 000 000. Milk yield started to decrease significantly when the somatic cell count was higher than 200 000/ml (4.51 vs 3.42 kg in morning milking); if the somatic cell count was higher than 1 000 000/ml pH value increased significantly (6.76 vs 6.88), otherwise, lactose content decreased significantly (4.66 vs 4.10). Fat, protein and casein content were affected mainly by the lactation phase. Rennet clotting time and curd firming time were significantly different when the somatic cell count passed from <200 000 to more than 1 000 000, curd firmness appeared not to be affected by udder health.

In Table 6 it can be noted that the milk samples with poor and very poor rennet ability were characterized by a higher somatic cell count with respect to milk samples with good and fairly good rennet ability (respectively 314 330 and 385 850 vs 203 260 and 231 330/ml).

It appears that the higher milk yield and the better chemical and technological characteristics were obtained when the somatic cell count was somewhere between 100 000 and 200 000/ml.

4. Bacterial count and milk quality

With regard to the bacterial count, European limits are the same as for Italy, i.e. 500 000/ml for products from raw milk. It has been reported that often raw milk delivered to cheese farms contains a high number of total germs (Galiero et al., 1996; Amante et al., 2001).
In Italy the price of buffalo milk is not dependent upon its fat and protein content and somatic cell and bacterial count, as is generally applied to cow's milk, but a poor hygienic quality of the milk could be one of the many factors affecting the shelf-life of Mozzarella, which is a fresh cheese. During the preparation of Mozzarella, a thermic treatment of curd provokes a decrease in the bacterial count, but it is not known if the shelf-life of the finished product varies according to the number and the type of germs present in the raw milk.

5. Conclusions

In buffaloes a high energy diet increases fat content as well as protein content. Fat added to diets in the first phase of lactation increases the milk yield. A very low level of dietary protein can cause a decrease in milk yield, protein content, and protein quantity and a destabilization of the milk freezing point. When the protein level of the diet is higher, the only effect on milk quality is an increase in the NPN content of the milk. The average somatic cell count of buffalo milk is not very high; a higher milk yield and better chemical and technological characteristics are obtained when the somatic cell count is approximately 200 000/ml. The high bacterial count is a critical issue with regard to buffalo milk and requires greater vigilance on the farm.

Table 1. Milk average yield(1), fat and protein content of buffaloes controlled in Italy (AIA).

Years	Head controlled	Yield (kg)	Fat (%)	Protein (%)
1977 - 1981	2 220	1 669	7.09	-
1982 - 1986	6 673	1 658	7.88	-
1987 - 1991	9 852	1 818	8.13	4.4
1992 - 1996	13 994	1 935	8.23	4.56
1997 - 2001	20 786	2 096	8.31	4.72

(1) Average yield of lactation length higher than 150 days

Table 2. Effect of different energy content of the diet on the milk yield and quality of the Italian buffalo.

	Monitoring		Monitoring			1st Experimental trial		2nd Experimental trial	
Period of treatment	**Whole lactation**		**Whole lactation**			**2nd to 5th month of lactation**		**2nd to 5th month of lactation**	
Milk FU/kg DM	0.78 0.82	0.70 0.72	0.82	0.77	0.73	0.78	0.68	0.83	0.77
Milk (kg/d)	9.77	9.39	10.46A	8.21B	7.27C	10.97	11.59	10.59	10.58
Fat (%)	8.79A	7.97B	8.83A	8.47B	8.83A	8.60	8.13	9.04	8.97
Protein (%)	4.53A	4.22B	4.77A	4.80A	4.70B	4.41	4.28	5.02	4.85
References	Bertoni et al., 1991		Bartocci et al., 2002			Verna et al., 1994; Tripaldi et al., 1997		Verna et al., 1994; Tripaldi et al., 1997	

A,B = P<0,001; a,b = P<0,01

Table 3. Effect of administration of fat to diet on milk yield and quality of the Italian buffalo (Zicarelli, 2001b, modified).

