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INVITED REVIEW

Prolonged oestrus as a cause of infertility in dairy cattle – A review

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Abstract: Prolonged oestrus is one of the conditions associated with perturbed follicular dynamics that culminates into substantial economic losses to dairy industries through low success rate of artificial insemination, increased inter-calving period, reducing total milk yield, lowering calf production, shortening reproductive life of the animals, lowering fertilisation rate and abnormal embryonic development. Prolonged oestrus is characterized by the exhibition of extended duration of oestrus in various breeds of cattle. Cows having prolonged oestrus need to be examined at frequent intervals for determining the correct time of artificial insemination. This makes it challenging to inseminate the animals at the correct time, which is a major inconvenience for the field veterinarians and farmers. Since the follicular dynamics in cows is intricate to comprehend, the disorders in which also often go unnoticed and not treated/managed wisely in the field conditions. Therefore, it is of paramount importance to understand the aetiology of prolonged oestrus so that strategies to improve fertility could be designed. Alterations in hypothalamo-hypophyseal-gonadal axis, which is also influenced by some other extrinsic factors like stress, nutrition, age, parity, breed etc., apart from the endocrine alterations could cause the prolonged dominance of follicle. The presence of suprabasal progesterone and luteal insufficiency are considered as the main cause of this condition among cattle. In this review, we attempted to analyse the prior art on the topic and to delineate the possible causes, and to suggest managemental strategies to mitigate the prolonged oestrus condition in dairy cows

Keywords: Cattle, Follicular dynamics, Fertility, Suprabasal progesterone, Prolonged oestrus, Repeat breeding

Introduction

The profitability of dairying depends to a large extent on the efficiency of reproduction. Maximizing reproductive efficiency requires the matching of genotypes to the production environment in order to ensure that the calving intervals are short and the rates of conception to artificial breeding are high. However, when the reproductive efficiency of the dairy cows are analysed, based on the records, it was observed that the conception rates are low (country's average is 35%) and the calving intervals are quite high. One of the important reasons for extended calving interval is repeat breeding. It has been reported that the incidence of repeat breeding in cattle ranged from 5.5 to 33%. It is also reported that one of the most significant reason for repeat breeding in high producing crossbred cows is prolonged oestrus and associated alterations in the endocrine profile.

Prolonged oestrus is a condition in which the duration of oestrus and the interval between the onset of oestrus and ovulation interval both are exceeding the normal range. In some cases, the duration of oestrus has been reported to be greater than 36 hrs, which can vary from 2-7 days (Bage et al. 2002; Bloch et al. 2006). At field conditions, the inseminators/veterinarians are baffled to precisely time the insemination. Prolonged oestrus condition can increase the number of services per conception to more than 4 against the optimum of <2. In addition, it also increases the inter-calving period and decreases the total milk yield, fertilisation rate, embryo development and calf production (Government of Kerala-Economic Review, 2015). Prolonged oestrus has been reported to be observed in 50% of repeat breeder cows (Cummins et al. 2012; Ghuman et al. 2014; Arun et al. 2020). The conception rate in cows showing prolonged oestrus decreased drastically as the duration of oestrus increased (Nebel et al. 2000). The

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conception rate for the animals exhibiting prolonged oestrus was reported as 70%, 80%, 41.66% in two, three- and four-days duration, respectively (Nath 2014). Although several treatment protocols have been evaluated for their effectiveness in treating the condition, their effect is variable and inconsistent (Nakao et al. 1984; Shelar et al. 2002; Shakir 2018). To develop a suitable management and therapeutic strategy for management of prolonged oestrus condition and to achieve high conception rates in cows affected with the condition, it is essential to understand the underlying aetiology. However, systematically organized and analysed reviews are not available on the topic; therefore, in this review we attempted to analyse the prior art on the topic and to delineate the possible causes, and to suggest managemental strategies to mitigate the prolonged oestrus condition in dairy cows.

Incidence and duration of prolonged oestrus

The incidence of prolonged oestrus in cattle of different regions are shown table 1. has been reported as 26.6 and 21.62% among crossbred cattle in Kerala maintained under field and farm conditions, respectively (Jeba-Sujana, 2005). Nearly, 30-40% of repeat breeder crossbred cows displayed prolonged oestrus ie, about 37-60 hrs against the normal duration of 24-36 hrs. Moreover, 70% of these repeat breeder animals had suprabasal plasma progesterone (>1ng/ml) at oestrus (Bage et al. 2002; Singh 2003, Dadarwal et al. 2005 and Singh et al. 2009). The low progesterone (1ng/ml) is vital for conception and favours typical fern pattern in cervical mucus, but when progesterone increases, the typical pattern changes to atypical fern pattern or nil pattern (Kumaresan et al. 2001). Parvathy (2015) reported that 21.78% of prolonged oestrus conditions among the crossbred cows reared under farm conditions. Bedi et al. (2007) observed that out of 1332 oestrus in crossbred cows, 21.9% of crossbred cows exhibited oestrus period for less than 24hrs, whereas 48.9% of cows exhibited oestrus period for 24-36 hrs and 30.05% of cows exhibited oestrus period for more than 36 hrs. Study conducted on crossbred cattle by Das (2017) recorded that the incidence of prolonged oestrus was about 14.24%, and 7.60, 8.04 and 1.06% of cows showed oestrus for 2-, 3- and 4-days duration, respectively. The duration of prolonged oestrus in cattle of different regions are shown **table 2.** The variation in the incidence of prolonged oestrus in crossbred cows may be due to differences in the breeds of cattle and their environment, level of nutrition and stress factor affecting the animals**.**

Suprabasal progesterone concentration and prolonged oestrus

Elevated progesterone concentrations during oestrus extended the secondary signs of oestrus due to persistence of preovulatory follicle (Duchens et al. 1995a). The marginal rise in serum progesterone level during oestrus affected the expression of normal oestrus signs, hormonal synchrony around oestrus and disturbances in ovulation leading to poor fertility in dairy cows. In addition, delay in ovulation due to increased plasma progesterone in oestrus leads to ageing of oocyte and reduced conception rate (Duchens et al. 1995a, Meier and Bruke 2010). An abnormal serum progesterone concentration was observed in repeat breeder cows with prolonged oestrus (0.5-1.0 nmol/L) against the normal basal value of <0.5 nmol/L during oestrus (Albihn et al. 1991; Båge et al. 1997; Gustafsson et al. 1986; Singh et al. 2009). Layek et al. (2013) stated an extended interval from oestrus to ovulation in cows, which had progesterone level greater than 1ng/ml, compared to the cows that had normal progesterone concentration (less than 1ng/ml) during oestrus. Parvathy (2015) observed that the animals exhibited prolonged oestrus had increased progesterone level during oestrus and luteal insufficiency during mid luteal stage.

Table 1 Incidence of prolonged oestrus in cattle of different region

Incidence	Breed	Place	Reference
30-40% repeat breeder cows	Swedish Red & White Breed	Sweden	Bage et al. 2002
46.1% delayed ovulation		Germany	Braun and Sarmento 2004
26.6 and 21.62%	Crossbred cows	India (Kerala)	Jeba-Sujana 2005
30-40% repeat breeder cows	HF x Sahiwal	India (Punjab)	Dadarwal et al. 2005
			Bedi et al. 2007
29.33% delayed ovulation in repeat breeder	Crossbred cows	India (Assam)	Das 2017
20-30%	Crossbred cows	India	Nanda and Singh, 2008
51.47% repeat breeder	Crossbred cows	India (Kerala)	Parvathy 2015
31.82%	Crossbred cows	Croatia	Zobel et al. 2009
19.86%	HF		
5.88%	Simmental		
50% repeat breeder	Crossbred cows	India	Ghuman et al. 2014
59.64%	Crossbred cows	India (Kerala)	Mathew et al. 2014
25.86%	Crossbred cows	India (Kerala)	Shakir 2018
14.24%	Crossbred cows	India (Guwahati)	Das 2017
16.75%	Crossbred cows	India (Assam)	Nath et al. 2019
25.96%	Crossbred cows	India (Kerala)	Arun et al. 2020

One of the foremost reasons for prolonged oestrus exhibition by the cattle is the abnormal prolongation of the life of dominant follicle, which ultimately leads to delayed ovulation. Normally the oestrus to ovulation interval in cows is about 25-35 hours; the onset of oestrus to LH surge occurs at about 9 hr i.e., about 18-26 hrs before ovulation (Saumande and Humblot, 2005). As far as when the chronology of different events is concerned, peak concentrations of estradiol-17â is observed at 6.8 hr, the LH surge occurs at 9.1 hr and the ovulation occurs at 29.4 hr after the onset of oestrus (Stevenson et al. 1998, Saumande and Humbolt, 2005). A follicle that persists beyond its normal time is called as prolonged dominant follicle and is the main reason for the prolonged oestrus condition. Delayed ovulation of the follicle can be assessed using three parameters, which include (i) the interval from oestrus to LH surge (ii) the interval from LH surge to ovulation and (iii) the interval from oestrus to ovulation (Saumande and Humbolt, 2005; Bloch et al. 2006; Meier & Bruke 2010).

In several studies, it has been stated that the interval between the onset of oestrus and ovulation was more variable than the interval between the LH peak and ovulation (Rajamahendran et al. 1989; Saumande and Humbolt, 2005; Niyas et al. 2019). The endocrine milieu in which the preovulatory follicle grows determines its persistency. The problem may lie at the level of hypothalamus to release normal GnRH, which in turn can delay the pituitary LH surge leading to delayed LH surge or untimely LH surge (Pursley et al. 1995; Saumande and Humbolt, 2005). In addition, the increase in the time period between the LH surge and the time of ovulation can be due to problems at the ovarian cellular level and its microenvironment, which may occur due to effect of suprabasal progesterone (Duchens et al. 1995a, Duchens et al. 1996).

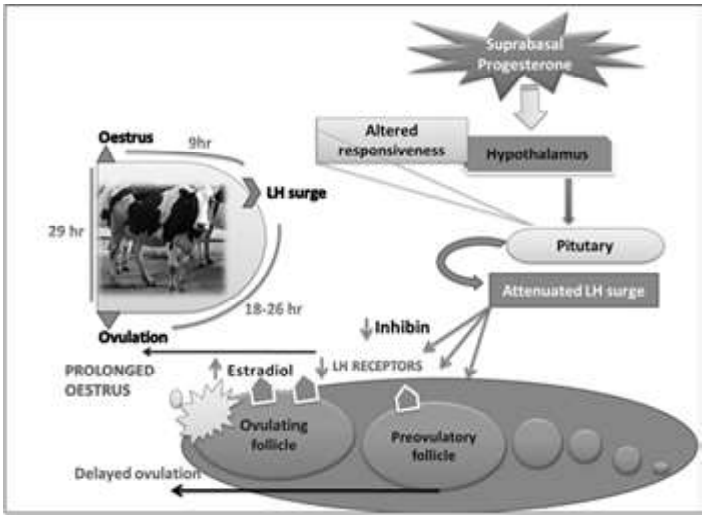

Fig. 1 Hypothetical model on the effect of suprabasal progesterone concentrations on hypothalamo-hypophyseal-ovarian axis and oestrus duration

The most accepted cause for prolonged oestrus in cattle is the presence of suprabasal progesterone of about >0.5ng/ml or the delayed ovulation (Singh et al. 2009). The elevated level of progesterone in turn results in altered LH pulse frequency (Bridges and Fortune, 2003). It is reported that suprabasal progesterone is due to the result of incomplete luteolysis and some extra-gonadal source like adrenal which can elevate the basal progesterone level when the cows are exposed to stress (Honparke et al. 2010). The effect of suprabasal progesterone on the hypothalamo-hypophyseal-ovarian axis, which alters interval between i) oestrus to LH surge, ii) LH surge to ovulation and iii) oestrus to ovulation to prolong the duration of oestrus by

Table 2 Duration of prolonged oestrus in cattle

Duration of prolonged oestrus	Breed	Place	Reference
65.3±15.3 hr	Swedish Red & White breed	Sweden	Bage et al. 2002
42.66±2.74 hr	Crossbred cattle	India (Punjab)	Singh, 2003
90.48±20 hr in repeat breeder cows	HF×Sahiwal	India (Punjab)	Dadarwal et al. 2005
58.0 ± 5.29 hr	Swedish Red and White breed	Sweden	Singh et al. 2005
65.65±2.57 hr	Crossbred cows	India (Kerala)	Jeba-Sujana, 2005
>36 hr	Crossbred cows	India (Punjab)	Bedi et al. 2007
33.27±1.56 hr in delayed ovulation	Crossbred cows	India (Guwahati)	Das et al. 2009
72.00±4.17 hr in delayed ovulation	Crossbred Jersey cows	India (Kashmir)	Bhat and Bhattacharyya 2012
36-80 hr Repeat breeder cows	Crossbred cows	India (Punjab)	Singh et al. 2012
37-80 hr repeat breeder cows	Crossbred cattle	India (Punjab)	Ghuman et al. 2014
>96 hr	HF crossbred cattle	India (Kerala)	Mathew et al. 2016
73.36±3.14 hr repeat breeder cows	Crossbred cattle	India (Tamilnadu)	Senthilkumar et al. 2017
84.0±6.26 hr	Crossbred cows	India (Tirupathi)	Radhika 2017

affecting the ovarian microenvironment is shown in figure 1. Nanda and Singh, (2008) stated that the factors like lactational and nutritional stress were the major reasons for the occurrence of suprabasal progesterone and prolonged oestrus among dairy cattle. This elevated suprabasal progesterone inhibits IGF-1 secretion and reduces responsiveness of follicular cells to LH (Omari et al. 2020). The maintenance of prolonged dominant follicle may cause premature maturation of the oocyte which causes the chromosomes to condense and the meiosis to progress to metaphase II before LH surge. It is also stated that embryos obtained from cows that ovulated from persistent follicles were compromised and could not reach the 16- cell stage (Ahmad et al. 1995).

In a study, it was reported that relative risk of a cow becoming repeat breeder was 58% and 42%, when the AI is performed at suprabasal progesterone level and basal progesterone level, respectively (Bage 2003). Delayed LH surge and extended LH pulse frequency delays ovulation and ultimately results in preovulatory ageing of the oocyte (Singh et al. 2005). The estrogen: progesterone ratio should be greater than 1 for a normal ovulation to occur. The presence of suprabasal progesterone reduces the tubal contractility and impairs transport of sperm from sperm reservoir (Bloch et al. 2006). Recently, a study conducted on the differential abundance of proteins in follicular fluids from preovulatory follicles of less fertile dairy cows, reported the increased abundance of TIMP2, which can disrupt the tissue remodelling necessary for ovulation which leads to delayed ovulation and suggested to have a prolonged heat onset to ovulation interval in low fertile cows (Zachut et al. 2016).

Predisposing factors for prolonged oestrus in cattle

Stress factors

Stress can be of several types which includes environmental, managemental, physical or social stress. These kind of stresses were stated to be the causative agent for sustained stimulation of adrenal glands, which could be a factor for occurrence of suprabasal progesterone during prolonged oestrus (Bage et al. 2000). Generally, heat stress is associated with a lot of physiological changes that cause immediate and delayed negative effects on secretion of gonadotropins, follicular dynamics, ovulation, corpus luteum development, steroidogenesis, oocyte developmental competence, embryonic survival, utero-placental function, lactation and post-natal development. Heat stress particularly reduces follicular dominance by inducing multiple large follicles as well as prolonged dominant follicles (Hansen 2009). Alteration in high tonic FSH availability disturbs normal follicular selection and dominance. Altered LH surge and negative energy balance during heat stress can disrupt the normal oocyte maturation (eg; premature meiosis) and reduces developmental competence (Mihm et al. 1994). Heat stress will depress follicular oestradiol due to reduced theca cell androstenedione production associated with low 17-alpha-hydroxylase expression and reduced aromatase activity in granulosa cells. Thereby, heat stress reduces follicular dominance by inducing multiple large follicles as well as prolonged dominant follicles (Wolfenson et al. 2000, Hansen 2009).

Heat stress causes some direct effects on oocytes like, oxidative damage, apoptotic cell death, irreversible changes on cytoskeleton and meiotic spindle, which will interfere with cell division, reduced mRNA and protein reserves for early embryonic development and altered membrane integrity which effects both the signal transduction and protein transport (Hansen 2009). In addition, heat stress causes major differences in gene transcript levels of DNA binding proteins, transcription factors, Erg-1, progesterone, and prostaglandin receptors ultimately leads to reduced oocyte competence which results in poor fertility rate after insemination (Wakayo et al. 2015). During hot season, in order to maintain body temperature, imbalances in energy metabolism occurs. This would lead to suppressed activity of aromatase enzyme in granulosa cells and retard the functionality of dominant follicles (Wolfenson et al. 2000). Roth et al. (2000) stated that follicular microenvironment was adversely affected by elevated exposure of temperature and will lead to deterioration of functioning dominant follicles. Thus, the ovulatory follicle might require more time and a larger size for attaining ovulation under heat stress and such follicles would be of inferior quality and lead to yield poor quality oocytes and subsequent infertility (Hansen 2009). Satheshkumar et al. (2015) studied natural influence of season on Indian crossbred cows and found that the ovulatory wave emerged significantly earlier (Day 11.5) and the dominant follicle of that wave had a prolonged growth phase (11days) during summer compared to the cold season (days 14.8 and 5.8 respectively). They concluded that increased incidence of two follicular wave cycles which accounted for the persistence and aging of the follicle at the time of ovulation and altered luteal endocrine activity might be the reasons for the diminished fertility in crossbred cattle during hot season. Lactational and nutritional stresses are also considered to be the major reasons for the occurrence of suprabasal progesterone and prolonged oestrus among dairy cattle (Nanda and Singh, 2008).

Age and Parity

Occurrence of prolonged oestrus among repeat breeder cows were also influenced by age and parity of the cows, mainly because of underlying endocrine disturbances, nutritional and production stress as the age and parity increases. In several studies, it was reported that, cows of parity four and above in an age group of 8-12 years were more prone to be repeat breeder exhibiting prolonged oestrus. These may occur due to negative energy balance which alters the insulin responsiveness of the follicle, altered prostaglandin synthesis, CL function, anatomical defects due to increased parity and age (Singh et al. 2012, Asaduzzaman et al. 2016, Arun et al. 2020).

Nutritional causes

Negative energy balance is also a cause for alteration of function, development, and maintenance of follicles. It is strongly correlated with low concentrations of glucose, insulin, IGF-1, and secretion of gonadotropins, which ultimately leads to low FSH and LH peaks and hence results in anovulation. IGF-1 has a key role in follicular development, reduction in its concentration during negative energy balance reduces ovarian responsiveness to LH stimulation. Decreased insulin can cause anovulation by interfering normal LH pulses and FSH pulses. Low IGF-1 and insulin together reduces the responsiveness of follicles towards LH and thereby suppress follicular oestradiol production (Omari et al. 2020).

The dairy cattle reared in small holder production system are deficit in most of minerals (Kumaresan et al. 2010), which can impact reproductive performance (Kumaresan et al. 2009). In addition, the minerals and their deficiency were area specific, especially the correlation between calcium level in soil and in cattle were reported (Kumaresan et al. 2010). Calcium-Calmodulin system participates in the regulation of steroidogenesis at different stages of granulosa cell differentiation, thereby affecting the growth of preovulatory follicles (Kendell et al. 2003). It has effects on gonadotropin regulation, independent of stage of follicular maturation and cellular differentiation. Calcium also has a role in influencing delivery of cholesterol by mitochondria or by stimulating conversion of pregnenolone to progesterone in the adrenal gland and ovaries (Wiederkehr et al. 2011). Calcium dependent mechanism has a role in the luteinising hormone release from the pituitary gland. Calcium also plays a key role in increasing the number and size of ovarian preovulatory follicles as well as ovulation rate (El-Shahat and Maaty 2010). Marginal deficiency of phosphorus could cause disturbances in the pituitary-ovarian axis including ovulation. Inorganic phosphorus is essential for normal phospholipid metabolism, cAMP synthesis, energy transformation at cellular level, and integral part of many coenzymes may be a key to its effect on reproduction.

The effect of dietary supplementation with calcium salts of long chain fatty acids with or without of L-carnithine on ovarian activity was studied by El-Shahat and Maaty (2010). They found that calcium played a key part in improving the number and size of ovarian preovulatory follicles as well as ovulation rate. Significantly ($p<0.01$) lower level of inorganic phosphorus (3.73 ± 0.29mg/dL) in infertile repeat breeder cows than normal cyclic cows (5.06 ± 1.19 mg/dL) was reported by Awasthi and Kharche, (1987). Das et al. (2009) observed that the serum inorganic phosphorus concentration was significantly higher in the animals with normal ovulation (5.45 ± 0.15mg/dl) than the animals with anovulation (4.45 ± 0.14mg/dl). However, Nath et al. (2014; 2019) recorded the mean serum calcium and phosphorus levels in prolonged oestrus cows did not differ among different days of prolonged oestrus.

Deficiency of zinc can cause reduction in GnRH secretion by hypothalamus and decreases the levels of FSH and LH and results in anovulation (Karaca et al. 2007). It is a co-factor for more than 300 metalloenzymes in DNA synthesis and gene transcription. Zinc in proteins can either participate directly in chemical catalysis or maintain protein structure and stability. Zinc finger proteins implicated in gene expression of receptors of the steroid hormones which are involved in reproduction. Das et al. (2009) reported the concentration of Zn (ppm) was significantly ($p<0.01$) lower in the animals with anovulation (0.85 ± 0.03) and delayed ovulation (1.41 ± 0.04) compared to the animals with normal ovulation (1.78 ± 0.02). Ahmed *et al*., 2017 reported that the plasma concentration of zinc (µg/dl) was significantly higher ($p<0.05$) in normal cyclic cows (26.4 ± 17.4) than in repeat breeder (18.8 ± 17.7) crossbred cows.

Strategies for improving fertility in cows exhibiting prolonged oestrus

The regulation of follicular dynamics by inducing the occurrence of three follicular wave cycles might be a potential target for therapeutic intervention of summer infertility syndrome in crossbred cows (Satheshkumar et al. 2012). The conception rate in prolonged oestrus crossbred cows was found to be 70%, 80%, 41.66% in two, three- and four-days duration of oestrus, respectively (Nath et al. 2014). The prolonged oestrus exhibited by the repeat breeder cows amplified the risk of poorly timed AI, which resulted in reduced conception rate (Nebel et al. 2000). A higher pregnancy rate was achieved when the period of dominance was restricted to 1-4 days, whereas dominance of >10 days was associated with no pregnancies and concluded that pregnancy rate decreases with the increase in duration of dominance (Viñoles et al. 2001). The duration of dominance for optimum fertility is less than 8 days. Reducing the period of follicle dominance by optimizing the ovulatory response to the initial GnRH injection of synchronization protocol improved early embryo development (Cerri et al. 2009). Reduction in embryo quality was observed even when concurrent extension of follicle dominance was of only 1.5 – 2 days (Cerri et al. 2009). Period of dominance is more important for early embryo quality in high producing lactating dairy cows than the endocrine steroidal milieu in which the ovulatory follicle develops. High producing cows with extended interval between follicle deviation to oestrus have reduced fertility because embryo quality is compromised when dominance of the ovulatory follicle is increased by as few as 1.5 days (Cerri et al. 2009). Pregnancy rate of the cows that ovulates 24 hr after the oestrus onset can be optimized through repeated insemination every 24 hr till the occurrence of ovulation (Van-Eerdenburg et al. 2002). However, the oocytes from delayed ovulating follicles are abnormal due to the extended growth period and therefore repeated AIs may be unsuccessful (Duchens et al. 1995b).

In crossbred cows the mean duration of oestrus before treatment with 1500 IU hCG was 70.50±4.20 hrs and after the treatment it was 47.25±2.39. The mean duration of oestrus was 48.62±1.84 hr when treated with 3000 IU hCG as against 69±4.39 hrs in the control (Mathew et al. 2016). Senthilkumar et al. (2017) studied 60 repeat breeder cows with the history of prolonged oestrus and noticed that the mean duration of oestrus before hormonal therapy was 75±3.71 hr. However, the duration was reduced to 48.21±3.12 hr after the treatment with 1500 IU hCG or 10µg GnRH analogue at the time of insemination. The slow ovulatory follicle growth and corpus luteum regression could be associated with the persistence of oestrus characters which can be optimised following PGF2á administration during early luteal phase of cows followed by timely insemination and improved conception rate (Ghuman et al. 2014; Shakir 2018).

GnRH or its analogues are indicated for inducing ovulation close to the time of insemination to enhance conception rates as they stimulate acute release of LH and FSH from anterior pituitary (Shaw 1999). Fertility improved in some studies when GnRH was administered between days 11 and 14 in lactating dairy cows (Drew and Peters, 1994) but not in other studies (Jubb et al. 1990; Stevenson et al. 1993, Bartolome et al. 2005). GnRH can be used in the prolonged oestrus cows as they are affected with delayed ovulation and/or suboptimal functioning of corpus luteum (Bedi et al. 2007). The conception rate in repeat breeder crossbred cattle having prolonged oestrus was 50% and 42.80% following intramuscular injection of 2.5 mL of Buserelin acetate after single and double insemination, respectively (Sharma et al. 2006). The double injection of Buserelin (GnRH) is efficient in improving the conception rates in prolonged oestrus repeat breeding crossbred cattle. The conception rate in the cows those were administered Buserelin (20µg) 6 hrs before AI and again on day 12 were significantly higher (52%) than those were given single Buserelin (20µg) 6 hrs before AI (34%) (Dadarwali et al. 2007).

Conclusions

Prolonged oestrus in dairy cattle baffles field veterinarians in deciding the appropriate time of insemination ultimately leading to reduced conception rates and repeat breeding. As a managemental measure the animal should be maintained under normal energy balance and minerals like calcium, phosphorus and zinc level should be maintained at adequate levels in the feed of cows. In cows showing prolonged oestrus, reducing the period of follicle dominance by administration of GnRH/LH will be advantageous. Pregnancy rate of the cows that ovulates 24 hr after the oestrus onset can be improved through repeated insemination every 24 hr till the occurrence of ovulation. Use of different regimens of GnRH shots were reported to have more significant improvement in the prolonged oestrus condition, by its action to induce ovulation and prevent luteal insufficiency. The 1500 IU hCG or 10µg GnRH analogue at the time of insemination can be tried to reduce the duration of oestrus in the cows having prolonged oestrus. However, further studies needed to determine the oocyte quality, probably in terms of genomics, in prolonged follicle and other risk factors associated with prolonged follicular dominance.

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RESEARCH ARTICLE

Study of the keeping quality of cheese dip stored in PET Bottles: Sensory, physico-chemical, textural and microbiological aspects

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Abstract: This study aimed to evaluate the storage stability of novel cheese dip (CD) and cheese dip with spices (SCD) stored in PET bottles at refrigeration temperature (4±1°C). The change in quality attributes in terms of sensory, chemico-physical (pH, acidity, thiobarbituric acid (TBA), acid degree value (ADV) and color (L*, a*, b*) textural and microbiological (total plate count, coliform and yeast and mold) were studied at an interval of 10 days. Also, linear regression models were developed to correlate the changes in attributes with storage time. Keeping quality of both CD and SCD decreased over the period of time. An increase in firmness, SPC, YM, TBA, and ADV was observed for both the samples, whereas pH, adhesiveness, and sensory scores decreased. On day 30, SCD exhibited significantly lower acidity, ADV, TBA, tyrosine, and microbial count than the CD. Also, the sensory scores of SCD were significantly higher compared to CD. Linear regression equations between days and quality attributes showed a determination coefficient (R^2) >0.85. This study clearly shows that keeping the quality of cheese dip depends on the composition and ingredients used for its manufacturing. In conclusion, the spoilage of CD is mainly accompanied by an increase in acidity due to the growth of microorganisms responsible for decreased sensory scores and changes in textural properties of the product. Accordingly, the heating time-temperature combination can be altered to further improve the keeping quality of the product at refrigeration temperature.

Keywords: Cheese dip, PET bottles, Preservation, Quality, Shelf life

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Introduction

Understanding the relationship between food storage and quality attributes is vital for several aspects from manufacturer to consumers. The study of change in sensory, physico-chemical, textural, and microbiological attributes of foods is strategic to predict the shelf life, selection of packaging material, and storage conditions required to maintain the quality of food products. Moreover, an appropriate shelf life endorses that quality food is available for consumers. Directly related and interlinked, food packaging is the concept of shelf life. CD known also called as processed liquid cheese, is one of the key variants of processed cheese (PC) in the market now-a-days. It has gained consumer acceptance because of its multifarious uses, from a pleasing appetizer to providing unique flavor to several dishes and foods (Saad et al. 2016; Salek et al. 2019; Szafrańska and Sołowiej, 2019). However, owing to the unorganized production of CD, there is no knowledge regarding the behavior of CD during storage.

According to Orbis Research (2017), the cheese sauce (CS) and cheese dip market will boom by several million dollars by 2022. Like other variants of processed cheese, the CD is an oil-in-water type emulsion that can be prepared by blending various dairy and non-dairy based ingredients (Bansal et al. 2017; Shalaby et al. 2017; Přikryl et al. 2018; Černíková et al. 2017). However, owing to higher moisture content, CD may have a lower shelf life than other variants of processed cheese. Furthermore, lower salt to moisture ratio (1.4-1.6%) and higher pH might cause faster deterioration of CD during storage. Moreover, due to no strict standards and legal definitions for CD, manufacturers use several dairy and non-dairy-based ingredients for their manufacturing which might affect the storage stability of the product. Researchers have utilized different ingredients (acid casein, whey proteins, skim milk curd, skim milk powder, soy protein concentrate, milk protein concentrate) for the manufacturing of CD or CS and have studied quality aspects during storage (Saad et al. 2015, 2016; Shalaby et al. 2017). Saad et al. (2015) tested the impact of different preservatives on the storage stability of CS.

The authors reported a significant increase in the shelf life of cheese dip with added nisin and potassium sorbate mixture.

No single study has been reported that shows the development and consequently storage stability of the product. Also, the effect of the addition of natural spices on the keeping quality of CD has not been reported in the literature. In a previous study (Bansal et al. 2017), the influence of different ingredients viz., cheddar cheese, milk fat, sodium caseinate, and whey proteins on the sensory, physico-chemical, textural, and rheological properties of CD were studied using rotatable central composite design of response surface methodology. The solution containing 6% cheddar cheese, 8.82% protein blend (20:80 of sodium caseinate to WPC-70), and 9.72% cream yielded maximum desirability.

The current study focused on the effect of storage on the sensory (flavor, BT, CA), hardness, and adhesiveness for optimized CD and CD with 0.3% roasted cumin powder as developed in a previous study by Bansal et al. (2017, 2019). The optimized products were hot filled in PET bottles for its stability evaluation at refrigeration temperature. The change in physico-chemical properties viz., moisture content, pH, acidity, TBA, ADV, and color (L*, a*, b*) were also evaluated. The microbiological profile including SPC, coliforms, and YM counts were observed throughout the storage period.

Materials and Methods

Materials

Cheddar cheese of three months old was procured from the Experimental Dairy Plant, ICAR-National Dairy Research Institute, Karnal. The cream was separated from mixed milk, and the fat content was adjusted to 60 ± 1%. In the current study, whey protein concentrate (WPC-70) and sodium caseinate were procured from Mahaan Proteins Pvt. Ltd., Mathura, Uttar Pradesh, India. Carboxy methylcellulose from Merck Specialties Pvt. Ltd., Mumbai, India, was used. In addition, tri-sodium citrate was obtained from Posy Pharmaceutical Ltd, Ahmedabad, Gujarat, India; Sigma Aldrich, Mumbai, India, supplied glycerol monostearate. Polyethylene terephthalate (PET) bottles of 180 ml capacity (M/s Alpha packaging, Surat) were procured from the local market of Karnal, Haryana, India.

Cheese dip manufacturing and storage

CD and SCD were manufactured as described by Bansal et al. (2017, 2019) with the optimized level of cheddar cheese (6%), protein blend (8.82%), and cream (9.72%) and 0.3% roasted cumin for SCD. A batch size of 10 Kg for both CD ad SCD was prepared in duplicate to carry out the analysis during storage. The products were hot-packed in pre-sterilized PET bottles and stored at refrigeration (4 ± 1° C) temperature till further analysis. The products were evaluated at an interval of 10 days for different quality parameters.

Sensory analysis

For the sensory evaluation, a descriptive score card was used. A 25 point score card with 12 points for flavor, 9 points for BT, and 4 points for CA was applied to compare CD during the storage. The evaluation was carried out by eight panelists well trained with the sensory evaluation of milk and milk products. All the panelists were working as scientists at the Dairy Technology Division of ICAR-NDRI, Karnal, India. The panelists received 20 g of sample in plastic cups at refrigeration temperature accompanied with a disposable spoon. The judging of products was carried out in booths with proper environmental surroundings.

Physico-chemical properties

The cheese dip samples were analyzed for moisture content according to the Association of Analytical Official Chemists (AOAC, 2005). The oxidation of milk fat in terms of Thiobarbituric acid value was measured as the method described by Tarladgis et al. (1960). The degree of lipolysis (Acid degree value) was evaluated as per the procedure delineated by Deeth-Fitzgerald and Wood (1975). The pH of the samples was measured by inserting a glass tip electrode of calibrated pH meter. The temperature of the sample was maintained at 25 ± 1° C before analyzing pH.

The change in color during storage was measured using a Hunter colorimeter (Reston, Virginia, USA). The instrument was calibrated each time before use. Samples were filled densely up to 1 cm in height in the glass sample container. Color values were analyzed by CIELAB software and presented in terms of L*, a*, b*. These values represent black (0) to white (100), red (+) to green (-), and yellow (+) to blue (-), respectively.

Textural properties

Instrumental textural attributes were measured on a Texture Analyser, model-TAXT2i (Stable Micro Systems, Godalming, Surrey, UK). A load cell of 25 kg with a P-25 cylindrical aluminum probe was used for measuring the textural attributes of CD and SCD. Samples were removed from the refrigerator and packed to a constant weight of 35.5 g in a sample container. Each sample was taken from the refrigerator and immediately compressed at 1 mms^{-1} to a strain of 30% with 0.10 N force. Textural properties viz. firmness and adhesiveness were computed from the force-time curve. The in-built software viz., texture expert for windows (version 1.20, Stable Micro System) was used to compute the firmness and adhesiveness. Samples were evaluated in triplicate at a storage temperature of the product (5 ± 1° C).

Microbiological profile

For microbiological analysis of cheese, 11 g of cheese sample was diluted with 99 mL dilution blank for making the first dilution.

All other dilutions were made successively using a 9 mL dilution blank. The dilution blanks were prepared by mixing 2% sodium citrate and 0.9% sodium chloride followed by autoclaving at 121° C for 20 minutes. The SPC, coliform, and YM counts were enumerated by surface plating in triplicate on plate count agar, violet red bile agar, and potato dextrose agar, respectively. The petri plates were kept at 37 °C for the growth of SPC and coliform and at 25° C for yeast & mold count. The growth of colonies was counted after 48 h for SPC and coliform, while yeast and mold counts were enumerated after 3-5 days. The results were presented in a number of colonies per gram of sample. All agar for surface plating was procured from HiMedia (Bombay, India).

Statistical analysis

Data were analyzed using the IBM SPSS Statistics 20 software package. A two-way Analysis of variance (ANOVA) was performed to find significant differences among various quality parameters during storage. Means with p-value < 0.05 were considered statistically different. Coefficient of determination was evaluated to validate the linear regression analysis.

Results and Discussion

Sensory analysis

Table 1 shows the change in flavor, BT, and CA scores of CD and SCD with the progress of the storage period. The flavor score of CD and SCD decreased significantly ($p<0.05$) from 10.83 to 8.12 and 11.37 to 10.37, respectively, on the 30th day of the storage period. During 30 days of study, change in flavor scores were 2.71 and 1.0 units, respectively, for CD and SCD. Moreover, it was observed that flavor scores were significantly different between the samples from day 1. Similarly, BT and CA scores decreased for both the samples throughout the storage study. The BT scores of CD decreased to 7.41 from 8.08, and for SCD, it decreased to 7.50 from 8.25. Thus, the decrease in BT scores for CD and SCD was significant ($p<0.05$) during the storage study. Sensory CA scores also decreased significantly for both the samples with storage time. However, there was a non-significant difference in CA scores among the samples on the same storage day.

The results are concomitant with the previous works reported by Saad et al. (2015) and Desouky et al. (2019) for processed cheese products. The decrease in sensory attributes with the progress of storage might be because of an increase in the bacterial count and consequently an increase in free fatty acids and acidity. Although, the lesser reduction in flavor scores of SCD might be owing to the preservative effect of cumin. Similar results have been testified by Cakir et al. (2016) for Erzincan Tulum cheese. The authors reported better liking of the samples containing cumin seeds in comparison to control cheese. In consistent to above results, previous studies have concluded that an increase in acidity might lead to a deterioration of the product (Kapoor and Metzger, 2008; Hamad and Ismail, 2013). Therefore, the present study results suggested that addition of cumin to CD might improve the sensory profile of the product due to a lesser increase in acidity over 30 days compared to CD without cumin.

Physico-chemical properties

Table 2 depicts the change in moisture content of samples over 30 days at refrigeration temperature. A significant ($p<0.05$) reduction in moisture content for both CD and SCD was observed with the progress of storage. A change of 1.21 units was observed from 1st day of storage to the 30th day of storage for CD. At the same time, the moisture content of SCD decreased by 0.85 units during 30 days of storage study. The non-significant differences in moisture content of both the samples might be because of the addition of cumin and consequently usage of the same amount of less water during product manufacturing as described in previous studies (Bansal et al. 2017; 2019). Further, a decrease in moisture content during storage might be owing to the evaporation of free available water in the sample. The results are in agreement with the previous study reported by Rafiq and Ghosh (2018). In another study, Ney (1988) stated that storage of PC for 30 days at 20° C could cause a weight loss of 2-5g/Kg.

Table 1 Change in sensory scores of CD and SCD over 30 days of storage

Dependent variable		Sample	Storage days		
		1	10	20	30
Flavor	CD	10.83 ± 0.25^{aA}	9.83 ± 0.31^{bB}	8.75 ± 0.23^{cB}	8.12 ± 0.49^{dB}
	SCD	11.37 ± 0.44^{aA}	11 ± 0.31^{abA}	10.83 ± 0.47^{bA}	10.37 ± 0.48^{cA}
BT	CD	8.08 ± 0.14^{aA}	7.75 ± 0.09^{bB}	7.54 ± 0.15^{cB}	7.41 ± 0.17^{cA}
	SCD	8.25 ± 0.11^{aA}	8.12 ± 0.09^{aA}	7.92 ± 0.07^{bA}	7.5 ± 0.09^{cA}
CA	CD	3.87 ± 0.08^{aA}	3.41 ± 0.23^{bA}	3.29 ± 0.13^{bA}	3 ± 0.09^{cA}
	SCD	3.9 ± 0.05^{aA}	3.7 ± 0.1^{bA}	3.5 ± 0.18^{cA}	3.12 ± 0.05^{dA}

Means with different small letter superscripts within a row shows significant difference ($p<0.05$) within the sample during storage study

Means with capital letter superscripts within a column shows significant ($p<0.05$) difference between the samples at same storage day

Table 2

The degradation of lactose to lactic acid and consequently change in pH and acidity of CD and SCD during 30 days of storage are represented in Table 2. With the progress of storage, it was noted that the acidity increased while pH decreased for all the samples. The pH of CD and SCD decreased from 6.58 to 6.03 and 6.34, respectively. The lactic acid content of CD and SCD increased to 0.46% and 0.31%, respectively, on the 30th day of storage from 0.20% on the 1st day of storage. Nevertheless, a significant ($p<0.05$) change in pH and acidity of CD and SCD during the storage period. Furthermore, the samples exhibited significant differences between them after the 10th day of storage. The decrease in pH with the progress of storage might be because of the breakdown of lactose to lactic acid by heat-resistant microorganisms in the product. In previous studies, similar trends have been testified by Shalaby et al. (2017) and Salek et al. (2019) for processed cheese sauces. Moreover, Awad et al. (2002) and El-Mahdi et al. (2014) specified that change in pH could also be because of change insoluble nitrogen and decomposition of emulsifying salts and their interaction with proteins. The lesser increase in acidity of SCD than CD might be due to the antimicrobial properties of cumin. Rabita et al. (2006) stated that the addition of spices to cheeses suppresses the growth of microbes and consequently increase in acidity.