Type of fatty acids	**Calcium salts of long chain fatty acids**		**Crio-crystallized fatty acids**							
Period of treatment	**First two months of lactation**		**Mid-late lactation**		**First two months lact.(fixed diet)**		**First two months lact. (cross group)**		**50-110 days in lactation**	
References	Di Palo, 1992		Polidori et al., 1997		Di Palo et al., 1997		Di Palo et al., 1997		Di Palo et al., 1997	
Milk FU/kg DM	0.905	0.866	0.935	0.906	0.923	0.851	0.923 0.851		0.944	0.875
Milk (kg/d)	14.02	12.63	7.70A 6.31B		9.83a	7.60b	10.31A	8.39B	8.71	8.50
ECM (kg/d) (1)	23.35a	20.39b	13.40A(2)	10.96B	16.09a	12.07b	15.85a 14.33b		14.14	12.5
Fat (%)	8.14	7.80	9.12	9.04	8.21	7.71	7.76	8.65	8.04a	7.49b
Fat (g/d)	1141a	985b			811a	588b	781	734	704a	621b
Protein (%)	4.72	4.62	4.69b	4.79a	4.41	4.48	4.18A	4.48B	4.36	4.42
Protein (g/d)	662	584			429a	339b	430	376	380	372
Estimated Mozzarella (kg/head/d)	3.60	3.14			2.42	1.85	2.40	2.13	2.11	2.02

A,B = P < 0,001; a,b = P < 0,01

(1) ECM = equivalent correct milk; (2) 4% FCM (kg/d) = fat corrected milk.

Table 4. Effect of different protein content of diet on milk yield and quality of the Italian buffalo (experimental trials).

Days in milk	Milk average yield (kg/d)	Protein content of diet (%)	Effect on milk yield and quality	References
30-150	10	12		(Verna et al., 1994; Tripaldi et al., 1997)
		14	NPN content	
132-214	10	9		(Campanile et al., 1998)
		12	→ milk yield → milk protein Increase of blood urea content and stabilization of milk freezing point	
164-246	7	9		
		12	Increase of blood urea content and stabilization of milk freezing point	
45-165	14	17		(Sarubbi et al., 2000)
		19	No significant differences in milk yield and quality	

Table 5. Daily average production, physical-chemical and technological characteristics of the Italian buffalo milk according to the somatic cell number (Tripaldi et al., 2003).

Somatic cells (n*103/ml)	<50	50÷99	100÷199	200÷299	300÷399	400÷499	500÷999	≥1000	RMSE
Milk yield (kg/d)	9.32ab	9.93a	9.55ab	8.96abc	8.62bc	8.61bc	7.89c	7.99c	3.70
pH	6.73dc	6.71dc	6.70d	6.72dc	6.74bc	6.77ab	6.80a	6.79a	0.11
Protein (%)(1)	4.84a	4.72ab	4.77ab	4.30abc	4.15bc	3.66c	3.64c	3.62c	0.45
Casein (%)(1)	3.90a	3.81ab	3.90a	3.26bc	3.07c	2.74c	2.71c	2.69c	0.35
Casein/ Protein (%) (1)	80.79a	80.31a	82.12a	79.36ab	77.18bc	74.86c	74.75c	74.31c	1.98
Rennet clotting time, r (min)	14.64b	14.27b	14.53b	14.73b	16.68a	16.93a	17.42a	17.03a	4.63
Curd firming time, K_{20} (min)	3.57bcd	3.45cde	3.05e	3.12de	3.48cde	3.95ab	3.66bc	4.20a	1.31
Curd firmness, A_{30} (mm)	42.64dc	45.77bc	50.28a	48.37ab	45.17bc	44.25dc	43.60dc	40.58d	12.12

a, b, c. d, e : $P<0.05$ (1)Data from 5 percent of samples

Table 6. Daily average production, physical-chemical and health characteristics and coagulating properties of the Italian buffalo milk samples regrouped according to their renneting ability (Tripaldi et al., 2003).