Changes in the chemical indicator of oxidative rancidity (TBA) in CD and SCD during storage are shown in Table 2. TBA value increased in all the cheese samples for 30 days of storage. Before day 10, samples did not show significant differences ($p>0.05$) in TBA value between CD and SCD. However, from day 10-30, all samples were statistically significant ($p<0.05$) in terms of TBA value. On day 30, TBA values of CD and SCD increased from 0.0097 to 0.034 and 0.026, respectively. These outcomes specify that cheese sauce with added cumin had better resistance to oxidative rancidity. Thippeswamy and Naidu (2005) studied the antioxidant potency of cumin and concluded that cumin is an effective inhibitor of free radical-mediated lipid oxidation. Nevertheless, the increase in TBA value with storage might be due to oxygen present in the packed product headspace and the diffusion of oxygen through the PET bottles. In previous studies, researchers have reported increased lipid oxidation during the storage of milk fat (Olmedo et al. 2013; Smet et al. 2008; Pettersen et al. 2005).

Lipolysis is one of the major chemical changes that deteriorates the quality of stored foods and limits shelf life. It can be observed from Table 2 that ADV increased for both the samples as the storage phase progressed. Moreover, the samples were significantly different ($p<0.05$) in ADV from day 10. The increase in FFA might be due to the proliferation of yeast and molds as storage progress. Similar results have been reported for processed cheese during 30 days of storage by Rafiq and Ghosh (2018). Many scientists have tried spices and their essential oils to reduce lipolysis during the storage of food products (Ayar, 2002; Quiroga et al. 2013). In another study, Halamova et al. (2010) testified the antifungal properties of cumin seeds quinones against dairy

Table 2 Effect of storage on physico-chemical attributes of CD and SCD

Dependent variable	Sample	Storage days			
		1	10	20	30
Moisture	CD	76.53 ± 0.11^{aA}	76.32 ± 0.14^{aA}	75.66 ± 0.09^{bA}	75.32 ± 0.18^{cA}
	SCD	75.91 ± 0.08^{aB}	75.73 ± 0.1^{abB}	75.44 ± 0.07^{bB}	75.06 ± 0.09^{cA}
pH	CD	6.58 ± 0.002^{aA}	6.31 ± 0.01^{bB}	6.17 ± 0.07^{cB}	6.03 ± 0.003^{dB}
	SCD	6.58 ± 0.002^{aA}	6.55 ± 0.02^{abA}	6.47 ± 0.01^{bA}	6.34 ± 0.04^{cA}
Acidity	CD	0.2 ± 0.002^{aA}	0.29 ± 0.003^{bA}	0.37 ± 0.011^{cA}	0.46 ± 0.004^{dA}
	SCD	0.2 ± 0.002^{aA}	0.22 ± 0.004^{bB}	0.25 ± 0.003^{cB}	0.31 ± 0.01^{dB}
TBA	CD	0.0097 ± 0.001^{aA}	0.015 ± 0.002^{bA}	0.024 ± 0.002^{cA}	0.034 ± 0.002^{dA}
	SCD	0.0097 ± 0.001^{aA}	0.013 ± 0.001^{aA}	0.019 ± 0.001^{bB}	0.026 ± 0.002^{cB}
ADV	CD	0.318 ± 0.078^{aA}	0.664 ± 0.09^{abA}	0.89 ± 0.22^{bA}	1.7 ± 0.295^{cA}
	SCD	0.318 ± 0.078^{aA}	0.503 ± 0.09^{abB}	0.63 ± 0.08^{abB}	0.85 ± 0.17^{bB}
*L**	CD	86.44 ± 0.04^{bA}	85.12 ± 0.018^{dA}	85.31 ± 0.02^{cA}	86.57 ± 0.017^{aA}
	SCD	75.89 ± 0.04^{bB}	74.98 ± 0.04^{dB}	75.33 ± 0.025^{cB}	76.91 ± 0.035^{aB}
*a**	CD	0.65 ± 0.01^{bB}	0.49 ± 0.03^{cB}	0.52 ± 0.035^{cB}	0.75 ± 0.014^{aB}
	SCD	0.98 ± 0.02^{bcA}	0.94 ± 0.015^{cA}	1.06 ± 0.05^{bA}	1.31 ± 0.016^{aA}
*b**	CD	12.62 ± 0.005^{bA}	11.96 ± 0.057^{dA}	12.37 ± 0.108^{cA}	13.11 ± 0.047^{aA}
	SCD	10.39 ± 0.006^{bB}	10.11 ± 0.03^{dB}	10.23 ± 0.026^{cB}	11.07 ± 0.03^{aB}

Means with different small letter superscripts within a row shows significant difference ($p<0.05$) within the sample during storage study

Means with capital letter superscripts within a column shows significant ($p<0.05$) difference between the samples at same storage day

spoilage yeasts. The authors concluded that it could be used as a natural preservative in the dairy industry.

Color is one of the most critical parameters that define the quality of foods, as it is the first impression to fit in the consumer's mind. The color values of CD and SCD differed significantly ($p<0.05$) throughout the storage period (Table 2). Between 1 and 10 days of storage, L*, a*, and b* values decreased, and after that, an increase in the values was observed. The whiteness values (L*) of SCD were lower compared to CD. This might be owing to the added roasted cumin, which gives a blackish tinge to the product. Moreover, the increase in a* and b* value with the progress of storage might be owing to the Maillard reaction and /or degradation of proteins throughout storage (Buňka et al. 2008; Banach et al. 2014). The increase in yellowness may also be because of a decrease in moisture content. The results are concomitant with Abd-Rabou et al. (2005) and Azzam (2007), where authors reported an increase in yellowness and a decrease in moisture content throughout the storage study. Also, with the progress of the storage phase, authors have reported lowering the whiteness of cheese (Abd-Rabou et al. 2005; Azzam, 2007). In contrast, Esmer et al. (2009) reported no particular pattern for L*, a*, and b* values in Crottin de Chavignol Cheese.

Textural attributes

The progress of storage was accompanied by a significant ($p<0.05$) increase in firmness and a decrease in adhesiveness through 30 days of storage (Table 3). Moreover, the samples were significantly different at each storage day for firmness and adhesiveness. The firmness of CD and SCD increased from 0.284 to 0.371 and 0.292 to 0.385, respectively, at the end of the storage period. Consequently, the adhesiveness of samples decreased from 0.192 to 0.132 and 0.17 to 0.11 for CD and SCD, respectively. The change in textural attributes of cheese is affected by several factors, including pH, moisture content, proteolysis, lipolysis (Fox et al. 2020; Kapoor and Metzger, 2008; Tamime, 2011). In general, it has been testified that the progress of storage is accompanied by a decrease in firmness (Delgado et al. 2011; Khosrowshahi et al. 2006) owing to the breakdown of protein into smaller peptides. However, in this study, an increase in firmness with storage time might presumably be because of dominating factor of pH in comparison to protein breakdown. Several authors have reported the increase in firmness and decrease in adhesiveness of cheese up to a certain period due to lowering of moisture content and decrease in pH with storage days (Tamime, 2011; Gutiérrez-Méndez et al. 2013; Barukčić et al. 2020). The increase in firmness with a reduction in pH might be owing to increased interactions between milk proteins at lower pH. Also, according to Awad et al. (2002), firmness increases with storage time, presumably because of the decomposition of emulsifying salts, which reduces the pH.

Microbiological profile

On day 1, the SPC count of CD and SCD was 9.77×10^2 cfu/g. The number of microorganisms detected increased to 2.88×10^4cfu/g for CD and 2.33×10^4cfu/g for SCD at the end of 30 days storage period. Further, the samples were significantly ($p<0.05$) different at each storage day for SPC (Table 3). The VRBA plate count method used to detect coliforms did not show any coliform throughout the storage period. Also, on day 1, YM was not spotted for both CD and SCD. However, with the progress of storage, the number of YM increased to 2.8 cfu/g for CD and 2.6 cfu/g for SCD on the 30th day of storage. There were significant ($p<0.05$) differences in the YM count among the samples at each storage day. The results indicate that the change in quality parameters of CD and SCD could be because of the growth of microorganisms over a period of time. Also, the non-presence of coliforms indicates the good hygienic practices followed in the course of product manufacture. Previous studies have also reported the increase in SPC of cheeses over the period of time (Papaioannou et al. 2007; Dermiki et al. 2008; Esmer et al. 2009a; Smigic et al. 2018). However, the statistical differences between SPC of CD and SCD testified that the addition of cumin to CD

Table 3 Textural and microbiological profile of CD and SCD during 30 days of storage

Dependent variable	Sample	Storage days			
		1	10	20	30
Firmness	CD	0.284 ± 0.005^{aA}	0.298 ± 0.007^{bA}	0.345 ± 0.008^{cA}	0.371 ± 0.002^{dA}
	SCD	0.292 ± 0.007^{aB}	0.318 ± 0.006^{bB}	0.365 ± 0.002^{cB}	0.385 ± 0.006^{dB}
Adhesiveness	CD	0.192 ± 0.004^{aA}	0.167 ± 0.002^{bA}	0.155 ± 0.003^{cA}	0.132 ± 0.005^{dA}
	SCD	0.17 ± 0.004^{aB}	0.13 ± 0.006^{bB}	0.12 ± 0.003^{cB}	0.11 ± 0.003^{dB}
SPC	CD	2.99 ± 0.003^{aA}	3.89 ± 0.008^{bA}	4.22 ± 0.01^{cA}	4.46 ± 0.002^{dA}
	SCD	2.99 ± 0.003^{aA}	3.81 ± 0.004^{bB}	4.07 ± 0.002^{cB}	4.37 ± 0.002^{dB}
YM	CD	————	0.15 ± 0.045^{a}	0.41 ± 0.015^{bA}	0.45 ± 0.04^{cA}
	SCD	————	————	0.30 ± 0.04^{aB}	0.41 ± 0.015^{bB}

Means with different small letter superscripts within a row shows significant difference ($p<0.05$) within the sample during storage study

Means with capital letter superscripts within a column shows significant ($p<0.05$) difference between the samples at same storage day

Table 4 Regression coefficients and R^2 values for the quality attributes of CD and SCD during storage

Indicator	Dependent variable	Sample	Regression[a]			
			β_0	β_1 [b]	Statistical difference	R^2
Sensory	Flavor	CD	10.83	-0.09475	A	0.98
		SCD	11.39	-0.03266	B	0.97
	B & T	CD	8.0421	-0.0228	A	0.95
		SCD	8.3345	-0.0254	A	0.94
	C & A	CD	3.8192	-0.0228	A	0.94
		SCD	3.9552	-0.0262	A	0.98
Physico-chemical	Moisture	CD	76.634	-0.0443	A	0.97
		SCD	75.983	-0.0294	A	0.98
	pH	CD	6.552	-0.01836	A	0.96
		SCD	6.612	-0.008295	B	0.94
	Acidity	CD	0.1949	0.008858	A	0.99
		SCD	0.1881	0.003728	B	0.95
	TBA	CD	0.0078	0.0008	A	0.99
		SCD	0.0083	0.0006	B	0.98
	ADV	CD	0.2035	0.0452	A	0.93
		SCD	0.3045	0.0178	B	0.99
Textural	Firmness	CD	0.2759	0.0032	A	0.97
		SCD	0.2888	0.0034	A	0.98
	Adhesiveness	CD	0.1916	-0.002	A	0.98
		SCD	0.1621	-0.0019	A	0.85

[a] Regression equations: $Y = \beta_o + \beta_1 X$; where, Y = Dependent variable; β_o = a constant that is equal to value of Ywhen the value of X = 0; β_1 = coefficient of X; X = independent variable (Storage time); R^2 = coeffieicnet of determination

[b] The slopes of each varaible and sample followed with the different letters in the column are significantly different at $\alpha = 0.05$

suppressed the growth of microflora and thus might aid in the storage stability of CD. Nayra et al. (2002) reported that the YM count in soft cheese was detected after 15 days of storage period. Further, Pereira-Dias et al. (2000) stated that spoilage of processed cheese occurs when the YM is more than 6-7 log cfu/g. This indicates that the change in quality parameters of CD and SCD might not be attributed to the growth of YM within the stipulated storage study.

Regression analysis

The linear regression equations between dependent variables (sensory, physico-chemical, and textural attributes) and independent variable (storage time) for CD and SCD are shown in Table 4. In general, the coefficient of determination for linear regression models was more than 0.85. This indicates that linear regression models were well adjusted and can forecast the change in quality attributes during storage. In CD, higher slopes (β1) of the linear regression equations were detected in the dependent variables (pH, acidity, TBA, ADV) associated with product deterioration to slopes of SCD regression equations. Also, the regression slope of CD flavor showed a higher negative value than SCD, indicating faster spoilage of CD than SCD. Thus, regression analysis demonstrates the preserving effect of cumin addition in the cheese dip. Also, Olmedo et al. (2013) and Asensio et al. (2011) testified the preserving effect of different spices viz., oregano, and olive oil in cheese. They testified statistically significant differences among the regression coefficient of the equations. On day 1, the SPC count of CD and SCD was 9.77×10^2 cfu/g. The number of microorganisms detected increased to 2.88×10^4cfu/g for CD and 2.33×10^4cfu/g for SCD at the end of 30 days storage period. Further, the samples were significantly ($p<0.05$) different at each storage day for SPC (Table 3). The VRBA plate count method used to detect coliforms did not show any coliform throughout the storage period. Also, on day 1, YM was not spotted for both CD and SCD. However, with the progress of storage, the number of YM increased to 2.8 cfu/g for CD and 2.6 cfu/g for SCD on the 30th day of storage. There were significant ($p<0.05$) differences in the YM count among the samples at each storage day. The results indicate that the change in quality parameters of CD and SCD could be because of the growth of microorganisms over a period of time. Also, the non-presence of coliforms indicates the good hygienic practices followed in the course of product manufacture. Previous studies have also reported the increase in SPC of cheeses over the period of time (Papaioannou et al. 2007; Dermiki et al. 2008; Esmer et al. 2009a; Smigic et al. 2018). However, the statistical differences between SPC of CD and SCD testified that the addition of cumin to CD suppressed the growth of microflora and thus might aid in the storage stability of CD. Nayra et al. (2002) reported that the YM count in soft cheese was detected after 15 days of storage period. Further, Pereira-Dias et al. (2000)

stated that spoilage of processed cheese occurs when the YM is more than 6-7 log cfu/g. This indicates that the change in quality parameters of CD and SCD might not be attributed to the growth of YM within the stipulated storage study.

Conclusions

The CD and SCD stored in PET bottles for 30 days showed stability in terms of sensory, chemico-physical, textural, and microbiological parameters. Although storage caused an increase in deterioration parameters for both the samples, these changes did not affect the acceptability of the products. Nevertheless, SCD showed greater stability in terms of textural, chemico-physical, and microbiological parameters, making it more acceptable compared to CD. The change in quality parameters with storage was verified with linear regression models as R^2 values for most of the dependent variables were higher than 0.95.

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RESEARCH ARTICLE

Effect of optimized formulation (combination of bronopol and kathon) on compositional and physico-chemical parameters of milk samples

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Abstract: The experiment was conducted to study the effect of optimized preservative formulation (combination of bronopol and kathon) on compositional and physico-chemical parameters of milk. The optimized formulation was added at the rate of 0.6% in milk samples and effect was observed on estimation of fat, protein, lactose and ash content during storage at 37°C. For comparison, the legally permitted preservative (by Food Safety and Standard Authority of India, 2011) formalin was also added at the rate of 0.4% in milk samples. There was no significant effect of optimized formulation on estimation of fat and lactose content for 45 days and total solid (TS), ash and protein content for 90 days. However, on addition of formalin, the fat and lactose values of milk decreased after 15 days of storage and no significant effect was observed on TS, ash, protein content for 90 days and lactose content for 60 days.

Keywords: Bronopol, Kathon, Formalin, Milk samples

Introduction

Authenticity of dairy products is a major concern for legal authorities as some unscrupulous traders are indulges into malpractice of adulterating milk to combat between great demand and scarce availability of milk. To control such malpractices in India, Food Safety and Standard Act, 2006 has been enacted and the relevant rules have also been framed in 2011, named as Food Safety and Standard Rules (FSSR, 2011). FSSR, 2011 provides minimum standards for all food and dairy products. To maintain the standards prescribed under the law, the food safety officers have been assigned the job of collecting samples from Food Business Operators and to send them to Food Analyst for analysis. The Food Analyst is usually deluged with a vast number of samples; therefore, delay in the analysis of the sample is bound to occur. As milk and milk products are perishable in nature, they get easily spoiled and since spoiled samples are considered unfit for analysis, the samples collected are, therefore, required to be preserved. Generally, the preservation methods involve physical and chemical techniques. The physical method includes preserving the product under varying the conditions of storage, and the chemical method includes preserving the product by an addition of some external reagents.

Physical method (e.g. refrigeration, chilling and heating) requires considerable economic investment and maintenance facilities and moreover, it is applicable only to preserve the food and dairy products for a short duration. Thus, the physical methods are not applicable for the preservation of food and dairy products for longer period of time. In India, FSSR (2011) only approved 0.4% formalin (37-41% formaldehyde) to be added in milk and milk products samples stored for chemical analysis. Formalin is antimicrobial in nature and used for preservation of organ specimens. It acts through alkylation of amino, carboxyl, or hydroxyl group, and probably damages nucleic acids. It inactivates all microorganisms, including spores. According to Haselkorn and Doty (1961) formalin interacts with the amino groups of adenine, cytosine, and guanine in the nucleic acid component, denaturing them and resulting in the inhibition of the growth of microorganisms. Preservation action of formalin is very effective but, in the literature, conflicting and contradictory reports are available regarding effect of formalin on the estimation of major milk constituents, particularly milk fat which mainly acts as a base for the commerce of milk and milk products. Singh and Shrivastava (2016) reported that the fat content of milk sample reduced from 6.0% to 5.45% after storage of 1 year with 0.4% formalin at room temperature. It was also concluded that when formalin concentration increased from 0.1% to 0.4%, fat content continuously decreased from 5.45% to 3.05% after 4-6 hr of

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storage at room temperature. However, few researchers (Sandhu et al. 1984; Jandal and Rai, 1988, 1989; Karmakar and Ghatak, 1995, 1997) reported no significant change in Gerber fat values of milk samples preserved with 0.4% formalin. Whereas others (Mulder, 1943; Pien et al. 1976; Krishnamurthy, 1974; Sivakova et al. 1976; Hussain et al. 1984; Des Raj and Singhal, 1988; Bajaj and Rai, 1992; Sharma and Sarwar, 2000) found low fat values in the formalin treated milk samples. The cross-linking between the formaldehyde and milk protein leads to the formation of a cross-linked matrix which in turn hinders the detection of milk fat values by Gerber and Rose-Gottlieb method, which are known to be used for chemical analysis of the milk. Hence, there is a need to develop suitable alternative or substitute of formalin for preserving the dairy products.

Different other preservatives such as mercuric chloride, potassium dichromate, hydrogen peroxide, bronopol™ and azidiol for preservation of dairy products. However, until now no such preservative is disclosed which can provide preservation along with maintaining the quality of the sample when used alone at ambient temperature. Among above mentioned preservatives 2-bromo-2-nitropropane-1,3-diol is most widely used (0.02-0.1%) as a milk sample preservative and does not interfere with the normal testing procedures. Bronopol™ is extensively used in Europe and the U.S.A. Kathon™ is a preservative used in cosmetics and hygienic products (Hofmann et al. 2018). Bumbadiya et al. (2017) reported that Bronopol and kathon have broad spectrum antimicrobial activity and have a potential to be used as preservative in milk and milk products.Since efficiency of analytical methods of fat estimation in formalin preserved milk is questionable, there is need to find out suitable alternative to formalin. As no single preservative can satisfy the entire necessary requirement, thus combination of preservative is a novel approach for efficient preservative action. So in the present investigation, combination of Bronopol and Kathon preservative was prepared and their effects on compositional & physico-chemical parameters of raw cow milk were analyzed.

Materials and Methods

Chemicals and reagents

Ammonia Solution (30%) and Iso Amyl Alcohol were purchased from S D Fine-Chem. Ltd., Mumbai, India. Boric Acid, Folin's Reagent, Hydrogen Peroxide, Petroleum ether, Methanol, Ethyl alcohol (95%), Diethyl ether, Isopropanol, Sulphuric Acid and Hydro chloric acid were purchased from Merck Darmstadt, Germany. Copper Sulphate Penta Hydrate, Mercuric Chloride, Phenolphthalein Indicator, Potassium Dichromate, Potassium Hydroxide, Sodium Carbonate and Sodium Hydroxide were purchased from SRL Chemicals Mumbai, India. Methyl Red and Methylene Blue were purchased from Qualigens Fine Chemicals Mumbai, India. Bronopol and Kathon preservatives were purchased from Sigma-Aldrich Inc., St. Louis, USA.

A formulation (combination of bronopol and kathon) is optimized and a patent (Patent application no. 201911032383 dated 09.08.2019) has been filed for the same.

Milk sample collection and addition of preservatives in milk

Milk samples (250 ml) were added with optimized formulation (combination of bronopol and kathon) at the rate of 0.6% and stored at 37°C for 3 months. For comparison, the milk samples added with 0.4% formalin (permitted by FSSAI) were also stored at 37°C for 3 months. The samples were analyzed after every 15 days intervals for compositional and physico-chemical parameters. Pooled raw cow milk samples were collected from livestock research centre of the institute (National Dairy Research Institute, Haryana, India) in cleaned autoclaved glass bottles.

Compositional analysis of milk

Estimation of fat in milk was done by Gerber method as given in BIS (IS: 1224-1, 1977) and Rose Gottlieb method as described in BIS (IS 1479-2, 1961). Protein content of milk sample was evaluated by (AOAC 991.23). For Lactose content, the procedure given in BIS (IS: 1479-2, 1961) was used. Estimation of TS was done as per the procedure given in Bureau of Indian Standards (IS: 12333, 1997). For the determination of ash of milk samples, the procedure given in BIS (IS: 1479, 1961, Reaffirmed 2003) was used.

Titratable acidity of milk

Titratable acidity of milk samples was analyzed as per the procedure described in BIS (IS: 1479-1 1960).

Estimation of free fatty acid (FFA) content in milk

Estimation of FFA content in milk samples was determined as per the method described by Deeth and Fitzgerald (Lipolysis in Dairy Products, 1976). Accurately 3 ml sample of milk was taken in test tube. Then 10 ml extraction mixture (iso propanol: petroleum ether: 4N H_2SO_4 40:10:1), 6 ml petroleum ether and 4 ml water were added and shaken vigorously for 15 second. Two layers were allowed to settle (5-10 min) and upper layer (usually 7.5 ml) was withdrawn and transferred to a small flask.2 drops of 1% methanolic phenolphthalein indicator was added and titrated with 0.02N methanolic KOH solution. Blank reading was obtained using water instead of milk. The FFA content was calculated by following equation.

$$FFA(miliequivalent/ml) = \frac{V \times N \times 10000}{3P}$$

Where,

V=Volume of 0.02N KOH used for sample

N=Normality of KOH,

P=Proportion of upper layer titrated

Estimation of Tyrosine value in milk

Estimation of Tyrosine value in milk samples was determined as per the method explained by Lowry (Protein Measurement with the Folin Phenol Reagent, 1951). In 5 ml of milk sample, 10 ml of 0.72 N trichloroacetic acid (TCA) and 1 ml of distilled water were added. The contents were shaken and kept undisturbed for 30 min. Then the mixture was filtered through whatman no. 42 filter paper. 0.5 ml of filtrate was transferred to a test tube and then 5 ml of alkaline reagent was mixed and kept at room temperature for 10 minutes. Then to it 0.5 ml of Folin's reagent was added and kept for 30 minutes at room temperature to develop colour. Run the blank test simultaneously using water instead of milk.. Colour of sample was measured at 750 nm in a spectrophotometer using the blank solution as reference.

Statistical Analysis

Data reported were expressed as mean values with standard errors. In experiments, wherever required, two-way analysis of variance (ANOVA) with a subsequent least significant difference (LSD) test was applied for multiple sample comparison to test for any significant differences ($P<0.05$) in the mean values of all the groups as described by Snedecor and Cochran (1994) using the statistical program of Microsoft® Excel Version 5.0 (Microsoft Corporation, Redmond, WA, U.S.A.). Graphs were prepared in software Graph Pad Prism version 5.0 (Graph Pad Software, Suite 230 La Jolla, CA 92037, U.S.A.).

Results and Discussion

Effect of preservatives on fat content of milk

The initial fat content of control milk sample was 4.08±0.05% and 4.10±0.11% by Gerber and Rose Gottlieb method, respectively. The initial fat content of milk samples preserved with 0.6% of optimized formulation was 4.07±0.04% and 4.10±0.04% by Gerber and Rose Gottlieb method, respectively. Addition of optimized formulation (combination of kathon and bronopol) did not have any influence on fat values of milk samples by Gerber and Rose Gottlieb method; however, it was noticed that fat values by Gerber as well as Rose Gottlieb method decreased on addition of 0.4% percent formalin. It is evident from Table 1 that an erratic trend was noticed in the fat content of milk samples added with formalin.

The initial fat content of milk samples preserved with 0.4% of formalin was 4.05±0.13% and 4.06±0.04% by Gerber and Rose Gottlieb method, respectively. The fat content in formalin preserved samples decreased significantly after 15 days of storage by both Gerber and Rose-Gottleib method. However, in case of milk samples added with optimized formulation, the fat content was found to decrease significantly after 60 days of storage at 37°C by both Gerber and Rose-Gottleib method.

Lower fat values were observed in formalin preserved samples mainly due to hardening of proteins in presence of formaldehyde. Formaldehyde reacts with milk proteins and form practically insoluble high molecular weight compounds (Fraenkel-Conrat and Olcott, 1946, 1948 a, b) and samples correspond to lower fat values due to incomplete dissolution of fat entrapped in milk proteins. In Gerber method, a difficulty was experienced in dissolving the butyrometer contents during shaking. The proteins did not dissolve completely but dispersed in fine particles which deposited below the fat layer after centrifugation. Similarly, in Rose Gottleib method, turbidity was observed in the fat extraction tubes in case of formalin treated milk samples which may be again be due to incomplete dissolution of proteins with ammonia.

The estimation of fat content in formalin added and stored milk samples indicated that repeatability is affected and no specific trend was observed. The results of the present study are also in consonance with the findings of Mulder, 1943; Pien et al. 1976; Krishnamurthy, 1974; Hussain et al. 1984; Des Raj and Singhal, 1987; Des Raj and Singhal, 1988; Bajaj and Rai, 1992, Chaudhary, 2013 who also reported lower estimations of fat in formalin preserved milk samples during storage of milk samples for variable time durations. However, the literature available on the effect of formalin on the fat estimation by Gerber method is contradictory and some workers (Sandhu et al. 1984; Jandal and Rai, 1988,1989; Karmakar and Ghatak, 1995, 1997) observed no significant change in the fat estimation by Gerber method in formalin preserved milk samples. Sanchez et al. (2005) observed no significant differences in fat content in case of bronopol-preserved goat milk samples stored at refrigeration temperature. Chaudhary (2013) also reported no change in the fat content (by Gerber method) of milk samples preserved with 0.4% kathon and 0.045% bronopol till 5th week of storage. In contrast, Bertrand (1996) reported higher milk fat of 4.43% vs. 4.37% in cow milk preserved with bronopol compared with that of unpreserved samples.

Effect of preservative on protein content of milk

The total protein content in control milk sample was found to be 3.88±0.01%. Immediately after addition of formalin and optimized formulation, the protein content of milk was found 3.79±0.05 and 3.83±0.03%, respectively. It is apparent from Table 1 that addition of optimized formulation and formalin did not have any effect on protein content of milk and also the protein content remained fairly constant during the storage period at 37°C in milk samples with preservatives (combined formulation and formalin). Similar results were reported by Bector and Narayanan (1973). They observed no significant difference in protein content of milk either on addition of formalin or during storage up to 6 months analyzed by Kjeldahl method. Furthermore, when formalin was added in cow (Karmakar and Ghatak, 1997) and buffalo milk (Karmakar and Ghatak, 1995) showed no change in milk proteins content by kjeldahl method during storage for one month at 7-8°C.

Effect of preservative on Total Solids of milk

In control milk sample the total solid content was found to be 13.67±0.01%. After addition of preservatives (optimized formulation and formalin), no significant change was observed in total solid content of milk in comparison to control milk. After addition of formalin and optimized formulation, the total solid content of milk was 13.67±0.01 and 13.72±0.02%, respectively. It is evident from Table 1 that the total solid content remained fairly constant all over the storage period at 37°C in milk samples with preservatives (combined formulation and formalin). This finding is in agreement with those found by Sandhu et al. 1984; Bector and Narayanan, 1973; Bajaj and Rai 1992 who confirmed that the total solid content analyzed using gravimetric method was not affected in formalin preserved milk. Gupta and Gupta (2010) also reported no significant change was observed in total solid content of milk preserved with 0.3% and 0.5% formalin during storage up to 48 h.

Effect of preservative on ash content of milk

The total ash content in control milk sample was found to be 0.70±0.01%. Immediately after addition of preservatives (optimized formulation and formalin), no significant variation was observed in total ash content of milk in comparison to control milk. After addition of formalin and optimized formulation, the ash content of milk was 0.71±0.02 and 0.70±0.01%, respectively. It is apparent from Table 1 that the ash content remained fairly constant all over the storage period at 37°C in milk samples with preservatives (optimized formulation and formalin).

Effect of preservative on lactose content of milk

In control milk sample the lactose content was found to be 4.62±0.02%. Immediately after addition of preservatives (optimized formulation and formalin), no significant variation was observed in lactose content of milk in comparison to control milk. After addition of formalin and optimized formulation, the lactose content of milk was 4.60±0.02 and 4.59±0.05% respectively.

It is evident from Table 1 that decrease in lactose content was observed in milk samples added with preservative (optimized formulation and formalin) during storage. A significant difference was observed after 60 days of storage and there after it remained constant till the end of storage period. At the end of 90 days of storage, the lactose content was found 4.44±0.05 and 4.48±0.06% in milk samples added with formalin and optimized formulation, respectively. The findings are similar with several workers (Bector

Table 1 Effect of addition of preservatives on compositional parameters of milk

Fat (%)				Gerber method				
	Days	0	15	30	45	60	75	90
	FPM	4.05±0.13^{aA}	3.88±0.03^{bA}	3.74±0.04^{cA}	3.76±0.05^{cA}	3.69±0.03^{cA}	3.79±0.01^{cA}	3.71±0.02^{cA}
	OPM	4.07±0.04^{aA}	4.09±0.05^{aB}	4.08±0.02^{aB}	4.06±0.12^{aB}	3.99±0.05^{bB}	3.93±0.02^{bB}	3.89±0.02^{cB}
				Rose Gottlieb method				
	Days	0	15	30	45	60	75	90
	FPM	4.06±0.04^{aA}	3.82±0.15^{bA}	3.78±0.07^{cA}	3.75±0.05^{cA}	3.65±0.15^{cA}	3.73±0.12^{cA}	3.68±0.07^{cA}
	OPM	4.10±0.04^{aA}	4.10±0.05^{aB}	4.09±0.02^{aB}	4.07±0.12^{bB}	4.04±0.05^{bB}	4.01±0.02^{bB}	4.01±0.05^{bB}
Protein (%)								
	Days	0	15	30	45	60	75	90
	FPM	3.79±0.05^{aA}	3.82±0.08^{aA}	3.85±0.05^{aA}	3.89±0.01^{aA}	3.89±0.04^{aA}	3.83±0.02^{aA}	3.82±0.06^{aA}
	OPM	3.83±0.03^{aA}	3.82±0.03^{aA}	3.89±0.02^{aA}	3.91±0.03^{aA}	3.98±0.03^{aA}	3.87±0.01^{aA}	3.85±0.04^{aA}
Total solid (%)								
	Days	0	15	30	45	60	75	90
	FPM	13.67±0.01^{aA}	13.67±0.02^{aA}	13.65±0.01^{aA}	13.66±0.02^{aA}	13.67±0.06^{aA}	13.68±0.09^{aA}	13.68±0.01^{aA}
	OPM	13.72±0.02^{aA}	13.73±0.04^{aA}	13.71±0.04^{aA}	13.74±0.01^{aA}	13.74±0.01^{aA}	13.72±0.08^{aA}	13.74±0.04^{aA}
Ash (%)								
	Days	0	15	30	45	60	75	90
	FPM	0.71±0.018^{aA}	0.71±0.004^{aA}	0.70±0.002^{aA}	0.70±0.008^{aA}	0.69±0.007^{aA}	0.70±0.012^{aA}	0.70±0.004^{aA}
	OPM	0.70±0.007^{aA}	0.70±0.004^{aA}	0.69±0.004^{aA}	0.70±0.008^{aA}	0.70±0.013^{aA}	0.70±0.005^{aA}	0.70±0.009^{aA}
Lactose (%)								
	Days	0	15	30	45	60	75	90
	FPM	4.59±0.02^{aA}	4.59±0.01^{aA}	4.56±0.03^{aA}	4.56±0.07^{aA}	4.51±0.06^{bA}	4.49±0.03^{bA}	4.44±0.06^{bA}
	OPM	4.59±0.02^{aA}	4.59±0.0.5^{aA}	4.53±0.04^{aA}	4.51±0.01^{aA}	4.49±0.04^{bB}	4.45±0.07^{bB}	4.48±0.05^{bB}

Data are presented as means ± S.E (n=3). a-c Means with different superscript are significantly different ($p<0.05$) from each other in row

A-B Means with different superscript are significantly different ($p<0.05$) from each other in column for each parameter

FPM: Formalin Preserved Milk, OPM: Optimized Formulation Preserved Milk

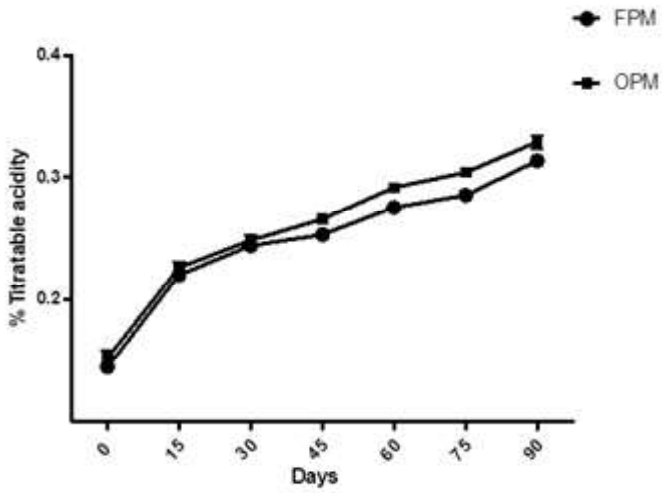

Fig. 1 Effect of preservatives on Acidity (% Lactic acid) of milk during storage at 37±1°C, Data are presented as means ± S.E. (n=3), FPM: formalin preserved milk, OPM: optimized formulation preserved milk

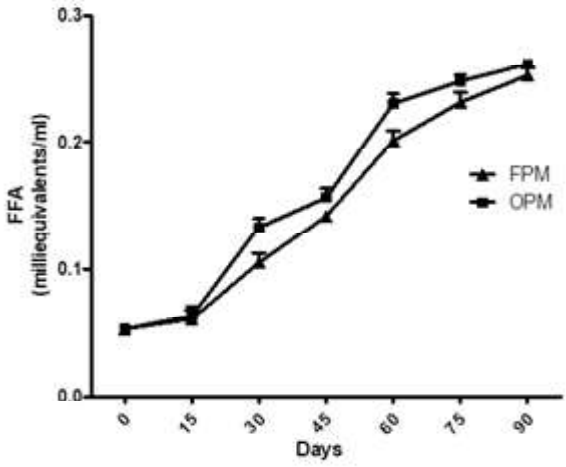

Fig. 2 Effect of preservatives on FFA (milliequivalents/ml) of milk during
Storage at 37±1°C, Data are presented as means ± S.E. (n=3), FPM: Formalin
Preserved Milk, OPM: Optimized Formulation Preserved Milk

and Narayanan, 1973; Sandhu et al. 1984; Bansal and Singhal, 1991; Bajaj and Rai 1993) who reported that storage of milk samples preserved with 0.4% formalin either at room or refrigeration temperatures for six months to 1 year had no effect on lactose content of the milk samples estimated by Lane-Eynon method.

Effect of preservative on Titratable Acidity of milk

The % titratable acidity of control milk sample was found to be 0.13±0.01% lactic acid. After the addition of optimized formulation, no major variation was observed, however on addition of formalin, the % titratable acidity was found to be slightly higher than % titratable acidity of control milk samples. This increase in initial acidity on addition of formalin was due to the liberation of hydrogen ions when formaldehyde reacts with primary amino groups, amide groups and guanidyl groups of protein (Jenness and Patton, 1969; Bansal and Singhal, 1991; Upadhyay et al. 2014). Although the acidity of milk increased after addition of formalin (0.4%) but statistically there was no significant difference in the initial acidity of control milk with that of the milk samples added with formalin (0.4%).

It is clear from Figure no 1 that an increasing trend was found in the % titratable acidity of milk samples added with optimized formulation and formalin. In case of 0.4% formalin preserved milk samples, the initial % titratable acidity was 0.14±0.02% lactic acid, which significantly increased after 15 days and continued to increase till 90 days of storage at 37°C. After 90 days the % titratable acidity was 0.31±0.05% lactic acid. Whereas, milk samples preserved with optimized formulation (0.6%), the initial % titratable acidity was found 0.13±0.06% lactic acid, which significantly increased after 15 days and continued to increase till 90 days of storage at 37°C. After 90 days the % titratable acidity was 0.33±0.09% lactic acid.

When the formalin was added, an instant increase in acidity of milk sample occurs, which continued during subsequent storage. Similar results were reported by Venkateswara rao et al. 1950; Armandola, 1969; Minzner and Kroger, 1974; Fahmi et al. 1982; Bansal and Singhal, 1991 who observed a gradual increase in acidity while preserving composite samples of cow and buffalo milk with 0.2, 0.3, 0.4, 0.5 % (v/v) formalin in glass bottles at room temperature (16-30°C) up to one year.

Effect of addition of preservative on FFA content of milk

The FFA content in control milk sample was found to be 0.05±0.001 milliequivalents/ml milk. No any significant difference was observed in FFA content of milk in comparison to control milk immediately after addition of preservative. It is evident from Figure no 2 that an increasing trend was found in the FFA content of milk samples added with optimized formulation and formalin.

In case of 0.4% formalin preserved milk samples, the initial FFA content was 0.05±0.006 milliequivalents/ml milk, which significantly increased after 30 days and continued to increase till 90 days of storage at 37°C. After 90 days the FFA content was 0.25±0.01 milliequivalents/ml milk. Whereas, In case of milk samples preserved with optimized formulation (0.6%), the initial FFA content was 0.05±0.002 milliequivalents/ml milk, which significantly increased after 30 days and continued to increase till 90 days of storage at 37°C. After 90 days the FFA content was 0.26±0.08 milliequivalents/ml milk.