	Group 1 Good rennet ability	**Group 2 Fairly good rennet abil.**	**Group 3 Poor rennet ability**	**Group 4 Very bad rennet ability**	**RMSE**
% Samples	71.92	18.64	3.76	5.68	
Daily average yield (kg/d)	9.51A	9.09A	7.86B	6.72C	3.55
pH	6.70C	6.78B	6.86A	6.87A	0.09
Somatic cells ($n*10^3$/ml)	203.26B	231.33B	314.33A	385.85A	296.86
Clotting time (min)	13.61C	18.71B	25.11A		3.59
Curd firming time (min)	2.81C	4.46B	5.92A		0.87
Curd firmness (mm)	52.24A	36.27B	17.44C		8.35

A, B, C: $P<0.01$

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Chapter X

BUFFALO CHEESE AND MILK INDUSTRY

Antonio Borghese

Istituto Sperimentale per la Zootecnia
(Animal Production Research Institute)
Via Salaria 31, 00016 Monterotondo (Rome), Italy

The buffalo products market is increasing in the same countries where buffalo numbers are increasing since both of these factors are linked to consumer demand. In Italy, in particular, the price of buffalo milk is much higher (€1.20/kg) than that for bovine milk (€0.30). Moreover mozzarella cheese consumption is increasing in Italy and in the world: 14 percent of the Italian production is exported to Germany, France, UK, Switzerland, USA and Japan.

This increase in demand is due to several factors: the D.O.P. (Denomination of Protected Origin) "Mozzarella di Bufala Campana" registered in the E.U., the high quality of mozzarella (Fig.1), very soft and tasty, rich in milk and flavours, and the spread of Italian cooking style using mozzarella in pizza, caprese and other dishes.

Figure 1. Mozzarella di Bufala Campana cheese
(Bubalus bubalis photo, 1999)

The market limits are linked with the organization of the cheese industry and with distribution, therefore many farmers or cooperatives manage and process their production in order to achieve additional and alternative profits, to be sure to sell the milk and also for producing different cheeses, not only mozzarella, which is limited by a very short life span, but other products, such as treccia, ricotta, crescenza, robiola, caciocavallo, butter and yoghurt.

One of the main marketing problems is due to the diversity between typical mozzarella, which is produced by small-scale industries, with natural yeasts and microbes and with a shelf-life of only three to five days, not preserved in the refrigerator, and large-scale industry and distribution that must produce long shelf-life mozzarella (30 days) for supermarkets and export, preserved in the refrigerator, but without live yeasts and microbes, and less soft and

juicy. Both these products have the same denomination and D.O.P. but are very different; both are useful for the market but can produce confusion for the consumer.

In the other European and Near East countries, no typical cheese exists which demands a good price on the global market: this is a limiting factor for the economic expansion of the animal network for the diffusion of quality products and for the technical development of buffalo livestock. In Bulgaria, Romania and Albania buffalo milk is almost completely processed into yoghurt which is the most requested product on local markets. Other countries produce butter and creams from the fat and following processing they also drink the skimmed milk: in Egypt a cream, called Queshta Mosakhana is the floating cream removed after boiling milk; Gaymar in Iraq is obtained both from spontaneous floating and from spinning; Quishada in Syria is obtained from raw or boiled milk and is sometimes heated to make it more concentrated. Ghee is obtained by boiling butter and is much appreciated in Egypt and Azerbaijian.

Many farms undertake their own processing of cheese and cream and sell the products directly.

Classifying the types of cheese according to water content (Borghese and Moioli, 2002), the following are typical products:

1. Soft cheese (water content > 45 percent): Karish, Mish and Domiati in Egypt; Madhfor in Iraq; Mozzarella in Italy; Alghab in Syria; Vladeasa in Romania.
2. Semi-hard cheese (water content 40-45 percent): Beyaz peyneri in Turkey.
3. Hard cheese (water content < 40 percent): Braila in Romania; Rahss in Egypt; White brine in Bulgaria; Akkawi in Syria.

Table 1 (Borghese et al., 2000) summarizes the cheeses of the Mediterranean area by dividing them according to their origin and the different stages of their technology and types of classification.

The most common classification of cheese is made according to the type of coagulation: either enzymatic coagulation (due to the rennet), or acid coagulation (after natural acidification or due to lactic bacteria). Many cheeses undergo a mixed coagulation (both acid and enzymatic) though in some of them the acid coagulation prevails, while in others the enzymatic prevails. Most of the cheeses produced in the Mediterranean area belong to the acid-enzymatic category, meaning that the acid coagulation prevails, which can be envisaged since the milk is left to acidify before adding rennet and because the coagulation times are long. Looking at Table 1, it is evident that the acid coagulation prevails in all cheeses. A general consideration should now be mentioned. Since technologies are the result of many factors and must fit with the overall conditions of each environment. Each technology is affected by climate, by the availability of animal or vegetable rennet and by the characteristics of the raw milk, which is also affected by climate and the sanitary conditions of the herds. The Italian buffalo cheese called Mozzarella originated from southern Italy, where temperatures are high and buffalo rearing systems were mainly on pastures; therefore the technology related to this cheese had to be adapted to a kind of milk that, when reaching the dairy plant, was already acidified. The same has probably occurred for most Mediterranean cheeses.

In some cheeses, milk undergoes only a spontaneous acidification (Domiati, Karish, Mish, Madhfor, Alghab). In other cheeses, acidification is encouraged by adding starters, i.e. lactic bacteria cultures (Vladeasa, Beyaz peyneri) or natural whey cultures (Mozzarella). Starters are also used in cheeses where enzymatic coagulation prevails, in order to favour rennet activity and following the processing stages (White brine cheese, Fresh cheese of Iraq, Braila).

Table 1 also includes other important aspects regarding the treatment of milk before processing and characteristics of the technology.

Pasteurization, i.e. heating treatment of milk with the purpose of killing pathogenic germs and reducing microflora, that can damage and result in losses in the final product, is only

performed in a few instances (White brine cheese, Domiati, Braila, Vladeasa, Beyaz peyneri).

Some cheeses are consumed fresh, i.e. only a few days after processing (Karish, Fresh cheese of Iraq, Mozzarella, Ricotta, Alghab), others are ripened and consumed even after several months. In this case, Mediterranean countries adopted the very wise practice of preserving cheese in brine, in order to guarantee excellent conservation without expensive investments, such as refrigerators. In fact, the ripening and preserving of cheese without refrigerators in hot climates, could not only damage cheese, but also be risky for the health of the consumer and might cause considerable losses of products. In one cheese (Mish) it was observed that acid buttermilk, skimmed milk and whey are added to the brine.

In Table 1 a few technology peculiarities are made evident. In Domiati and Akkawi cheese, salt is added to the milk before processing. This practice is very common in Egypt and Syria, deriving from the need to add bacteriostatics to milk, in order to limit spontaneous microflora during processing. In two cheeses (Madhfor, in Iraq, and Mozzarella, in Italy) after the curd has been cut into pieces, it is left to acidify. Acidification is strictly dependent on the temperature, because it is caused by lactic bacteria which grow best at high temperatures. After acidification, in the case of Mozzarella, the curd which has reached a pH of 4.8 4.9, is stretched and then it is moulded. The stretching phase is typical of Mozzarella: no other buffalo cheese is produced in this way. The stretching phase includes several actions: hot water is poured on the curd while the curd is kneaded and stretched. Then whey is removed and further hot water is added several times, while the kneading and stretching continue. This technology phase is the crucial step in the making of Mozzarella and produces the typical stretched texture of the cheese. Stretching can be done either manually (Fig. 2) or mechanically; however, even in the bigger dairy plants, manual stretching is preferred because it improves both the quality and yield of the product. The stretching phase is also important because the hot water together with the curd acidity help to improve the sanitary conditions of the product. The subsequent phase, the moulding in pieces of various weight (from 15 g to 500 g) and shapes (egg-shaped or braided) can also be performed either manually or mechanically (Fig. 3). During the stretching phase, either salt can be added or the moulded pieces can be left in brine at 10-18 percent NaCl for a short time (from a few minutes to a few hours). Mozzarella is then preserved for a few days in acidified brine (2-3 percent NaCl).