Effect of addition of preservative on tyrosine value of milk

The Tyrosine value in control milk sample was found to be 42.39±0.74 mg/100 ml milk. After addition of formalin (0.4%), no significant variation was observed in tyrosine value of milk, however on addition of optimized formulation (0.6%), the tyrosine value was found to be 28.80% higher than tyrosine value of control milk samples. The higher values of tyrosine may be due to reaction of isothiazoline group of Kathon™ with folin's regent.

It is evident from Figure no 3 that the tyrosine value of milk samples added with optimized formulation and formalin was increased significantly. In case of 0.4% formalin preserved milk samples, the initial tyrosine value was 42.83±1.39 mg/100 ml milk, which significantly increased after 15 days and continued to increase till 90 days of storage at 37°C. After 90 days the tyrosine value was 167.05±1.97 mg/100 ml milk. In case of milk samples preserved with optimized formulation (0.6%), the initial tyrosine value was 54.60±0.49 mg/100 ml milk, which significantly increased after 15 days and continued to increase till 90 days of storage at 37°C. After 90 days the tyrosine value was 345.33±4.96 mg/100 ml milk.

Conclusions

From this study, it was ascertained that during storage at 37°C, there was no significant effect of optimized formulation (0.6%) on estimation of fat (Gerber and Rose Gottlieb) and lactose content for 45 days, on estimation of total solids, ash and protein content for 90 days. On addition of formalin (0.4%), the fat values of milk decreased significantly after 15 days of storage and also during storage at 37°C, no specific trend was observed. The lactose content was also found to decrease significantly after 60 days of storage. However, there was no any significant effect of formalin (0.4%), on estimation of total solids, ash and protein content of milk for 90 days. The addition of optimized formulation (0.6%) and formalin (0.4%) did not have any significant effect on titratable acidity and FFA content of milk. However, on addition of optimized formulation (0.6%), the tyrosine value was found to be 28.80% higher than tyrosine value of control milk samples. Therefore, it may be concluded that the optimized formulation is able to prevent deterioration of milk upto 45 days at ambient temperature without interfering with the chemical composition of the same and it can serve as a suitable alternative of formalin as preservative.

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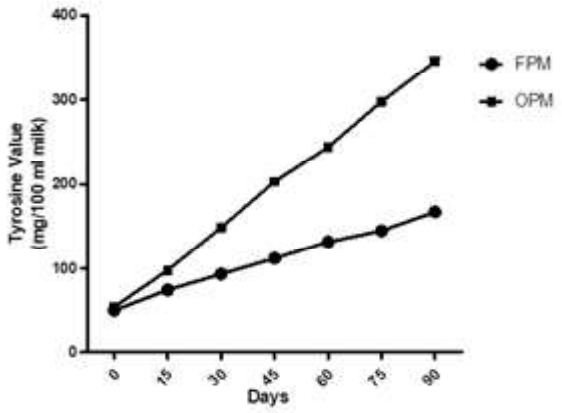

Fig. 3 Effect of preservatives on Tyrosine Value (mg/100 ml) of milk during Storage
at 37±1°C, Data are presented as means ± S.E. (n=3) FPM: Formalin Preserved Milk,
OPM: Optimized Formulation Preserved Milk

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RESEARCH ARTICLE

In vitro assessment of antioxidative potential of goat milk, casein and its hydrolysates: Comparison of goat milk with bovine and buffalo milk

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Abstract: The present study was executed with an aim to explore the antioxidative potential of goat, cow, and buffalo milk. Buffalo milk has showed highest antioxidative potential than goat and cow milk as measured by ABTS, ORAC, and DPPH assays, whereas goat milk has showed better antioxidative potential than cow milk when measured by ORAC and DPPH. Further, the effect of temperature on the antioxidative potential of goat milk was assessed. An increase in temperature has a negatively affect the antioxidative potential of goat milk. The antioxidative potential of goat milk was in the following order: raw milk > pasteurized milk > boiled milk. Casein derived from goat milk by isoelectric precipitation was hydrolyzed by gastrointestinal enzymes pepsin (P), trypsin (T), chymotrypsin (C), and their combinations PT, PC, TC, and PTC. Among all the casein hydrolysates, the maximum antioxidative potential was found in PT hydrolysate, further fractionated by 10, 3 and 1 kDa ultrafiltration membranes. 3-10 kDa fraction exhibited maximum antioxidative potential in comparison to other fractions of PT hydrolysate. Our results suggested that antioxidative potential of goat milk and its hydrolysates could be an important mean to obtain natural antioxidative peptides.

Keywords: Antioxidative potential, Buffalo milk, Casein hydrolysates, Cow milk, Goat Milk

Introduction

Oxidative stress is a physiological disproportion of evolvement of reactive oxygen species (ROS) and their neutralization by the antioxidative system. The uncontrolled production of free radicals in organisms can turn into oxidative stress. This imbalance has been closely linked with alzheimer's, atherosclerosis, cardiovascular disease, diabetes, huntington, rheumatoid arthritis, other age-related diseases and break down of vital biochemical compounds such as lipids, cellular proteins, DNA in the body (Fitzpatrick et al. 2009). Proper intake of antioxidants in the form of food enriched in antioxidants may protect the body from abovementioned diseases. Thus, antioxidant-enriched food products are becoming increasingly popular. Previous studies have demonstrated that food components, including ascorbic acid (vitamin C), β-carotenes, tocopherols, polyphenols, and bioactive peptides, have protective effects against oxidative stress and, therefore, act as antioxidants (Simos et al. 2011). Goat milk production has shown an upsurge worldwide and highly preferable owing to its anti-allergenicity especially in infants while comparing with cow milk (Park, 2009; Simos et al. 2011) also anti-platelet, antibacterial, immunomodulatory and antioxidative capacity (Simos et al. 2011). Additionally, goat milk has manifested diverse biological properties in comparison to different species milk including human, such as high buffering capacity, dissimilar alkalinity (Park, 2009), higher digestibility because of small size fat globules (Meena et al. 2014) and higher amount of short and medium chain fatty acids which are linked with hypocholesterolemic function in humans, by preventing cholesterol deposition and enhancing its movement and excretion (Kalyan et al. 2018). Therefore, due to goat milk's nutritional and bio-functional properties, it has received extensive attention. The antioxidative properties of goat milk are reported higher than bovine and donkey milk (Simos et al. 2011). These beneficial effects occur due to the release of bioactive compounds as goat milk is readily digestible and absorbed in the gut and small intestine, respectively (Park, 2009).

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Bioactive peptides from bovine milk are extensively known, but in recent times, studies have also been conducted on non-bovine species such as camel, sheep, donkey and goat (Mudgil et al. 2018; Li et al. 2013). Goat milk proteins have shown presence of peptides with antimicrobial, antioxidative, antidiabetic, ACE inhibitory and immune-modulating properties (Mudgil et al. 2018; Li et al. 2013; Park, 2009). A few studies have reported antioxidative peptides from goat milk protein. A total five antioxidative peptides were identified in goat milk casein hydrolysate after digestion with two enzymes namely, Alcalase and Pronase however, four reported peptides didn't correspond to sequences in goat casein databases (Li et al. 2013). Kusumaningtyas et al. (2016) performed enzymatic digestion with pepsin, trypsin, and chymotrypsin and by *Bacillus* sp.E.13 protease indicating antimicrobial and antioxidative potential of *Bacillus* sp.E.13 protease against other enzymes. The bioactive peptides of non-bovine species milk and their milk products were comprehensively reviewed recently (Guha et al. 2021). Previously, studies pertaining to antioxidative potential of goat milk has been performed. However, in present study all the commonly accepted antioxidative assays (FRAP, ABTS, DPPH and ORAC) have been performed to compare the antioxidative potential of goat milk with cow and buffalo milks. Alongwith, the effect of different degree of thermal treatment on antioxidative capacity of goat milk has also been performed. Further, antioxidative potential of goat milk casein and its hydrolysates and most potent antioxidative fraction of goats' milk casein obtained by enzymatic digestion with pepsin, trypsin and chymotrypsin individually and in combination.

Materials and Methods

Procurement and processing of milk

Raw milk of goat (crossbred of *Alpine* × *Beetal*), cow (*Sahiwal* breed) and buffalo (*Murrah* breed) were procured from the Livestock Research Centre, ICAR-National Dairy Research Institute, Karnal Haryana (India), and fat content was adjusted to 3% for all samples owing to variability in fat-soluble antioxidants such as tocopherols, retinol and carotenoids with varying fat percentage (Chauveau-Duriot et al. 2010).The SNF content of the fat adjusted milk were measured as 8.5, 8.5 and 9.0 % for cow, buffalo and goat milk respectively. The antioxidant potential of raw, pasteurized (63°C/30 min) and boiled milk (100°C for no-hold) were further assessed. All the chemicals were procured from Sigma-Aldrich Chem. Co., St Louis, MO, USA except the pepsin which was procured from Sisco Research Laboratories, Mumbai, India.

Assays to measure Antioxidative capacity of milk from different species

Antioxidant components from milk samples were isolated as per the method of Alyaqoubi et al. (2014). The following four different assays were employed for measuring the Antioxidative potential of milk from different species.

ABTS assay

ABTS assay was executed as per the methodology indicated by Re et al. (1999). Trolox standard was used in the range of 50 to 500 µM at an interval of 50 µM. Trolox equivalent antioxidant capacity (TEAC) of the samples was evaluated from the standard curve ($y = 0.187x - 3.818$ (R^2- 0.994)) of Trolox (50 to 500 µM) based on the inhibition percentage.

Oxygen radical absorbance capacity-Fluorescein (ORAC-FL) assay

ORAC assay was performed via fluorescein as per the procedure of Davalos et al. (2004). The trolox standard was used in the range of 10 to 100 µM at an interval of 10 µM. The standard curve equation was $y = 0.328x + 1.635$ (R^2- 0.991).

Ferric Reducing Antioxidant Power (FRAP) activity

The FRAP activity was performed as per the methodology of Benzie and Strain (1996). The trolox standard was used in the range of 0.08 to 0.72 µM at an interval of 0.08 µM. The standard curve for Trolox was $y = 1.017x + 0.022$ (R^2 - 0.989).

DPPH activity

The antioxidant capacity established on DPPH radical for milk samples was analyzed by methods defined by Zhidong et al. (2013). Trolox standard was used in the range of 100-800 µM. The standard curve equation was $y = 0.107x + 3.529$ (R^2 - 0.993).

Screening of casein hydrolysates for Antioxidative activity

Isolation of goat milk casein

Isoelectric precipitation method was utilized for separating the casein of goat milk (Davies and Law, 1977). The concentration of goat milk casein was evaluated by Lowery method using the standard curve i.e., $y = 0.001x + 0.054$ (R^2- 0.992). The purity of the casein derived from goat milk was tested by Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) in a vertical slab gel electrophoresis system (Laemmli et al. 1970).

In vitro hydrolysis of casein protein

Isoelectrically precipitated casein was hydrolyzed with pepsin, trypsin, chymotrypsin, and their different combinations (Jinsmaa and Yoshikawa, 1999). Before carrying out the hydrolysis of protein by the enzymes and their combinations, the time duration of complete hydrolysis by each enzyme was standardized. Pepsin, trypsin, and chymotrypsin were mixed with pre-incubated casein solution (1 $mgmL^{-1}$) in the ratio of 1:100 (E:S), followed by incubation at 37ºC for 8 h using a water bath with continuous

mixing. Thereafter, samples were collected at varying time breaks (0, 1, 2, 4, 6 and 8h). The degree of hydrolysis was calculated by evaluating the free amino groups released during hydrolysis by the OPA method (Church et al. 1983). Thereafter, casein was hydrolyzed by the enzymes and their combinations for 3h by following the same procedure as described above. For pepsin combination with other enzyme, firstly the casein was hydrolyzed with pepsin at 2.0 pH for 3h followed by increment of pH to 8.0 using 1.0N NaOH and then allowing the digestion with other enzymes subsequently for 3h each. After incubation, the reaction was terminated by enhancing the pH to 8.0 by 1.0N NaOH for pepsin, while trypsin and chymotrypsin activity was inhibited by raising the temperature to 98°C for 10 min. Peptide concentration in the hydrolysates was estimated by Bicinchoninic acid (BCA) assay (Smith et al. 1985). Briefly, 10 µL of sample was mixed with 190 µL BCA reagent (1:19) in 96-well ELISA plate and incubated at 37°C for 30 min. ELISA plate was cooled to room temperature and the absorbance was recorded at 562 nm. The peptide concentration in the hydrolysates was evaluated using the standard curve of BSA ($y = 0.048x + 0.023$ with an R^2 of 0.987). Further, Tricine Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (Tricine SDS-PAGE) was performed to detect low molecular weight peptides in hydrolysates (Pardo and Natalucci, 2002). Furthermore, isolated casein was used for further studies.

In vitro evaluation of the casein and its hydrolysates

Antioxidative activity of the casein and its hydrolysates was evaluated by ABTS, ORAC and DPPH assay as described above.

Ultrafiltration of Pepsin-Trypsin hydrolysates

Pepsin-Trypsin hydrolysates of casein with maximum Antioxidative activity were purified using ultrafiltration. Pepsin-Trypsin hydrolysate of casein were ultrafiltered by 10, 3 and 1 kDa molecular weight cut-off (MWCO) membrane which fractionated the casein hydrolysate based on their size and also eliminated the enzyme and non-hydrolyzed proteins from the reaction mixture (Segura-Campos et al. 2011). The ultra-filtrated peptide fractions so obtained were labelled as >10 kDa (10 kDa retentate), 3-10 kDa (3 kDa retentate), 1-3 kDa (1 kDa retentate) and <1 kDa(1 kDa permeate) as described in Supplementary Fig. 1.The antioxidant activity of each fraction was measured as discussed previously.

Statistical analysis

The differences among the treatments were evaluated using one way ANOVA and considered statistically significant at 5% level of significance.

Results and Discussion

Antioxidative potential of milk

The antioxidative properties of milk were measured in terms of TEAC using ABTS, ORAC, FRAP and DPPH assay. Results revealed improved antioxidative potential of buffalo milk as compared to cow and goat milk using all the four assays (Table 1). However, goat milk showed better antioxidative potential than cow milk when evaluated using ORAC and DPPH assay. Besides the contribution of water-soluble compounds namely phenols, ascorbate, and low-molecular weight thiols, it is well established that TEAC also depends on milk fat components, which encompasses various fat-soluble antioxidants such as tocopherols, retinol, and carotenoids (Chauveau-Duriot et al. 2010), casein and whey proteins (Pihlanto, 2006), although in present study fat was adjusted to constant value of 3% for all milk samples. Therefore, the detected variations in TEAC might be attributed to variation in species-specific milk composition particularly, protein and other potential antioxidative compounds (Niero et al. 2018). The differences among diverse assays could also be due to their quantifying parameters. Huang et al. (2005) reported that antioxidative assays are majorly of two types i.e., dependent on hydrogen atom transfer reactions and electron relocation reactions. Thus, it is suggested that ABTS, FRAP and DPPH should be utilized to measure antioxidant reducing capacity and ORAC assay to evaluate peroxyl radical scavenging potential of antioxidant. Our results are in confirmation with Alyaqoubi et al. (2014) who had found significantly higher ($p<0.05$) antioxidant activity of goat milk than cow milk using DPPH and FRAP assay. Similarly, Simos et al. (2011) claimed better antioxidative capacity of goat (Prisca breed) milk as compared to cow milk reflecting higher bioavailability of antioxidant compounds in goat milk. In present study, FRAP assay showed converse result with goat milk possessing significantly ($P<0.05$) higher antioxidative activity in comparison with buffalo milk but non-significantly ($P>0.05$) different from cow milk (Table 1).

Table 1 TEAC (µmolmL^{-1}) of goat raw, pasteurized and boiled milk

Assay	Type of milk				
	Cow raw	Buffalo raw	Goat raw (GR)	Goat pasteurized (GP)	Goat boiled (GB)
ABTS	5430 ± 39.48^{a}	5571 ± 32.32^{b}	5416 ± 18.11^{a}	4964 ± 36.44^{b}	4749 ± 21.22^{c}
ORAC	3968 ± 3.23^{b}	4242 ± 33.95^{c}	4135 ± 2.64^{a}	4076 ± 35.02^{a}	4052 ± 40.53^{a}
FRAP	0.28 ± 0.00^{ab}	0.26 ± 0.01^{b}	0.31 ± 0.01^{a}	0.30 ± 0.00^{ab}	0.29 ± 0.00^{b}
DPPH	131.9 ± 0.91^{b}	207.3 ± 10.80^{c}	180.3 ± 2.77^{a}	166.2 ± 6.39^{b}	151.6 ± 3.19^{c}

Values are expressed as mean ± SEM (n=3). ^{abc} Dissimilar superscript within a row within an attribute indicate significant differences ($P<0.05$).

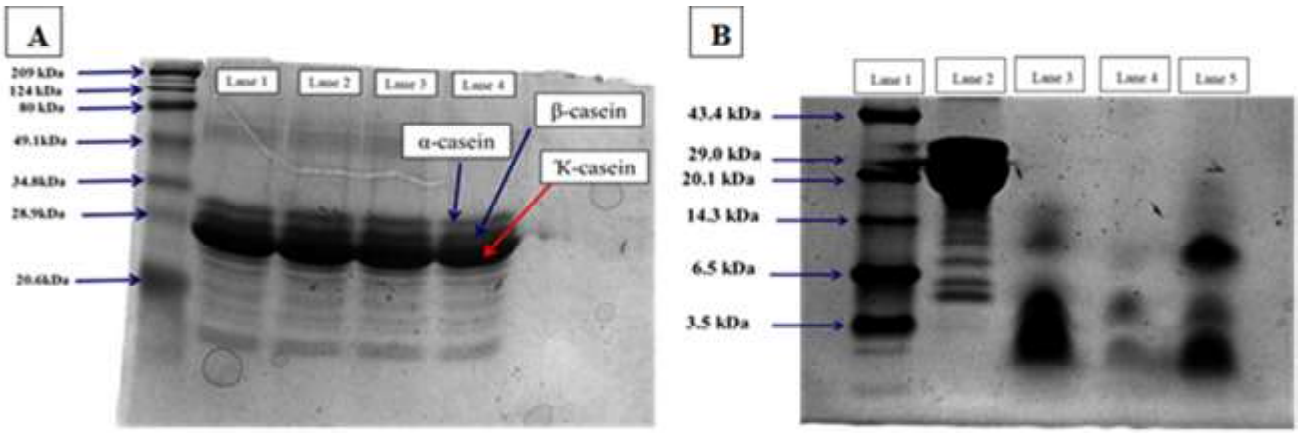

Fig. 1 (A) SDS-PAGE of Isoelectric Casein isolates (Lane 1 – Low molecular weight markers: Myosin (209 kDa), β-galactosidase (124 kDa), Bovine serum albumin (80 kDa), Ovalbumin (49.1 kDa), Carbonic anhydrase (34.8 kDa), Soybean trypsin inhibitor (28.9 kDa) and Lysozyme (20.6 kDa) from top to bottom; Lane 2-5 – Casein) (B) Tricine SDS-PAGE of casein and its hydrolysates. (Lane 1- Low molecular weight markers; Lane 2- Casein; Lane 3- Casein hydrolyzed by pepsin; Lane 4- Casein hydrolyzed by trypsin; Lane 5- Casein hydrolyzed by chymotrypsin)

Further, antioxidative potential of pasteurized and boiled goat milk declined in comparison to raw goat milk when estimated by ABTS, ORAC, FRAP and DPPH assay. The antioxidative potential by ABTS of pasteurized goat milk and boiled goat milk was 4964 ± 36.44 and 4749 ± 21.22 μmolmL^{-1} respectively thus signifying inverse relationship between antioxidative potential and degree of heat treatment. Raw goat milk had the highest antioxidative activity followed by boiled and pasteurized goat milk as measured by ABTS and DPPH assay (Table 1). Heat treatment has detrimental effects on the antioxidative properties of goat milk and it is reported that higher temperature short time treatments can deplete the overall antioxidative properties of milk. Contrarily, high heat treatment facilitates the Maillard reaction between nucleophilic group of amino acids and carbonyl group of reducing sugars and these Maillard reaction products i.e., brown melanoidins, enhance recovery and possible elevation of antioxidative properties (Zulueta et al. 2009). Alyaqoubi et al. (2014) assessed the impact of heat treatment on antioxidative potential of milk and reported lower antioxidative potential of pasteurized milk than unpasteurized milk and reduced phenol and flavonoids content in pasteurized milk.

Preparation and screening of casein hydrolysates

Goat milk casein was isolated by isoelectric precipitation and concentration in goat milk sodium caseinate was 270 mgmL^{-1}as determined by Lowry method. Then, the purity of the casein samples was evaluated by 15% Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (Fig. 1A). Different digestive enzymes such as pepsin (P), trypsin (T), chymotrypsin (C) and their combinations were employed to hydrolyze isoelectrically precipitated casein for peptides formation. The hydrolysis rate of casein protein by trypsin and chymotrypsin increased with time and almost got saturated after 3 h of incubation (Fig. 2A), but hydrolysis by pepsin persisted even after 3 h, although the hydrolysis rate was slightly lower. Consequently, duration of hydrolysis was standardized to 3h for the experiments owing to maximal degree of hydrolysis during this duration (Fig. 2A). Shanmugam et al. (2015) also reported similar findings in their study. Similarly, the antioxidant peptides were liberated maximally at 3h while digesting casein with pepsin and trypsin (Ming and Zhi-he, 2012). The degree of hydrolysis of goat milk casein hydrolyzed by P, T, C, PT, PC, TC and PTC were 2.84 ± 0.210%, 5.29 ± 0.194%, 5.85 ± 0.048%, 6.27 ± 0.127%, 5.07 ± 0.073%, 7.19 ± 0.250% and 8.53 ± 0.127%, respectively (Fig. 2B). The extent of hydrolysis of intact casein using trypsin, chymotrypsin and all the combinations was significantly higher as compared to pepsin. The degree of hydrolysis was in the range of 7.10 to 17.60 % for buffalo milk casein hydrolyzed by P, T, C singly and their combinations (Shanmugam et al. 2015). Variations were attributed to enzyme specificity for cleavage site, enzyme rate of reaction, substrate affinity and the quantum of sites available in respective proteins (Mudgil et al. 2018). Zhirong et al. (2021) reported that efficiency of enzyme also depends upon denaturing conditions mainly due to heat treatment or pH thereby better accessibility to internal cleavage sites of protein. Preferential cleavage of a peptide bond between hydrophobic amino acids residues of leucine, phenylalanine, and tyrosine is catalyzed by pepsin (Dunn, 2002). Similarly, chymotrypsin hydrolyses the protein sites having hydrophobic amino acids (Cassidy et al. 1997). The specificity of trypsin issolely limited to hydrophilic amino acid residues of arginine and lysine (Olsen et al. 2004). The yield (%) of the peptides after hydrolysis by P, T, C, PT, PC, TC and PTC were 19.51 ± 0.565, 22.24 ± 1.012, 20.15 ± 0.548, 18.90 ± 0.308, 17.58 ± 0.356, 18.19 ± 0.638 and 17.83 ± 0.116% respectively (Fig. 2C) estimated by BCA method. Casein hydrolysis was visually evaluated by tricine SDS-PAGE to determine the low molecular weight peptides in hydrolysate and to ascertain hydrolysis of casein by different

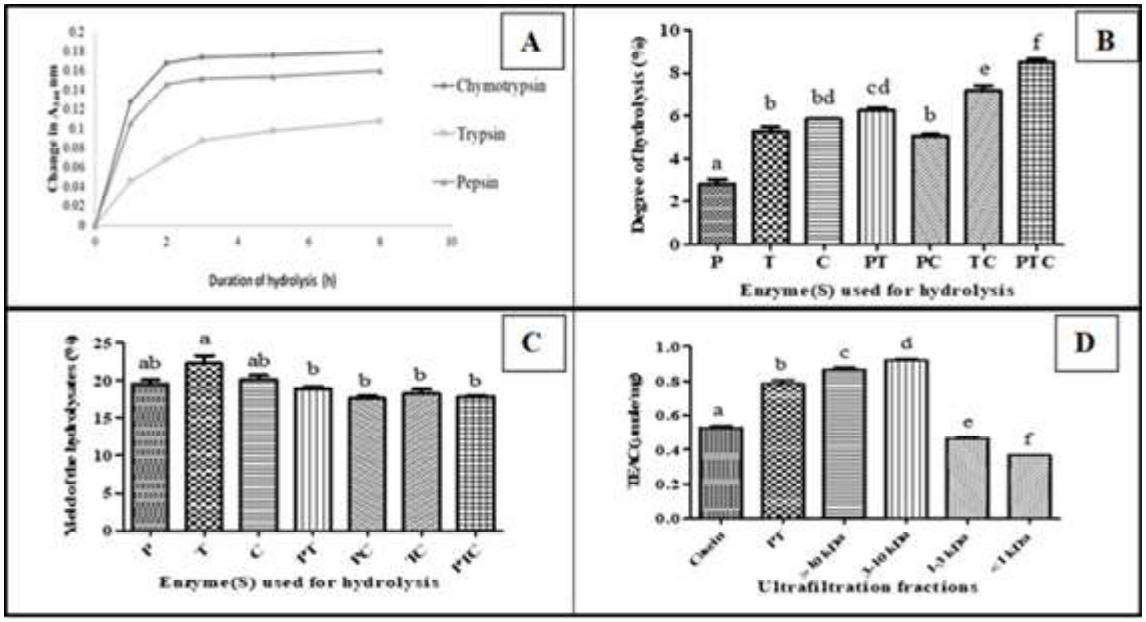

Fig. 2 (A) Rate of hydrolysis of casein by pepsin (P), trypsin (T) and chymotrypsin (C). (B) (a) Degree of hydrolysis of casein hydrolyzed by pepsin (P), trypsin (T), chymotrypsin (C) and their combinations (PT, PC, TC and PTC) as estimated by OPA method. (C) Yield (%) of casein hydrolyzed by P, T, C, PT, PC, TC, and PTC. (D) Antioxidant activity of different fractions (>10 kDa, 3-10 kDa, 1-3 kDa &< 1 kDa) of pepsin–trypsin (PT) hydrolysates. The bars bearing different alphabets differ significantly (P<0.05).

Table 2 TEAC values (μmolmg^{-1}) of casein and its hydrolysates by different assays

Groups Assay	Casein	P	T	C	PT	PC	TC	PTC
ABTS	0.52 ±0.01^{a}	0.73±0.01^{b}	0.74± 0.01^{b}	0.77±0.01^{bc}	0.78± 0.02^{c}	0.80±0.01^{c}	0.74± 0.02^{b}	0.79±0.01^{bc}
ORAC	0.34±0.0^{a}	0.40±0.01^{b}	0.39±0.00^{bd}	0.38±0.0^{cd}	0.37± 0.00^{c}	0.36± 0.00^{c}	0.35±0.00^{ac}	0.36± 0.0^{ac}
DPPH (x10^{-3})	1.59±0.18^{a}	2.74±0.07^{b}	4.15± 0.05^{c}	2.76±0.12^{b}	4.37± 0.14^{c}	2.81±0.15^{b}	4.38± 0.07^{c}	3.96± 0.01^{c}

Values are expressed as mean ± SEM (n=3).^{abc} Different superscript within a row within an attribute indicate significant differences (P<0.05).

enzymes as previously determined by OPA method. It was observed that casein was completely hydrolyzed during this digestion process by pepsin, trypsin, and chymotrypsin as depicted in Fig. 1B.

Antioxidative property of casein hydrolysates

The antioxidative potential of casein hydrolysates was analyzed on the basis of free radical scavenging activity using ABTS, DPPH, and ORAC (Table 2). In ABTS, ORAC, and DPPH assay, TEAC of hydrolysates was between 0.736 to 0.804 μmolmg^{-1}, 0.352 to 0.402 μmolmg^{-1} and 2.745 to 4.381 x 10^{-3} μmol mg^{-1} of the casein hydrolysates, respectively, whereas the values of unhydrolyzed casein were 0.528, 0.347, and 1.598 x 10^{-3} μmol mg^{-1}as measured by ABTS, ORAC, and DPPH assay, respectively. Similarly, the antioxidative activity of casein hydrolysates obtained from P, T, C, and their mixture got enhanced significantly in comparison to unhydrolyzed casein when evaluated using ABTS and DPPH assay (Table 2). The TEAC values of buffalo milk based hydrolyzed casein obtained by pepsin, trypsin and chymotrypsin enzymes were higher than unhydrolyzed buffalo milk casein (Shanmugam et al. 2015). The ABTS assay yielded higher antioxidative activity of the hydrolysates obtained from PT and PC than P, T, C, TC, and PTC hydrolysates. However, the antioxidative assay of PC hydrolysate was significantly (P<0.05) lower than PT hydrolysate when analyzed using DPPH assay, therefore PT hydrolysate was opted for further purification.

Purification of pepsin–trypsin (PT) hydrolysate of casein by ultrafiltration and assessment of antioxidative potential of different fractions

PT hydrolysate of casein possessed maximum antioxidative activity which was further purified using ultrafiltration. The obtained fractions were further analyzed for their antioxidative capacity. The PT hydrolysate was also ultrafiltered using 10 kDa, 3 kDa, and 1 kDa MWCO to separate enzyme and non-hydrolyzed protein from the reaction mixture and fractionation of casein

hydrolysate based on their size. Antioxidative activity of ultrafiltration fractions of PT hydrolysate was assessed by ABTS assay. Antioxidative activity of 3-10 kDa fraction was significantly ($P<0.05$) higher than all the other fractions and PT hydrolysate (Fig. 2D). Our findings are in agreement with Shanmugam et al. (2015), who also chose PT hydrolysate of buffalo milk casein for ultrafiltration and reported highest antioxidative activity of 1 kDa permeate as evaluated using ABTS assay. Sun et al. (2011) reported that out of four fractions the PHH-IV fraction that is 3 kDa permeate has higher inhibitory effect of lipid peroxidation and scavenging effect on superoxide radical. Similarly, Kusumaningtyas et al. (2016), observed the variations in the antioxidative capacity of goat milk protein hydrolysates based on the technique used and reported the maximum activity of 10-30 kDa than 3 kDa as analyzed using ABTS and DPPH, respectively. The higher activity of < 1 kDa peptides in antioxidant assays may be due to higher amount of total hydrophobic and aromatic amino acids as these aminoacids' types exhibited more antioxidative activity (Ajibola et al. 2011).

Conclusion

The present study revealed better antioxidative potential of goat and buffalo milk in comparison to cow milk. However, it could not be ascertained that which milk species has higher antioxidative potential in totality as TEAC depends on number of compounds besides proteins and their composition. Temperature adversely influences the antioxidative capacity of goat milk and antioxidative capacity decreases with increased temperature. Hence, it can be inferred that antioxidative potential of goat raw milk is better than any heat treatment given. Although, due to their intrinsic pathogenic microbiota, goat raw milk consumption is restricted, which has been related to foodborne disease. Antioxidative potential increased upon hydrolysis of goat milk casein. Further, pepsin-trypsin (PT) hydrolysate of goat milk casein exhibited the maximum antioxidative potential than other hydrolysates. PT hydrolysate fraction 3-10 kDa fraction could be utilized to develop physiologically functional foods or therapy drugs. The identification of more potent antioxidative peptides of 3-10 kDa fraction of PT hydrolysate and their bioavailability and antioxidative effect has to be studied both *ex vivo* and *in vivo*.

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RESEARCH ARTICLE

Application of response surface methodology in process optimization of Soytofu and their sensory evaluation

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Abstract: The objective of this investigation was to optimize the levels of four salt and three acid coagulants and coagulating temperature of soy milk so as to prepare quality soytofu by employing Response Surface Methodology (RSM) and to select an ideal one among the seven coagulants. To optimize the level of coagulant and temperature, Three Level Factorial Design was used. This included three levels of salt (0.2, 0.4 and 0.6%, w/v on soy milk basis) and acid (0.15, 0.25 and 0.35%, w/v on soy milk basis) coagulants with three levels of coagulating temperature (75, 82.5 and 90°C). Thus 13 RSM experiments including five central and eight axial points were conducted to produce soytofu samples with each of the coagulant. Soytofu samples were evaluated organoleptically on nine point hedonic rating scale. Results revealed that the optimum concentration of coagulants (% w/v, on soymilk basis) and coagulating temperature (°C) of soymilk for making quality soytofu was 0.6, 86.55, 0.57, 84.9, 0.6, 87.23, 0.46, 86.18, 0.35, 89.33, 0.3, 80.25 and 0.15%, 87.45°C, respectively, for $CaCl_2$, $CaSO_4$, $MgCl_2$, $MgSO_4$, lactic acid, acetic acid and citric acid coagulants. Preparation of soytofu sample from RSM optimized level and their organoleptic evaluation indicated that among the seven coagulants calcium chloride was best for making quality soytofu and it contained 68.59, 50.3, 12, 5.9 and 15.35% moisture, protein, crude fat, ash and carbohydrates, respectively.

Keywords: Coagulants, Optimization, Soybean, Soytofu, Temperature

Introduction

Tofu is usually considered as salt or acid coagulated water based gel, with soy lipids and proteins trapped in the gel network (Kohyama et al. 1995). It is an inexpensive, nutritious and versatile meat or cheese substitute with bland taste and porous texture. On the moisture free basis, tofu contains about 50% protein and 27% oil, and the remaining constituents are carbohydrates and minerals (Tripathi and Mishra, 2005). Yield and quality of tofu are important parameters which should be kept in mind during the production of tofu. It is influenced by environmental as well as processing factors such as cultivar (Sun and Breene, 1991, Tanwar et. 1998, Krishna et al. 2002), soaking time (Choi and Kim 1983), type of coagulant (Sutar et al. 2009, Shokunbi et al. 2011), concentration of coagulant (Sun and Breene, 1991), temperature of coagulant (Shih et al. 1997, Agrahar-Murugkar 2015), pressure applied (Gandhi and Bourne 1988) and processing methods (Agrahar-Murugkar 2015).

Till now many coagulants have been used in soytofu preparation like calcium chloride, calcium sulphate, magnesium chloride, lemon juice, Epsom salt and top water of fermented maize (Sun and Breene 1991, Obtalu 2008, Sutar et al. 2009, Shokunbi et al. 2011). There is dearth of information regarding usage of acid coagulants (citric acid, acetic acid and lactic acid). So, in present investigation response surface methodology (RSM) was applied to optimize the level of coagulants and coagulating temperature of seven different alt and acid coagulants. As, RSM is a valuable statistical tool that has been widely used in process optimization as well as in recipe formulation and processing conditions (Sharma et al. 2005, Deshpande et al. 2008, Karuppaiya et al. 2009 and Granato et al. 2010).

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Materials and Methods

The present investigation was carried out in the Department of Food Science and Technology, G.B. Pant University of Agriculture and Technology, Pantnagar, U.S. Nagar (Uttrarakhand). Mature variety of soybean PS-1347 was procured from Crop Research Centre of G.B. Pant University of Agriculture and Technology, Pantnagar. Soybean seeds were cleaned manually and stored in a cool and dry place until required. Seven coagulants (calcium chloride, calcium sulphate, magnesium chloride, magnesium sulphate, lactic acid, acetic acid and citric acid), and other chemicals employed in the present study were of AR grade. The physiochemical properties of soybean seeds were also analyzed.

Physico-chemical analysis of soybean

Soybean used in research was test for various physical parameters such as thousand grain weight, hull content, hardness, bulk density, true density and porosity as per the following formula:

$$\text{Bulk density (g/cc)} = \frac{\text{Weight of soybean in gram}}{\text{Volume of soybean in cubic centimeter}}$$

$$\text{True density (g/cc)} = \frac{\text{Weight of grain in gram}}{\text{Volume of toluene displaced in cubic centimeter}}$$

$$\text{Per cent porosity} = \frac{\text{True density} - \text{Bulk density}}{\text{True density}} \text{X } 100$$

Hardness of 10 randomly taken seeds of soybean was determined with a Hardness Tester (Kiya Seisakusho Ltd., Japan). Hardness represented the force required to break the seed vertically and horizontally and average value was expressed in kg. While moisture, fat, protein, total ash, calcium, phosphorus and iron was estimated by Ranganna (1996).

Design of experiment

The investigation comprised of two experiments. In the first experiment, seven coagulants were employed individually for preparation of soytofu. Soymilk was coagulated at three different concentration of each coagulant. Thirteen soytofu samples were prepared with each coagulant by using RSM and level of each coagulant and coagulating temperature were optimized so as to produce soytofu having maximum yield and ideal organoleptic characteristics. In the second experiment, seven soytofu samples were prepared as per the RSM optimized levels for each of the coagulant and corresponding coagulating temperature. These soytofu samples were subjected to organoleptic evaluation so as to find out the best coagulant among the seven and proximate composition of the best soytofu sample was also determined. The experiments were planned using RSM with Three Level Factorial Design. A full second order model was fitted into each response. The adequacy of the model was tested using coefficient of determination (R^2) and Fisher's F-test.

Preparation protocol

Whole soybeans were immersed in water (14-16 hours) and were washed thoroughly by rubbing them manually between the palms, the hulls were removed completely. Then dehulled soybeans were then ground at a Mixer-Grinder (Philips, HL 1632) in hot water (95°C) for 3 min. During extraction the ratio (bean to water) was 1:8 (w/v). The resultant suspension was passed through a double-layered fabric of muslin. The soymilk thus obtained was boiled to inactivate trypsin inhibitor and other anti-nutritional factors for five minutes (Sutar et al. 2009).

The volume of coagulant needed as per the RSM was dissolved in 20 ml of distilled water and transferred to a 1 liter size stainless steel tank. Freshly prepared 500 ml of soymilk was brought to the coagulating temperature required and then gently poured into the vessel containing coagulant for soytofu preparation. Then for about 30 minutes the contents were left undisturbed and allowed to cool to room temperature. The content was passed through a double layered cheese cloth to separate curd and whey. The curd was then transferred into a wooden box lined with a double layered cheese cloth. Cloth corners were folded over the contents and a constant pressure of 28 g/cm^2 was exerted for 15 minutes on the contents. The box had holes to drain off the whey at its sides and the edges. The prepared soytofu was then removed from the wooden box and weighed and stored in chilled water at refrigeration temperature until organoleptic analysis. Freshly prepared samples of soytofu were tested organoleptically within 24 hours (Table 1).

Screening of ideal coagulant

For the screening of best coagulants, seven soytofu samples were prepared from each coagulant separately as per the RSM optimized coagulant concentration and corresponding coagulating temperature. An ideal coagulant was selected on the basis of yield and sensory parameters. A soytofu sample was prepared as per the ideal selected coagulant and it was analyzed for proximate composition (moisture, crude protein, crude fat, ash and carbohydrate) as per AOAC methods.

Organoleptic evaluation

The acceptability of soytofu samples prepared from soybean variety PS 1347 was determined organoleptically. The taste panel was comprised of 10 members drawn from staff and PG-students of Department of Food Science and Technology. The panelists evaluated soytofu samples on 9-point hedonic scale for sensory

attributes (appearance, colour, odour, taste, texture and overall acceptability).

Statistical analysis

Optimization of levels of each coagulant and coagulating temperature was done using the RSM (Design-Expert 8.0.7.1). Data recorded in the experiments were analyzed and the response functions were developed using the multiple regression. ANOVA was used to analyze the models as described by Snedecor and Cochran (1986).

Results and Discussion

The physico-chemical characteristics of soybean variety PS-1347 are shown in table 2. Similar values with regard to fat content in soybean were also observed by Krishna et al. (2003). The value of ash content in whole soybean was in accordance with those reported by Sutar et al. (2009). The carbohydrate content is slightly lower than reported by Sutar et al. (2010). Jain and Mittal (1992) and Krishna et al. (2003) also estimated calcium in different Indian varieties of soybeans which ranged from 62.38 to 298.47, 252.91 to 304.17 mg/100 g, respectively.