Figure 2. Mozzarella manual stretching (Borghese photo, 2003)

Figure 3. Mozzarella mechanical moulding (Borghese photo, 2003)

Interesting by-products from dairy plants are Ricotta (produced only in Italy, where the production regulation was approved in order to obtain the DOP, Fig. 4) and similar products in Syria and Egypt (Alkarish). They are made from the whey after the processing of cheese. It is surprising that these by-products, which exploit the proteins lost in the whey (very rich in sulphurate amino-acids), are not produced in other countries. It is possible to speculate that either the residual whey is too acid to let whey proteins precipitate or that, being too fresh and with low acidity, it is subject to alterations.

In Turkey a drink with water and yoghurt (AYRAN) is widely consumed. Creams also show a variability in production; in Egypt, Queshta Mosakhana is the floating cream removed after boiling milk. Gaymar in Iraq is obtained both from spontaneous floating or from spinning, in this case it is then pasteurized.
In Italy, cream from buffalo milk is obtained after spinning; then it is pasteurized. After thermo-acid coagulation (citric acid) of cream, another dairy product is obtained which is called Mascarpone.
Quishada (a product of Syria) is obtained from raw or boiled milk; sometimes, this cream is heated to make it more concentrated.

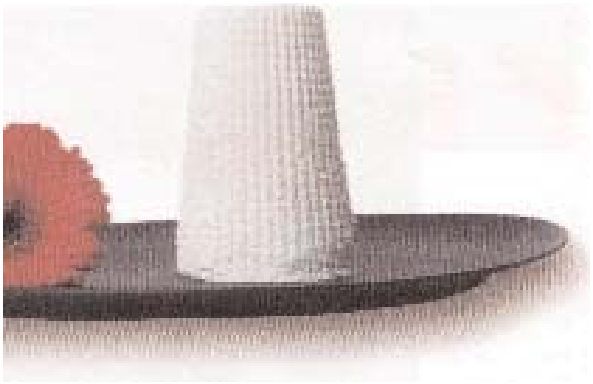

Figure 4. Italian ricotta (Bubalus bubalis, 2004)

Figure 5. Azerbaijan ghee and soft cheese (Borghese photo, 2003)

Industrial butter is produced by churning cream, often after pasteurization. The home-made product is obtained simply by churning acidified milk. The peculiarity of buffalo butter is its colour, which is much whiter than cow butter, due to the lack of carotenoids.
Ghee is obtained by boiling butter. It is very popular in Egypt and valued by the baking industry. In Azerbaijan, Ghee is the only product obtained from buffalo milk fat. Some very simple soft cheeses are also produced in Azerbaijan (Fig.5).

It is evident that the dairy products of the Mediterranean area need to be studied more closely: the variability of their technologies is in fact an important part of global biodiversity.

Among all Mediterranean cheeses, the Italian Mozzarella is the cheese which registers the highest quantity of production and forecasts predict that production is going to increase. At present the actual production is about 38 million kg/year. The success of this cheese is correlated on the one hand to the consumer appreciation of this kind of cheese which includes three types: the whole buffalo milk Mozzarella, the mixed (buffalo-cow), and the whole cow milk Mozzarella and, on the other hand, to the evolution of buffalo farming. In fact, the increasing demand for buffalo milk together with the excessive production of cow milk all over Europe, have encouraged buffalo farmers to increase buffalo milk production through the improvement of management and feeding conditions, as well as through milk recording and the selection of best breeding animals. In fact, in Italy, 27.8 percent of buffaloes (ANASB, 2003) are submitted to official milk recording and genealogy registration. The average milk production of milk recorded buffaloes during standard lactation is over 2 100 kg milk and a good number exceed 5 000 kg. It is only recently that the buffalo sector has started to develop, and relations between buffalo farming, milk processing and scientific expertise are very intense.

Over the past few years, the consumption and market demand for fresh cheeses such as Mozzarella has increased considerably. In order to distinguish and protect the Mozzarella made from buffalo milk, buffalo milk producers have created an association that has drafted official regulations for the production of Buffalo Mozzarella cheese, and has succeeded in obtaining the formal approval of the Italian Government with two laws in 1979 and 1993. Regulations now impose the following requirement in order to utilize the name "Mozzarella di Bufala Campana"

D.O.P. (Denomination of Protected Origin): "Only raw, whole buffalo milk must be used, the origin of the milk must be from areas where buffaloes have been raised for centuries, and the particular processing that has been performed for centuries in these areas must be followed". This regulation was approved in Brussels for all countries in the European Union. Therefore cheeses made from bovine milk or mixed milk cannot be called Mozzarella D.O.P. The yield of buffalo mozzarella is 24 percent in comparison to the 13 percent of the bovine variety and the buffalo mozzarella is richer in fat and proteins.