Effects of different levels of salt coagulants and temperature on yield and sensory profile of soytofu

Soytofu prepared by 0.6% $CaCl_2$ was added to soymilk at 82.5°C had maximum product yield of 27.9% while addition of 0.2% $CaCl_2$ in soymilk at 90°C resulted in minimum soytofu yield of 22.4%. Soytofu yield was maximum (29%) when $CaSO_4$ was used at a level of 0.4% while the coagulating temperature of soymilk was 82.5°C. Results indicate that yield of soytofu was maximum when 0.6% $MgCl_2$ was used at 75°C. An observation of yield by coagulation through lactic acid (0.6%, 82.5°C), acetic acid (0.35%, 75°C) and citric acid (0.35%, 90°C) was noted as 27.5%, 25.7% and 22%, respectively. The sensory score of the prepared soytofu was done on the basis of nine-point of hedonic scale. Maximum overall acceptability score of soytofu at different coagulant and temperature levels were $CaCl_2$ (8.75) $CaSO_4$ (8.25), $MgCl_2$ (9) $MgSO_4$ (9), lactic acid (9), acetic acid (8.87) and citric acid (9). Yield was calculated by following formula

$$\text{Yield (\%)} = \frac{\text{Soytofu obtained}}{\text{Soymilk taken for coagulation}} \text{ X } 100$$

Calculated F values of soytofu yield based on ANOVA depicted that yield is significantly affected by the level of coagulant and temperaure at 1% level of significance when coagulated by $CaCl_2$ and $MgCl_2$, while in case of citric acid and lactic acid yield is only affected by coagulant concentration at 1% level of significance. Product yield was affected by temperature at 1% and 5% level of significance when coagulant was $MgSO_4$ and $CaSO_4$, respectively but at 5% level of significance soytofu yield was affected by using only acetic acid.

When $CaCl_2$ was used at 0.6% concentration with temperature of soymilk as 82.5°C, the sensory parameters (odour, taste and texture) was maximum as compare to other combination of processing variables, while appearance of soytofu had highest score at 0.4% level of $CaCl_2$ and 82.5°C. The F value suggests that the effect on appearance and taste are significant at 5% level with values of 5.63 and 5.74, respectively, odour texture and overall acceptability are significant at 1% level with values 12.05, 20.35 and 27.44, respectively. However effect on the color of soytofu is nonsignificant with F value of 2.44.

It can be seen that soytofu produced by 0.6% $CaSO_4$ with coagulating temperature of 82.5°C of milk was rated superior (overall acceptability score 8.25). The ratings of this soytofu sample with regard to odour (8.5), taste (8.5) and texture (8) were also appreciably higher. The effect on colour of soytofu due to

Table 1 Response Surface experiments to prepare soytofu by employing salt and acid coagulants

Experiment	Salt coagulant		Acid coagulant		Coagulating temperature	
	%	coded value	%	coded value	°C	coded value
1	0.2	-1	0.15	-1	75	-1
2	0.6	+1	0.35	+1	82.5	0
3	0.4	0	0.25	0	82.5	0
4	0.2	-1	0.15	-1	90	+1
5	0.6	+1	0.35	+1	75	-1
6	0.4	0	0.25	0	75	-1
7	0.6	+1	0.35	+1	90	+1
8	0.4	0	0.25	0	82.5	0
9	0.2	-1	0.15	-1	82.5	0
10	0.4	0	0.25	0	82.5	0
11	0.4	0	0.25	0	82.5	0
12	0.4	0	0.25	0	90	+1
13	0.4	0	0.25	0	82.5	0

Table 2 Physico-chemical characteristics of soybean seeds

Physical characters	Values
Hull content (%)	7.98±0.289*
Grain hardness (kg)	8.75±0.238
	19.1±0.185
Bulk density (g/cc)	0.71±0.004
True density (g/cc)	1.21±0.079
% Porosity	43.63±0.312
Colour	Light yellow
1000 grain weight (g)	132.03±0.026
Moisture (%)	10.3±0.395
Protein (%)	39.98±0.036
Crude fat (%)	19.55±0.195
Ash (%)	6.23±0.193
Carbohydrate (by difference) (%)	23.94
Calcium (mg)	257±1.155
Phosphorus (mg)	421.72±1.938
Iron (mg)	11.18±0.068

*Average value ± Standard Deviation

processing variables was negligible. The ratings for odour, taste, texture and overall acceptability of soytofu increased due to increasing level of $CaSO_4$ from 0.2 to 0.6% irrespective of temperature level while appearance of the product is improved when soymilk was coagulated at 75 and 82.5^{oC} temperature.

Soytofu prepared using 0.4% $MgCl_2$ and 90^{oC} temperature of soymilk recorded highest ratings for its appearance (9), odour (8.5), taste (8.75) and texture (9). In general, soytofu sample produced with 0.2% coagulant at 75^{oC} was disliked most as indicated by its lowest organpleptic score with regard to appearance (4), taste (5.25), texture (4.25) and overall acceptability (4.5). The F value suggests that the effect on appearance odour, taste and overall acceptability are significant at 1% level with values of 27.74, 77, 11.3 and 11.64, respectively while texture is significant at 5% level with F value of 6.51. However, color is not affected significantly. The R^2 values were found to be 95.26, 97.88, 88.86, 82.31, 89.09% for appearance, odour, taste, texture and overall acceptability, respectively while it was was 58.6% for color which further indicate that color was not influenced significantly due to variations in concentrations of $MgCl_2$ (0.2-0.6%) and temperature (75-90^{o}C) as employed in making soytofu. Ratings for appearance, taste and texture of the product are improved due to either increasing the concentration of $MgCl_2$ or temperature and both were independent of each other. Rating of odour is maximum with the use of 0.4% $MgCl_2$. The odour of soytofu is improved with increasing the coagulating temperature of soymilk and use of $MgCl_2$ at the level of 0.4 and 0.6%.

With the use of 0.4% $MgSO_4$ and temperature of soymilk as 90^{o}C registered maximum organoleptic ratings with regard to appearance (8.5), odour (8.75), taste (8.75) and texture (9). Improvement in odour, texture and overall acceptability of the product due to increasing the coagulating temperature of soymilk at all the levels of $MgSO_4$ (0.2 to 0.6%) were used in the preparation of soytofu. The appearance score of soytofu is maximum at 82.5^{o}C temperature of soymilk and that is independent of concentration of $MgSO_4$. However, variations in temperature with any of the concentration of $MgSO_4$ were not affecting the taste of the soytofu.

Table 3 Optimum values of coagulant concentration and temperature of soymilk to produce ideal soy soytofu

Coagulants	Coagulant concentration (%)	Temperature of soymilk (^{o}C)
$CaCl_2$	0.6	86.55
$CaSO_4$	0.57	84.9
$MgCl_2$	0.6	87.23
$MgSO_4$	0.46	86.18
Lactic Acid	0.35	89.33
Acetic Acid	0.3	80.25
Citric Acid	0.15	87.45

Data indicate that overall acceptability score of the soytofu sample by lactic acid was maximum (9). Furthermore, ratings for the other sensory attributes i.e. odour, taste and texture were also highest (9, 9 and 8.85, respectively) for this soytofu sample. Soytofu produced with use of 0.25% lactic acid and temperature of soymilk as 82.5^{o}C was of poorest sensory value due to its least overall acceptability score of 3. The model F value suggests that the effect on appearance, odour and taste was significant at 1% level with values of 54.1, 37.7 and 13.33, respectively while texture and overall acceptability, was significant at 5% level with values 6.05, and 6.49, respectively. The R^2 values were found to be 97.51, 96.33, 90.87, 81.19 and 82.22% for appearance, odour, taste, texture and overall acceptability, respectively while it was 43.38% for color which further indicate that color was not influenced significantly due to variations in concentration of lactic acid (0.15-0.35%) and temperature of soymilk (75-90^{o}C) as used in making soytofu. Lack of Fit for the significantly influenced sensory attributes (appearance, odour, taste, texture and overall acceptability) was non significant.

Organoleptic ratings in respect of appearance, odour, taste, texture and overall acceptability were 8.75, 8.88, 9, 9 and 8.75, respectively for soytofu sample wherein acetic acid concentration and soymilk temperature were 0.25% and 82.5^{o}C, respectively. By regression analysis model F value suggests that the effect on appearance, odour, taste, texture and overall acceptability are highly significant at 1% level with values of 80, 153, 19.29, 15 and 15.9, respectively. The R^2 values were found to be 98.35, 99.15, 93.03, 91.52 and 92% for appearance, odour, taste, texture and overall acceptability, respectively while it was was 72.11% for color which indicate that color was not influenced significantly due to variations in concentration of acetic acid (0.15-0.35%) and temperature of soymilk (75-90^{o}C) as used in making soytofu. Lack

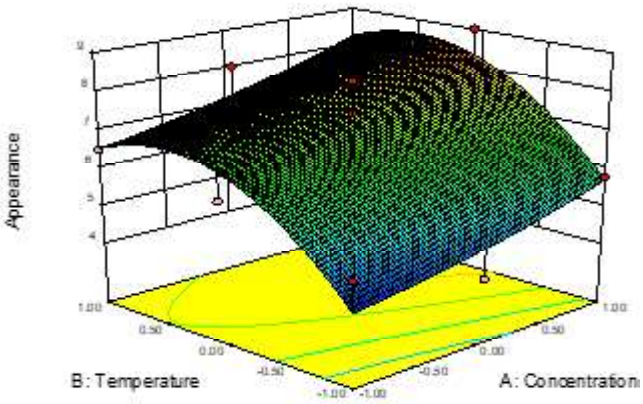

Fig.1. Response surface plot of appearance score of tofu prepared by calcium chloride

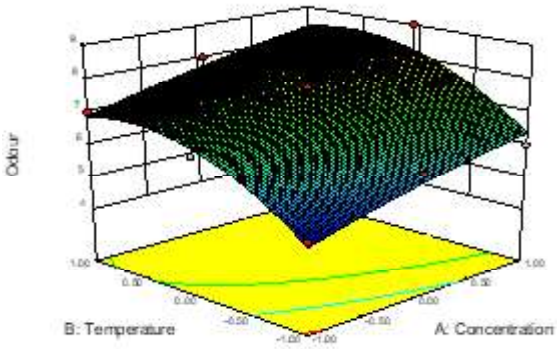

Fig.2. Response surface plot of odour score of tofu prepared by calcium chloride

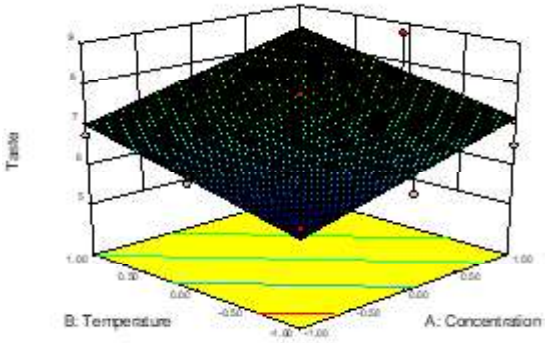

Fig. 3. Response surface plot of taste score of tofu prepared by calcium chloride

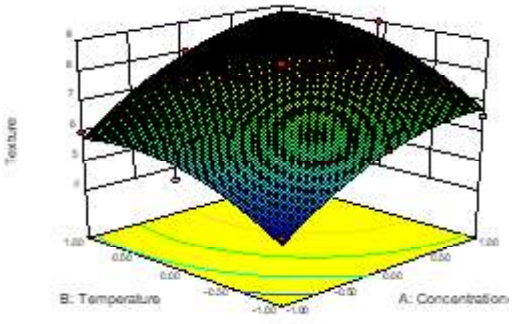

Fig. 4. Response surface plot of texture score of tofu prepared by calcium chloride

Table 4 Yield and organoleptic score of soytofu prepared using seven coagulants at optimized levels of coagulant concentration and temperature of soymilk

Responses	Coagulants						
	$CaCl_2$	$CaSO_4$	$MgCl_2$	$MgSO_4$	Lactic Acid	Acetic Acid	Citric Acid
Yield (%)	27	27.02	25.78	26.91	25	21.93	20
Appearance	8.71	7.29	8.29	7.86	7.29	6.14	6.14
Colour	9	7.43	8.43	7.86	8.86	8	6.86
Odour	8.57	7.8	7.29	7.3	6.57	4.86	7.86
Taste	8.72	7.57	7.86	7.11	7.57	5.43	5.43
Texture	8.71	7.1	7.29	8	7.43	5.57	5.79
Overall acceptability	8.73	6.29	7.57	8.01	7.43	5	6.14
F-value	**	**	**	**	**	**	**
S.Em±	-	0.35	0.43	1.64	0.19	0.55	0.48
CD at 1%	-	0.85	0.95	1.85	0.60	1.0	1.0

** Significant at 1% level of significance

of Fit for the significantly influenced sensory attributes (appearance, odour, taste, texture and overall acceptability) was non significant.

Soytofu occupied first position with regard to overall acceptability score (9) at 0.15% citric acid at 82.5°C. The ratings for appearance, taste and texture in respect of this sample was highest (9). The model F value suggests that the effect on appearance, odour, taste, texture and overall acceptability are highly significant at 1% level with values of 9.42, 7.61, 16.81, 9.91 and 9.58, respectively. The R^2 values were found to be 87.22, 84.46, 92.29, 87.63 and 87.21% for appearance, odour, taste, texture and overall acceptability, respectively while it was 31.26% for color which further indicate that color was not influenced

significantly due to variations in concentration of citric acid (0.15-0.35%) and temperature of soymilk (75-90°C) as used in making soytofu. Ratings for appearance, taste, texture and overall acceptability declined with increase in concentration of citric acid at any of three coagulating temperatures used in the study. Odour score is unaffected due to variations in the levels of citric acid at all the three levels of temperature. Texture and odour of the soytofu sample are improved with increase in temperature of soy milk and variations in the concentration of coagulant used. Increase in coagulating temperature of soy milk does not affect the score of appearance at all the three concentrations of citric acid.

Variations in yield and sensory attributes of soytofu prepared by coagulating soymilk at different temperature with different types of coagulants might be ascribed to nature of coagulating agent and their variable mode of formation of soy gel. Two types of coagulants as employed in this investigation for formation of soytofu were salt type and acid type. Formation of protein gel due to addition of salt in soymilk depend upon ionic strength, water affinity, molecular weight, molecular size, ionization ability of forming cross linkage etc. of coagulants. Similarly, formation of protein gel by incorporation of different acid coagulants is governed by type of acid and their pH lowering effect. Gel strength is influenced by coagulating temperature, type of coagulants and its concentration. Softness or compactness of gel is affected by coagulants water binding or water trapping property of gel which inturn control the yield as well as sensory properties of gel. Several previous researches have also recognized different factors which affect the yield and sensory quality of soy soytofu. Gandhi (2009), Sun and Breene (1991), Shih et al. (1997), Choi et al. (1983), Lim et al. (1990), Sun and Breene (1991), Yuwono (2013), Shih et al. (1997), Gandhi and Bourne (1988), reported that yield of soy soytofu depends upon soybean variety, processing method, soaking time, type of and concentration of coagulants, temperature of soymilk for coagulation, stirring speeds and time of coagulation and pressing of curd, respectively. Similarly, Narayan et al. (1997), Tanwar et al. (1998), Yuwono (2013), Li et al. (2014), Smith et al. (1960) and Wang (1967) also bring into being that soybean variety, storage condition of soybean, method of production, type and concentration of coagulant and pressing of soytofu affect the sensory profile of soytofu.

Yield and sensory evaluation of soytofu prepared by optimized levels of coagulants and temperature of soymilk

Yield and organoleptic score of soytofu prepared using selected coagulants at optimized levels shows that use of calcium sulphate and calcium chloride as coagulant gave maximum yield 27.02 and 27%, respectively as compared to other coagulants. Soymilk coagulated with citric acid produced minimum yield (20%) of soytofu. Krishna et al. (2002) also observed similar results of calcium chloride in preparation of soytofu (Table 4).

Soytofu was evaluated by the panelists to determine its acceptability. It was evaluated for appearance, colour, odour, taste, texture and overall acceptability on nine-point hedonic scale. Soytofu prepared from calcium chloride at concentration of 0.6% and 86.55°C temperature of soymilk gives highest scores for appearance, colour, odour, taste, texture and overall acceptability with values 8.71, 9, 8.57, 8.72, 8.71 and 8.73, respectively. Statistical analysis of the data showed significant difference at 1% level among the soytofu samples prepared from different coagulants. Soytofu prepared from calcium chloride was found to be most satisfactory as it had utmost overall acceptability(Fig.1-4). This was followed by magnesium sulphate, magnesium chloride and lactic acid. Previous researches reported that soytofu prepared with calcium salts had very superior general acceptability in terms of colour, texture, taste and overall acceptability (Table 4).

Soytofu prepared by using $CaCl_2$ as a coagulant was found to be finest for making soytofu. The moisture, protein, crude fat, ash and carbohydrate content of soytofu were recorded as 68.59 ± 0.02, 50.3 ± 0.302, 12 ± 0.120, 5.9 ± 0.911 and 15.35 ± 0.03%, respectively. Shokunbi et al. (2011) also determined proximate composition of soytofu which were slightly higher than present investigation. The ash content reported in this work (5.9%) is slightly higher than reported by Shih et al. (1997). Such variations can be attributed to the variation in preparation methods as well as the types of soybean used to produce soytofu. However, Obiegbuna et al. (2014) recorded lower values for proximate composition of soytofu.

Conclusions

On the basis of above observations it can be concluded that the concentration of coagulants and levels of coagulating temperature of soymilk were different for making ideal soytofu. The optimized levels of coagulants and coagulating temperature for making quality soytofu was 0.6, 86.55, 0.57, 84.9, 0.6, 87.23, 0.46, 86.18, 0.46, 0.35, 89.33, 0.3, 80.25 and 0.15%, 87.45°C, respectively for $CaCl_2$, $CaSO_4$, $MgCl_2$, $MgSO_4$, lactic acid, acetic acid and citric acid coagulants. Preparation of soytofu sample from RSM optimized level and their organoleptic evaluation indicated that among the seven coagulants calcium chloride was best for making quality soytofu with higher and it contained 68.59, 50.3, 12, 5.9 and 15.35% moisture, protein, crude fat, ash and carbohydrates, respectively.

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RESEARCH ARTICLE

Probiotication of *Bifidobacterium Spp*. in dairy and non-dairy mango juice medium – A Green approach

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Abstract: Mango (*Mangifera indica L.)* is a climacteric, tropical drupe of the *Anacardiaceae* family. This study aims at determining the ideal synbiotic combination using mango as a natural prebiotic source in two different mediums, dairy, and non-dairy, for two different probiotics *Bifidobacterium infantis* and *Bifidobacterium lactis*. Skimmed milk and sugarcane were observed on analysis to promote the growth of these probiotics and were the chosen dairy and non-dairy substrates used in combination with mango. The ideal synbiotic combination of Mango with dairy /non-dairy medium was optimized on the basis of sensory evaluation supported by total viable counts. Fermentation for 4 hours of the ideal synbiotic combinations with 0.1% inoculum of probiotic cultures was done. The 03:07 ratio of mango: skimmed milk and mango: sugarcane with 4 hours of incubation gave desirable results with increasing microbial counts, drop in pH and nominal increase in acidity as fermentation time progressed. This ideal combination of Probiotics with Mango in both dairy and non-dairy medium could be a promising blend in the food and beverage industry for formulating naturally functional products.

Keywords: Beverage, Green approach, Mango, Prebiotics, Probiotics, Synbiotics, Sugarcane juice, Skim milk

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Introduction

Consumers show interest in ingesting diets to maintain the health and prevent the degenerative or chronic diseases such as diabetes, cancer, hypertension (Granato, Branco et al. 2010). The growing awareness of the relationship between diet and health has led to an increasing demand for food products that support health above and beyond providing basic nutrition i.e., functional foods. Functional foods comprise of conventional foods containing naturally occurring bioactive substances, foods enriched with bioactive substances and synthesized food ingredients introduced to traditional foods (Grajek W, Olejnik A et al. 2005). Prebiotics and probiotics are becoming leaders in the functional foods category as it is considered to exert positive effects on our body (Prado FC, Prada JL et al. 2008).

Probiotics have been a functional food of interest since the earliest of times. Probiotics are nonpathogenic microorganism which improves the intestinal microbial balance, provides health benefits and prevents some diseases (FAO, 2002). Prebiotics are non-digestible dietary components that pass through the digestive tract to the colon and are a potential substrate for fermentation by the microbiota. High potential is attributed to the simultaneous use of probiotics and prebiotics. The term "synbiotic" was first used in 1995, to describe a combination of synergistically acting probiotics and prebiotics (Bengmark, S., 2005).

Fruits & vegetables such as banana, asparagus, beans, cereals, onion, garlic, Jerusalem artichoke, chicory, are natural prebiotic sources. Apart from being a prebiotic source, fruits and vegetables also have other functional properties which is an added benefit. Moreover, the fruit juices also have a good refreshing taste and are a consumption choice for people of all age groups since they are perceived as healthy foods. (Jankovic, Sybesmaet.al., 2010).

With the idea of utilizing locally available indigenous fruit as a natural prebiotic source, this study explores the use of mango in dairy and non-dairy medium as a substrate for growth of Bifidobacterium species. Mango could be a better, greener and

safer alternative as a prebiotic for use in the ideal synbiotic combination.

This study aims at determining the ideal synbiotic combination using mango as a natural prebiotic source in two different mediums, dairy, and non-dairy, for the probiotics *Bifidobacterium infantis* and *Bifidobacterium lactis*

Materials & methods

Materials

Fresh mangoes were procured from the local market (Banganapalli variant). Fresh sugarcane juice was purchased from the local vendor and stored under refrigeration condition prior to use. Branded skim milk in tetra pack was procured from the supermarket and used for the study.

Probiotic strain

Proven probiotic strains of *Bifidobacterium infantis* (UBBI-01) & *Bifidobacterium lactis* (UBBLa-70) was obtained in the lyophilized forms from Unique biotech, Hyderabad for the study.

Probiotic Culture

The cultures were prepared by incubating the probiotic strains in Bifidobacterium broth (Himedia No. M1395) overnight at 37°C under anaerobic conditions to obtain the working culture. This was maintained at 5°C and sub cultured in Bifidobacterium broth.

Total Plate Count-TPC

Dilutions were placed on selective Bifidobacterium agar (Himedia No. M1396) and grown under anaerobic conditions in Anaerobic jars (GasPak System) for 48 hours at 37°C (Vinderola & Reinheimer, 1999).

Turbidimetric method

Turbidimetric method is an indirect, rapid method used for following growth of bacterial cultures. A spectrophotometer can be used for measurement of cell mass and in this indirect determination method the absorbances were estimated before and after incubation (Pelczar M. J.et al. ,2003). The optical density of the samples was checked at the wavelength of 600nm

Gram staining

Representative colonies were taken and subjected to Grams staining techniques (RajanS. & Christy R., 2010) and observations were recorded.

Assessing the chosen dairy and non-dairy substrates for promoting Probiotic growth

Skimmed milk and sugarcane juice were checked for their suitability as a potential dairy and non-dairy substrate for *B. lactis* and *B. infantis,* by inoculating the probiotic cultures (100 µl in 10 ml of substrate), incubating for 24 hours and determining the bacterial viable counts and change in absorbance.

Assessing prebiotic efficacy of mango

The prebiotic potential of mango was observed by inoculating probiotic cultures (100 µl in 10 ml of substrate), incubating for 24 hours and determining the bacterial viable count and change in absorbance.

Sample Preparation

The selected mango fruits (Banganapalli variant) were washed and cleaned with potable water. The inedible parts like seed, rind and peel were removed. The fruit pieces were ground in a blender and the pulp of the fruits were used in the study. The pulp was stored frozen for further use. Mango pulp, skimmed milk, sugarcane juice were individually heated at 80 ºC for 20 minutes and were used to prepare different combination.

Optimization of the ideal blend ratio

The dairy blend was prepared with skimmed milk and mango pulp, while the non-dairy blend was prepared with sugarcane juice and mango pulp in different ratios as given in Table 1

Sensory evaluation

The different ratios of fruit and the dairy medium/fruit and non-dairy medium were subjected to sensory evaluation using a five points hedonic scale. The sensory attributes were evaluated by 30 panelists. The best combination acceptable to the consumers was determined and the ideal ratio of mango: dairy, mango: non-dairy was chosen for further study.

Table 1: Blend ratios

	Blend ratio of mango based dairy medium			
Blend ratio	D1	D2	D3	D4
Mango: Skimmed milk	01:09	02:08	03:07	04:06
	Blend ratio of mango based non-dairy medium			
Blend ratio	ND1	ND2	ND3	ND4
Mango: Sugarcane juice	01:09	02:08	03:07	04:06

Assessing the chosen substrates for promoting probiotic growth

The viable count was determined by plate count technique.100 µl of the *B. infantis* was inoculated into all the combinations. The blends were enumerated on selective Bifidobacterium agar &grown under anaerobic conditions in for 48 hours at 37^{0}C (Vinderola & Reinheimer, 1999). Turbidimetric technique was also used as the indirect method of measuring the growth (Pelczar et al. 2003). The viable count of *B. lactis* was also similarly determined by total plate count and turbidimetric technique.

Analysis of the ideal synbiotic combination

Assessing the Growth pattern

The ideal combination was freshly prepared and 0.1 % of each probiotic culture was inoculated for the study. Viable counts and turbidity were determined at 0 hours and 4 hours.

Measurement of specific chemical changes produced

pH and acidity were determined by pH meter, titration (AOAC, 2016) respectively. Both pH and TA values of the formulated beverages was measured at 0, 2 and 4 hours during the incubation period.

PCR

PCR is used a novel approach to confirm the probiotic growth in mango-based combinations.

Genomic DNA from the culture& probiotic product was extracted using a DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol Genomic DNA prepared was used as a template for PCR amplification. The amplification was done as mentioned by Yeon S, 2007 & the amplified products were analyzed by electrophoresis on a 2% agarose gel containing 1µl of ethidium bromide, and the DNA bands were visualized under UV illumination and photographed

Results and Discussion

Turbidimetric method

The optical density of *B. infantis* and *B. lactis* before incubation was found to be 0.377±0.039, 0.53±0.022 and after incubation was found to be 0.721±0.108, 0.928±0.017 respectively (shown in figures 1 & 2).

TPC

Using the plate count method, *B. infantis* and *B. lactis* was found to have 9.9242±0.029, 9.0492±0.062 log cfu/ml in the working culture used in the study.

Gram staining

Representative colonies were taken and subjected to Grams staining techniques and it was observed that both *B. infantis* and *B. lactis* were stained purple, indicating that both are gram positive and rod shaped.

Assessing the chosen substrates for promoting probiotic growth

The ability of the selected dairy and non-dairy substrates (milk and sugarcane juice) to promote the growth of probiotics *B. infantis* and *B. lactis* individually was evaluated. It was observed that the viable count of *B. infantis* and *B. lactis* in the substrate milk, after 24 hours, was found to have increased from initial values of 9.9242±0.029, 9.0492±0.062 log cfu/ml to 11.1289±0.019 and 10.3388±0.019 log cfu/ml respectively. This was also supported by the increase in OD values determined by turbidimetric determination. The optical density of *B. infantis* and *B. lactis* in the substrate milk, before incubation was found to be 0.514±0.016 , 0.512±0.04 and after incubation was 1.212±0.012, 1.123±0.01 respectively. Milk, the dairy medium used in Mango-dairy combination is a proven substrate which promotes probiotic growth. Dairy-based fermented products and yogurts have been utilized as potential probiotic products since the ancient times (Bansal S et al. 2016).

The viable count of *B. infantis* and *B. lactis* in the substrate sugarcane was found to increase to 11.5021±0.080 log cfu/ml and 10.7695±0.008 log cfu/ml respectively. Similarly there was also an optical density of *B. infantis* and *B. lactis* in the substrate sugarcane, from 0.521±0.005 to 1.618±0.006 and 0.502±0.008 to 1.311±0.005 respectively.

An increase in the viable count of Bifidobacterium strains in native soymilk medium was observed by Havas et al. (2015). The fermentation processes cultured with initial cell concentrations in 10^5-10^7 cfu/ml resulted in 10^8 cfu/ml after 8–12 h of incubation in soymilk, and were kept viable up to the end of fermentation (48 h). Volumetric productivities of *B. bifidum* (B-3.2), *B. bifidum* (B-7.1) & *B. breve* (B-9.14) were 1.6×10^{10} cfu/L.h, 4.5×10^{10} cfu/L.h and 7.6×10^9 cfu/L.h, respectively. The values of *B. lactis* (Bb-12) &*B. longum* (Bb-46) probiotic strains were 2.7×10^9 cfu/L.h and 1.0×10^{10} cfu/L.h.

Assessing prebiotic efficacy of mango

The exact component responsible for mango's prebiotic capacity is unknown. Its prebiotic potential could due to the presence gallic acid, dietary fiber, resistant starch & also pectin content (Yadav et al. 2010; Ramulu et al. 2003; Chatterjee et al. 2016; Holscher, 2017; Slavin, 2013). The Prebiotic potential of mango was observed by bacterial viable count and turbidimetric method. The viable count of *B. infantis* and *B. lactis* was found have increased to 11.2508±0.024 and 10.3660±0.012 log cfu/ml on incubation. This was also supported by the increase in OD values

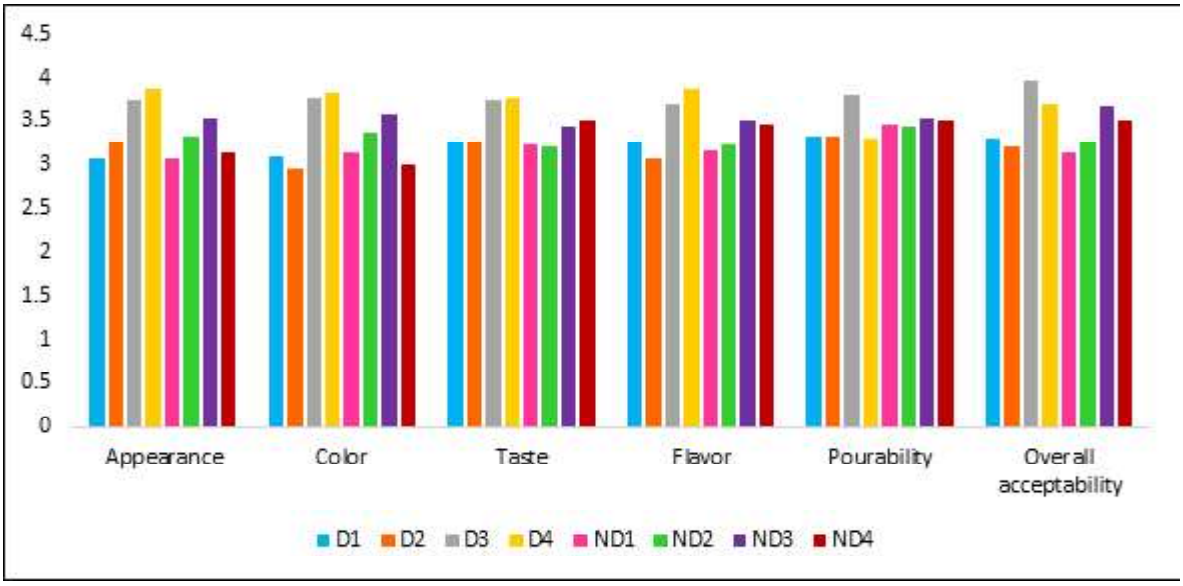


Fig. 1 Sensory evaluation of Dairy Blend

which was found to increase from 0.934±0.002 to 1.458±0.006 for *B. infantis* and from 1.222±0.01 to 1.423±0.01 for *B. lactis.*

Optimization of the ideal blend ratio used in the study

Sensory evaluation

Fruit juices have been suggested as an ideal medium for the functional health ingredients because they inherently contain beneficial nutrients, they have taste profiles that are pleasing to all the age groups (Vasudha and Mishra, 2013). It could be clearly observed that the panelists showed more preference as the proportion of mango juice increased (figure 1).

Statistical analysis of the results revealed that significant difference existed between the overall acceptability score ($p<0.05$) of samples between the different blend ratios in both dairy and non-dairy mediums.

Assessing the ability of the probiotic culture to utilize the blends as a prebiotic substrate

Turbidimetric method

The change in optical density before and after incubation for 24 hours, in the different mango-skim milk and mango-sugarcane juice blends inoculated with *B. infantis* was observed to increase and the highest of variations was found in D3I, ND3I.

Similarly the change in optical density in different mango-skim milk and mango-sugarcane juice blends inoculated with *B. lactis* was observed and the highest variations was seen in ND3I, ND3L (table 2).

TPC method

After 24 hours increase in total viable count of *B. infantis* inoculated in the different mango-skim milk and mango-sugarcane juice blends was observed. It can be observed that D3I and ND3I show higher log cfu values (11.9173±0.003), (12.0816±0.004). Statistical analysis showed that the viable count of dairy and non-dairy combination inoculated with *B. infantis* was found to have no significant difference, hence comparable.

Similarly, after 24 hours an increase in total viable count of *B. lactis* inoculated in the different mango-skim milk and mango sugarcane juice blends was observed. D3L and ND3L showed higher log cfu values (10.6117±0.003), (11.0792±0.045) when compared to other samples (see Table 2). Statistical analysis showed that the viable count of dairy and non-dairy combination inoculated with *B. lactis* is found to have no significant difference, hence comparable.

The results of this study were similar with the results of study by Bujna et al. (2018) which showed that all investigated *Bifidobacterium* and *Lactobacillus* strains were able to grow well in the apricot juice without supplement of any nutrients meaning this matrix in itself was suitable medium for propagation of probiotic bacteria. In all cases the cell numbers at 24 h of fermentation were higher than 10^8 cfu/mL, and cell yields varied from 1.15×10^{10} cfu/L h to 1.78×10^{10} cfu/L h.

Analysis of the ideal synbiotic combination

The ideal synbiotic combination of 3:7 mango with dairy /non-dairy medium was optimized on the basis of the sensory score, supported by highest total viable counts in this ratio.

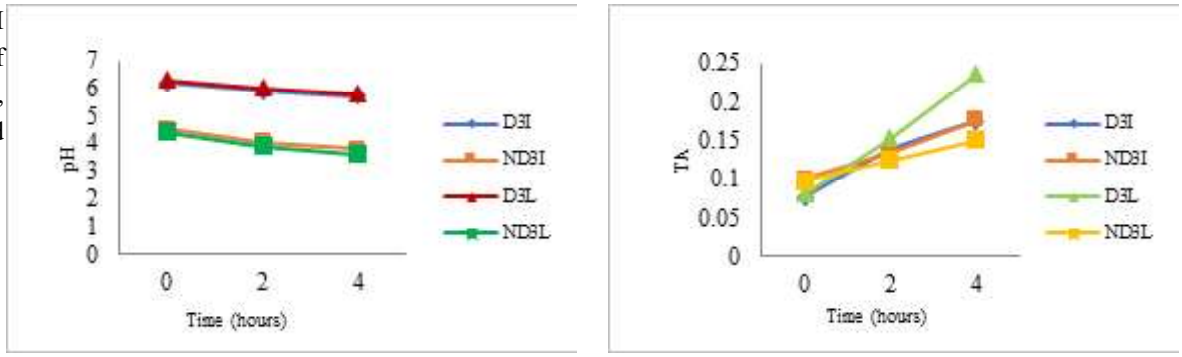


Fig. 2 pH & TA of D3I, ND3I, D3L and ND3L

D3I- 03:07 mango skim milk blend inoculated with *B.infantis*; ND3I-03:07 mango sugarcane blend inoculated with *B.infantis*; D3L- 03:07 mango skim milk blend inoculated with *B.lactis*; ND3L-03:07 mango sugarcane blend inoculated with *B.lactis*

Table 2 Total plate count and variation in OD values of the dairy and non-dairy blends

	TPC	OD
	Dairy and non-dairy mango blends inoculated with *B infantis*	
D1I	10.6018±0.072	0.167±0.002
D2I	10.6086±0.030	0.234±0.003
D3I	11.9173±0.003	0.808±0.007
D4I	11.3848±0.018	0.661±0.004
ND1I	10.5433±0.032	0.287±0.002
ND2I	10.5289±0.057	0.577±0.001
ND3I	12.0816±0.004	0.978±0.005
ND4I	10.5595±0.035	0.554±0.021
	Dairy and non-dairy mango blends inoculated with *B.lactis*	
D1L	9.2709±0.014	0.316±0.013
D2L	9.3603±0.031	0.451±0.006
D3L	10.6117±0.003	1.136±0.004
D4L	10.4493±0.033	0.516±0.02
ND1L	9.4771±0.002	0.42±0.011
ND2L	9.4722±0.008	0.21±0.003
ND3L	11.0792±0.0457	0.489±0.005
ND4L	9.4771±0.001	0.374±0.007

The values are the means of triplicate determination. ± indicates the standard deviation of the mean

Table 3 OD values at 0th hour and after 4 hours

	0 Hour	4 Hour
D3I	1.486±0.002	1.726±0.004
ND3I	1.444±0.003	1.716±0.002
D3L	1.536±0.002	1.8775±0.001
ND3L	1.417±0.001	1.785±0.005

The values are the means of triplicate determination. ± indicates the standard deviation of the mean

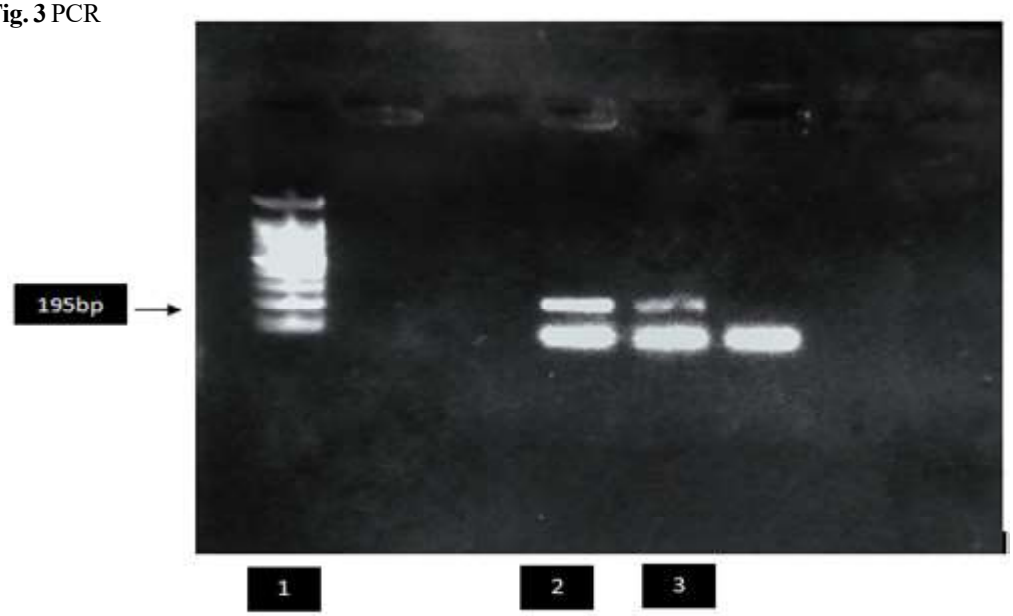
Assessing the growth pattern

The growth pattern of the culture in the combinations during 4 hours of incubation was assessed by determining colony forming unit and optical density at various time intervals. The colony count of D3I increased from 10.1785±0.024 to 10.4413±0.010 & for ND3I the colony count increased from 10.150±0.007 to 11.4771±0.001. Similarly, the colony count of D3L increased from 9.7898±0.017 to 10.2936±0.013 & for ND3L the colony count increased from 9.7505±0.019 to 10.3658±0.020. The change in optical density of D3I, ND3I, D3L, ND3L from 0 hour to after 4 hours is shown in table 3.