Buffalo Mozzarella is different from other types of Mozzarella because of its typical texture and juicy consistency, apart from its special taste. Outside the original area of production (Naples and nearby provinces) Buffalo Mozzarella is considered a top quality product, to be consumed on special occasions. Thanks to the official stamp (green and red), printed on the wrapping paper, the buyer can immediately differentiate Buffalo Mozzarella from other similar cheeses in the shop. Furthermore, the existence of approved regulations in addition to the special stamp allows the detection and repression of fraud.

The efforts of buffalo milk processing plants have led to the expansion of demand for this cheese on international markets. Mozzarella is a component of a typical Italian food, the Pizza, which is known and appreciated all over the world and the success of Mozzarella was boosted by the increase of Pizza consumption everywhere.

The development of the buffalo sector in Italy has contributed to the creation of new job opportunities both at the farm and at the dairy processing level. The development of buffalo in an area of Italy, where unemployment was high due to it being less industrialized, has favoured, in particular, the employment of young people who in Italy were not consistently present in agriculture (Borghese, 2003). Research in dairy technology has demonstrated that buffalo milk is suitable for processing into various dairy products which could be exported all over the world. In Latin America over the last few years the number of farms that supply milk to produce "Caso Blanco", mature cheese and Mozzarella, besides producing buffaloes for slaughter, has steadily increased, even if the magnitude of increase is hard to estimate: Amazonia, where the species is continuously reproductive, is the area most suited to Mozzarella production (Zicarelli, 2001). In Asia, where 96 percent of total world buffalo milk is produced, the cheese industry is also developing to satisfy food demands particularly those for human protein requirements. Dairy technology is expanding substantially in India, where, following the "White revolution" milk production is the highest in the world (about 134 million tons), mostly due to buffalo farming: 55 percent of total milk produced in the country and 65 percent of global buffalo milk. The chemical superiority of buffalo milk over that of other species makes it preferable for processing as fluid milk and for use in the manufacture of several Indian and western dairy products. Generally speaking, buffalo milk is more suitable for the manufacture of the following dairy products (Patil and Nayak, 2003).

Concentrated milk

Buffalo milk is as stable to heat as cow milk in its concentrated form; kheer is an indigenous cereal-based concentrated milk product mainly prepared from buffalo milk for immediate consumption.

Fat-rich milk products

Buffalo cream churns much faster at higher fat levels and gives higher overrun than cow cream. Due to the bigger size of globules and higher proportion of solid fat in buffalo milk, the separation of the cream and the churning of the cream is easier and the loss of fat in skimmed milk and buttermilk is far less. Buffalo milk produces butter with a significantly higher yield due to its higher fat content compared to cow milk. Further, in keeping quality tests, butter from buffalo cream displayed more stability than that from cow cream, due to the more solid fat and slower rate of fat hydrolysis in the former cream. This might explain why during storage, cow milk fat is more vulnerable to hydrolytic rancidity. The texture of buffalo ghee is better than cow ghee due to its bigger grain size, which, in turn, may be due to a higher proportion

(9-12 percent) of high melting triglycerides compared to only about 5 percent in cow milk fat (Patil and Nayak, 2003).

Heat-desiccated milk products

Buffalo milk is preferred in India for the manufacture of heat-concentrated milk products like khoa, rabri, kheer and basundi. Evidence has revealed that buffalo milk always results in high yields and a superior quality of condensed milk products compared to cow milk. Khoa (a heat-desiccated indigenous milk product), a product of great commercial importance due to its use as a base for the preparation of a variety of indigenous milk sweets such as burfi, peda, milk cake, gulabjamun, etc. throughout the country. Since buffalo milk gives greater yield and has a more desirable softer body and smooth texture because of the presence of a proportionately higher fat content, the quality of khoa made from buffalo milk is superior to that made from cow milk as the product has a moist surface and a sticky and sandy texture (Reddy, 1985). Ramamurthy (1976) claimed that the higher emulsifying capacity of buffalo milk fat is due to the presence of higher proportions of butyric acid (50 percent) containing triglycerides compared to only 37 percent in cow milk fat, a factor responsible for the smooth and mellow texture of buffalo milk khoa. In addition, the standards for khoa prescribed under the Prevention of Food Adulteration (PFA) rules in India, is heavily slanted towards the use of buffalo milk. Moulick et al. (1996) reported that, in terms of chemical, microbiological and sensory attributes, the overall quality of kalakand was superior from buffalo milk to that from cow milk.

Heat-acid coagulated milk products

The quality of buffalo milk paneer (an acid coagulated milk product) is superior to that of cow milk paneer. The cow milk paneer is too soft, weak and fragile and after cooking its pieces loose their identity (Sachdeva et al., 1985). The low proportion of solid fat, the smaller size of casein micelles and fat globules, and the lower colloidal calcium could be the reason for the inferior quality of paneer from cow milk. Indian paneer is produced through acidifying milk by adding acidified curd or citric acid or lemon juices; following this it is boiled for a few minutes and coagulation is obtained; the curd is filtered and pressed without salt. Paneer (Fig. 6) must be consumed within three days, preferably mixed with spicy sauces made from various vegetables and spices: pepper, chilly, ginger, cumin, garlic, tamarind, Greek hay etc. Indian people normally do not use cutlery to consume paneer and eat it with a special flattened, round and low leavened bread, called "ciabatti". An addition of 0.3 percent sodium citrate to buffalo milk was found to be effective in producing chhana similar to chhana from cow milk in terms of springiness and quality of Rasogolla prepared from the same (Rao, 1986).

Fermented milk products

The superior body and texture of buffalo milk dahi could be attributed to the higher total solids content, especially fat and protein, the casein micelles and the large fat globules and higher calcium content in the colloidal state (Sindhu and Singhal, 1988). Ghosh (1986) reported that misti dahi made from buffalo milk is popular in the Eastern belt of India. Buffalo milk is also appropriate for making yoghurt with improved body and texture, because of its higher total solids content (13-17 percent) as compared to cow milk. In addition, when buffalo milk is used it requires no prior concentration or addition of milk powder to obtain optimum body. It is reported that the growth of yoghurt starter culture is faster in buffalo milk and produces more acetaldehyde, a key flavour component, than in cow milk and thus has a high organoleptic quality in the final product (Singh and Kaul, 1982). Chakka, a base material of Shrikhand, is preferentially prepared from buffalo milk since the curd obtained from cow milk is soft, weak and of low curd tension but the curd from buffalo milk is hard, smooth and mellow. The yield of Shrikhand from buffalo milk is about 15-20 percent higher than that from cow milk. Shrikhand and chakka made from buffalo milk are extremely nutritious and are popular among the Indian population (Patil and Nayak, 2003).

Frozen milk products

In comparison to cow milk, ingredients from buffalo milk viz. skim milk powder and whey solids, produce better body and texture in ice cream (Patel and Mathur, 1982). The higher

protein content in buffalo milk may help to make ice cream more compact and smooth and has a tendency to prevent a weak body and coarse texture. Hence, use of buffalo milk solids in ice cream may improve sensory appeal especially in vanilla ice cream where no colouring is added.

Dehydrated milk products

Buffalo milk and cream are intrinsically whiter and more viscous. Hence, buffalo milk is more appropriate for the production of tea and coffee whitener powders. The whey proteins of buffalo milk are more resistant to heat denaturation compared to the whey proteins of cow milk and thus dried buffalo milk may be preferred to dried cow milk for those technological applications where higher levels of undenatured whey proteins are more desirable.

Figure 6. Indian Paneer with spices (Borghese photo, 2003)

Figure 7. Indian soft cheese (Borghese photo, 2003)

Figure 8. Pastillas de leche and other Philippine milk products (Barile photo, 2004)

Figure 9. Italian mozzarella and ricotta (Borghese photo, 2003)

Cheeses

In India there are many simple buffalo soft cheeses (Fig. 7) obtained from direct acid coagulation, without adding salt, that must be consumed fresh within three days of their preparation. No old tradition of cheese making exists in India, either with regard to technologies or to ripening techniques and hence cheese consumption is also a relatively new habit.