In the study by Nguyen BT et al. (2019) pineapple juices were inoculated with different probiotic bacteria (Lactobacillus acidophilus, Lactobacillus plantarum, and Bifidobacterium lactis) at an initial cell density of about 10^6-10^7 cfu/ml. All investigated Lactobacillus and Bifidobacterium strains were able to grow well in the pineapple juice. In the case of bifidobacteria it reached a level of 10^9 cfu/ml after 24 of fermentation.

Physiochemical analysis: pH & titrable acidity

Fig. 3 PCR



(1) 100bp ladder (2) *Bifidobacterium lactis* culture (3) Mango inoculated with *B. lactis*

Both pH and titrable acidity values of the formulated combinations were measured at 0, 2 and 4 hours during the incubation period. The pH of all the four combinations was found to decrease gradually over the hours (shown in figure 2). The percentage TA of mango in dairy and non-dairy medium (*B. infantis)* observed at 0, 2, 4 hours recorded an increase and for mango in dairy and non-dairy medium (*B. lactis)* the percentage TA was found to increase as incubation time increases (figure 2). Andrade et al (2020) has also reported a similar decrease in pH from the initial 5.28 & 6.47 of jerivá and macaúba palm fruit pulps to 4.76 and 5.20 respectively during the fermentation with *B. lactis*

The results obtained of increased log cfu/ml and acidity combined with drop in pH are indicative that 3:7 ratio of mango:skimmed milk and mango: sugarcane were utilized as a prebiotic source for growth by *B. lactis and B. infantis*.

PCR

The presence of probiotic culture *B. lactis* was confirmed using specific primers, by conventional PCR. The sequence of the *B. lactis* primer used is forward primer: CCCTTTCCACGGGTC-CC and reverse primer: AAGGGAAA-CCGTGTCTCCAC The PCR product was represented by a single band in the corresponding region of the DNA marker ladder (figure 3).

Freeze dried powder of the ideal symbiotic combinations

Freeze-drying is a technique adopted for successful long-term preservation of material. The 3:7 blend of the ideal synbiotic combinations were freeze dried and preserved for further use.

Conclusion

Mango as a prebiotic source in dairy and nondairy medium showed promising and comparable results for both the bifidobacterium species. The blend ratio of 3:7 for the both the mediums was observed to be the ideal synbiotic combination which was optimized on the basis of the sensory score, supported by highest total viable counts in this ratio. It was observed that the ideal synbiotic combination of mango: dairy and mango: non-dairy medium containing 0.1% of *B. infantis or B. lactis* with a fermentation time of 4 hours provided desirable results for viable growth of probiotic culture and activity in terms of pH and acidity and hence can be used in probiotic product formulations using a green approach.

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RESEARCH ARTICLE

Process optimization for the manufacture of red rice (*Oryza sativa* L.) *kheer*

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Abstract: Red rice contained 13.92±0.13 % moisture, 2.11±0.07 % fat, 1.80±0.01% total ash and 144.45 ± 1.36 (mgGAE/100g) total phenolic content, respectively. The present study was aimed to prepare *kheer* using red rice @ 18g (T_1), 20g (T_2) and 22g (T_3). Coconut sugar was used @15 g in all the combinations. Fresh toned milk (3.0 % fat and 8.5 % SNF) was used with broken red rice (Half broken) grains. Cleaned broken rice was soaked in water (rice to water ratio as 1: 2.5) and cooked at 93°C for about 15 minutes. *Kheer* prepared using 20 g of rice (T2) was selected as optimized one on the basis of sensory evaluation. The developed red rice *kheer* was acceptable upto10 days of storage at refrigerated temperature. Optimized *kheer* contained 3.02 ± 1.21% fat, 56.16 ± 2.11% moisture and 43.84 ±3.05 % total solids and 356.24± 3.45 Total Phenolic Content (mgGAE/100g)

Keywords: Red rice, *Kheer*, Coconut sugar, Shelf- life, Total phenolic content

Introduction

Kheer is very popular traditional dairy product consumed all over India. *Kheer* is considered as nutritious dairy dessert. But its production is limited to household level and unorganized sector only. Red rice is superior to white rice due to its nutritional profile and health benefits. So, the use of red rice in place of white rice will not only offer opportunities to develop value added food products but also enhance the nutritional and functional properties of *kheer*.

Red rice is rich in antioxidant, minerals and protein as compared to white rice. Due to its lower glycemic index (63.15 ± 2.63mg/dl) than white rice, red rice can be a part of the diets of diabetics as well as persons suffering from other non-communicable diseases. Red rice is a storehouse of nutritional excellence and is a healthier alternative to white or polished rice (Raghuvanshi et al. 2017). Due to the high fibre content, red rice possesses the ability to keep healthy metabolic function and bowel function also. It can help in weight management due to anthocyanins. Red rice is highly useful in treating and controlling different ailments due to its nutritional value and its use is very common among the practitioners of traditional medicines and communities. (Rathna Priya et al. 2019).

Increasing threats of ailments such as obesity, hypertension and diabetes mellitus etc. has become a serious concern for the people suffering with such ailments due to the high intake of sugar in food and confectionary products. Production of sweeteners made naturally with low Glycemic Index (GI) can be a solution for such ailments. Only red rice as a main ingredient won't serve the purpose of preparing a healthy *kheer*, so coconut sugar was incorporated in the *kheer* because of its nutritional profile. Coconut sugar was reported to have glycemic index of about 35 (Kusumawaty et al. 2012). Foods with low glycemic index (GI) are important for diabetes, obesity, heart disease and hypertension (Jenkins et al. 1981). Coconut sugar is a good source of minerals like zinc, iron, calcium, phosphorous, potassium and magnesium. Hebbar et al. (2015) also reported that coconut sugar is good source of vitamins, such as vitamin C, B complex, antioxidants, polyphenols and dietary fibres. Now- a -days, coconut sugar becomes very popular among health conscious consumers. Milk is deficient in iron, vitamin C and other minerals are also found in less quantity. So far no work has been carried out on the utilization of red rice for preparation of traditional dairy products; hence this work has been planned for optimization a process for manufacture of *kheer*. Therefore, red rice, coconut

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sugar and milk will formulate a perfect, balanced and healthy combination for *kheer*.

Materials and Methods

Red rice of Truefarm and coconut sugar of Tropicoco Kokos Natural was purchased via online from Flipkart. Toned milk of Amul with 3.0% fat and 8.5% SNF was purchased from the local market of Varanasi.

Proximate composition of red rice

By following the method of AOAC (2000), moisture and fat content was calculated by taking 5g of sample. The ash content of finely ground sample of red rice was estimated by following the protocol of (AOAC 2000). Total phenolic content was determined by using Folin Ciocalteau procedure of (Singleton and Rossi, 1965).

Formulation of red rice *kheer*

All the ingredients were mixed together in required quantities. Red rice was incorporated in three different quantities which were 18, 20 and 22g, respectively. Coconut sugar was incorporated @ 15 g. Fresh toned milk was used in constant quantity of 500 ml in all the three treatments. *Kheer* prepared by adding white rice 12.5g into 500ml of toned milk was taken as control. Notations for control and different treatments are shown below:

T_0 = White rice (12.5g) + Sugar (25g) + Toned Milk (500ml) as control

T_1 = Red Rice (18g) + Coconut Sugar (15g) + Toned Milk (500ml)

T_2 = Red Rice (20g) + Coconut Sugar (15g) + Toned Milk (500ml)

T_3 = Red Rice (22g) + Coconut Sugar (15g) + Toned Milk (500ml)

Preparation of *Kheer*

Initially, clean and dry red rice grains were broken (half) into a mixer grinder. *Kheer* was prepared by using the standard method (Kumar et al. 2005) with some modifications. Red rice was soaked in water at room temperature(rice: water as 1:2.5) for 30 minutes and precooked at 93°C for 10 min. Precooked red rice was added to boiled toned milk (3% fat and 8.5% SNF) with continuous agitation of the mixture (**Fig.1 & Fig.2)**.

Sensory evaluation of red rice *kheer*

Sensory evaluation of red rice *kheer* was done by panel of 20 semi -trained judges in respect of colour & appearance, flavour, body & texture and overall acceptability. Score card was provided to all judges comprising 9 points Hedonic Scale.

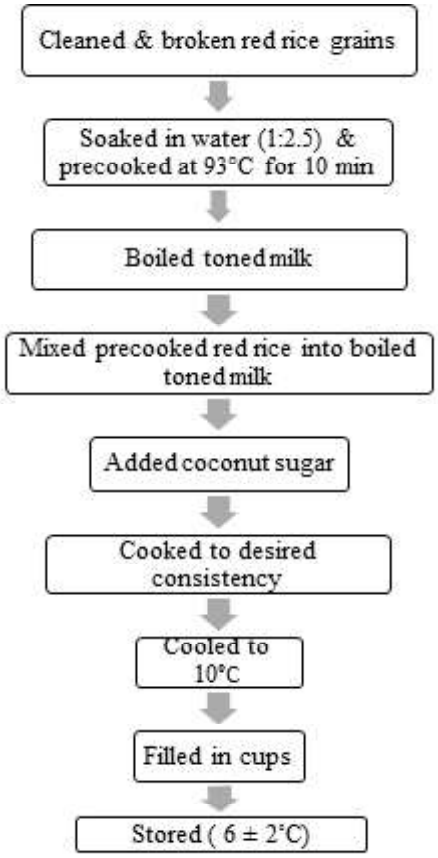

Fig.1 Flow diagram for preparation of red rice *kheer*

Chemical composition of red rice *kheer*

By following the method of AOAC (2000), moisture content was calculated by taking 5g of sample. After the determination of moisture, the left residue was taken for calculation the total solid content.

Moisture content was calculated by the formula:-

$$\text{Moisture (\%)} = \frac{(W2 - W1)}{(W1 - W)} \times 100$$

Where,

W = Weight of empty dish

W1 = Weight of dish with the sample

W2 = Final weight of dish

Statistical Analysis

T^1 T^2 T^3

Fig. 2 Different formulations of red rice *kheer*

Table1 Proximate composition and total phenolic content of red and white rice

Constituents	White Rice	Red Rice
Moisture (%)	12.85±0.15	13.92±0.13
Crude fat (%)	0.67±0.01	2.11±0.07
Total Ash (%)	0.57±0.04	1.80±0.01
Total Phenolic Content (mgGAE/100g)	25.09 ± 1.10	144.45 ± 1.36

Data represents as mean ± Standard Deviation (n = 3) at (p>0.05)

The data obtained during the course of investigation were subjected to statistical analysis. One-way analysis of variance (ANOVA) was applied to analyze test of significance.

Results and Discussion

Shelf- life of red rice *kheer*

Based on the sensory characteristics of the different combinations T2 was selected for shelf- life study with control sample of white rice *kheer. Kheer* was packed in polystyrene cups of 100 g capacity and stored at 6±2°C in refrigerator. The pH, acidity and microbial parameters of red rice *kheer* were determined at an interval of 2 days for a period of 10 days

Proximate composition of red rice

The moisture and fat per cent content of red rice was 13.92 ± 0.13, 2.11± 0.07 which was on the higher side then the control i.e. 12.85 ± 0.15 and 0.67 ± 0.01, respectively (Table 1). Similar results were reported by (Raghuvanshi et al. 2017). They found that red rice have 12.75% moisture, 1.53 % ash and 1.81% fat. The ash content of red rice was also higher than white rice as red rice is highly rich in fibre as compared to wheat and many other vegetables. (Gopalan et al. 2007). The total phenolic content of red rice was 144.45 ± 1.36 mg catechin equivalent/g observed higher than that of white rice 25.09 ± 1.10 mg catechin/g. Sompong et al. (2011) estimated the total phenolic content of total ten different red rice varieties ranging between 79.2 and 691.4 mg FA equivalent/100g. The total phenolic content and total flavonoids content of red rice was found to be 143.38 mg GAE/100 gm and 120 mg R.E. /100 gm respectively (Raghuvanshi et al. 2017).

Sensory analysis

Table 2 depicts that the sensory score for body and texture of T_2 formulation was found to score highest i.e. 8.50 ± 0.24 which was found to be statistically non-significant over other treatments at (P>0.05). The sensory score for overall acceptability of T_2 formulation was highest 8.11 ± 0.19 which was found to be statistically non-significant over other treatments at (P>0.05). Therefore, T_2 was the optimized product on the basis of sensory evaluation. Addition of more than 20 g of red rice in *kheer* resulted in highly viscous product which results in poor score. Similar results were observed by Sandey et al. (2009). They found that flavour score of cereal based dairy product prepared with 10% and 20% of little millet flour were statistically at par and product with 10% addition of little millet resulted in smooth and soft body and texture.

Chemical composition of red rice *kheer*

Optimised red rice-based *kheer* has total solids content of 43.84± 3.0, whereas the total solids per cent of control was 44.57 ± 2.78. Similar results were reported by (Mor et al. 2017). The optimised product has the higher total solid content than that of control which was significantly not different from each other (Table 3). The statistical data shows non-significant difference between values (p>0.05). Deshmukh et al. (2017) estimated the total solid content of poppy seeds *kheer* which was observed as 41.42%.

Coconut sugar also influenced the free radical capacity significantly (P<0.05). Low et al. (2015) studied the antioxidant activity of probiotic ice cream by incorporating different levels of cane sugar and coconut palm sugar. Total phenolic content of

Table 2 Sensory evaluation of red rice *kheer*

Treatments	Body and Texture	Colour and appearance	Flavour	Overall acceptability
T0	7.50± 0.31	7.00± 0.23	8.50± 0.17	7.66± 0.23
T1	7.50± 0.26	7.50± 0.29	7.50± 0.14	7.50± 0.24
T2	8.50± 0.24	7.83± 0.18	8.00± 0.16	8.11± 0.19
T3	6.88± 0.31	7.25± 0.25	7.00± 0.26	7.04± 0.27

Values are mean ± Standard Deviation (n=20)

Table 3 Chemical composition of optimized red rice *kheer*

Constituents	Control (T^0)	Optimised (T^2)
Fat (%)	2.45± 0.89	3.82± 1.21
Moisture (%)	55.43± 2.88	56.16 ± 2.11
Total Solids (%)	44.57± 2.78	43.84± 3.05
Total Phenolic Content (mgGAE/100g)	28.21± 1.08	356.24± 3.45

Data represented as mean ± Standard Deviation (n = 3) at (p>0.05)

Table 4 Changes in pH, acidity and microbial count of red rice *kheer* during storage

Storage duration(days)	pH	Acidity (%)	SPC (log CFU/g)
0	Control : 6.34±0.40	Control : 0.34 ± 0.01	Control : Nil
	Optimized: 6.40 ± 0.60	Optimized: 0.21 ± 0.03	Optimized: Nil
2	Control : 6.30± 0.42	Control : 0.36 ± 0.03	Control : 1.08 ± 0.40
	Optimized: 6.37± 0.53	Optimized: 0.22 ± 0.10	Optimized: 1.02 ± 0.20
4	Control : 6.23± 0.72	Control : 0.40 ± 0.12	Control : 2.45± 0.12
	Optimized: 6.32 ± 1.01	Optimized: 0.21 ± 0.40	Optimized: 2.32 ± 0.42
6	Control : 6.24± 0.71	Control : 0.45 ± 0.03	Control : 3.40 ± 0.22
	Optimized: 6.27 ± 0.50	Optimized: 0.20 ± 0.08	Optimized: 3.53 ± 0.23
8	Control : 6.20± 0.22	Control : 0.50± 0.06	Control : 4.35 ± 0.21
	Optimized: 6.24 ± 1.01	Optimized: 0.22 ± 0.20	Optimized: 6.12 ± 0.20
10	Control : 6.10± 0.64	Control : 0.54 ± 0.05	Control : 4.90 ± 0.31
	Optimized: 6.20 ± 0.65	Optimized: 0.21 ± 0.31	Optimized: 4.42 ± 0.45

Values mentioned as mean ± Standard Deviation, (n=3) at (p>0.05)

optimized *kheer* was significantly higher (P<0.05) as compared to control *kheer* i.e. 58.21 ± 1.08 and 356.24 ± 3.45 mgGAE/100g, respectively. Mudoi and Das (2019) analyzed sixteen varieties of red rice for various phytochemicals, antioxidant activities and a few mineral contents. They reported that the antioxidant activities were observed to be the highest 96.00±0.26% in 'Negheribao' (for brown form of rice)and 86.35± 3.88% in 'Kenekuabao' (for polished form of rice) and the lowest 81.54±0.23% in 'Betu'(for brown form of rice) and 59.65±4.64 % in 'Ranga Dariya' (polished rice), respectively. Red rice as well as coconut sugar has influenced the phenolic content of *kheer*. Similar results have been reported by Victor and Orsat (2018) who studied that palm sugar has appreciable amount of antioxidant activity and total phenolic content also.

Shelf- life of red rice *kheer*

During storage, the pH of red rice *kheer* was decreased and acidity was increased significantly (P< 0.05) and their interaction effect was found non-significant on pH and acidity of product (Table 4). Similar results were reported by (More et al. 2017). They reported that during storage, little millet *kheer* at refrigerated temperature (6±1°C) the pH decreased significantly while acidity and viscosity increased. Standard plate count (SPC) count of *kheer* was increased significantly during storage. Yeast & mould count and coliform count was found nil during the storage period.

Economic Analysis

To assess and evaluate the overall impact of a project in monetary and quantifiable terms, cost analysis of red rice *kheer* was done by taking all the used ingredients into consideration. In order to determine the feasibility of the study, cost of production was calculated for optimized *kheer* (Table 5). Cost of raw materials was added along with packaging cost and marketing and distribution expenses. 100g (one cup) of red rice *kheer* was prepared in approximately Rs. 15.16. The profit margin at 25% of

Table 5 Cost analysis of red rice *kheer* (100g)

Ingredients	Quantity	Cost (Rs.)
Red rice	20g	4.0
Coconut Sugar	15g	13.5
Toned Milk	500ml	22.0
Total (Quantity of *kheer*)	300g	39.5
	Quantity/100g /cup	13.16
Packaging cost	1 cup + Aluminium foil	1.0
	Total	14.16
Processing cost	Per cup	1.0
	Total	15.16
Marketing and distribution expenses @25% of product	Per cup (100g)	3.29
	Total	Rs. 18.45

cost of product is also applied which took overall price of *kheer* to a very nominal price of Rs. 18.45.

Conclusion

Kheer is consumed by wide group of population. In the present study, red rice *kheer* with coconut sugar as a natural sweetener was prepared which has higher amount of protein, fiber, minerals as compare to white rice. It can be concluded that the treatment T_2 of red rice *kheer* prepared by mixing 20g of red rice and 15g of coconut sugar with 500 ml of toned milk (3% fat and 8.5% SNF) was found to be the most acceptable. Both red rice and coconut sugar has low glycemic index which is good for the consumers suffering from diabetes. This product was prepared with the aim to provide nutrition to consumers of every age group.

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RESEARCH ARTICLE

Isolation and characterization of *Staphylococcus aureus* from bovine mastitis in Andhra Pradesh

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Abstract: *Staphylococcus aureus* is the key causative agent for contagious mastitis and responsible for subclinical infections leading to economic loss to dairy industry worldwide. Hence, the present work was planned to isolate and characterize the *Staphylococcus aureus* from bovine mastitis cases of *S. aureus* origin. From non therapeutic areas, a total of 381 staphylococcal isolates were recovered out of 438 milk samples with percentage positivity of 86.98, whereas, out of 60 milk samples in selected therapeutic areas 40 staphylococcal isolates with positivity of 66.67% were recovered based on cultural and biochemical tests. A total of 290 out of 381 and 30 out of 40 culturally positive isolates respectively from non therapeutic and therapeutic areas were confirmed as *S. aureus* by PCR method targeting the 16S rRNA with an expected amplicon size of 229bp. Sequencing and nucleotide analysis of 16S rRNA amplicons of non therapeutic area isolates showed 99.3% identity whereas therapeutic isolates showed 95-100% identity with gene bank reference strains. On phylogenetic analysis, isolates form non therapeutic area were not closely related to reference gene bank strains whereas isolates of therapeutic area were shown close evolutionary relationship with gene bank reference strains. Further, Peruru isolates of therapeutic area have shown close evolutionary relationship with Spain isolate KX348312.1 and formed separate group in cladogram.

Keywords: Contagious mastitis, *Staphylococcus aureus*, Subclinical mastitis, 16srRNA, Phylogenetic analysis

Introduction: Mastitis is the predominant intra mammary infection in dairy cattle in India causing huge economic loss to the dairy industry. Both clinical and subclinical bovine mastitis are responsible for reduction in milk production, deterioration of quality of milk and milk products, increased amount of health care expenditure, financial loss due to culling of sick animals and even mortality of cows. *S. aureus* is the major contagious agent of bovine mastitis. *S. aureus* is the most important and lethal agent (Jaradat et al. 2014) causing chronic and deep infections in mammary tissue and becomes difficult to treat successfully and is responsible for dairy scourge in the livestock industry (Husain et al. 2012a; Raza et al. 2013). It originates from the cows environment and infect the udder via the teat canal (Padhy et al. 2014). The staphylococci have been adapted to survive in the udder, usually establish chronic, subclinical infection and are shed in the milk becoming the source of infection for other healthy cows during milking process. The main source of infections for *S. aureus* mastitis is the udder of infected cows which is transferred via milkers hands, utensils, towels and the environment (floor) where the cows are kept (Radostitis et al. 2007).

The bacterial culturing of the raw milk is the standard procedure for diagnosis of the bacterial pathogens, but the method is time consuming. Several approaches regarding phenotyping and genotyping are being used to subtype the *S. aureus* isolates recovered from animals and human (Kalorey et al. 2007; Saei et al. 2009). The molecular diagnosis could be the most suitable technique for the identification of various circulating strains of pathogens which are difficult to identify by conventional methods. The molecular based techniques are much effective in pursuing the spread of bacterial infections and developing diagnostic methods. The 16S rRNA genes are highly conserved throughout bacterial evolution; hence it was targeted for molecular identification of *S. aureus* using PCR method.

Extensive and indiscriminate use of antibiotics in the treatment and control of mastitis lead to accumulation undesirable of drug residues in milk, emergence of antibiotic resistant bacteria and

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necessary withholding period. To avoid indiscriminate use of antibiotics and to reduce the cost of treatment, practice of antibiotic sensitivity testing should be adopted before initiation of treatment. Antimicrobial therapy is a primary tool for controlling Staphylococcal mastitis. Wide spread and indiscriminate use of antimicrobial agents has resulted in the emergence and transfer of resistant organisms to humans through milk. The emergence of resistance to antibiotics in gram-positive pathogens has become a major global issue (WHO, 2015). Hence, the present study was undertaken to isolate and identify *S. aureus* from bovine mastitis cases by both cultural and molecular methods. Further, antibiogram of the recovered *S. aureus* isolates was conducted to know the antibiotic of choice, sensitivity and resistance pattern for effective treatment and control of mastitis.

Materials and Methods

Collection of milk samples

A total of 498 milk samples were collected randomly from milch cattle in non therapeutic and therapeutic areas of Andhra Pradesh during the period from November, 2017 to July, 2018 shown in Table 1.

Isolation and Identification of *Staphylococcus aureus*

Processing of mastitis milk samples

Milk samples from bovine mastitis cases were subjected for isolation and identification of *Staphylococcus aureus* using conventional and cultural isolation methods. The collected milk samples were inoculated into BHI broth (5ml) and incubated at 37^0C for 24 hrs (Cruickshank et al. 1975). After incubation a loopful of inoculated broth culture was streaked on to the selective differential media like Blood agar, MacConkey's agar and selective media like Mannitol salt agar media and incubated at 37 ºC for 48 hrs in incubator and observed for morphology, cultural characteristics, Gram's staining, Biochemical confirmation by Catalase, Oxidase and Coagulase tests etc (Quinn et al. 2004).

Extraction of bacterial DNA

DNA was extracted according to the method of Arora et al (2006). Single pure bacterial colony was picked and dissolved in 50µl of distilled water in 1.5ml eppendorf tubes. The mixture was boiled at 96°C for 8 min in water bath. The tubes were removed and immediately chilled by placing on to the ice for 10min. All the bacterial lysates were centrifuged at 10,000 rpm for 5 min in refrigerated centrifuge at 4°C. From this snap chilled bacterial lysate, the supernatant containing genomic DNA was collected and stored at -20^0 c for conducting PCR.

Molecular Detection of *Staphylococcus aureus*

The culturally and biochemically positive *S. aureus* isolates were further confirmed genotypic ally using PCR with genus specific primers according to method of Pati and Reena Mukherjee (2016).

Primers

Oligonucleotide primers were obtained from Sigma Aldrich Chemical India Pvt.Ltd., Bangalore. Details of the primer sequences are shown in Table 2.

Polymerase chain reaction (PCR)

The PCR method is standardized according to the method of Lovesth et al 2004. The total reaction volume was set up to 25µl consisting of 2.5 µl 10x PCR buffer, 0.5 µl of 10mM dNTP mix, 0.3 µl of Taq polymerase, 100µM concentration of forward and reverse primer (0.5µl each), 1.5µl of MgCl2 (25mM), 3µl of template DNA and remaining volume was made up with nuclease free water. The DNA template obtained by boiling and snap chilling extraction technique was subjected to PCR with genus specific primers, for the identification of *S. aureus*.

Steps and thermal cyclic conditions for 16SrRNA gene PCR

The amplification reactions were carried out in 0.2ml micro centrifuge tubes using programmable thermal cycler (Proflex PCR, applied bio-systems life technologies, Singapore) with initial denaturation at 95°C for 5min, followed by 35 cycles with

Table 1 Details of the milk samples screened against subclinical & clinical mastitis in non therapeutic and therapeutic areas of Andhra Pradesh

Area	No. of milk samples collected	No. of milk samples tested positive	Percent Positive
Non Therapeutic area	438	381	86.98
Therapeutic area	60	40	66.67
Total	498	421	

Table 2 Details of Oligonucleotide primers for amplification of 16SrRNA gene of *Staphylococcus aureus*

Target Gene	Primer Sequence (5'-3')	Amplicon Size (bp)	Reference
16S rRNA	16S- F-GTAGGTGGCAAGCGTTACC 16S- R CGCACATCAGCGTCAG	229	Lovesth et al. 2004

denaturation at 95°c-1min, 72°c-30sec and 72°c -90 seconds. A final extension step was also carried out at 72°c, for 90Sec.

Analysis of PCR products

Amplified PCR products were subjected to agarose gel electrophoresis as described by Sam brook and Russell (2001). PCR amplified product specific to *S. aureus* was visualized under U.V trans-illuminator and photographed with gel documentation system (BIO-RAD, Gel Doc™).

DNA sequence analysis

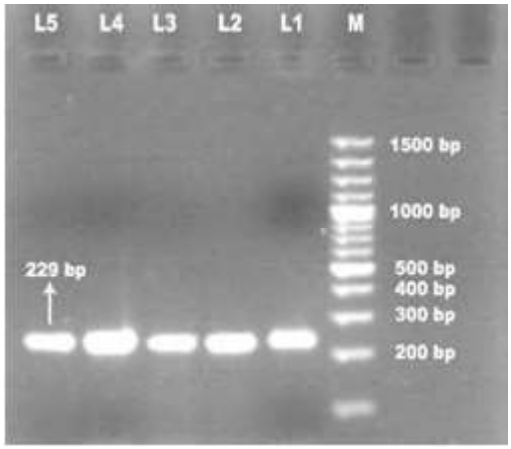
Amplified PCR products of 16SrRNA gene were sequenced and sequence analysis was done using NCBI BLAST for similarity and percent identity searches with the gene sequences available in the Gene bank. After confirmation of *Staphylococcus aureus,* the nucleotide sequences were submitted to gene bank through sequin submission portal for obtaining accession numbers. Nucleotide sequencing and Phylogenetic (Cluster) analysis was done by multiple sequence alignment using online software Clustal X 2.1. The phylogenetic analysis was done using the MEGA 6.06 program.

Results and Discussion

Cultural isolation

A total of 381 *Staphylococcal* isolates were recovered on cultural isolation with positivity of 86.98% from 438 milk samples collected from non therapeutic areas. Similarly, a total of 40 *Staphylococcal* isolates from therapeutic areas were obtained from 60 milk samples on cultural isolation with positivity of 66.67% (Table 1). Various workers reported the positivity rate of 80.98% from Maharastra (Yadav 2018) and Sanotharan et al. 2016 from Srilanka (90.5%). However, Qudri et al.2017 recorded low positivity rate of 42.2 from Srinagar. Inconsistency and irrational use of antibiotics indicated the variations in positive percent of our findings and earlier studies from non therapeutic areas. Whereas, the positivity rate (66.67%) of our study in the therapeutic areas were in coincidence with the earlier reports of Jyothi et al. (2018) from Hyderabad with 62.9%; Bhat et al. (2017) from Jammu with 60.80% and Mubarak et al.(2012) from Tamilnadu with60.80%. However, high prevalence was reported by Mpatswenumugab et al. (2017) from Rwanda with 72.1% and Pati and Reena Mukherjiee (2016) from Uttar Pradesh with 71.87%. Whereas low prevalence rate was recorded by Harini and Sumati 2011 from Karnataka (58%) and 46.3% by Jena et al. 2015 from Rajasthan.

The variability in occurrence of *S. aureus* in mastitis cows among different reports might be that *S.aureus* is a contagious pathogen transmitted from one cow to another in a herd by contact via unhygienic milking procedures and poor farm management practices, environmental inconsistency and lack of awareness among dairy farmers.



Lane M: 100bp DNA ladder
Lane 1-5: positive *S. aureus* isolates

Fig. 1 PCR amplification of 16SrRNA gene of *S. aureus* isolates-Non therapeutic areas

All the 421 isolates from non therapeutic and therapeutic areas were shown hemolytic colonies on blood agar media; on mannitol salt agar media, yellow color round glistening colonies indicative of Coagulase Positive; pink color colonies indicative of Coagulase Negative and no growth on Macconkey's agar suggesting positive for *S. aureus*. On Gram's staining reaction all positive isolates revealed gram positive cocci with grape like clusters and biochemically positive to catalase test and negative to oxidase test.

Similarly, 271 isolates of *S. aureus* (71.0%) out of 381 isolates from non therapeutic areas and 27 isolates out of 40 isolates from therapeutic areas were positive for Coagulase test

Molecular Characterization of *S.aureus* isolates

All the *S. aureus* isolates which positive phenotypically were subjected to genotypic confirmation using 16SrRNA PCR. Out of 381, 291 (73.5%) *S. aureus* isolates from non therapeutic areas (Fig 1) and 30 isolates (75%) out of 40 *S.aureus* isolates from therapeutic areas yielded expected amplicon size of 229 bp (Fig 2). It has been used by several workers (Pati and Reena Mukerjee, 2016; WHO 2015; Lange et al.2015; Ciftci et al. 2009; Zhang et al. 2004; Krimmer et al. 1999). During the present study the identification rate of 73.5 to 66.67% was reported and it was less than those reported by earlier workers Lange et al. 2015 (95%); Pati and Reena Mukerjee 2016 (83.8%) and Mellmann et al. 2006 (91%) respectively.

The results of multiple nucleotide alignment of 16SrRNA gene of all the isolates (291) from non therapeutic areas showed nucleotide identity in the range of 99.3% with reference gene bank strain of Assam (Accession no.KP337596.1), Bihar strain (Accession no .MH 255793.1),UttarPradesh (Accession no. KX 181851.1), German (GU 459255.1), Spain (Accession no.KX 348312.1) and USA strain (KJ 83377.1). Similarly multiple nucleotide alignment of 16Sr RNA Gene of all the isolates from therapeutic

Lane M: 100 bp DNA ladder
Lane 2-7: positive *S. aureus* isolates
Lane 1: Negative control

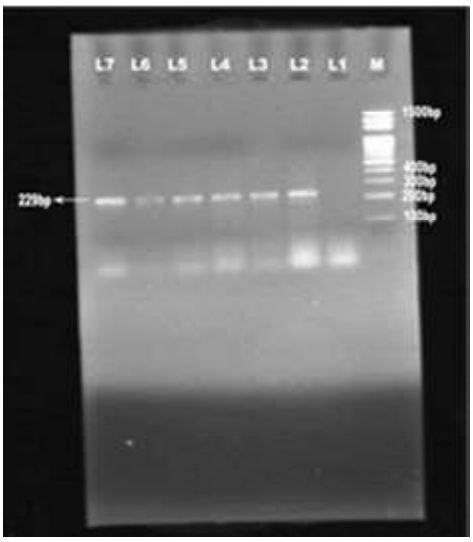


Fig. 2 PCR amplification of 16S rRNA gene of *S. aureus* isolates -Therapeutic areas

areas (LFC,SKP&Peruru) showed a nucleotide identity in the range of 95-100% on comparison with reference strains in the gene bank database. The partial 16Sr RNA Sequencing differentiated 95% of the isolates tested for species identification in the present study. The identification rate of 95% obtained in this study was higher than those reported by Mellmann et al. (2006) with 91%.

On phylogenetic analysis, isolates from non therapeutic areas were not closely related to reference gene bank strains (Fig 3). Whereas isolates of therapeutic areas viz., Livestock Farm Complex (LFC) and Sorakayalapalem (SKP) shown close evolutionary relationship with gene bank reference strains. However, Peruru isolates have shown close evolutionary relationship with Spain isolate KX348312.1 and formed separate clade.

During the study, antibiotic sensitivity test was conducted on S. aureus isolates recovered from non therapeutic areas and selected therapeutic areas. The antibiotic sensitivity test results revealed that the isolates recovered from non therapeutic areas showed higher sensitivity to Amoxicilli 60.38% (64/106) followed by Ampicillin 56.60% (60/106) ,Enrofloxacin 53.77% (57/106),Ciprofloxacin 46.23% (49/106),Gentamycin 26.42% (28/106),Tetracycline 20.75% (22/106),Streptomycin 12.26%(13/106) ,Kanamycin 9.43% (10/106) and Amikacin 7.55% (8/106%) but were resistant to Amikacin 92.45%(98/106) followed by Kanamycin 90.57%(96/106),Streptomycin 87.74% (93/106), Tetracycline 79.25%(84/106),Gentamycin 73.58% (78/106), Penicillin-G 56.60% (60/106), Ciprofloxacin 53.77% (57/106),Enrofloxacin 46.23% (49/106) Ampicilln 43.40% (46/106) and Amoxicillin 39.62% (42/106) respectively. This was in accordance with reports of Qayyum et al. (2016); Husssain et al. (2012a) and Idriss et al (2014). But in therapeutic areas higher sensitivity to Enrofloxacin 66.67%

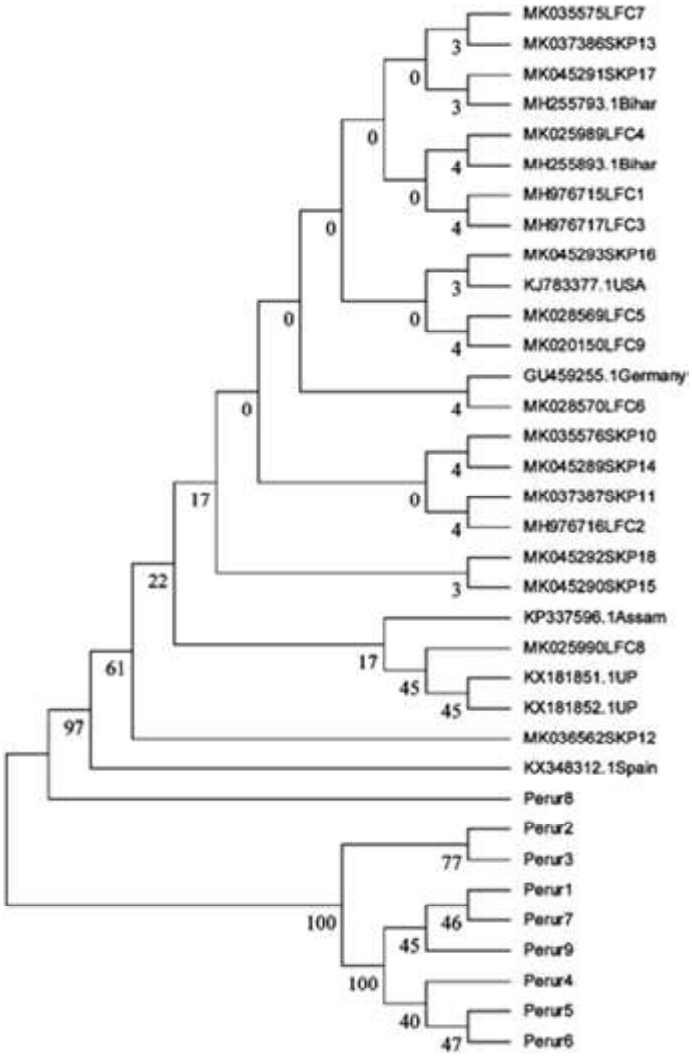


Fig.3 Phylogenetic analysis of 16S rRNA gene of *S. aureus* isolates

followed by Ciprofloxacin 59.25%, Amoxicillin 44.44% and Ampicillin 40.74%. Similar sensitivity pattern of *S. aureus* isolates to Enrofloxacin was reported by Baghel et al.2018; Dar et al. 2014; Bhanot et al. 2012 ; Kumar and Sharma 2002 ; Sahoo et al. 2009; Joshi et al. 2006 and Ranjan et al. 2010. It could be due to lesser usage of these antibiotics in majority of districts of Andhra Pradesh in treating the mastitis cases.

In the present study among Quinolones, Enrofloxacin 66.67% Ciprofloxacin 59.25% and beta lactamase antibiotics like Amoxicillin 63.7% and Ampicillin 56.6% were found to be effective in therapeutic study areas. The extensive use of antibiotics in treatment and control of mastitis has possible implications on human heath through emergence of antibiotic resistant bacteria. The antibiotic sensitivity profile of isolated pathogens can serve as a guide for field veterinary practitioners to provide effective and timely treatment of mastitis cattle.

Conclusions

The present work was undertaken with an objective of isolation and characterization of *S. aureus* from clinical and subclinical cases of bovine mastitis and to conduct antibiogram of all *S. aureus* isolates to know the antibiotic of choice for the treatment of bovine mastitis caused by S.aureus. Hence, the present study revealed the isolation, identification and phenotypic characterization of *S. aureus* strains by cultural and biochemical tests; genotypically using 16SrRNA PCR. Further, it was reported that 16SrRNA PCR was found to be powerful and simple tool for the identification of *S. aureus* from bovine mastitis. Thus the findings of this study in non therapeutic and therapeutic areas would be helpful in detecting the existence and emergence of *S. aureus* isolates, antibiotic resistant pathogens and useful in selection of isolate for preparation of vaccine for effective control of clinical and sub clinical mastitis caused by *S. aureus*.

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RESEARCH ARTICLE

Effects of genetic and non genetic factors on production performance of primiparous Kankrej cattle

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Abstract: The present investigation included the records of 294 Kankrej cows, sired by 66 sires spread over a period of 20 years (1996 to 2015) maintained at Livestock Research Station, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat. The effects of genetic and non genetic factors on performance traits (Daily Milk Yield, 305 Days Milk Yield, Total Milk Yield and Lactation Length) of primiparous Kankrej cows was studied. The least square means of daily milk yield, 305 days milk yield, total milk yield and lactation length were calculated and found 6.71±0.14, 1893.42±60.00, 1983.29±72.47 kg and 290.78±7.33 days, respectively. The season and period of calving did not affect these traits, while effects of sire was significant (*P<0.05) on 305 DMY, but it did not have any effect on the traits such as DMY, TMY and LL of primiparous Kankrej cow. On the basis of these observations, we can suggest that proper management practices as well as use of better sire will help to improve the production performance of the herd.

Keywords: Kankrej cow, Genetic factors, Performance traits

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Introduction

Livestock sector plays an important role in the livelihoods security of dairy farmers in the country. The rural economy mainly depends on agriculture and allied sector, where animal husbandry and dairy development plays an important role through supplementing the income of rural households, particularly, the landless, small and marginal farmers. Milk production is the enterprise in which the small scale farmers can easily engage themselves in order to improve their livelihood and regular income from selling of milk moves them from subsistence to a market based income. India continues to be the largest producer of milk in the world, since last decades, where government initiated several measures to increase the productivity of livestock, which has resulted in increasing the milk production significantly from the level of 17.0 million ton. in 1951 to 187.7 million ton during 2018-19 (Anonymous, 2019). At present availability of milk per head per day in the country is 394 grams (2018-19), which was only 132 grams in 1951.

The viability of a dairy farm is depends upon the performance of the animals reared for milk production, where daily milk yield records are important to know the milk ability of individual animals. It is also important that success of dairy enterprise is depends upon efficiency of production performance of the herd. There are several non genetic factors which influence the phenotypic value of economic traits, and the absence of accurate value of these traits make it difficult to estimate genetic parameters of the traits which determine optimum selection criterion for planned improvement of the animals (Dass and Sadana, 2000). Therefore, in present investigation was undertaken to study the effects of various genetic and non-genetic factors on (Daily Milk Yield, 305 Days Milk Yield, Total Milk Yield and Lactation Length) of primiparous Kankrej cow.

Materials and Methods

The present study was conducted to know the effects of genetic and non genetic factors on production performance traits of 294 Kankrej cattle maintained at Livestock Research Station, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat. Geographically, Livestock Research

Station, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar is located in the north Gujarat. The climate of the farm is semi-arid in nature. Management practices followed on the farm were uniform for the herd. All animals were housed under loose housing system with adequate sheds for shelter against sun, rain and extreme winter. All animals at the farm were stall fed with dry roughages, green fodder and concentrate in proper proportion. The animals having lactation length less than 100 days, incomplete lactation due to sale or death during lactation, abortion and still birth etc. were considered as abnormal lactation and not included in the study. The data pertaining Kankrej cows was maintained over a period of 20 years from 1996 to 2015. The data was grouped into 4 periods with duration of 5 years *viz*. P1: 1995-2000, P2: 2001-2005, P3: 2006-2010 and P4: 2011-2015. Each year was delineated into 3 seasons each with duration of 4 months *viz.* S1: Nov.- Feb (winter)., S2: March- June (summer) and S3: July- Oct. (monsoon). The traits included in the study were Daily Milk Yield, 305 Days Milk Yield, Total Milk Yield and Lactation Length.

The period of calving and season of calving were considered as fixed effects and sire was considered as random effects for all traits. Single trait analyses was done by fitting a general linear model (GLM) to study the effect of various fixed effects on each production traits using SPSS v.20.0 software. To study the effects of genetic and non-genetic factors on DMY, 305 DMY, TMY and LL record of Kankrej cattle. The following model was used:

$$Y_{ijkl} = \mu + A_i + B_j + C_k + e_{ijkl}$$

Y_{ijkl} = Observation for the trait

μ = Overall mean

A_i = fixed effect of i^{th} period of calving

B_j = fixed effect of j^{th} season of calving

C_k = Random effect of k^{th} sire

e_{ijkl} = residual random error (l), NID $(0, \sigma^2)$

The pair-wise comparison of subclass means within each fixed effect was done by Duncan's Multiple Range Test (DMRT).

Results and Discussion

The present investigation was conducted at Livestock Research Station, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, District-Banaskantha Gujarat to know the effects of genetic and non genetic factors evaluate the performance of Kankrej herd maintained from 1996-2015. The climatic conditions of the region is semi arid, where summer is dry and hot and temperature goes up to 40ÚC, while winter is not stressful as average temperature remains from 10Ú C to 30Ú C with relative humidity 69 % and 48 % in morning and afternoon, respectively. The data pertaining to DMY, 305 DMY, TMY and LL was recorded from the records of the farm and analyzed the effects of non genetic factors like, season, period and genetic factors like sire was studied and result are mentioned in table: 1.

Daily Milk Yield (DMY)

The average Daily Milk Yield (DMY) of primiparous Kankrej cow based on 291 number of observation was 6.71 ± 0.14 kg (Table: 1). The values of DMY are in close agreement with Singh and Singh (2016) as 5.94 ± 0.10 kg for Sahiwal cattle. The value was contrast with the findings of Ageeb and Hayes (2000) and Ayeneshet et al. (2018) for cattle.

The performance of Kankrej cow, calving during summer, monsoon and winter season was 6.80 ± 0.18 kg, 6.65 ± 0.22 kg and 6.66 ± 0.18 kg, respectively. It was highest during summer season (6.80 ± 0.18 kg), and least during monsoon season (6.65 ± 0.22 kg). The DMY values were not significantly differ (Table: 1) for primiparous cow. The effect of season of calving was non-significant on DMY for primiparous Kankrej cattle and it is in close agreement with Singh and Singh (2016) and Vinoo et al. (2005) for Sahiwal cattle and Ongole cattle respectively, while the results are in contrast with findings of Ayeneshet et al. (2018). In, table 1. result reveled that DMY of primiparous Kankrej cattle for different seasons was not significantly differ from each other.

Similarly, DMY of the Kankrej cattle was 7.19 ± 0.53 kg, 6.73 ± 0.31 kg, 6.16 ± 0.27 kg and 6.74 ± 0.27 kg during different periods of calving *viz;* P1, P2, P3 and P4 respectively. The highest DMY was observed during P1 period (7.19 ± 0.53 kg), whereas it was least during P3 period (6.16 ± 0.27 kg) in primiparous cow. The period of calving have non-significant effect (Table 1) on DMY and it is in agreement with Bala et al. (2017) for White Fulani cow. These findings are contradictory with the findings of Singh and Singh (2016) for Sahiwal cattle. In, Table 1. result revealed that DMY of primiparous Kankrej cattle for different periods was not significantly differ from each other. Similarly, sire had non-significant effect on DMY for primiparous Kankrej cow (Table 1).

305 Days Milk Yield (305 DMY)

The least square means of 305 DMY of primiparous Kankrej cow based on 294 number of observation was 1893.42 ± 60.00 kg (Table: 1). These values of 305 DMY are in agreement with Ekka (2014) as 1854.18 ± 84.56 kg for Kankrej cattle. The finding value was contrast with the findings of Rehman et al. (2008), Singh et al. (2018) and Rathod et al. (2020) for Indigenous cattle.

The performance of primiparous Kankrej cow, during summer, monsoon and winter season was 1910.98 ± 74.27 kg, 1899.78 ± 90.74 kg and 1870.41 ± 71.28 kg, respectively. It was highest during summer season (1910.10±74.27 kg), and minimum during winter season (1870.41 ± 71.28 kg). However, effect of season of calving

Table: 1 Least Square Means of production traits of primiparous Kankrej Cows

Factors	DMY (Kg)	305 DMY (Kg)	TMY (Kg)	LL(days)
Estimated Mean (μ ± S.E.)	6.61 ± 0.08(291)	1902.97 ± 31.42(294)	1992.46 ± 37.76(294)	297.76 ± 3.62(293)
Population Mean	6.71 ± 0.14(291)	1893.42 ± 60.00(294)	1983.29 ± 72.47(294)	290.78 ± 7.33(293)
Season of calving	NS	NS	NS	NS
Winter(S-1)	6.66 ± 0.18(116)	1870.41 ± 71.28(119)	1954.02 ± 86.07(119)	288.14 ± 8.70(118)
Summer(S-2)	6.80 ± 0.18(127)	1910.10 ± 74.27(127)	2001.68 ± 89.69(127)	291.63 ± 9.09(127)
Monsoon(S-3)	6.65 ± 0.22(48)	1899.78 ± 90.74(48)	1994.19 ± 109.57(48)	292.55 ± 11.08(48)
Period	NS	NS	NS	NS
1996-2000(P-1)	7.19 ± 0.53(35)	1975.23 ± 220.54(37)	2084.35 ± 266.32(37)	280.61 ± 26.94(37)
2001-2005(P-2)	6.73 ± 0.31(72)	2015.90 ± 130.66(72)	2139.34 ± 157.79(72)	313.55 ± 15.99(72)
2006-2010(P-3)	6.16 ± 0.27(93)	1705.76 ± 114.74(94)	1752.23 ± 138.55(94)	282.97 ± 14.07(93)
2011-2015(P-4)	6.74 ± 0.27(91)	1876.83 ± 114.73(91)	1956.77 ± 138.55(91)	285.97 ± 14.01(91)
Sire	NS	*	NS	NS

Figures in parenthesis indicates number of observation/records., **P<0.01 highly significant; *P<0.05 significant; NS= Non significant; S.E.= Standard Error; N= Subclass means with different superscripts are significantly different from each other

on 305 DMY of primiparous cow was non significant (Table 1) and it is in close agreement with Ekka (2014); Shingare et al. (2015) and Gaikwad et al. (2018) for Kankrej cattle, Deoni cattle and Phule Triveni cattle, respectively. Whereas, it is in contrast with the findings of Kakati et al. (2017). In Table 1 result also revealed that 305 DMY was not differed significantly with each other.

The average 305 DMY of the primiparous Kankrej cow during different periods of calving *viz;* P1, P2, P3 and P4 was 1975.23 ± 220.54 kg, 2015.90 ± 130.66 kg, 1705.76 ± 114.74 kg and 1876.83 ± 114.73 kg, respectively. The highest 305 DMY was observed in P2 period (2015.90 ± 130.66 kg) and lowest during P3 period (1705.76 ± 114.74 kg), but these 305 DMY values were non-significant effect (Table: 1). These findings are in close agreement with Singh et al. (2016) for Karan-Fries cattle. The present results are not match with the findings of Gaikwad et al. (2018). In table 1. result revealed that 305 DMY of primiparous Kankrej cattle for different periods was not significantly differing from each other.

The least square analysis has revealed sire have significant (P<0.05) effect on 305 DMY of primiparous Kankrej herd and it is in close agreement with the findings of Singh et al. (2020) in HF cattle. These results are in contrast with the findings of Mandal and Sachdeva (2001) for crossbred cattle.

Total Milk Yield (TMY)

The average TMY of primiparous Kankrej cow based on 294 number of observation was 1983.29 ± 72.47 kg (Table 1). These values are in accordance with the reports of Kishore et al. (2016) and Kumar et al. (2016a) as 2021.08 ± 42.80 kg, and 2177.61 ± 62.06 kg for Tharparkar cattle and Crossbred cows. The present findings are contradictory with the findings of Chaudhary (2016 *lo-cit*), Bhutkar et al. (2014) and Rathod et al. (2020) for indigenous cattle.

The performance of primiparous Kankrej cow, during summer, monsoon and winter season was 2001.68 ± 89.69 kg, 1994.19 ± 109.57 kg and 1954.02 ± 86.07 kg, respectively. It was highest during summer season (2001.68 ± 89.69 kg) and lowest during winter season (1954.02 ± 86.07 kg). The value of TMY was not significant (Table 1) among primiparous cow. These findings are in close agreement with Ekka (2014) for Kankrej cattle and Singh and Singh (2016) and Narwaria et al. (2017) for Sahiwal cattle. In contrast to present results the findings of Singh et al. (2017) found that season of calving have significant effect on TMY. In Table 1 result revealed that TMY of Kankrej cows was not differ significantly with each other.

The average TMY of the primiparous Kankrej cow during different periods of calving viz; P1, P2, P3 and P4 was 2084.35 ± 266.32 kg, 2139.34 ± 157.79 kg, 1752.23 ± 138.55 kg and 1956.77 ± 138.55 kg respectively. The highest TMY was observed during P2 period (2139.34 ± 157.79 kg) and lowest during P3 period (1752.23 ± 138.55 kg). The period of calving have non-significant effect on TMY (Table 1) in primiparous Kankrej cow and it is in close agreement with Dongre et al. (2017) and Parmar et al. (2019) for Deoni cattle and crossbred cattle, respectively. These findings are not in agreement of Narwaria et al. (2017) for Sahiwal cattle. In Table 1 result revealed that TMY of Kankrej cattle during different period was not differed significantly with each other. The least square analysis has revealed non-significant effect of sire on TMY in primiparous Kankrej cattle in comparison to 305 Days Milk Yield due to differences in the days which affecting the milk yield (Table 1).

Lactation Length (LL)

The least square means of lactation length (LL) of primiparous Kankrej cow based on 293 number of observation was 290.78 ± 7.33 days (Table 1). These findings are in close association with the reports of Al-samarai et al. (2015) as 298.28 days for Holstein

cow, Chaudhary (2016 *lo-cit*) as 300.90 ± 10.31 days for Kankrej cow, Mishra et al. (2016) as 294.93 ± 2.58 days for Tharparkar cattle and Kumar et al. (2017) as 288.55 ± 19.41 days for Sahiwal cattle. The results are contrast with the reports of Sawant et al. (2016); Chakravarthi et al. (2017) and Parmar et al. (2019).

The average LL of primiparous Kankrej cow, calvings during summer, monsoon and winter season was 291.63 ± 9.09, 292.55 ± 11.08 and 288.14 ± 8.70 days, respectively. It was highest during monsoon season (292.55 ± 11.08 days), and minimum during winter season (288.14 ± 8.70 days). However, effects of season of calving on LL of primiparous cow were non significant (Table: 1). The present findings are in close agreement with Ekka (2014) for Kankrej cattle, Bhutkar et al. (2014) for Deoni cattle and Dongre et al. (2017) for Deoni cattle. The present findings are contrast with the findings of Dangi et al. (2013) and Mishra et al. (2016). In table 1. result showed that LL of Kankrej cattle was not differed significantly with each other. Similarly, LL of the primiparous Kankrej cow were 280.61 ± 26.94 days, 313.55 ± 15.99 days, 282.97 ± 14.07 days and 285.97 ± 14.01 days during different periods of calving viz; P1, P2, P3 and P4, respectively. The highest LL was observed during P2 period (313.55 ± 15.99 days) and lowest during P1 period (280.61 ± 26.94 days) in primiparous cow but these values were non-significant (Table 1). These findings are supported by the Bhutkar et al. (2014); Kumar et al. (2014); Dongre et al. (2017) and Parmar et al. (2018). They reported that period of calving did not have any effect on LL. In table 1. result showed that LL of Kankrej cows was not differ significantly with each other. The effects of sire on LL of Kankrej cow (Table: 1) was observed non-significant. These finding are supported by the observations of Al-samarai et al. (2015) as they reported that sire effect on LL was non-significant.

Conclusion

The effects of non-genetic factors (season of calving, period of calving) on performance of primiparous Kankrej herd shows non-significant, whereas genetic factor (sire) has significant effect on 305 DMY under semi arid region of north Gujarat. On the basis of these observations, we can suggest that proper management practices as well as use of pedigreed/progeny tested sire will help to improve the production performance of the herd.

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RESEARCH ARTICLE

Effect of propionic and potassium sorbate supplementation on quality and performance of sugarcane tops silage

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Abstract: The objective of present study was to evaluate the effects of chemical additives on the quality parameters and nutritive value of sugarcane tops (SCT) silages. The trial was carried out in a completely randomized experimental design with total twelve treatments including two controls, C1 (without additive) and C2 (with common additives *viz.,* 0.5% Urea, 0.5% NaCl and 2% molasses on wet basis). However, ten treatments (C2+ other chemical additives *viz.,* SB; Soidum benzoate, PS; Potassium sorbate, PA; propionic acid and their combinations) were applied @ 0.1% (wet basis) onto the fresh SCT and ensiled for 30 days in 3.5 L vacuum sealed plastic jars in two replicates of each treatment. The analyzed variables were: chemical composition, quality parameters and nutritive values. Selection of most promising additive/combination was done with Flieg point and fitness values. The addition of chemical additive before ensiling produced silages with better quality indices (pH, lactic acid, Flieg point and fitness values), higher nutritive value (crude protein and ME) and low NDF as compared to control with additive (C2). Ammonia Nitrogen (NH_3N% TN) and protein fractionation (NDICP and ADICP) were also considered for the identification of best SCT top silage and chemical additive effect. The results of present study indicate that PA and PS+PA were the most promising additive treatments for the improving the SCT silage quality and nutritive value. Furthermore, information extended from the present study is giving future direction towards animal feeding trials with SCT silage with chemical additive.

Keywords: Propionic acid, Quality, Sodium sorbate Nutritive values, Sugarcane tops Silage

Abbreviation

ADF, acid detergent fiber; ADICP, acid detergent fiber insoluble protein; CP, crude protein; DM, dry matter; EE, ether extract; FM, fresh matter; LA, lactic acid; ND, no detected; NDF, neutral detergent fibre assayed with a heat stable amylase and expressed inclusive of residual ash; NDICP, neutral detergent fibre insoluble protein, SCT, sugarcane tops SB, Soidum benzoate; PS, Potassium sorbate; PA, propionic acid

Introduction

Fodder management plays a pivot role in successful dairy farming and the utilisation of alternate fodder resources or crops reduces the stress on existing conventional feed resources. The sugarcane (*Saccahrum officinarum*) is among the largest commercial crop of India, over 282 lakh tons of sugarcane and 34.6 MMT sugarcane tops (SCT) are produced annually (ISMA, 2019). However, a large portion of this remained left as such on field or burnt in the field (Pandey et al. 2009). Ensiling is post-harvesting management and find most effective on improving digestible energy content of poor quality fodder by solubilising the fibrous (NDF and ADF) structure. Although, uncontrolled yeast and mold risk and clostridia growth, poor aerobics stability, high NH_3N%-TN, and butyric acid concentration are common with sugarcane tops silage preparation (Khanal et al. 1995; Kebede et al. 2018). This might be a resultant of poor fermentation process, the low water soluble sugar contents and high buffering capacity, which in turn increases dry matter losses and lowers its nutritive value (Soundharrajan et al. 2021).

Sugarcane tops are poor quality lignin-rich roughages having limited cell wall degradability, low protein and energy

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content (Akinbode et al. 2017). Kebede et al. (2018) prepared SCT silage with urea (1% FM) and molasses (4% FM) and reported nitrogen content and nutritional value improvements. Use of additives and nutrient supplementation during ensiling can improve the silage quality parameters; secondly, ensiling would facilitate the utilisation of poor quality fodder of higher NDF and ADF in animal diet. There is a negative relation between higher NDF and ADF with fibre digestibility and dry matter intakes in animals (Mahyuddin 2008). Alli et al. (1982) reported ADF levels reduced from 43.1% to 29.9% in sugarcane tops silage prepared with additive treatment. Similarly, Pedroso et al. (2011) reported an improvement in energy content of sugarcane silage on addition of sodium benzoate (1g/kg fresh matter basis) as compared to untreated silage. Data availability on improving the sugarcane tops silage quality with the use of chemical additives is still restricted and inconclusive. Therefore, present *invitro* study was conducted to identify the most promising chemical additives that can improve quality, fermentation characteristics and nutritive value of SCT silage and can also provide most promising chemical additives for successful crop residues ensiling.

Materials and Methods

Forage harvesting for silage preparation

The study was conducted at the National Dairy Research Institute, Karnal, Haryana, India. Sugarcane tops were procured after cutting stem during the harvesting period. Whole sugarcane tops were chaffed into 2-4 cm particle length using an electrical chaff cutter. At the time of ensiling DM content was the 30.0%. Urea (0.5%), NaCl (0.5%) and molasses (2%) were added in all treatment groups as common additive. Before this, molasses was treated with dilute sulphuric acid @2% FM basis which hydrolyses large sucrose molecule to glucose and fructose.

Schedule of ensiling experiment

The chopped sugarcane tops were evenly mixed with chemical additives and respective chemical additive divided into two replicates and packed in plastic jar silos vacuum-sealed containers of 3.5 L capacity (CELLO, Packing Co. Ltd., India). A total of 24 jars (12 sets of SCT silage including two controls (C1 and C2) ×2 replicates) were packed and kept in the laboratory at ambient temperature (at 21 ± 1ºC), for 30 days of ensiling. The two controls were (C1) without additives and (C2) added with common additives (0.5 % NaCl, 0.5% Urea and 2% molasses) treatments. Excluding the two controls C1 and C2, total 10 treatments were prepared out of which seven were mixed with common additive plus different chemical additive combinations [Sodium benzoate (SB), Potassium sorbate (PS) and Propionic acid (PA), SB+PS, SB+PA, PS+PA and SB+PS+PA] added at the dose rate 0.1% as such basis. Other three treatments were prepared with 0.5% NaCl, 0.5% urea and 2% Molasses, respectively on as fresh basis.

Proximate and cell wall constituent analysis

DM contents were determined by oven drying at 65ºC for 72h and ground to pass a 1-mm screen. Dry matter loss was determined by ashing of fresh fodder and silage samples (Dickerson et al. 1991; Ashbell and Weinberg, 1992)

$$\text{DM loss (\%)} = [\ 1- (\text{ash}_{\text{fresh}}/\text{ash}_{\text{silage}})] *100$$

Crude protein (CP) and ether extract (EE) were analysed according to standard procedures detailed by the AOAC (2005). The neutral detergent fibre (NDF) and ADF were analysed as per Van Soest et al. (1991). Acid detergent insoluble protein (ADICP), neutral detergent insoluble protein (NDICP) was estimated as per Licitra et al. (1996). The TDN content and ME contents of silage samples were estimated as per the equations given by NRC (2001).

pH, buffering capacity, flieg point and fitness value analysis

The pH was measured by using a Eutech pH meter (Oakton Instruments, IL USA) and the buffering capacity of the sample was done as per the method of Playne and McDonald (1996). 10 g of fresh silage material was macerated with 250 ml of distilled water and filtered through Whatman filter paper no.1. The pH of this extract was immediately measured. For buffering capacity, the macerated sample was first titrated to pH 3 with 0.1 N-hydrochloric acid to release bicarbonate as carbon dioxide and then titrated to pH 4 and then to pH 6 with 0.1N sodium hydroxide. Buffering capacity was expressed as mill equivalent of alkali required to change pH from 4 to 6 per 100 g DM. To assess the quality of the silage, Flieg points from the pH value and DM of silage were measured at the end of the fermentation period with the following equation (Moselhy et al. 2015).

Flieg points= 220 + [(2*DM"15)] "40*pH

The suggested score by authors were as follows, very bad for < 20, bad with a score between 21 to 40; to be medium with a score between 41 to 60; to be good (61 to 80) and to be very good when it had a score above 81. The estimation of lactic acid in silage samples was done as per the method of Barker and Summerson (1941) and modified by Barnett (1951) and for NH_3-N estimation, 2 ml of water extract was taken in micro Kjeldahl apparatus and contents made alkaline with 40% NaOH solution. Steam distillation was done using KEL PLUS - N analyzer (Pelican, India) and the NH_3 evolved was collected in a boric acid solution having a mixed indicator and titrated against N/100 H_2SO_4.

Experimental design and statistical analysis

The data was subjected to one -way analysis of variance (ANOVA) with the fixed effects of additives and ensiling period using the general linear model procedure of SPSS (20.0). Pairwise comparisons of the mean values were tested by Duncan multiple

range test (Duncan 1955) and the Hypothesis testing was done at a 5% signiûcance level.

Results and Discussion

Chemical composition

The dry matter, NDF and ADF contents (Table 1) of fresh sugarcane tops are in agreement with the values reported in literature. Chemical composition of fresh sugarcane tops reported in several studies varies with working cultivars of sugarcane (Andrade et al. 2002; Santos et al. 2009). Dry matter content ranged between 24 and 37%, NDF between 36 and 56% and ADF between 21 and 36%, although, the crude protein content is within the range of 3.80 to 6.70% of the dry matter (Kebede et al. 2018).

After 30 days of ensiling, chemical composition (DM, CP, EE, OM and NDF) of sugarcane tops silages prepared with different chemical additive are presented (Table 2). Results suggested that DM content increased in control C2 (with common additive) and all treatments with chemical additives combination, highest in SB+PA, PS+PA, SB+PS, SB+PS+PA followed by PS, SB and PA. This might be due to added mass of different salts. Present study has shown that the total ash (%DM) content was higher ($P < 0.05$) in treatment groups SB+PS+PA, SB+PS. Other studies have also reported that the ash content was increased on addition of chemical additive (Santos et al. 2009). In relation to control C1 (without additives) SCT silage, the CP content was ($P < 0.05$) found higher in all additive treatments and control C2. The CP content was increased ($P < 0.05$) by 62.80, 54.84, 38.91% in SB+PS, PS, SB+PS+PA treatments with respect to the control C1 (without additive). Increase in silage nitrogen content might be due to urea addition; Schmidt et al. (2007) have reported at 0.5% FM urea addition, nitrogen recovery was 75 percent. EE content ranged from 1.88 to 3.22 (%DM) among all the treatments. Buffering capacity in all additive treatment groups remained similar to the control (C1 and C2), it ranged from 97.33 to 52.69 (meqNaOH/100g DM), however, it significantly ($P < 0.05$) increased in urea treated SCT silage. SCT silage prepared with

Table 1 Chemical composition (g/100g DM), pH, buffering capacity of fresh sugarcane tops

Parameter	Mean (±standard deviation)
DM (%FM)	30.00±0.58
Crude protein (% DM)	6.61±0.09
Ether extract (% DM)	2.66±0.08
Organic matter (% DM)	92.54±0.01
Total ash (% DM)	7.46±0.01
NDF (% DM)	77.37±0.19
ADF (% DM)	44.40±0.07
Hemicellulose (% DM)	32.97±0.16
NDICP (% DM)	1.9 ± 0.49
ADICP (% DM)	0.93 ± 0.01
ADL (% DM)	6.16±0.42
TDN (% DM)[a]	58.01±1.02
ME (MJ/kg DM)[a]	8.48±0.84
pH	6.82 ± 0.04
Buffering capacity (Meq % DM)	36.16±2.33

DM, dry matter; FM, fresh matter; NDF, neutral detergent fiber; ADF, Acid detergent fibre, NDICP, neutral detergent fiber insoluble protein; ADICP, Acid detergent insoluble protein, ME, metabolisable energy, TDN, total digestible nutrient estimated by NRC, 2001,

Table 2 Chemical composition and nutritive value of sugar cane treated with additives at the time of ensiling

Treatment	DM(Freeze dried)	CP (% DM)	EE (% DM)	Buffering capacity (meqNaOH % DM)	ASH (% DM)	NDF (% DM)	ADF (% DM)
SB	34.27^{c}	8.41^{abc}	2.90^{ab}	84.92^{ab}	10.36^{ab}	67.20^{fj}	40.29^{f}
SB+PS	34.39^{c}	9.41^{a}	2.47^{abc}	75.96^{b}	14.52^{c}	67.83^{ef}	40.23^{f}
SB+PA	36.38^{a}	8.37^{abc}	2.49^{abc}	68.66^{bc}	11.96^{b}	65.79^{k}	38.29^{j}
PS	33.47^{cd}	8.95^{ab}	3.22^{a}	72.00^{bc}	9.72^{a}	66.53^{jk}	41.45^{d}
PS+PA	35.32^{b}	7.51^{bc}	2.86^{abc}	66.28^{bc}	9.3^{a}	67.05^{fj}	38.09^{j}
PA	32.36^{e}	9.25^{a}	2.47^{abc}	69.12^{bc}	8.53^{a}	68.05^{ef}	40.53^{ef}
SB+PS+PA	34.27^{c}	8.73^{ab}	2.64^{abc}	67.17^{bc}	14.03^{c}	68.54^{de}	40.82^{e}
Urea	28.14^{f}	8.04^{abc}	3.37^{a}	97.33^{a}	10.79^{ab}	73.83^{a}	43.50^{a}
Molases	32.48^{e}	7.01^{cd}	2.60^{abc}	68.15^{bc}	8.92^{a}	71.68^{a}	42.16^{bc}
NaCl	28.12^{f}	9.16^{ab}	2.40^{abc}	69.28^{bc}	9.32^{a}	71.90^{b}	41.74^{cd}
C1	28.30^{f}	5.78^{d}	2.01^{bc}	52.69^{bc}	8.53^{a}	70.41^{c}	42.35^{b}
C2	33.09^{de}	8.96^{ab}	1.88^{c}	79.68^{ab}	9.67^{a}	69.45^{d}	40.55^{ef}
SEM	0.47	0.21	0.10	2.33	0.32	0.41	0.26

Means within a column which are not followed by a common superscript letter are significantly different ($P < 0.05$)
SEM= Standard error of mean MSS; C1, without additive, C2 0.5% NaCl, 0.5% Urea and 2% molasses on FM basis
SB, Sodium benzoate; PS, Potassium sorbate; PA, Propionic acid added @ 0.1% on FM basis

Table 3 Quality parameters of sugarcane tops silage after 30 day of ensiling with deferent chemical additive treatments

Treatment	pH (30 d)	Lactic acid(g/100g DM)	DM loss (%)	NH -N (% of total-N)	NDICP (% CP)	ADICP (% CP)
SB	5.09^{d}	5.69^{bcd}	4.35^{cde}	19.29^{c}	27.83^{a}	15.41^{ab}
SB+PS	4.92^{e}	4.98^{cd}	3.70^{e}	26.36^{b}	18.81	9.94^{c}
SB+PA	4.82^{e}	5.23^{bcd}	5.46^{abcd}	39.58^{a}	17.64	10.45^{c}
PS	4.57^{f}	6.00^{bc}	3.88^{de}	36.33^{a}	15.88	8.68^{c}
PS+PA	4.45^{f}	6.58^{b}	5.08^{abcde}	38.72^{a}	19.03	10.56^{c}
PA	4.22^{j}	8.57^{a}	4.75^{abcde}	29.97^{b}	17.83	9.71^{c}
SB+PS+PA	4.47^{f}	6.16^{bc}	4.30^{cde}	26.09^{b}	18.04	11.84^{bc}
Urea	5.94^{b}	5.45^{bcd}	6.24^{ab}	16.37^{c}	18.05	12.08^{b}
Molasses	5.75^{c}	5.34^{bcd}	4.84^{abcde}	19.58^{c}	18.29	8.67^{c}
NaCl	4.51^{f}	6.61^{b}	6.90^{a}	42.59^{a}	19.93	17.11^{a}
C1	5.94^{b}	4.53^{d}	5.62^{abc}	39.02^{a}	24.00	10.43^{c}
C2	6.14^{a}	4.33^{d}	4.92^{abcde}	37.09^{a}	18.67	10.01^{c}
SEM	0.11	0.19	0.17	1.56	0.82	0.50

Means within a column which are not followed by a common superscript letter are significantly different ($P < 0.05$)

ADICP, acid detergent fiber insoluble protein; NDICP, neutral detergent fiber insoluble protein; NH3-N, ammonia nitrogen % total nitrogen

Table 4 Nutritive values and Flieg point values of sugarcane tops silage under deferent additive treatments

Treatment	TDN (% DM)	ME(MJ/kg DM)	Flieg point	Fitness value
SB	58.15^{ab}	9.06^{a}	69.95^{f}	0.49^{cd}
SB+PS	58.63^{ab}	8.97^{a}	76.97^{e}	0.51^{c}
SB+PA	59.21^{a}	9.02^{a}	85.17^{cd}	0.48^{de}
PS	59.94^{a}	9.19^{a}	89.34^{c}	0.53^{b}
PS+PA	59.43^{a}	9.02^{a}	97.65^{ab}	0.53^{b}
PA	58.76^{ab}	9.00^{a}	100.79^{a}	0.56^{a}
SB+PS+PA	59.38^{a}	8.82^{ab}	94.73^{b}	0.56^{a}
Urea	52.04^{e}	7.75^{c}	23.87^{k}	0.46^{ef}
Molasses	56.82^{bc}	8.51^{b}	39.96^{j}	0.50^{cd}
NaCl	51.60^{e}	7.71^{c}	80.84^{de}	0.44^{f}
C1	54.99^{cd}	8.11^{c}	24.20^{k}	0.53^{b}
C2	53.34^{de}	8.04^{c}	25.44^{k}	0.50^{cd}
SEM	0.50	0.09	4.93	0.01

Means within a column which are not followed by a common superscript letter are significantly different ($P < 0.05$)

Means within columns with different superscripts differ ($P < 0.05$)

ME, Metabolisable energy; TDN, total digestible nutrient estimated by NRC, 2001.

different treatment had ($P < 0.05$) variations in cell wall components (NDF, ADF), which has been depicted in the Table 2. Cell wall component (NDF, ADF) in all additive treated SCT silage was found lower ($P < 0.05$) than control (C1 and C2). The reduction in NDF, ADF was higher in chemical additives SB+PA, PS, PS+PA, SB+PS treated sugarcane tops silages, which is an indicative effect of chemical additives can degrade structural carbohydrates to water soluble carbohydrate and improve silage quality and fibre digestibility. Similar to our findings Siqueira et al. (2007) has observed higher hemicellulose solubilisation and degradation in sugarcane silage on sodium benzoate (0.1 FM) treatment and resulting in increased ($P < 0.05$) IVDMD in treatment groups i.e. 61.1 against 39.4% to the control.

Quality parameters

Silage samples were analysed on 30th day of ensiling for pH and lactic acid content, the values are presented in Table 3. The pH content of ensiled SCT silage ranged from 4.45 to 6.14 respectively. In relation to control (C1 and C2) all the additive treatment group has low ($P < 0.05$) pH values and were minimum in treatments PA, PS+PA, SB+PS+PA and PS sugarcane tops silages. The pH value of additive treated SCT silage was within the ideal range, indicating a good fermentation characteristic (McDonald et al. 1995, Kebede et al. 2018). Buffering effect of urea attributes the slower decline in pH (Kebede et al. 2018). Results from present study suggested clear relationship between

decline in pH and increase ($P < 0.05$) in Lactic Acid content (g/100g DM) in all additive treated SCT silages as compared to control C1 and C2. A similar result of lower pH values was reported by Siqueira et al. (2007), their study in which sodium benzoate was added at 0.1 percent FM in sugarcane tops silage. In the present study, mean Dry matter loss (DML % DM) of control SCT silage was approximately similar to the other studies by Pedroso et al. (2007) and Kebede et al. (2018). However, the dry matter loss on chemical additive treatment was higher and the values ranged from 31.12 to 12.85 (% DM). This might be a cumulative loss of dry matter occurred during fermentation, or due to urea treatment. Additionally, Kung and Shaver (2001); Kebede et al. (2018) has reported urea treatment of SCT at 0.5% FM accomplished a significant higher DM loss than control silage, while adding molasses with urea can counteract the DML by urea. In contrary to this, Pedroso et al. (2011) in their study also reported DML in sugarcane silage reduced by 31% when added with 0.5% urea FM.

In present study, NH3-N (% TN) increased ($P < 0.05$) in C2 (with common additive) and chemical additive treatment as compared to the control C1 (additive free) SCT silage. Obtained results indicate that ammonia nitrogen increased by addition of urea in all groups. However, SCT silage treated with propionic acid had the lowest NH_3N (% TN) as similar to control without additive treatment (C1). These finding on propionic acid addition from present study agreed with previous report (Winters et al. 2001) and the explanation that the addition of propionic acid during ensiling suppresses the proteolysis and plant enzyme activity which simultaneously improved amino acid balance and reduces the NH_3-N concentrations. ADICP is one of the important parameters of silage quality and was used to assess the successful ensiling technique. The ADICP of the silage was expressed in terms of ADICP (% CP) of SCT silage is presented in Table 3. The results indicated that ADICP (% CP) obtained for SCT silage sample was significantly higher in urea (0.5% FM) and other chemical additive added SCT silage, while other treatments were similar to the control C1 and C2. ADICP content is defined as the fraction of crude protein which remained unavailable to the animals, ADICP content > 12% CP suggests excess heat damaged protein produced or Maillard reaction occurred between carbohydrate and ammonia due poor ensiling (Yu and Thomas, 1976).

Nutritive values and Flieg point

Nutritive value especially energy content, TDN (%DM) and ME (MJ/kg DM) of sugarcane tops silage ranged from 51.60 to 59.94 and 7.71 to 9.19 respectively (Table 4). Results from the study depicts that the TDN and ME content ($P < 0.05$) increased in all chemical additive treated SCT silage as compared to the control SCT silage C1 and C2. The values were highest for treatment PS followed by PS+PA, and PA and lowest in NaCl and C1 treatment SCT silage. The ME value of intact SCT was reported to be 7.0 MJ/kg DM (McKenzie and Griffiths, 2007). Akinbode et al. (2017) have reported ME of SCT silage was increased on ensiling as attributed to the increased NFC content compared to fresh sugarcane tops. Pedroso et al. (2011) assessed the effect of various chemical and biological additives for quality sugarcane silage and reported that silage treated with sodium benzoate (1g/kg fresh matter basis) had a higher TDN content compared to untreated silage, which indicates that sodium benzoate treatment improved the nutritional value of sugarcane silage. Flieg point values of C1 and C2 SCT silage was 24.20 and 25.44, respectively, all additive treatments were higher ($P < 0.05$) in Flieg point than control C1 and C2 SCT silage. According to Flieg point score (Moselhy et al. 2015) all the additive treated SCT silage was of very good quality. Flieg point and fitness value were highest in treatment PA followed by PS+PA. The Fitness value was also higher ($P < 0.05$) in all additive treated SCT silage, which suggested for PA and PS+PA additives were the most promising additive for the improving the silage quality and nutritive value. Results from the study can provide important information on selection of best chemical additive and considering for feeding trail in dairy animals.

Conclusion

Chemical additive supplementation before ensiling can improve sugarcane tops silage quality parameters and nutritive values. Addition of chemical additive improved protein and energy content by fibrous solubilisation by chemical additive treatment. The values of ammonia nitrogen (NH_3N% TN), ADICP, flieg point and fitness value are considered tool for the selection most promising chemical additives. The potassium sorbate and propionic acid and their combination were found most promising chemical additive in the study, added at the dose rate 1 g per kg sugarcane tops on wet basis. Furthermore, feeding trail recommended for assessing effect of these chemical additives on feed intake and nutrient utilisation in animals.

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RESEARCH ARTICLE

Support of *Desi* cows in the daily livelihood of farm households in Karnataka

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Abstract: Desi cows along with crossbred have major contribution in fulfilling the demand of milk by the growing population of India. Achieving food security, nutrition security and income security for the farmers and by the farmers is a priority concern of national and state governments in India. In order to fulfill the national goal of doubling farmers income by 2022, animal husbandry in general and dairy farming in particular are considered as potential activities. In this backdrop, the present study was carried out in six districts of Karnataka based on higher population of desi cows with a sample size of 240 farm households. The study revealed that the sample households had more number of milking desi cows as compared to crossbred cows. Average quantity of green fodder and concentrates fed per animal in case of desi cows was less compared to crossbred cows. Thus, total feed cost and expenditure per animal was less in case of desi cows than crossbred cows. Desi cows required less expenditure on health per day/anim., but the net return per animal, per farm and per liter was less in desi cows due to low productivity as compared to crossbred cows. Crossbred cows supplied more nutrients to farm i.e. NPK kg/year/animal compared to desi cows. However, protein, fat and calcium nourishment per animal to the family was more in case of desi cows as compared to crossbred cows. Employment generation (Man days/year) per animal was more in case of crossbred cows as compared to indigenous cows. Security for uncertainties and status symbol was more in case of crossbred cows as compared to desi cows.

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Keywords: Desi cows, Crossbred cows, Dairy farming, Livelihood.