In the Philippines the Nueva Ecija Federation of Dairy Carabao Cooperatives (NEFDCCO) has been established, this is a federation which collects milk, and processes and sells the various products: raw milk, pasteurized fresh milk, aromatized milk, ice creams, pastillas de leche (milk pastilles), kesong puti and paneer cheeses (Fig.8).

Table 1. Dairy products in European and Near East Countries (Borghese et al., 2000)

Type of cheese	Country of origin	Employed milk	Type of production	Pasteurization	Acidification of milk	Type of coagulation	Pressing	Technology peculiarity	Ripening	Water content
White brine cheese	Bulgaria	Buffalo	Industrial	yes	Through starters	Enzymatic-acid	3-4 hours		In brine at 22-23% NaCl, for 35-40 days	Hard cheese
Domiati	Egypt	Buffalo or cow+buffalo	Industrial	yes	Light acidification before coagulation	Acid-enzymatic	yes	Salt is added to milk (6-14%)	In brine for 9 months	Soft cheese
Karish	Egypt	Buffalo + skimmed cow milk or buttermilk after acidification of cream			Natural acidification for 1-3 days	Acid				Soft cheese
Mish cheese	Egypt		Home-made		Natural acidification for 1-3 days	Acid		Preserved in acid brine either with buttermilk, acid skimmed milk, or whey	In acid brine	Soft cheese
Rahss	Egypt	Buffalo or cow+buffalo	Industrial			Acid-enzymatic	yes		2-3 months at 12-18 °C	Hard cheese
Fresh cheese	Iraq	Skimmed buffalo milk			Through starters	Enzymatic	yes			Soft cheese
Madhfoor or Dhafayer	Iraq				Light acidification before coagulation	Acid-enzymatic	yes	Acidification of curd till pH 5.2	In brine at 10% NaCl, for 2 months	Soft cheese

Type of cheese	Country of origin	Employed milk	Type of production	Pasteurization	Acidification of milk	Type of coagulation	Pressing	Technology peculiarity	Ripening	Water content
Mozzarella	Italy	Buffalo	Home-made and industrial		Through addition of whey from the processing of the previous day. Whey undergoes a natural acidification at room temperature.	Acid-enzymatic		Acidification of curd till pH 4.8-4.9 and stretching in hot water	Preserved only a few days in its whey + 2-3% NaCl, lightly acidified	Soft cheese
Ricotta	Italy	Whey from mozzarella processing	Home-made and industrial			Thermo-acid		Protein precipitation of whey at 85-90 °C	4-6 °C for a few days	Over 60% water content
Braila cheese	Romania	Buffalo		yes	Through starters	Enzymatic	yes		In brine at 10-12% NaCl, for 1 month	Hard cheese
Vladeasa, Bucedis, Home made cheese	Romania	Buffalo or cow+buffalo		yes	Through starters	Acid-enzymatic		There are two types: high and low fat content	At 5-10 °C for 3 weeks	Soft cheese

Type of cheese	Country of origin	Employed milk	Type of production	Pasteurization	Acidification of milk	Type of coagulation	Pressing	Technology peculiarity	Ripening	Water content
Alghab/Hama cheese	Syria	Buffalo or cow+buffalo			Natural acidification for 3-4 hours	Acid-enzymatic	yes	Coagulation for 3-4 hours		Soft cheese
Akkawi	Syria	Buffalo or cow+buffalo						Salt is added to milk (10-12%)	In brine	Hard cheese
Al Karish	Syria - Egypt	From whey after Alghab processing				Thermo-acid		Protein precipitation of whey through boiling		By product with higher water content
Beyaz peyneri	Turkey	Buffalo or buffalo+ sheep/goat		yes	Through starters	Acid-enzymatic		Coagulation for 1.5-2.5 hours	In brine at 12-14% NaCl, for 4-6 months	Semi-hard cheese

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