Introduction

Livestock plays an important role in Indian economy. About 20.5 million people depend upon livestock for their livelihood. Livestock provides livelihood to two-third of rural community. It also provides employment to about 8.8 % of the population in India. India has vast livestock resources. Livestock sector contributes 4.11% GDP and 25.6% of total Agriculture GDP. India is World's highest livestock owner at about 535.78 million. Out of this 192.49 million is cattle population i.e. 151 million desi and 39.732 million crossbred cows. In 2017-18, Indian dairy sector produced 176.3 million tons of milk. The per capita availability of milk has also increased from 112 grams per day in 1968-69 to 290 grams per day in 2017-18 (Basic Animal Husbandry Statistics, 2018). Cows have major contribution in the fulfilling the demand of milk by the growing population of India.

Most of the desi cows (about 80%) are non- descript and only 20 per cent belong to desi breeds. Desi cows are considered to be robust and resilient and are particularly suited to the climate and environment of their respective breeding tracts. They are endowed with qualities of heat tolerance, resistance to diseases and the ability to thrive under extreme climatic stress and less than optimal nutrition (GOI, 2014). Most of the desi cows possess A2 allele of beta casein as compared to crossbred cows. Reportedly A1 milk is associated with some metabolic disorders like diabetes, heart diseases etc., whereas, A2 milk from desi breeds does not have any such association. Cow is a source of subsidiary income for many families in India especially the resource poor who maintain few heads of animals.

In Karnataka, more than 60 per cent of its population lives in rural areas and depends on agriculture for their source of income (Government of Karnataka, Census, 2011). Nearly 90 per cent of the population in the semi-arid region depends on agriculture for livelihood and livestock rearing as subsidiary occupation. In difficult situations, livestock, especially desi cows rescue the farmers from economic crisis. During drought and hot environmental conditions desi cows are reported to thrive on

poor quality roughages and acts as the buffer in the crisis. Therefore, it becomes essential to know the exact role played by desi and crossbred cows in the livelihood of resource poor farmers. Considering the above facts, present study entitled "Support of Desi Cows in the daily livelihood of farm households in Karnataka" was undertaken.

Materials and Methods

As per National Bureau of Animal Genetics Resources (2020), there are six desi cows breeds in Karnataka, namely; Amrithmahal, Deoni, Hallikar, Khillari, Krishna Valley and Malnad Gidda along with good number of crossbred cows population. So, the present study was carried out purposively in the Karnataka. The sampling scheme adopted for this study was three-stage stratified random sampling without replacement. The study was conducted in six districts of Karnataka based on highest population of desi cows. From each of the selected districts, two taluks were selected based on highest density of desi and crossbred cows in that locality, as per the secondary data. From each of the selected taluk, based on similar criteria, a cluster of three village panchayats and a total of 36 village panchayats formed the study area. From each selected cluster of villages, 20 households owning desi and crossbred cows and from each selected household, one adult member or head of the household actively engaged in management of desi and crossbred cows was considered as the respondent. Thus, 20 cow owners from each cluster of villages, made a total of cow owners sample size to 240.

The data were collected through semi-structured interview schedule. The respondents were asked to give information on milk production and livelihood security parameters. The data so collected were analyzed for estimating the costs, returns from milk production and contribution in livelihood security of farmers from desi and crossbred cows. The returns were calculated over variable cost i.e. gross margin. The statistical significance of differences in milk production parameters were tested by using 'z' test with the help of SPSS software. Livelihood security is operationalized as contribution made by desi and crossbred cows in terms of income generation, nourishment to the family, nutrients to farm, employment generation, security during uncertainties and social status symbol. The index developed by Biradar et al (2013) was used with required modifications as given below:

- Contribution to the total household income: The net return was measured by collecting information on different production values of each cow and average values of each parameter were calculated.
- Nourishment to the family: Based on the daily average milk consumed by the family, the nutrients were computed in terms of protein, fat and calcium as suggested by Gopalan et al. (1971).
- Nutrients to the farm: The average farm yard manure applied to their respective farm was converted in terms of N, P and K by following the conversion factors suggested by Gautam (2007), that is, one ton of farm yard manure was equivalent to 8 Kg N, 4 Kg P_2O_5 and 16 Kg K_2O.
- Employment generation: Number of hours engaged in desi and crossbred cows rearing for one year were collected. Total hours spent in a year were divided by 8 hours to convert them in to man-days. Total number of man-days contributed was expressed as mean values.
- Security during uncertainties: Number of households having used desi and crossbred cows to face the uncertainties in the past two years.
- Status symbol: The number of households who regard keeping desi and crossbred cows as symbol of social status.

Results and Discussion

To ascertain role of desi and crossbred cows in dairy production of farmers, the average values of some of the milk production parameters were calculated and are presented in Table 1. Sample households had more number of milking desi cows (2.03) as compared to crossbred cows (1.66). Average quantity of green fodder and concentrates fed per animal in case of desi cows was 9.82 & 0.83 kg respectively. This was less as compared to crossbred cows (17.47 & 3.11 kg resp.). Although average quantity of dry fodder fed per animal in case of desi cows was more (7.13 kg) as compared to crossbred cows (7.06 kg), total feed cost and expenditure per animal was less in case of desi cows (Rs.56.61 & 67.11 resp.) than crossbred cows (Rs.104.13 & 138.11 respectively). In all the cases, desi cows were producing less milk (2.86 L/day) as compared to crossbred cows (6.83 L/day). Desi cows required less expenditure on health per day/anim. (Rs.0.5) but the net return/day per animal (Rs.12.98) was less due to low productivity as compared to crossbred cows (Rs.53.10).

As majority of the cows owners used own farm grown dry and green fodder to fed their cows or from grazing. Also, dung produced (19.45 & 25.23 Kg/day/anim. in case of desi & crossbred cows) was used for manure of his/her own farm including drought power (1.95 & 0.03 hrs/day in case of desi & crossbred cows). Milk for nourishment to family was higher from desi breeds (1.40 L/day) as compared to crossbred cows (0.19 L/day). Without considering cost of fodder as shown in Table 1, total feed cost (Rs/anim./day) was less in case of desi cows (13.35) as compared to crossbred cows (49.70). Thus, total expenditure (Rs/anim./day) was less in case of desi cows (23.85) as compared to crossbred cows (83.68). Net return/day per animal (Rs.56.24) was less in case of desi cows due to low productivity as compared to crossbred cows (Rs. 107.53).

Similarly, contribution of dairy animals in Western Maharashtra has been assessed by Kolekar et al. (2015), wherein gross return per animal, per farm and per liter was more in case of private dairy farmers as compared to cooperative dairy farmers. The 'z' test

was used to test the difference between the milk production parameters perceived for desi and crossbred cows. Analysis showed that there was a significant difference between all the milk production parameters of two cow breeds.

To ascertain contribution of desi and crossbred cows to the farmers livelihood, the average values of the types of contribution were calculated and presented in Table 2. From the Table 2, it is clear that net return/day per animal (Rs.12.98), per farm (Rs.27.18) and per liter (Rs.3.00) was less in desi cows due to low productivity as compared to crossbred cows (Rs. 53.10, 91.62 & 5.69 resp.). Majority of the cows owners used own farm grown dry and green fodder to feed their cows or from grazing. Also, dung produced used for manure of his own farm including drought power & some milk for nourishment to family. Protein, fat and calcium nourishment per animal to the family gm/day was less in case of crossbred cows (5.92, 7.59 & 0.22, respectively) as compared to desi cows (44.90, 57.52 & 1.68, respectively). Nutrients supply to farm i.e. NPK kg/year/animal was more in case of crossbred cows (73.66, 36.83 & 147.33, respectively) as compared to desi cows (56.79, 28.40 & 113.59, respectively). Employment generation (Man days/year) per animal was more in case of crossbred cows (80.02) as compared to desi cows (42.34).

Table 1 Milk production parameters of desi vs crossbred cows perceived by households

Parameter	Desi Cows		Crossbred Cows		P value
	Mean	SD	Mean	SD	
Total milking animals (no.)	2.03	1.80	1.66	1.29	0.008
Total milk production (L/day)	5.85	5.57	11.55	10.78	0.000
Total milk production (L/anim./day)	2.86	0.69	6.83	1.25	0.000
Total dry fodder fed (kg/anim./day)	7.13	2.42	7.06	2.05	0.001
Total daily green fodder fed (kg/anim./day)	9.82	4.39	17.47	5.39	0.001
Total concentrate fed (kg/anim./day)	0.83	0.51	3.11	0.79	0.000
Total feed cost (Rs/anim.)	56.61	14.66	104.13	18.51	0.013
Labour cost (Rs/anim./day)	10.00	.000a	30.00	.000a	Na
Health cost (Rs/anim./day)	0.50	0.00	3.98	0.18	Na
Total expenditure (Rs/anim./day)	67.11	14.66	138.11	18.49	0.014
Net return/anim. (Rs./day)	12.98	10.30	53.10	28.67	0.000
Milk nourishment to the family (L/day)	1.40	0.72	0.19	0.50	0.000
Dung production (Kg/day/anim.)	19.45	3.16	25.23	3.55	0.004
Draught animal power (hrs/day)	1.95	2.38	0.03	0.39	0.000
Without considering cost of fodder					
Total feed cost (Rs/anim./day)	13.35	8.08	49.70	12.64	0.000
Total expenditure (Rs/anim./day)	23.85	8.08	83.68	12.62	0.000
Net return/anim. (Rs./day)	56.24	15.75	107.53	33.43	0.000

Table 2 Contribution of desi vs crossbred cows to the farmers livelihood

Type of contribution	Unit	Values		P Value
		Desi Cows	Crossbred Cows	
Income from cows	Net return/anim./day (Rs.)	12.98	53.10	0.000
	Net return/farm/day (Rs.)	27.18	91.62	0.000
	Net return/L (Rs.)	3.00	5.69	0.003
Income from cows	Net return/anim./day (Rs.)	56.24	107.53	0.000
(Without considering cost	Net return/farm/day (Rs.)	114.01	182.48	0.011
of fodder)	Net return/L (Rs.)	14.03	11.93	0.000
Nourishment to the Family	Protein (gm/day/family)	44.90	5.92	0.000
	Fat (gm/day/family)	57.52	7.59	0.000
	Calcium (mg/day/family)	1683.60	222.00	0.000
Nutrients to the Farm	N kg/year	56.79	73.66	0.004
	P kg/year	28.40	36.83	0.004
	K kg/year	113.59	147.33	0.004
Generating Employment	Man days/year	42.34	80.02	0.000
Security for Uncertainties	Percentage	18.83	78.00	0.000
Status Symbol	Percentage	26.33	81.00	0.000

Security for uncertainties and status symbol was more in case of crossbred cows (78% & 81%, resp.) as compared to desi cows (18.83% & 26.33%, resp.). Similar contribution of livestock to the livelihood of farmers in Western Maharashtra has been assessed by Biradar et al. (2013), wherein contribution of livestock to the household income ranged from 18.60 to 33.90 per cent and 63 per cent of dairy farmers opined livestock farming is a symbol of higher social status and concluded that livestock system contributed economically and socially to enhance sustainable livelihoods. Chaminuka et al. (2013) studied the livelihood role of cattle in South Africa and revealed about 11 per cent of the local household households owned cattle and cattle income constituted 29 per cent of total household income. The study inferred that cattle production has important livelihood roles, but is not sufficient as a driver of economic development. Radder et al. (2012) studied the livelihood systems of dairy farmers in Karnataka using Nine-Square-Mandalas' model and revealed that dairy activities integrated into several other economic activities performed by households. The role of dairy varied from a major source of economic livelihood sustenance to the minor system of subsistence milk production. Income from dairy was the source for purchasing food grains and to meet family cash needs. The 'F' & 'Chi-square' test was used to test the difference between the types of contribution perceived by farm households in case of desi and crossbred cows. Analysis showed that there was a significant difference between all types of contribution of desi and crossbred cows.

Conclusion

The significant differences in milk production parameters and components of livelihood security in case of desi and crossbred cows were due to low productivity in case of desi cows as compared to crossbred cows. Also, one of the reasons for greater contribution of crossbred cows to livelihood security was most efficient and scientific management as compared to desi cows. However, Protein, fat and calcium nourishment per animal to the family gm/day was more in case of desi cows (44.90, 57.52 & 1.68, respectively) as compared to crossbred cows (5.92, 7.59 & 0.22, respectively). The potential to enhance the productivity of the desi breeds of India through professional farm management and superior nutrition is immense. Desi cows productivity can be improved with organized breeding programs, better management practices along with crossbred cows to hasten the efficiency of milk production and livelihood security of resource poor farmers.

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RESEARCH ARTICLE

Micro-level evaluation of socio-technological interventions to address climate change-induced stresses in dairy enterprises

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Abstract: The paper aims at micro-level evaluation of dairy centric socio-technological interventions under the Technology Demonstration Component (TDC) of the National Innovations in Climate Resilient Agriculture (NICRA) Project in Karnal, Yamunanagar and Sirsa districts of Haryana. The interventions were introduced to address the identified climate stresses under the project for enhancing resilience in dairy production. The study was conducted during 2015-16, employing Participatory Rural Appraisal (PRA) techniques to assess the farmers' feedback on climate change perception and their acceptance and satisfaction of introduced interventions, from the NICRA adopted village in each district. Ten socio-technological interventions were evaluated each based on two dimensions of perceived attributes and benefits sub-evaluation parameters. The reason based satisfaction was recorded on a farmer rating for each intervention. Among six introduced technological interventions, improved fodder crop varieties (total score 28 and 30) and area specific mineral mixture supplementation (total score 28 to 29) were perceived as highly beneficial in terms of sustained milk production even during periods of heat stress followed by moderate beneficial of interventions *viz*., Vitamin E supplementation, mustard oil supplementation and safe dung disposal through composting. Silage making intervention was perceived as low beneficial (total score 16). The Village Climate Risk Management Committee (VCRMC) was found as a highly beneficial (total score 27 to 30) social intervention, followed by women participation, contingency plan for climate change preparedness in fodder crops and preparation of season-centric milk products as moderate beneficial. The VCRMC was found highest satisfactory as rated by the farmers.

Keywords: Climate stresses, Dairy centric Interventions, Micro-level Evaluation, NICRA Project

Introduction

Animal husbandry continues to be a cornerstone for economic development as it accounts for about 26.2 per cent of the Gross Value Added (GVA) of agriculture and allied sector (GoI, 2019). The dairy farming sector is sensitive to climate which poses as a serious long-term challenge. The small holder farmers bear the major brunt of the undesirable effects of climate change and climate variability, as they often lack the adequate resources for adaptation to climate change. This severely curtails farm production, threatening the livelihood, food and nutritional security of the already marginalized households. The impact of climate change has predicted a fall in agricultural productivity of northern India to a tune of 25 per cent, between 2003 and 2080s (Cline, 2007). Further, the increase in frequency and intensity of extreme weather events cause severe socio-economic consequences particularly to the marginalized groups in society (Huvisa, 2012). Climate change exerts long term impacts on dairy farming in various ways such as heat stress in animals which could result in reduced feed intake and milk production, increase in disease incidence and decreased reproductive performance (Chakravarty et al. 2012). The most vulnerable people to climate change are most often the poorest, who lack effective coping strategies to deal with shocks and stresses and who have had to resort to ineffective responses (Daze, 2011).

The National Innovation in Climate Resilient Agriculture (NICRA) project was launched in 2011 by the Indian Council of Agricultural Research (ICAR) with the funding from the Ministry of Agriculture and Farmers Welfare, Government of India. The specific objective

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of the Technology Demonstration Component (TDC) of NICRA is to enhance the resilience of Indian agriculture (crops, livestock and fisheries) to climatic variability and climate change through demonstration of site-specific technological interventions on farmers' fields (Ponnusamy et al. 2019). Rural communities have always been working to adapt to change in climate as it gradually occurred over centuries, but farmers in their specific agricultural systems, would benefit highly from support to develop sound and location specific adaptation strategies (Allara et al. 2012). It is therefore important to analyze, the level of accrued benefits of dairy centric interventions from the farmer's perspective in the rural households (Ponnusamy and Pachaiyappan, 2018) in the project site. Such type of study has so far not been conducted to assess the interventions introduced in the livestock component under the umbrella of the NICRA project. Therefore, the present study was conducted to evaluate the dairy centric socio-technological interventions at micro-level and also assess the level of satisfaction of the farmers on those socio-technological interventions for further adaptation planning in the TDC-NICRA adopted villages.

Materials and Methods

Sampling

The study was carried out in Karnal, Yamunanagar and Sirsa districts of Haryana, under the umbrella of the Technology Demonstration Component of the NICRA project during 2015-16. Three villages *viz.,* Sohana in Karnal, Radauri in Yamunanagar and Rupana khurd in Sirsa district, adopted under the TDC-NICRA project were purposively selected and 30 households were selected from each village.

Under the Technology Demonstration Component of the NICRA project, the climate related stresses were identified in the respective study area. Climate stress such as heat stress in dairy animals and fodder scarcity in Sohana village, long dry spell, terminal heat stress, depletion of ground water in Radaur village and drought, heat wave, salinity in Rupana khurd village were the major identified climate induced stresses in the livestock farming by the respective project team.

Data Source

The study employed mainly primary data from 90 households, collected by personal interview through a developed and pre-tested schedule encompassing the socio-economic profile *viz*., landholding, herd size, annual income from dairy farming and gross annual income from all sources of each farmer from the selected villages. Data on farmer feedback based micro-level evaluation, were collected through the Participatory Rural Appraisal (PRA) techniques using a semi-structured interview schedule and checklist on various parameters pertaining to components of perceived attributes and benefits of the introduced socio-technological interventions. For conducting the PRA, representative group of farmer participants comprising 15 farmers in each district who had participated in the technology demonstration (TD) programme was selected by ensuring at least three participants for each intervention. To gain insight to the climate related stresses impacting the district, the Principal Investigators of the TDC-NICRA project in the three districts were also interviewed.

Socio-Technological interventions in the project site

Ten socio-technological interventions in dairy enterprise were considered of which six technological interventions comprises of area specific mineral mixture supplementation, improved fodder crop varieties, vitamin E supplementation, supplementation of mustard oil, silage making and safe dung disposal through composting while four social interventions included Village Climate Risk Management Committee (VCRMC), preparation of season centric dairy products for farm women, women participation and contingency plan for climate change preparedness in fodder crops. Area specific mineral mixture supplementation was introduced in each adopted village of all the three districts. Improved fodder crop varieties were introduced in the same adopted village in Karnal (BL 42, Oats Kent, Sorghum SX 17, Bajra-Napier grass) and Yamunanagar (Berseem varieties HB1, HB2) district, three technological interventions (vitamin E supplementation, supplementation of mustard oil and safe dung disposal through composting) were introduced in the adopted village of Karnal district and silage making was introduced in the adopted village in Yamunanagar district. Among the social interventions, Village Climate Risk Management Committee (VCRMC) was considered for each adopted village of all the three districts and two social interventions were studied in the adopted village in Karnal and one in Yamunanagar district.

Evaluation parameters for socio-technological interventions

Seven parameters were considered for the evaluation of each technological intervention at micro level. Farmer feedback on interventions was generated on the perceived attributes of the interventions *viz*., ease in use (X_1), farm suitability (X_2), triability (X_3) and relative advantage (X_4) and perceived benefits in terms of low input requirement (Y_1), high or sustained production (Y_2) and more income (Y_3). Each of these parameters were allotted a highest score of 5.0, thus, perceived attributes $(Z1) = \sum X_{i(1 \text{ to } 4)}$ Perceived benefits $(Z2) = \sum Y_{i(1 \text{ to } 3)}$ and total score for each technological intervention $= (Z1 + Z2) = \sum X_{i(1 \text{ to } 4)} + \sum Y_{i(1 \text{ to } 3)}$. Each technological intervention was thus evaluated based on maximum possible score of 35. Seven parameters were also considered for the evaluation of each social intervention at micro level. The perceived attributes of the social interventions pertained to number of households benefitted in the village (X1), inclusion of all categories of farmers (X2), linkage required with

government and other agencies (X3) and gender equity (X4) and the perceived benefits included perceived sustainability (Y1), benefits in terms of incentives / subsidies / services / information support / training (Y2) and relative advantage (Y3). Each of these parameters were allotted a highest score of 5.0, thus, perceived attributes (Z1) = $\sum X_{i(1 to 4)}$; perceived benefits (Z2) = $\sum Y_{i(1 to 3)}$ and total score for each social intervention = (Z1+ Z2) = $\sum X_{i(1 to 4)} + \sum Y_{i(1 to 3)}$. Each social intervention was thus evaluated based on maximum possible score of 35. The intervention was categorized as high (total score > 26), moderate (17.5 to 26) and low (< 17.5) based on < 50 %, > 50-75 % and > 75 % beneficial using mean and standard deviation.

Rating of satisfaction on the introduced Socio-Technological interventions

The satisfaction of the farmers on each socio-technological interventions in the study area was rated on a four points scale of 0 to 3 (no satisfaction, low satisfaction, moderate satisfaction and high satisfaction), on each intervention and their reasons of response were identified from the respective districts.

Results and Discussion

Socio-economic profile of farmers

The socio-economic profile of the farmers in the study area revealed that most of them belonged to the middle income category based on their dairy enterprise (43.33 %) and the total gross annual income of the most of the farmers (40.00 %) was found to be low (Table 1). Though a majority of the farmers (60 %) were rearing medium herd size, yet, income was found to be low. One of the major reasons for this could be the climate adversities affecting the district. To ameliorate the climate impact, the introduction of socio-technological interventions was envisaged to promote resilient production and thereby help to sustain farmers' income.

The technological interventions were introduced in all animal holding categories ranging from small (up to 2.65 Standard Animal Unit) to large herd size (more than 6.41 Standard Animal Unit). For introduction of improved fodder varieties to address fodder scarcity, land was a prerequisite and all categories of land holding farmers were included ranging from marginal farmers (upto 1 ha) to large farmers (more than 10 ha).

Technological interventions: The technological interventions introduced for dairy enterprises are presented in Table 2.

Area specific mineral mixture supplementation: This technological intervention had been introduced in the NICRA adopted villages in three districts. Total score based on four perceived attributes and three perceived benefits as per farmer feedback ranged between 28 to 29 and the technology was rated high by the farmers in all the villages of three districts. Due to heat stress experienced by the dairy animals, their fertility was reduced and milk yield was decreased. The area specific mineral mixture components for Cu, Mg, Ca and Zn supplemented @ 50gms/day specially, during transition period, proved beneficial. Fertility rate of dairy animals was also reported to increase.

Improved fodder crop varieties: Improved fodder crop varieties of berseem (BL 42), Oat (Kent), Sorghum (SX 17), Bajra-Napier grass were demonstrated in the same NICRA adopted village in

Table 1 Distribution of respondents according to annual income from dairy farming, annual income from all sources, herd size and land size (n=90)

Category	Annual Income from Dairy Farming (Rs.)	f	%
Low	Upto 8595	28	31.11
Middle	> 8595 to 25109	39	43.33
High	More than 25109	23	25.56
Category	Annual Income from All Sources (Rs.)		
Low	Upto 128516	36	40.00
Middle	> 128516 to 245954	29	32.22
High	More than 245954	25	27.78
Category	Standard Animal Unit (SAU)		
Small	Up to 2.65	18	20.00
Medium	> 2.65 to 6.41	54	60.00
Large	More than 6.41	18	20.00
Category	Land holding (ha)		
Marginal	Upto 1 ha	10	11.11
Small	1.1 to 2 ha	18	20.00
Semi-medium	2.1 to 4 ha	29	32.22
Medium	4.1 to 10 ha	17	18.89
Large	More than 10 ha	16	17.78

Karnal district. As per farmers' feedback, total score based on four perceived attributes and three perceived benefits was 30 and the technology was rated high in the adopted village of Karnal district. Improved fodder crop varieties (Berseem varieties HB1, HB2) were also demonstrated in the same adopted village in Yamunanagar district. As per farmers' feedback total score based on same perceived attributes and benefits was 28 and the technology was also rated high in the adopted village of Yamunanagar district too since the traditional berseem variety Mascavi suffered due to increased stem borer attack and HB 1 and HB 2 Berseem varieties were reportedly found to be resistant to stem borer attack, besides being fast growing and yielding nutritious and palatable fodder.

Supplementation of Vitamin E and Mustard Oil: The supplementation of Vitamin E @ 5 gm/day/animal and Mustard Oil supplementation of 50 ml/day/animal were introduced in the NICRA adopted village in Karnal district only. As per farmers' feedback, the total score based on similar perceived attributes and benefits was 24 for Vitamin E supplementation and 26 for Mustard oil supplementation. Both the interventions were rated as moderate as per farmers' feedback. Feeding mustard oil during summer season for 3 months helps in overcoming negative energy balance and help in sustaining milk production. Problems such as the high incidence of mastitis, poor reproductive efficiency and suppressed immune status of the animals were reported to be improved as Vitamin E supplemented during the transition period, works as an antioxidant and improves the immunity.

Silage making: The silage making was introduced only in the NICRA adopted village in Yamunanagar district for ensuring the availability of fodder during lean season as a result of the impact of climate change on agriculture. The intervention was however rated low as per farmers' feedback as the total score based on similar perceived attributes and benefits was only 16 as, it scored less on ease of use, farm suitability and triability under perceived attributes and low on low input and more income under perceived benefits, too.

Safe dung disposal through composting: The intervention was introduced in the NICRA adopted village in Karnal district to discourage open dumping of dung on the road side and mitigating the menace of mosquitoes. In addition, this could also result in methane reduction and boost the crop yields due to better nutrient availability. The intervention was rated as moderate as based on perceived attributes and benefits the total score was only 22. However, the farmers perceived this intervention to be cumbersome as it requires additional land and labour input.

Table 2 Evaluation of technological interventions introduced for dairy enterprise

Technological intervention in dairy enterprise	District	Total perceived attributes (Z1)	Total perceived benefits (Z2)	Attributes+ Benefits (Total Score) Z1 + Z2)
Area specific mineral mixture	Karnal	19	10	29
supplementation	Yamunanagar	18	10	28
	Sirsa	18	10	28
Improved fodder crop varieties	Karnal	19	11	30
	Yamunanagar	17	11	28
Vitamin E supplementation	Karnal	15	9	24
Mustard oil supplementation	Karnal	16	10	26
Silage Making	Yamunanagar	9	7	16
Safe dung disposal through composting	Karnal	14	8	22

Table 3 Evaluation of social interventions introduced for dairy enterprise

Social intervention in dairy enterprise	District	Total perceived attributes (Z1)	Total perceived benefits (Z2)	Attributes+ Benefits (Total Score)Z1 + Z2
Village Climate Risk	Karnal	15	14	29
Management Committee (VCRMC)	Yamunanagar	16	14	30
	Sirsa	14	13	27
Preparation of season centric dairy products for farm women	Karnal	12	12	24
Women participation	Yamunanagar	15	10	25
Contingency plan forclimate change preparedness in crops and fodder crops	Karnal	15	10	25

Social interventions: Four social interventions like the Village Climate Risk Management Committee (VCRMC), preparation of season centric dairy products for farm women, women participation and contingency plan for climate change preparedness in fodder crops were introduced for dairy enterprises as presented in Table 3.

Village Climate Risk Management Committee (VCRMC): Introduction of the VCRMC was mandatory under the NICRA project and introduced in the three NICRA adopted villages, one in each district. The intervention was highly rated as the total score based on the similar perceived attributes and benefits as per the feedback of farmers, were ranged from 27 to 30 in three districts. Many households of the village benefitted through the custom hiring service with active participation of women farmer members in the VCRMC.

Preparation of season centric dairy products for farm women: In the NICRA adopted village of Karnal district, women were trained on the preparation of season centric dairy products (Ponnusamy *et al*, 2020). The intervention was rated moderate as the total score based on perceived attributes and benefits as per feedback of farmers were 24. They desired more women to participate and more needs of women to be addressed in the project. Similar was the view of Salula (2012), that interventions such as integration of gender and vulnerable groups related issues into climate change initiatives, ensuring that climate change researches generate gender disaggregated data on impacts and response needed to be introduced.

Women Participation: Participation of women was encouraged in the NICRA adopted village of Yamunanagar district as women were trained on the use of fungicides and fungicides were distributed only through women. The intervention was moderately rated as the total score based on perceived attributes and benefits as per feedback of farmers were 25, securing moderate score in some sub parameters of evaluation but top score on gender equity.

Contingency plan for climate change preparedness in fodder crops: Due to unseasonal rain in the Karnal district during 2016, a contingency plan for climate change preparedness in fodder crops was developed and introduced in the NICRA adopted villages. The intervention scored moderate on number of farm families benefitted and inclusion of all farmer categories, securing a total score of 25.

Farmer satisfaction on the Socio-Technological interventions: Since the interventions were introduced to address the climate stresses affecting dairy enterprises in the districts, it was imperative to know the perceptions of the farmers towards climate change and climate variability in their area as it would affect their acceptance and satisfaction on the interventions. The satisfaction of the farmers was rated on a four point scale of 0 to 3, on each intervention and their reason for response is presented in Table 4.

As a result of area specific mineral mixture supplementation, the farmers of Karnal and Yamunanagar districts were highly satisfied

Table 4 Farmer satisfaction on the socio-technological interventions introduced in the NICRA adopted villages

Socio-technological intervention	Farmer satisfaction(0-3)	Reason for response
Area specific mineral mixture supplementation	3 2 (Sirsa)	Though it sustains high milk production, it is expensive
Improved fodder crop varieties	3 3	These are suitable, triable and more advantageous Fodder is good and stem borer does not attack HB1 &HB2 Berseem varieties in Yamunanagar district
Vitamin E supplementation	3	It is triable and easy to use
Supplementation of mustard oil	3	Easy to use and low input
Silage making	1	Though relative advantage is good, but, restricted land size and small herd size make it uneconomical
Safe dung disposal through composting	2	Though relative advantage is high, it requires some input
VCRMC	3	Through the VCRMC, many households of the village are benefitted and there are three women farmer members in the VCRMC
Preparation of season centric dairy products for farm women	2	It is high in relative advantage, more women need to be trained for scaling up the benefits to households
Women participation	2	The intervention is good, more needs of women may be addressed.
Contingency plan for climate change preparedness in fodder crops	2	All categories of farmers were included and relative advantage was also good

(score 3) by the sustained milk production during heat stress and the farmers of Sirsa district were moderately satisfied too, they found it more expensive, therefore, scored at 2. In both Karnal and Yamunanagar districts, farmers were highly satisfied with the improved fodder crop varieties, hence assigned with a top score of 3, as the new introduced varieties were found to be triable and more advantageous. Gitz and Meybeck (2012) also reported that greater forage production, more efficient use of land resources and enhanced profitability is essential, especially considering potential degradation of climate conditions such as increased risk of drought. Farmers were slightly more satisfied with mustard oil supplementation (score 3) over Vitamin E supplementation (score 3) as mustard oil is not only easy to use but also demands low input.

Acceptance of silage making was low (score 1) despite its relative advantage and role in sustained production of milk during fodder scarcity periods (April-May and August-September) as; restricted land size and small herd size render it unsuitable economically. In the same way, the farmers had failed to realize the importance of safe dung disposal through composting and opined a comparatively moderate satisfaction (score 2) because the intervention requires some input like land and labour for digging the manure pit and turning up the contents of the pit in 15-20 days for better quality of compost.

The farmers were highly satisfied with the introduction of the Village Climate Risk Management Committee (VCRMC) and rated at 3, in all the districts. On preparation of the season centric dairy products for farm women in adopted village of Karnal district, farmers gave a moderate satisfaction score of 2 as the intervention is high in relative advantage and more women need to be trained for scaling up the benefits to households. The farmers gave a moderate satisfaction score of 2 for women participation intervention as more needs of women may be addressed and contingency plan for climate change preparedness in fodder crops was also given a moderate rating of 2, on the farmer satisfaction.

Conclusion

The findings of the study disclosed the importance and influence of attributes of socio-technological interventions on sustained adoption to address the climate change issues in dairy farming. While the ease of adoption and its utility emerged as a point for further refinement of introduced technologies, convincing the farmers with appropriate extension interventions hold a greater promise for encouraging a strong climate resilient and profitable dairy farming.

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SHORT COMMUNICATION

Changes in hormones of the somatotropic axis associated with postpartum reproductive infections in Murrah buffaloes (*Bubalus bubalis*)

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Abstract: This study was designed to investigate the risk association of hormones of the somatotropic axis with postpartum infections/ disorders in Murrah buffaloes. A total of six (n=6) healthy pregnant Murrah buffaloes and 23 symptomatic buffaloes with symptoms indicative of metritis (n=5), endometritis (n=6) mastitis (n=8), and retained placenta (RP) (n=4) were selected from the ICAR-NDRI Cattle herd. Blood samples were collected from each healthy buffalo on days -21, -14, -7, 0, +7, +14, +21 relative to calving. Blood samples were collected from unhealthy buffaloes twice on alternate days as and when the symptoms of reproductive abnormalities were noticed. The results revealed that the plasma growth hormone was significantly ($P<0.05$) elevated in buffaloes exhibiting symptoms of metritis (7.40±0.96 ng /mL), mastitis (9.23±1.60 ng /mL), and RP (6.63±0.80 ng/mL). Similarly, plasma insulin level was significantly higher ($P<0.05$) in buffaloes infected with endometritis (1.53 ±0.21 ng/ml), mastitis (1.32 ±0.26 ng/mL), and RP (1.24 ±0.15 ng/mL). But, the plasma IGF-1 was significantly lower in buffaloes exhibiting symptoms of metritis (128.43±5.38 ng/mL), endometritis (112.65 ±4.38 ng/ mL), and mastitis (123.61 ±3.99 ng/mL) except in RP (122.21±5.10 ng/mL) than normally calved buffaloes (123.80±4.96 ng/ mL). From the experiment, it could be inferred that the levels plasma growth hormone, insulin, and IGF-1 differed significantly among buffaloes with and without postpartum infections and could be used for the risk assessment of postpartum infection in buffaloes.

Keywords: Buffaloes, Growth Hormone, Insulin, Insulin like Growth Factor-1, Postpartum Infections

The period around parturition in dairy cattle is characterized by negative energy balance (NEB), insulin resistance, reduced feed intake, hypocalcemia, and impaired immune functions (Mili et al. 2014, Sundrum, 2015, Mili et al. 2015a). The changes of hormones of the somatotropic axis (i.e., GH-insulin-IGF-1-glucose signaling pathway) help in energy homeostasis during the transition period in dairy cattle. However, the inability of dairy cows to cope with metabolic demands, hormonal milieu coupled with impaired immune functions make them susceptible to infectious diseases (metritis, endometritis, mastitis, RP, etc.), and thereby production performance of the dairy animal is seriously affected in terms of economic loss to farmers. Deviation of hormones of the somatotropic axis could be helpful as risk predictors for diagnosing metabolic and postpartum infections/ disorders in dairy cows. Therefore, the present study was aimed to find out the association of crucial hormones of the somatotropic axis with postpartum infections/ disorders in buffaloes.

The present experiment was conducted between September 2011 till May 2012 at ICAR-National Dairy Research Institute (NDRI), Karnal. The institute is located at an altitude of 250 m above mean sea level, latitude and longitude position 29°42N and 79° 54E, respectively. The maximum ambient temperature in summer goes up to 45° C, and the minimum temperature in winter comes down to 0° C with a diurnal variation in the order of 15-20° C. The average annual rainfall is 700 mm from early July to mid-September.

A total of six (n=6) numbers of healthy pregnant Murrah buffaloes and 23 symptomatic buffaloes with symptoms indicative of metritis (n=5), endometritis (n=6), mastitis (n=8), and retained placenta (n=4) were selected from the institute cattle herd. Metritis, endometritis, and mastitis infections were diagnosed by the institute's herd veterinary officer based on symptoms described by Sheldon et al. (2006). Metritis was diagnosed by the presence of systemic signs of sickness, including fever, red-brown watery, foul-smelling uterine discharge, dullness, elevated heart rate, and low production. In contrast, clinical endometritis was diagnosed

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Table 1 Plasma hormone concentration in buffaloes exhibiting postpartum infections / disorders

Parameters	Healthy	Postpartum infections			Day 0 (Normally calved)	RP (After 12h of parturition)
		Metritis	Endometritis	Mastitis		
IGF-1(ng/mL)	134.34±8.20[a]	128.43±5.38[b]	112.65 ±4.38[b]	123.61 ±3.99[b]	123.80±4.96	122.21 ±5.10
Insulin(ng/mL)	1.07 ±0.06[a]	0.86 ±0.12[b]	1.53 ±0.21[b]	1.32 ±0.26[b]	0.63 ±0.14[a]	1.24 ±0.15[b]
GH(ng/mL)	1.48 ±0.15[a]	7.40 ±0.96[b]	1.66 ±0.18	9.23 ±1.60[b]	2.47 ±0.55[a]	6.63 ±0.80[b]

Bearing different superscripts (a, b) in the mean value for the metritis, endometritis, mastitis indicated significant differences at $P < 0.05$ with that of healthy buffaloes, whereas RP compared to normally calved buffaloes (Day 0).

by the presence of purulent (>50% pus) or mucopurulent (approximately 50% pus, 50% mucus) uterine exudates in the vagina, 21 days or more postpartum. Clinical mastitis was diagnosed by an elevated somatic cell count in milk and visual signs of inflammation such as clumpy, watery, bloody, or yellowish milk. The buffaloes that did not shed the fetal membrane within 12 hours of parturition were considered as cases of RP. All these buffaloes were maintained under general managemental practices as followed at the institute.

A blood sample (15ml) was drawn in sterile heparinized vacutainer tubes by jugular venipuncture from each healthy buffalo on days -21, -14, -7, 0, +7, +14, +21 relative to calving. Blood was collected on the day of diagnosis of the infections and on the alternate day in the infected group. The heparinized samples were centrifuged at 3000 rpm for 15 minutes, plasma aliquoted, and stored at -20°C for further analysis.

Growth hormone and Insulin was measured in plasma using a bovine Growth hormone ELISA test kit purchased from endocrine technologies, inc. USA and Endocrine Technologies, 35325 Fircrest Street, USA, respectively. IGF-I activity was quantified by Bovine IGF-1 ELISA test kit" obtained from Life Science Inc, Wuhan 430056, and P.R. China, with detection limit range between 2.4 ng/ml-60 ng/ml.

The data for healthy buffaloes were analyzed by one-way analysis of variance through graph prism version 5 to quantify postpartum variations for peripheral concentrations of growth hormone, Insulin, and IGF-1. Since prepartum and postpartum values were not statistically significant between days except IGF-1(already published Mili et al. 2015b), the data from day 7 to 21 postpartum values were served as the reference value for healthy buffaloes, whereas the day of calving (day 0) values of healthy buffaloes was taken as a control for RP. Similarly for unhealthy buffaloes, all the values were expressed as mean ± standard error (SEM). The unpaired student "t" test using GraphPad prism version 5 was applied to compare the data of healthy and infected buffaloes.

The changes in plasma growth hormone, insulin, and IGF-1 levels in buffaloes infected with metritis, endometritis, mastitis, and RP compared to healthy ones is presented in Table 1. Plasma IGF-1 concentrations was significantly lower in buffaloes with metritis (128.43±5.38 ng/mL), endometritis (112.65±4.38 ng/mL), and mastitis (123.61±3.99 ng/mL) as against the healthy (134.34±8.20 ng/mL) ones expect RP, whereas plasma IGF-1concentration was not statistically different among the two groups. These results are in agreement with previous studies (Kikukawa et al. 2002; Nikolic et al. 2003; Kasimanickam et al. 2013; Giuliodori et al. 2013; Beltman et al. 2020). Earlier, a low IGF-1 concentration was registered in mastitis cows (Nikolic et al. 2003; Huszenicza et al. 2004), and cows with uterine infections (53.8 ± 4.4 ng/mL) compared to healthy (66.9 ± 3.2 ng/mL) cows (Beltman et al. 2020). Kasimanickam et al. (2013) revealed that the IGF-1 concentrations remained high in cows infected with subclinical endometritis whereas these hormone levels were low in cows with clinical endometritis and metritis.

Plasma insulin concentration was significantly higher ($P<.05$) in buffaloes infected with endometritis (1.53±0.21 ng/mL), mastitis (1.32±0.26 ng/mL) and RP (1.24 ±0.15 ng/mL) when compared to healthy (1.07±0.06 ng/mL) and normally calved buffaloes (0.63 ±0.14 ng/ mL), respectively, except for buffaloes with metritis, where it was significantly lower than the healthy animals. Our findings are in agreement with earlier reports on mastitis (Nikolic et al. 2003; Huszenicza et al. 2004), metritis, and endometritis (Kasimanickam et al. 2013). Earlier, higher insulin levels were recorded in cows infected with mastitis (Nikolic et al. 2003; Huszenicza et al. 2004). Likewise, significantly higher insulin levels were recorded in subclinical endometritis cows compared to clinical endometritis, and metritis (Kasimanickam et al. 2013).

The plasma growth hormone was significantly elevated in metritis (7.40±0.96 ng/mL), mastitis (9.23±1.60 ng/mL), RP (6.63 ±0.80 ng/ mL) when compared to healthy (1.48 ±0.15 ng/ mL) and normally calved buffaloes (2.47 ±0.55 ng/mL), respectively except endometritis buffaloes, where it was no significant changes than the healthy animals. Our findings are in agreement with earlier reports on mastitis (Gubbiotti et al. 2007). They revealed 2.5 times higher levels of growth hormones in Mastitis cows compared to healthy cows. An elevated level of growth hormones is expected for energy homeostasis during the transition period in buffaloes due to NEB. Earlier reports have indicated that the prolonged periods of NEB are associated with a decrease in insulin secretion

by the pancreas, which results in a lesser concentration of growth hormone receptors during the transition period (Pell and Bates, 1990). Therefore, an increased circulatory level of growth hormones with a lower IGF-1 level is expected due to uncoupled growth hormone-insulin-IGF-1-glucose signaling pathway for energy homeostasis (Mili et al. 2015b). These hormonal changes combined with alteration in humoral and cellular immune responses coupled with low antioxidant defense system during the transition period (Mili et al. 2015a), might explain the deviations of metabolic hormones are the predisposing risk indicators of postpartum infections and RP in buffaloes.

Conclusions

Hence, it can be inferred that plasma growth hormone, insulin, and IGF-1 differed significantly among buffaloes with or without postpartum infections and hence could be used for the risk assessment of postpartum infection. However, large-scale studies are required to determine the crucial threshold levels of these attributes to trace out the onset/ early identification of postpartum reproductive infections.

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SHORT COMMUNICATION

Effect of oral dosing of live and autoclaved culture of *Ruminococcus flavefaciens* FD-1 on rumen bacterial and fungal populations in Murrah buffaloes

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Abstract: Dietary interventions aiming at increasing the number and activities of beneficial gut microbes could enhance digestive functions as well as the long-term welfare of dairy animals. In the present experiment, the effect of supplementation of a novel bacterial culture *Ruminococcus flavefaciens* FD-1 isolated from the rumen liquor of Murrah buffaloes was studied for its effect on rumen microbial populations enumerated using the most probable number technique. Three permanently fistulated buffaloes maintained on a high fibre diet were used as the source of rumen fluid for bacterial isolation that was subsequently tested in vivo. Twelve healthy mid-to-late lactating buffaloes in their second to third parity were divided into two similar groups of six each with a mean body weight of 601.5 kg. One group (LBC) was dosed with 300 mL of live bacterial culture, whereas an equal volume of autoclaved bacterial culture was dosed in another group (ABC) for a period of one month. The treatment-wise effect of supplementation of *R. flavefaciens* FD-1 culture was evident in an increase ($P<0.05$) in total rumen fungal population by 2.7 times in LBC than ABC in the post-dosing period. However, the period-wise comparison revealed that the population of bacteria augmented ($P<0.05$) by 2.16 and 3.17 times in both the groups ABC and LBC, respectively; whereas the magnitude of increase in fungal population was 5.04 and 10.3 times, respectively for both the groups. Post-dosing effects were evident for only group LBC in both bacteria and fungi, respectively increasing ($P<0.05$) the population by 2.16 and 8.28 times than pre-dosing period. These preliminary results may foster scope for developing species-specific (autochthonous) bacterial probiotic/direct-fed microbial based on *R. flavefaciens* FD-1 for large ruminants maintained on fibrous diets under tropical production systems.

Keywords: Direct-fed microbial, Fibrolytic bacterial isolates, Fungi, Murrah buffaloes, Rumen bacteria, *Ruminococcus flavefaciens*

Harnessing the potential of ruminants through natural feed additives targeting an improved gut microbial balance is growingly exigent today with a heightened research interest worldwide (O'Hara et al. 2020). Additive-led improvement in production performance would be of special interest for ruminants raised under agricultural residue-based low input system that is prevalent in India. Since rumen microbiota is an integral part of feed digestion and nutrient metabolism, it is imperative to maintain an optimum population of multifarious rumen microbes (Liu et al. 2021). When bacterial culture in the form of probiotic or direct-fed microbial are supplemented, betterment in performance outcome and health status has been obtained through a better gut microbial balance (Mazon et al. 2020; Mahesh et al. 2021; Singh et al. 2021).

Ruminococcus are the predominant cellulose-digesting bacteria in rumen (Wanapat and Cherdthong, 2009; Israeli-Ruimy et al. 2017; Patra, 2020; Wang et al. 2020). A shift in microbial population was noted previously when *Ruminococcus* spp. are supplemented (Krause et al. 2001). However, it is also established

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that successful results are more likely to be obtained when bacterial culture originating from the host species (autochthonous) are supplemented (Patra, 2020; Singh et al. 2021). While the multitude of research explorations on various bacterial cultures is available, there seems limited published information on isolating fibrolytic bacteria from buffalo rumen and its subsequent evaluation on microbial population dynamics *in vivo*. Therefore, it was hypothesised in the present research that when the in-lab grown live culture of *R. flavefaciens* is orally administered, it survives in the rumen, multiplies as well as stimulates other beneficial microbes that co-exist in the ruminal consortium. Therefore, the main objective was to enumerate bacterial and fungal populations of Murrah buffaloes receiving either live or autoclaved cultures of *R. flavefaciens* before, during, and after the administration.

This research was approved by the Institutional Animal Ethics Committee of ICAR – National Dairy Research Institute, Karnal, India.

Fibre-degrading bacteria were isolated from the rumen liquor of three permanently fistulated adult male buffaloes maintained on a diet with a concentrate-to-wheat straw ratio of 40:60. Total fourty two numbers of bacterial isolates were isolated using Hungate's anaerobic roll tube technique (Hungate, 1969) using carboxymethylcellulose as the substrate in the culture medium. The culture medium for isolation of anaerobic fibrolytic bacteria was prepared as described by Bryant and Burkey (1953). Pure cultures were obtained by repeated roll tube preparation on agar medium, picking off the single bacterial colony, and subsequent sub-culturing into the broth medium. The fibre-degrading characteristics of the bacterial isolates were tested by their in vitro fibre-degradation potential on pure neutral detergent fibre using *in vitro* Hohenheim gas production technique and based on *in vitro* fibre degradability (Kumar and Sirohi, 2013[a]). The three most potent isolates (NB-1, NB-2 and NB-3) were selected and identified by molecular technique. The molecular characterisation of the isolates was done using conventional PCR technique. The genomic DNA extracted from the isolate was amplified using universal as well as gene-specific primers for ruminal fibre-degrading bacteria (Kumar and Sirohi, 2013[b]). All the isolates were found to be of genus *Ruminococcus*. The amplified product of three most potent fibrolytic isolates was sequenced and nearly the complete sequence data were obtained for all three isolates. Similarity values of 16S rRNA gene sequences retrieved from the select isolates have been shown in Table 1. All three isolates have shown similarity with the *Ruminococcus flavefaciens* strains. The isolate NB-1 showed 97% similarity with *Ruminococcus flavefaciens* strain FD-1 and was the most potent fibre degrader that was used later to supplement experimental Murrah buffaloes.

Twelve lactating Murrah buffaloes of mid-to-late lactation were selected from the buffalo herd of the institute and randomly distributed into two groups of six each according to their milk yield (mean: 5.9 kg/d) and live body weight (mean: 601.5 kg). The animals were housed in a well-ventilated open shed having arrangements for individual feeding and watering. Animals of both the groups were fed an experimental diet comprising of conventional concentrate:wheat straw:green maize in the proportion of 40:30:30. The overall diet had a nutrient profile of 12.1% crude protein, 2.6% ether extract, 55.6% neutral detergent fibre, 31.5% acid detergent fibre, and 9.2 MJ/kg of predicted metabolisable energy/kg dry matter (Owens et al. 2010).

Table 1 Similarity values of 16S rRNA gene sequences retrieved from three most potent ruminal fibre-degrading bacterial isolates

Isolate	Strain I.D.	Nearest relative taxon	Similarity (%)	Query coverage (%)	Gene bank accession no.
NB-1	NB-01	*Ruminococcus flavefaciens* strain FD-1	97	96	JN222390.1
NB-2	NB-02	*Ruminococcus flavefaciens* strain LP-C14	96	99	JN222391.1
NB-3	NB-03	*Ruminococcus flavefaciens* strain 007	96	97	JN222392.1

NB: NDRI buffaloes

Table 2 Ruminal populations of bacteria and fungi upon oral administration of *R. flavefaciens* FD-1 in Murrah buffaloes at different time intervals

	Bacterial population[1]		Fungal population[2]	
	ABC	LBC	ABC	LBC
Pre-dosing	$11.1^{aA} \pm 4.29$	$7.58^{aA} \pm 3.59$	$2.40^{aA} \pm 1.72$	$1.22^{aA} \pm 0.68$
During dosing	$24.0^{aB} \pm 0$	$24.0^{aB} \pm 0$	$12.1^{aB} \pm 2.60$	$12.6^{aB} \pm 3.89$
Post-dosing	$15.5^{aA} \pm 4.80$	$16.4^{aC} \pm 3.52$	$3.75^{aA} \pm 1.55$	$10.1^{bB} \pm 4.47$

[1]Expressed as cells $\times 10^{11}$/mL of rumen liquor; [2]Expressed as cells $\times 10^{5}$/mL of rumen liquor

[a,b]Values with different superscripts within each row, separately for bacterial and fungal population, differ significantly ($P<0.05$)

[A,B]Values with different superscripts within each column, separately for bacterial and fungal population, differ significantly ($P<0.05$)

After 21 days of adaptation, animals of treatment (LBC) and control (ABC) groups were orally dosed with live and autoclaved culture of *Ruminococcus flavefaciens* strain FD-1, respectively at the rate of 300 mL every alternate day. The bacterial culture contained 3×10^{12} cells per mL, and hence the total number of bacterial cells dosed per animal was 9×10^{14} cells on every alternate day continuously for a period of one month. Autoclaving of the culture was performed at 121°C for 15 minutes, thus arresting the activity of *R. flavefaciens* FD-1 and hence acted as control, and any effect detected in treatment group could be ascribed to the live cells of *R. flavefaciens* FD-1.

Rumen liquor sample was drawn individually from all the animals of both groups with the help of a stomach tube before feeding. i.e., pre-dosing (prior to the start of the dosing), during dosing (immediately after dosing), and post-dosing (after one month of completion of dosing) period for the quantification of total ruminal bacteria and fungi.

10 mL rumen liquor was taken in a sterilised beaker and transferred to a pre-gassed (CO_2) mixer-grinder containing 90 mL of anaerobic dilution medium. The mixture was thoroughly churned for 3-minutes in presence of CO_2 to dislodge microbes from feed particles. The dilution of the contents thus obtained was marked as 10^{-1}. Then, 1 mL of diluted sample was taken into dilution tube (containing 9 mL of sterilised anaerobic dilution fluid) to make the dilution to 10^{-2}. Likewise, serial dilutions were made up to 10^{-12}. The dilution ranged from 10^{-8} to 10^{-12} and 10^{-2} to 10^{-6}, and was used for the total bacterial and fungal count, respectively. Each dilution was in triplicate, and un-inoculated control tubes were also kept in triplicate. All tubes were incubated at 39°C for two weeks. After 2 weeks, pH was recorded, and a decrease of >0.3 unit pH when compared with control was considered positive for microbial growth. Three consecutive dilutions were selected in such a way that the maximum dilution contained negative tubes as well. The number of cells was enumerated from the most probable number (MPN) and the MPN media for total bacterial and fungal estimation were prepared in accordance with Grubb and Dehority (1976).

Data analysis was carried out separately for each bacterial and fungal population using one-way analysis of variance with the software package of Sigma Plot 11.0. The data were presented as mean ± SE, and the difference in mean values between ABC and LBC was tested for significance at $P<0.05$.

The treatment-wise effect of supplementation of *R. flavefaciens* FD-1 culture was evident in an increase ($P<0.05$) in total rumen fungal population by 2.7 times in LBC than ABC in the post-dosing period. In the both pre-dosing and dosing periods, the differences between groups ABC and LBC were non-significant for both bacterial and fungal populations. However, the period-wise comparison revealed that the population of bacteria augmented ($P<0.05$) by 2.16 and 3.17 times in both the groups ABC and LBC, respectively; whereas the magnitude of increase in fungal population was 5.04 and 10.3 times, respectively for both the groups. Post-dosing effects were evident for only group LBC in both bacteria and fungi, respectively increasing ($P<0.05$) the population by 2.16 and 8.28 times than pre-dosing period.

Our objective was to test whether or not the bacterial culture of *R. flavefaciens* could able to colonise the rumen and in turn stimulate other gut microbes so that its potential to use as a direct-fed microbial/probiotic for ruminants could be envisaged further.

Although *R. flavefaciens* dosing did increase microbial abundance, the effect of live and autoclaved cultures did not differentially influence microbial numbers during the dosing phase. It could be reasonable that certain residual nutrients contained in the growth media along with autoclaved cell fragments of test bacteria might have acted similar to the live culture in stimulating rumen bacteria and fungi. Such effects have already been known in ruminants, for instance, with yeast products (Shurson, 2018; Mahesh et al. 2021). In addition, our result on augmenting bacterial numbers corroborates with Ebtehag et al. (2016), who observed a significant increase in various bacterial populations in lambs after supplementation of bacterial isolate. Indeed, real-time quantification further deduced an increase in the bacterial population (Brishketu and Sirohi, 2012[b]). It could be seen that *R. flavefaciens* live culture exhibited carry-over effect by increasing fungal number many-fold, even after one month of withdrawal of supplementation. This indicates that fibrolytic bacterial culture stimulates the multiplication of fungal zoospores within rumen, albeit the mechanism underpinning such response remains unclear. Consistent with our findings, Krause et al. (2001) also documented changes in rumen microbial populations measured with16S-rRNA-based probes upon administration of *Ruminococcus* spp. Furthermore, given the highly recalcitrant lignin-cellulose structure found in the crop residue-based diets in India (Mahesh and Mohini, 2013), the present results appear to hold promise in practically enhancing the digestion of complex forage fibre mediated by rumen fungi (O'Hara et al. 2020; Hartinger and Zebeli, 2021), which could result in better ruminant performance. Supportively, when dairy cattle were drenched with rumen bacterial culture *Megasphaera elsdenii,* the milk yield of cows was increased along with better rumen health attributes (Mazon et al. 2020). Nevertheless, a slight variation in the absolute number of microbial population than the previous studies could be due to differences in rumen microbial composition between cattle and buffaloes (Wanapat and Cherdthong, 2009; Wang et al. 2020).

It is also important to select the origin of fibrolytic bacteria in obtaining a desired response. Varel et al. (1995) could not observe multiplication of proven fibrolytic bacteria *Clostridium* spp. isolated from bison and pigs when administered to fibre-rich hay

diets in bovines. Hence, it was suggested that autochthonous (native) microbes seem advantageous over allochthonous (outside the native habitats) species for quick adaptation and establishment (Patra, 2020), as also demonstrated recently in tropical buffaloes (Singh et al. 2021).

Conclusion

The quantification of rumen microbial populations using the MPN technique showed that there was a significant increase in total fungal and bacterial population after oral administration of *R. flavefaciens* FD-1. Although the carry-over effect was persistent with live culture for the fungal population over a period of one month, it is necessary to investigate the long-term sustainable effects. Nonetheless, these preliminary findings clue that there is a potential to increase fibre digestion in ruminants with this isolate, which may open-up an opportunity to develop *R. flavefaciens*-based DFM for ruminants maintained on fibrous diets. However, in order to practically merit its use, economic feasibility needs further assessment when applying the results from lab to farm.

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SHORT COMMUNICATION

Impact of different housing modification systems on growth performance and feed intake of Gir calves during winter season

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Abstract: The current study was carried out to investigate the impact of different housing modification systems on growth performance and feed intake of Gir calves during winter season. Selected 24 Gir calves of 12 months old were randomly divided into three homogenous modified housing groups containing with eight calves in each *viz.,* group GC-1: conventional barn (during night) + open paddock (during day), GC-2: conventional barn + rubber mat and GC-3: loose house + curtains (during night). The concentrate supplement was given 1.5 kg/calve/day as pellet feed, green lucerne (*Medicago sativa*) 2.5 kg/calve/day and wheat straw (*Triticum aestivum*) *ad lib* as dry fodder. During the statistical analysis, the mean of morning minimum temperature (P<0.05) was higher in GC-2 (11.81°C) over than GC-3 (11.27°C) and GC-1 (10.64°C) and the morning maximum temperature was significantly higher in GC-3 (22.97°C), which was above than groups GC-2 (22.39°C) and GC-1 (22.21°C). However, the mean value of the evening minimum temperature was significantly lower in GC-1 (11.67°C) followed by GC-3 (11.86°C) and GC-2 (12.40°C) and the evening maximum temperature was highest in GC-1 (24.95°C) followed by GC-3 (23.98°C) and GC-2 (23.54°C). The average mean of both relative humidity (RH) percent and temperature humidity index (THI) along with morning and evening times was (P<0.05) higher in GC-2 than GC-1 and GC-3 groups. However, total dry matter intake, voluntary water intake and feed conversion efficiency were significantly higher in the GC-1 group compared to the calves in the GC-2 and GC-3 groups. The initial body weight of the claves was similar as 122.37, 121.13 and 121.88 kg in GC-1, GC-2 and GC-3 groups. However, the final body weight of the calves was significantly (P<0.05) highest in group GC-1 (170.12 kg) followed by GC-2 (163.61 kg) and GC-3 (162.36 kg) respectively. The average daily weight gain was significantly achieved by GC-1 (530.90 g/day) followed by GC-2 (472.05 g/day) and GC-3 (449.88 g/day). It was concluded that the housing modification system can be effectively used to improve the growth performance, feed intake and sheds micro-climate of Gir calves during the winter season.

Keywords: Body weight; Feed efficiency; Feed intake; Gir calves; Housing modification; Micro-climate

Housing is one of the foremost requirements of dairy animals for better production, health and welfare. Housing protects animals from adverse climatic conditions, provides comfort for resting, eating, drinking, walking and prevents injury and disease (Anderson, 2002). The calves growth may be adversely affected by the environmental stress during management practices (Laxmi et al. 2013). Dairy cow shelters will be designed to modify the micro environment and protect the animals from severe weather conditions to reduce peak stress levels (Sinha et al. 2018). Kumat et al. (2016) reported that loose housing modified shed were improved productivity during the autumn and winter seasons. Therefore, the present investigation was conducted to assess the impact of different housing modification systems on growth performance and feed intake of Gir calves during winter season.

The current study was carried out on the dairy farm of Sri Karan Narendra Agriculture University, Jobner (Rajasthan) from November 2019 to March 2020, with the total duration of the experiment being 105 days, i.e. first preliminary (adaptation) period

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for 15 days and the next 90 days of actual trail period. Twenty-four healthy Gir calves of both sex with approximately the same body weight (121.79±18.76 kg) and 12 months old were selected. The calves were randomly divided into three identical modified housing groups with eight calves in each group *viz.,* group GC-1: conventional barn (during night) + open paddock (during day), GC-2: conventional barn + rubber mat and GC-3: loose house + curtains (during night). The following changed housing conditions were used for all groups during the experimental period: GC-1 group of calves were tied at the neck with iron-chains, fed individually and allow open paddocks during the day (08: 00 am to 05: 00 pm) and closed at night in winter (05: 00 pm to 08: 00 am). GC-2 group the conventional barn is completely closed structure as roofed and walls are also complete with windows and ventilators to get proper ventilation and lighting. The calves were tied to their neck with iron-chains and the rubber mat was laid on the floor in this barn to protect them from the cold. The calves in the GC-3 group were kept in the loose house for around 24 hours. The loose house had a covered area with simple asbestos sheet roofs and an open area paved with kaccha brick, which surrounded on three sides by 1.5 meters high walls, but gunny bag curtains were fitted in the open front side, only made available to the claves at night. Twice a day (10:30 am and 4:00 pm) the experimental calves received a basic diet with dry fodder as wheat straw (*Triticum aestivum*) *ad lib* and green lucerne (*Medicago sativa*) 2.5 kg/calve/days as green fodder and 1.5 kg concentrate as pellet feed with free access to water. The concentrate mixture contained 19.26% crude protein, 3.60% ether extract and 6.22% crude fiber.

Meteorological observations such as maximum, minimum, dry and wet bulb temperatures of different modified sheds were recorded daily at 07:30 am and 02:30 pm during the experiment. The relative humidity (RH) was calculated using the hygrometric tables (Indian Meteorological Department, Pune) from the readings of the dry and wet thermometer. The Temperature Humidity Index (THI) was calculated according to the formula of McDowell (1972).

THI = 0.72 (dry bulb temperature ^{o}C + wet bulb temperature ^{o}C) + 40.6

The amount of green fodder (lucerne), dry fodder (wheat straw) and concentrate (pellet feed) were offered to the animals and the leftover was weighed on two consecutive days at weekly intervals during the experiment for analysis of dry matter intake (DMI). The samples of feed and fodder were analyzed for proximate principles (AOAC, 2000). The calves voluntary water intake was recorded once a week, morning and evening by offering a measured quantity of clean and fresh water through a measured/ marked bucket during the experiment. All calves were weighted at the beginning and monthly intervals as well as at the end of the experiment. The body weights of the calves were recorded in the morning at 08:00 am before providing water and feed. Fasting before weighing was done in an attempt to reduce the gut fill and thereby minimizing the weight fluctuations.

In the present study, the mean of the morning minimum temperature was significantly (P<0.05) higher in GC-2 (11.81^{o}C) over than GC-3 (11.27^{o}C) and GC-1 (10.64^{o}C) groups. The morning minimum temperature was higher in the conventional barn (GC-2) as compared to the loose house (GC-3) due to the protection against the cold through the maximum area enclosed by a wall. The average morning maximum temperature was significantly higher (P<0.05) in GC-3 (22.97^{o}C) than in GC-2 (22.39^{o}C) and GC-1 (22.21^{o}C). However, the mean value of evening minimum temperature was recorded significantly lower in GC-1 (11.67^{o}C) followed by GC-3 (11.86^{o}C) and GC-2 (12.40^{o}C). While the average means value of the evening maximum temperature was noted significantly in GC-1 (24.95^{o}C) then followed by GC-3 (23.98^{o}C) and GC-2 (23.54^{o}C). The average mean Relative Humidity (RH) percent in both morning and evening times was significantly

Table 1 Average dry matter intake (DMI) and voluntary water intake (VWI) in Gir calves

Parameters	GC-1	GC-2	GC-3
DMI, kg/day			
Wheat straw	2.32^{a}±0.01	2.04^{b}±0.01	1.92^{c}±0.03
Green lucerne	0.61^{a}±0.00	0.61^{a}±0.00	0.61^{a}±0.00
Concentrate mixture	1.35^{a}±0.00	1.35^{a}±0.00	1.35^{a}±0.00
Total DMI	4.28^{a}±0.01	4.00^{b}±0.01	3.88^{c}±0.01
DMI kg/100 kg BW	2.93^{a}±0.08	2.80^{b}±0.09	2.77^{bc}±0.13
VWI, lit			
Morning	4.64^{a}±0.04	4.51^{ab}±0.03	3.65^{c}±0.03
Evening	7.55^{a}±0.04	6.65^{b}±0.04	6.68^{b}±0.04
VWI/day	12.19^{a}±0.05	11.16^{b}±0.05	10.33^{c}±0.04
VWI/kg DMI	2.85^{a}±0.02	2.81^{ab}±0.02	2.71^{c}±0.03

a, b and c means with different superscripts are significantly different (P<0.05) in a row

Table 2 Feed conversion efficiency in Gir calves (twelve months to fifteen months)

Attributes	GC-1	GC-2	GC-3
Initial weight (kg) at 365 days	122.37^{a}±4.92	121.13^{b}±6.32	121.88^{bc}±8.96
Final weight (kg) at 455 days	170.12^{a}±14.16	163.61^{b}±12.65	162.36^{c}±10.98
Total gain (kg) in 90 days	47.78^{a}±0.53	42.48^{b}±0.54	40.48^{c}±0.76
Average daily gain (g/day)	530.90^{a}±24.21	472.05^{b}±38.84	449.88^{c}±26.49
Total feed intake (wheat straw + green lucerne + concentrate) (kg) in 90 days	385.20^{a}	360.00^{b}	349.20^{c}
Feed efficiency (%)	12.40^{a}	11.80^{b}	11.59^{c}

a, b and c means with different superscripts are significantly different (P<0.05) in a row

(P<0.05) higher in GC-2 (80.92% and 62.10%) over than in GC-1 (77.44% and 59.20%) and GC-3 (75.78% and 57.60%). The wide gap in the morning relative humidity may be due to frequent water supplies and morning washing of the animal shed. The values of the Temperature Humidity Index (THI) were recorded both in the morning and evening times significantly (P<0.05) higher in GC-2 (61.35 and 70.00) than in the GC-1(60.15 and 66.68) and GC-3 (59.11 and 65.47) groups respectively. Similar trends were observed in calves and heifers through various houses modifications in winter concerning temperature, RH and THI by (Jat et al. 2003; Jat and Yadav, 2010; Kailash et al. 2018). While, Shekhawat and Choudhary (2012) reported different trends during the winter season in their study of lactating crossbred cows, which could be due to variations in the place or the location system of housing.

The average daily total dry matter intake (TDMI) of calves in different groups is presented in (Table 1). However, the TDMI (kg/day) and DMI (kg/100kg BW) during the experiment were significantly (P<0.05) higher in GC-1 (4.28±0.01 kg and 2.93±0.08 kg) compared to calves of the GC-2 and GC-3 groups. The higher DMI in both groups GC-1 and GC-2 than in the GC-3 group may be due to protection from cold winds that resulted in better growth by diverting the nutrients for growth. Similar results were obtained in the different studies by (Jat et al. 2003; Kailash et al. 2017). However, in another study according to (Lowe et al. 2019) floor type, as a completely slatted concrete floor or a completely slatted concrete floor covered with rubber strips, had no significant effect on forage or total dry matter intake over the entire trial period.

The results (Table 1) showed that the average daily voluntary water intake (lit/day) and voluntary water intake (kg/DMI) were significantly (P<0.05) higher in both groups GC-1(12.19±0.05 lit. and 2.85±0.02 lit.) and GC-2 (11.16±0.05 lit. and 2.81±0.02 lit.) than in group GC-3 (10.33±0.04 lit. and 2.71±0.03 lit.). The VWI is mostly connected to the DMI of the animals, there was a positive correlation between the water intake and the DMI of Gir calves. Similar results were obtained by Jat et al. (2003) and Kailash et al. (2017).

The results of table 2 revealed that the initial (365 days of age) body weight of Gir calves were similar as 122.37±4.92, 121.13±6.32 and 121.88±8.96 kg in group GC-1, GC-2 and GC-3 respectively and the corresponding final body weight at 455 days of age in groups GC-1, GC-2 and GC-3 has significantly differed 170.12±14.6, 163.61±12.65 and 162.36±10.98 kg. The findings of the present result are in agreement with Kailash et al. (2018) who reported that the body weight of claves significantly improved conventional barn with an open shed during the winter season under semi-arid environmental conditions.

The average daily weight gain (ADG) as depicted in table 2 indicates that the significantly (P<0.05) higher rate in GC-1 (530.50±24.21 g/day), which was over than GC-2 (472.05±38.84 g/day) and GC-3 (449.88±26.49 g/day) groups. Similarly, Jat and Yadav (2010) and Kailash et al. (2017) observed that protecting buffaloes and cross-bred cows calves from cold stress during the winter season improved calves growth performance.

The total feed intake during 90 days found out highest in Gir calves of GC-1 (385.20 kg) followed by GC-2 (360.00 kg) and lowest in GC-3 (349.20 kg) respectively (Table 2). However, the highest feed conversion efficiency percent was recorded in GC-1 (12.40%) over than GC-2 (11.80%) and GC-3 (11.59%) groups. This feed conversion efficiency percent was better in GC-1 due to the higher growth rate as well as the physical comfort and higher feed intake of these calves the during winter season. The findings of the present study agree with Yadav et al. (1990) and Jat and Yadav (2010) observed that during the winter season a higher feed intake and body weight were observed in buffaloes calves in a conventional closed barn with open house rearing. However, Lowe et al. (2019) reported that using concrete slats or rubber covered slats did not affect bulls feed conversion efficiency.

Conclusions

Therefore, it is concluded that the conventional barn during night time and open paddock during day time ameliorate the cold stress and improved feed intake, feed conversion efficiency, water intake, body growth and average daily gain of Gir calves compared to other housing modified systems during the winter season in the semi-arid zone of Rajasthan.

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SHORT COMMUNICATION

A study of centrifugal pressing of curd for *paneer* production

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Abstract: *Paneer* is one of the popular heat-acid coagulated indigenous milk product in Indian cuisine. Traditionally, it is prepared by removing whey from acidified milk by pressing method. The present investigation was performed to study the effect of independent parameters like initial thickness of curd (1.5, 3.0 and 4.5 cm), speed of rotation of centrifuge (500, 1000 and 1500 rpm) and time of rotation (5, 10 and 15 min.) on centrifugal pressing of curd for *paneer* making. The effect of initial thickness, speed of rotation and pressing time of curd on moisture, solid loss and hardness ratio of centrifugal pressing were determined. A dimensionless number called as 'centrifugal pressing number' was formed to assess the changes occurred during pressing. It was found that pressing time had the maximum effect on solid loss and hardness ratios but the rotational speed had maximum effect on moisture ratio. The thickness of curd had the least effect on moisture and solid loss ratio of pressing.

Keywords: Centrifuge, Hardness ratio, Moisture ratio, Paneer, Solid loss ratio

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Paneer is obtained by acid coagulation of heated milk with citric acid and contains nearly all milk proteins, fat, insoluble salts and colloidal matter. It is similar to an unripened variety of soft cheese which is extensively used in the preparation of a variety of culinary dishes and snacks (Kumar et al. 2014). *Paneer* is usually white in appearance with sweetish acidic nutty flavor, close knit texture and spongy body (Bandyopadhyay and Mathur 1987). Due to increasing demand of cost-effective high quality product, there is need for upgrading and scaling-up of technology especially for continuous production of *paneer* with higher product yield. Aheer (1986) used bowl of basket centrifuge with perforations on its periphery for removal of whey from coagulated mass. Agrawal and Das (2001) studied the phenomena of pressing of curd by analysing the delayed method of pressing of curd, which is being practiced in traditional method of *paneer* manufacturing. Mudgal and Agrawala (2010) measured the textural characteristics and overall sensory of *paneer* made from buffalo milk. Halder et al. (2011) developed kinematic half-turn nut *paneer* pressing mechanism for medium scale application. Considering the limited amount of studies carried out, the present study was undertaken to study the effect of centrifugal pressing of curd for production of *paneer*.

The buffalo milk (7 ± 0.3% fat; 16 ± 0.5% TS) was procured from local market. Milk was first heated to 95°C and then cooled to 70°C. It was then coagulated by mixing 22.7 g/l citric acid solution previously heated to 70°C in the ratio of 1:5 (acid:milk). Residence time of 1 min was allowed before straining of whey by muslin cloth. The pressing of curd was carried out using Sorvall centrifuge swinging bucket rotor (make: Thermo Fisher Scientific). The operating parameters were initial thicknesses of curd (1.5, 3.0 and 4.5 cm), speed of rotation of centrifuge (500, 1000 and 1500 rpm) and time of rotation (10, 20 and 30 min). The pressed curd samples were rotated with chilled water for 15 min in same centrifuge. The control sample was prepared by traditional method (Agrawal and Das 2001). The centrifugal pressing method was evaluated by quantity of whey removed i.e. moisture ratio of pressing, M_{rp}, (ratio of moisture content of pressed curd to the moisture content of curd before pressing), Solid loss ratio of pressing, S_{rp} (ratio of weight of solid lost with whey to the initial dry weight of curd) and Hardness ratio of pressing, H_{rp} (ratio of hardness of pressed curd to the hardness of curd before pressing)

Table 1 ANOVA for the effect of independent variable on various pressing ratios

Source of variation	Pressing ratios					
	Moisture		Solid loss			
	Mean sum of squares	F-value	Mean sum of squares	F-value	Mean Hardness sum of squares	F-value
Thickness	00.400	17.51**	0.000093	62.427**	0.726	30.856**
Speed of rotation	0.02318	104.04**	0.000168	112.339**	0.705	29.958**
Time	0.01900	84.45**	0.000338	225.940**	1.333	56.667**
Error	0.00020		0.000001		0.024	

F-value (1, 23) at 1% = 7.88 **Significant at 1%

were taken as the dependent variables. These parameters were considered as the measures for evaluating the pressing process. The recovery of milk solids in curd, R_c (kg milk solids in curd/kg solids in milk) and yield of *paneer*, Z_c (kg paneer/kg milk) (Agrawal and Das 2001) were also calculated. For analyzing the centrifugal pressing a dimensionless number called 'Centrifugal Pressing Number' N_{cp} was conceptualized which has combination of all independent input variables.

$$N_{cp} = \frac{[2\pi^2 n^2 (r_2^2 - r_1^2)\theta_p^2]}{B^2} \quad (1)$$

Where, n = Speed of rotation of centrifuge, rps; r_1=Distance of whey drainage surface of the curd from axis of rotation (outer), m; r_2 = Distance of free surface of the curd from axis of rotation (inner), m; $\hat{o}_p$ =Time of run of centrifuge for pressing of curd, s and B = Initial thickness of curd, m. The moisture, solid loss and hardness ratios were converted into coded values to bring the variation of all ratios in a single range of non-dimensional code values to compare their behavior against Centrifugal pressing number. Therefore, the ratios of moisture, solid loss and hardness were brought to uniform range of -1 to +1 by converting their variation in terms of coded values, N_c, using the following equations:

$$N_c = \frac{(X - X_{mean})}{(X_{mean} - X_{min})} \quad (2)$$

Where, x = Observed value of variable; x_{mean} = Mean value of variable; x_{min} = Minimum value of variable. *Paneer* is known for its excellent chewing properties. Higher values of hardness will lead to higher chewiness (Mudgal and Agrawala 2010). Therefore, during pressing curd should attain high value of moisture and hardness ratios alongwith low value of solid loss ratio. The moisture content of milk, whey, curd, pressed curd and *paneer* were determined by drying at 102 ± 2 °C (IS: 5162, 1980) and fat (IS: 1224, 1977). Hardness of curd, pressed curd and *paneer* were determined using Steven's Texture Analyzer. A 3^3 factorial experimental design was adopted to evaluate the combined effect of selected independent variables (Snedecor and Cocharan, 1967). The expulsion of whey due to centrifugation caused average reduction of 0.015 (in fraction) in recovery of milk solids from original value of recovery of milk solids 0.701 curd (in fraction) which was obtained after straining of whey from coagulated mass.

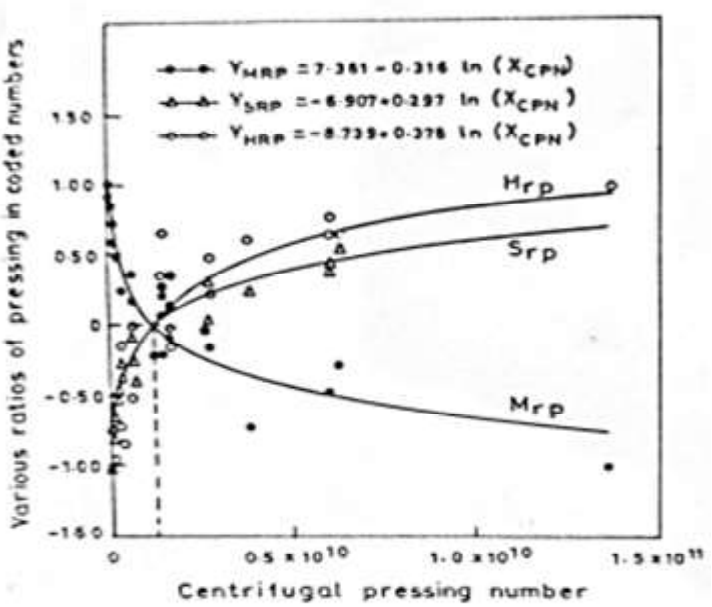

Fig. 1 Effect of centrifugal pressing number during centrifugal pressing of curd on various pressing ratios

The ANOVA for the effect of process variables on moisture, solid loss and hardness ratios of pressing is shown in Table 1. The effect of all the pressing variables on the moisture ratio, solid loss ratio and hardness ratio were highly significant ($p<0.01$). The initial thickness had the least significant effect on moisture and solid loss ratio. The selected levels of pressing time had highest effect on the solid loss and hardness ratio. The speed of rotation of centrifugal machine had the most significant, significant and least significant effects on moisture, solid loss and hardness ratios, respectively. It came to notice that the high speed of rotation caused more moisture removal and solid loss without much gain in hardness of pressed curd.

The variation of coded values of these ratios with respect to centrifugal pressing number is shown in Fig. 1. It showed that with the increase in centrifugal pressing number, the moisture ratio decreases. This might be due to centrifugation of curd at high speed with longer time leads to more removal of moisture from curd. The solid loss ratio increased with the increase in centrifugal pressing number. This might be due to longer time of centrifugal pressing of curd at high speed. The hardness ratio also increased with the increase in centrifugal pressing number of centrifugal pressing of curd. The rate of decrease in moisture ratio and increase in solid loss and hardness ratios decreased with the increase in centrifugal pressing number.

The desired combination of pressing ratios was obtained from point of intersection. The intersection of all three pressing ratios at centrifugal number of $1.25 * 10^{10}$ was found to be equal to 0.000 (in terms of coded value). The desired pressing ratios in coded values (0.000) were converted back to original values which were the mean values of moisture (0.808), solid loss (0.029) and hardness (1.731) ratios. In the present set of experimentation, the recovery of solids in *paneer* varied from 0.563 to 0.624 kg solids/kg solids in milk and yield of *paneer* varied from 0.193 to 0.233 kg *paneer*/kg milk. Similar result was also reported by Bhattacharya et al. (1971) that was 0.209 kg/kg of milk.

Conclusion

In this study the centrifugal pressing number was developed to optimize the independent variables (initial thickness of curd, speed of rotation and time of rotation of centrifuge) to ensure minimum solid loss and desired hardness of centrifuged pressed *paneer*. If the levels of independent variables are known, the different pressing ratios can be estimated with the help of Centrifugal pressing number. The developed empirical mathematical relationship is a useful tool for predicting the behavior of curd pressing phenomena, when different combination of parameters is adopted in centrifugal pressing.

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